GHENT UNIVERSITY

FACULTY OF BIOSCIENCE ENGINEERING

EARLY DETECTION OF *MYCOPLASMA HYOPNEUMONIAE* IN PIGS UNDER FIELD CONDITIONS

dr. Frédéric VANGROENWEGHE

Thesis submitted in fulfilment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences

Promoters
Prof. dr. ir. Olivier THAS
Prof. dr. Dominiek MAES

2018

The cover of the doctoral thesis was designed by Roger Verzele

This work was printed by University Press B.V.B.A. – Zelzate – www.universitypress.be

Early detection of *Mycoplasma hyopneumoniae* in pigs under field conditions

F. Vangroenweghe, 2018, Ghent University

ISBN 9789463571272

D/2018/10.412/1

To my dearest family, Ellen, Victor, Arthur & Elise-Marie

TABLE OF CONTENTS

LIST OF FREQUENTLY USED ABBREVIATIONS	1
CHAPTER 1 – *MYCOPLASMA HYOPNEUMONIAE*: PATHOGENESIS, EPIDEMIOLOGY, DIAGNOSIS AND CONTROL: A REVIEW	5
CHAPTER 2 – STUDY OBJECTIVES	69
CHAPTER 3 – HEALTH ADVANTAGES OF TRANSITION TO BATCH MANAGEMENT SYSTEM IN FARROW-TO-FINISH PIG HERDS	73
CHAPTER 4 – ASSESSMENT OF LITTER PREVALENCE OF *MYCOPLASMA HYOPNEUMONIAE* IN PREWEANED PIGLETS UTILIZING AN ANTEMORTEM TRACHEOBRONCHIAL MUCUS COLLECTION TECHNIQUE AND A REAL-TIME POLYMERASE CHAIN REACTION ASSAY	89
CHAPTER 5 – *MYCOPLASMA HYOPNEUMONIAE* INFECTION IN PERI-WEANED AND POST-WEANED PIGS IN BELGIUM AND THE NETHERLANDS: PREVALENCE AND ASSOCIATIONS WITH CLIMATIC CONDITIONS	101
CHAPTER 6 – SHORT COMMUNICATION: CONFIRMATION OF *MYCOPLASMA HYOPNEUMONIAE* IN A BREEDING HERD THROUGH TRACHEO-BRONCHIAL SWAB SAMPLING AND PCR	117
CHAPTER 7 – CASE REPORT: USE OF TRACHEO-BRONCHIAL SWAB qPCR TESTING TO CONFIRM *MYCOPLASMA HYOPNEUMONIAE* SEROPOSITIVITY IN A SPF BREEDING HERD	127
CHAPTER 8 – GENERAL DISCUSSION	141
REFERENCES	157
SUMMARY	189
SAMENVATTING	193
DANKWOORD	197
CURRICULUM VITAE	201
SPONSORS	205

LIST OF FREQUENTLY USED ABBREVIATIONS

A. pleuropneumoniae	*Actinobacillus pleuropneumoniae*
A. suum	*Ascaris suum*
AI/AO	all-in/all-out
ANOVA	analysis of variance
ATP	adenosine triphosphate
BALF	broncho-alveolar lavage fluid
BALT	bronchus-associated lymphatic tissue
BMS	batch management system
CCU	color changing units
CFT	complement fixation test
CFU	colony-forming units
CMI	cell-mediated immunity
Ct value	threshold value
DNA	desoxyribonuclease
DNT	dermo-necrotic toxin
ELISA	enzyme-linked immunosorbent assay
FCR	feed conversion rate
H. parasuis	*Hemophilus parasuis*
IFA	immunofluorescence assay
IHC	immunohistochemistry
ISH	*in situ* hybridization
L. intracellularis	*Lawsonia intracellularis*
LAMP	loop-mediated isothermal amplification
LS	laryngeal swab
LTB	heat-labile enterotoxin
M. hyopneumoniae	*Mycoplasma hyopneumoniae*
MDA	maternally derived antibodies
MEW	medicated early weaning
MLST	multilocus sequence typing
MLVA	multiple-locus variable number tandem repeat analysis
nPCR	nested PCR

OF	oral fluid
OR	odds ratio
P. multocida	*Pasteurella multocida*
PCMV	Porcine Cyto Megalo Virus
PCR	polymerase chain reaction
PCV-2	Porcine Circo Virus type 2
PCVD	Porcine Circo Virus disease
PFGE	pulsed-field gel electrophoresis
PIA	porcine intestinal adenomatosis
PRCV	Porcine Respiratory Corona Virus
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus
qPCR	quantitative PCR
RAPD	random amplification of polymorphic DNA
RFLP	restricted fragment length polymorphism
RNA	ribonucleic acid
rtPCR	real-time polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Se	sensitivity
SIV	Swine Influenza Virus
Sp	specificity
SPF	specific pathogen free
SPR	surface plasmon resonance
TBL	tracheo-bronchial lavage
TBS	tracheo-bronchial swab
USA	United States of America
VNTR	variable number tandem repeat

CHAPTER 1 – *MYCOPLASMA HYOPNEUMONIAE:* PATHOGENESIS, EPIDEMIOLOGY, DIAGNOSIS AND CONTROL: A REVIEW

CONTENTS

1.	General perspective of porcine respiratory disease and *Mycoplasma hyopneumoniae*
2.	The porcine respiratory tract
2.1.	Anatomy of the porcine respiratory tract
2.1.1.	Macroscopic anatomy
2.1.2.	Histology
2.2.	Function of the normal respiratory tract
2.3.	Conditioning and filtering by the conducting airways
3.	Porcine respiratory disease complex
3.1.	Dual infections involving *M. hyopneumoniae* and viral pathogens
3.2.	Dual infections involving *M. hyopneumoniae* and bacterial pathogens
3.3.	Dual infections involving *M. hyopneumoniae* and parasites
4.	*M. hyopneumoniae* - etiology
5.	*M. hyopneumoniae* - pathogenesis
6.	*M. hyopneumoniae* - epidemiology
6.1.	Role of wild boar
6.2.	Early infections
6.3.	Infection levels after weaning
7.	*M. hyopneumoniae* - risk factors
7.1.	*M. hyopneumoniae* - environmental risk factors
7.1.1.	Meteorological conditions
7.1.2.	Housing and stable climate conditions
7.2.	*M. hyopneumoniae* - biological risk factors
7.3.	*M. hyopneumoniae* - management risk factors
8.	*M. hyopneumoniae* - diagnosis
8.1.	Diagnosis
8.1.1.	Non-specific diagnosis
8.1.1.1.	Clinical symptoms
8.1.1.2.	Impact on performance
8.1.1.3.	Lung lesions
8.1.1.3.1.	Macroscopic lung lesions
8.1.1.3.2.	Microscopic lung lesions
8.1.2.	Specific diagnosis
8.1.2.1.	Host-response to the pathogen: serology
8.1.2.1.1.	Serological tests on blood

8.1.2.1.2.	Serological tests on other fluids: BALF and OF
8.1.2.2.	Pathogen or parts of the pathogen
8.1.2.2.1.	Isolation
8.1.2.2.2.	Detection of *M. hyopneumoniae* antigen
8.1.2.2.3.	Molecular detection methods
8.1.2.2.3.1.	*In situ* hybridisation
8.1.2.2.3.2.	Polymerase Chain Reaction
8.1.2.2.3.3.	Molecular typing techniques
8.2.	Further diagnostic considerations
8.2.1.	Diagnosis of clinical disease or monitoring: selection of representative animals
8.2.2.	Appropriate sampling strategies
8.2.2.1.	Serial sampling
8.2.2.2.	Cross-sectional sampling
8.2.2.3.	Targeted sampling
8.3.	Type of samples
8.4.	Test characteristics
8.4.1.	Epidemiological test characteristics
8.4.2.	Lab-technical test characteristics: detection limit and analytical sensitivity
9.	*M. hyopneumoniae* - antimicrobial treatment options
10.	*M. hyopneumoniae* - control measures
10.1.	Management and biosecurity
10.2.	Vaccination
10.2.1.	Commercial *M. hyopneumoniae* vaccines
10.2.2.	Experimental *M. hyopneumoniae* vaccines
11.	*M. hyopneumoniae* - eradication strategies

1. GENERAL PERSPECTIVE OF PORCINE RESPIRATORY DISEASE AND *MYCOPLASMA HYOPNEUMONIAE*

Porcine respiratory disease and *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) have been a major problem in the intensive pig production over the last decades (Maes *et al*., 2017). The disease complex, characterized by both acute and chronic respiratory symptoms depending on the situation and the pathogens involved, is responsible for major economic losses due to decreased productivity, *i.e.* daily weight gain and feed conversion rate (FCR), increased number of runt pigs, higher mortality and increased antibiotic use (Maes *et al*., 2008). Indeed, antibiotic use has been an important shift in focus during the last years, mainly due to problems with general antimicrobial consumption and decreased antimicrobial sensitivity in animal and human pathogens (Speksnijder *et al*., 2015). *Mycoplasma hyopneumoniae* plays a major role within the Porcine Respiratory Disease Complex (PRDC) as the principial etiological agent of enzootic pneumonia, a chronic respiratory disease that mainly affects finishing pigs (Sibila *et al*., 2009).

In order to maintain pig production within an acceptable economic perspective, taking into account the consumer's concerns towards responsible and reduced use of antimicrobials, the diagnosis and prevention of PRDC occurrence in general and *M. hyopneumoniae* in particular remains an issue under practical production conditions. Throughout the last decade, a rapid diagnostic evolution towards *M. hyopneumoniae* has taken place in relation to sampling methods and subsequent sample analytical methods, which has created more accurate and fast diagnostic possibilities (Sibila *et al*., 2009; Maes *et al*., 2017; Pieters *et al*., 2017). A recent diagnostic approach, namely tracheo-bronchial swab (TBS) sampling, has been developed and evaluated under experimental conditions for *M. hyopneumoniae* diagnostics (Fablet *et al*., 2010). However, further evaluation on practical application under field conditions was lacking until now.

Early diagnosis of *M. hyopneumoniae* infection from the perspective of piglet age and interval between infection and clinical symptoms is key in order to limit economic losses and to plan the appropriate curative and preventive interventions necessary to resolve the disease. In case of treatment, an early intervention can limit the amount of antimicrobials used to support the healing of the pigs, whereas from a prevention point of view, correct early diagnosis can help to apply the appropriate preventive measures to omit future use of antimicrobials. These different

aspects of early diagnosis for efficient prevention of *M. hyopneumoniae* forms the basis for the current work.

2. THE PORCINE RESPIRATORY TRACT

2.1. Anatomy of the porcine respiratory tract

2.1.1. Macroscopic anatomy

The respiratory tract is composed of the nasal cavity, nasopharynx, larynx and the tubular conducting airways including trachea, extra- and intra-pulmonary bronchi, bronchioles, and the gas exchange system including terminal bronchioles and alveoli. In the lungs, two separate blood-conducting systems are present, the pulmonary artery system supplying venous blood from the right ventricle to the capillary plexus surrounding the pulmonary alveoli and the bronchial tree vascularing the supporting structures around the trachea, bronchi and bronchioles. The intimate association of the large vasculature network and the large airspace of the alveoli holds a risk for entry of pathogens into the body (Van Alstine, 2012). A thin, translucent membrane, the pleura, covers the lungs and chest cavity.

A cartilaginous septum is dividing the nasal cavity longitudinally with on both sides two turbinate bones. These turbinate bones divide each of the two halves of the nasal cavity into three meatuses: dorsal, middle and ventral part. The length of the nasal cavity varies between different breeds. The nasal cavity represents an easily accessible site for sampling to detect potential presence of respiratory pathogens under field conditions.

The trachea is relatively short and divides posteriorly into two primary bronchi, one for the left lung half and one for the right lung half. A separate, small bronchus branches from the trachea leading to the right cranial (apical) lobe. Bronchi branch into the left cranial (apical) lobe, left and right middle (cardiac) lobes, left and right caudal (diaphragmatic) lobes, and the right accessory (intermediate) lobes (Judge *et al.*, 2013). The next branches of the tubular system are the bronchioles, each dividing into alveolar ducts and alveoli (Figure 1).

The lungs are divided into seven lobes: the right lung is composed of the cranial, middle, and caudal and accessory lobes; the left lung is composed of the cranial, middle, and caudal lobes (Figure 2). The lobes are separated by deep fissures, except the left cranial and middle lobes, which

are separated only by the cardiac notch. Lung lobes vary in size and weight (Garcia-Morante *et al*., 2016).

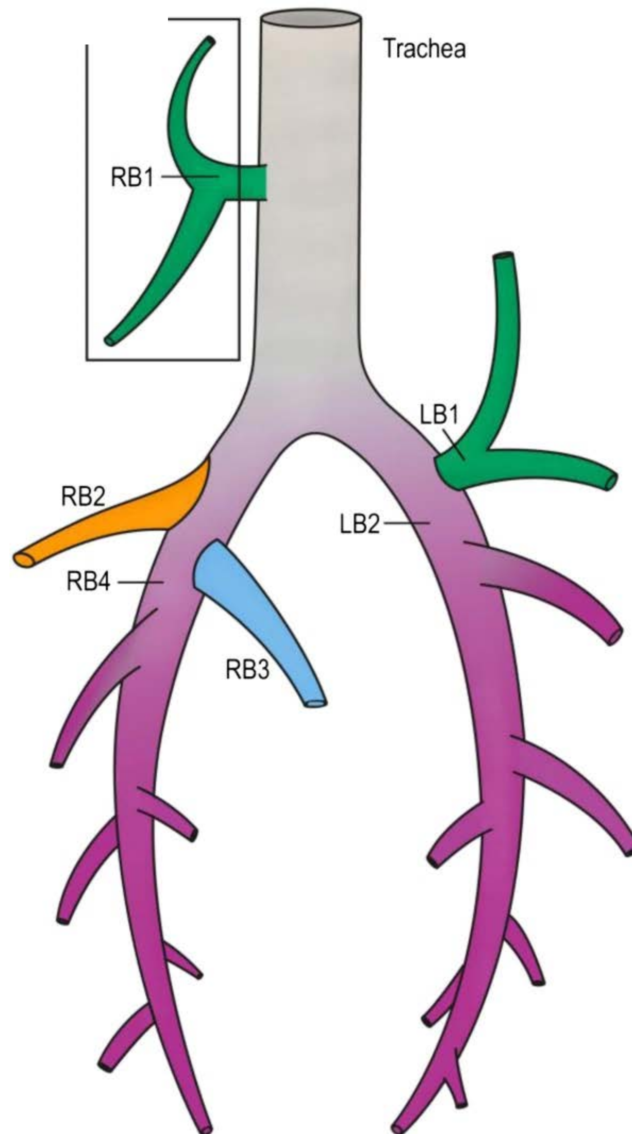

Figure 1. Nomenclature of the porcine airway tree (ventral aspect). Larger airways with different colors denoting different lung lobes (*green*, cranial lobes; *orange*, right middle lobe; *blue*, right accessory lobe; *purple*, caudal lobes). LB1, left cranial lobe bronchus; LB2, left caudal lobe bronchus; RB1, right cranial lobe bronchus; RB2, right middle lobe bronchus; RB3, right accessory lobe bronchus; RB4, right caudal lobe bronchus (Judge *et al*., 2013).

The lobes are subdivided by solid interlobular septa into separate lobules each served by a bronchiole. Collateral ventilation between lobules is minimal. As a result of this lobulation, exudate within alveoli, as seen with a bronchopneumonia, is often retained within lobules, giving the lungs a sharp line of demarcation between affected and unaffected lobules.

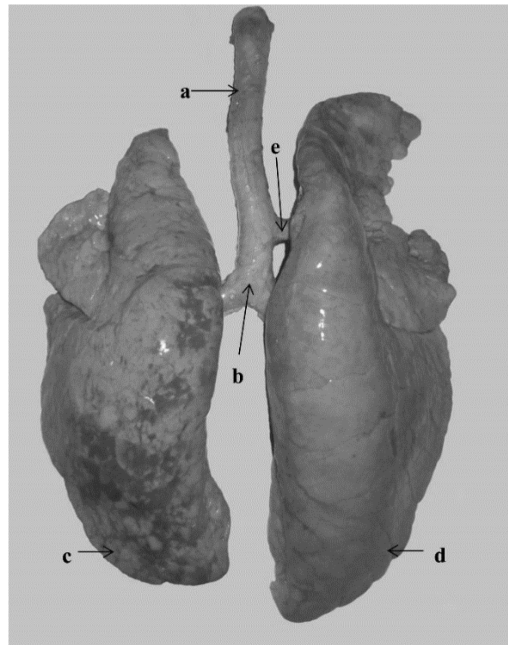

Figure 2. Porcine lung anatomy. Photograph (dorsal aspect) of lungs from a pig (age, ~ 22 wk; size, ~ 105 kg) showing trachea (*a*), carina (*b*), left lung (*c*), right lung (*d*), and cranial lobe bronchus (*e*) (Judge *et al*., 2013).

2.1.2. Histology

The vestibular region of the nasal cavity is lined with stratified squamous epithelium. The remainder of the nasal cavity is lined by ciliated pseudostratified epithelium with mucus-producing Goblet cells. This ciliated epithelium is covered by a bi-layered mucus coating produced by the Goblet cells and mucosal glands, and this epithelium continues through the pharynx, larynx, trachea, and bronchi. The epithelium of the bronchioles is lower in height, and is more squamous deeper in the lung towards the alveoli. Alveoli are lined by single-layered epithelial cells (type I pneumocytes) and by a small percentage of cuboidal epithelial cells (type II pneumocytes). Type II pneumocytes produce pulmonary surfactant and serve as progenitor cells for replacement and

turn-over of type I pneumocytes. Pneumocytes in the alveolar wall are very intimately attached to the capillary plexus, and together they form the blood–air barrier.

2.2.Function of the normal respiratory system

Gas exchange occurs in the pulmonary alveoli. During each breath, only a small portion of the total alveolar air volume is renewed. In the resting pig, 10–15% of the alveolar air is exchanged during each inspiration. The normal respiratory rate (breaths/minute) varies according to age of the animal: piglets and growing pigs 25–40; finishing pigs 25–35; sows in gestation 15–20.

The mucosal surface of the respiratory tract provides a critical interface between the pig and its environment. The alveolar surface area is very large (in humans more than 100 m^2) and is permanently exposed to the environment through inhaled air. Therefore, the respiratory tract is equipped with a potent and specialized defense system.

2.3.Conditioning and filtering by the conducting airways

Air is well filtered and properly conditioned before it reaches the alveoli. Inspired air is warmed by the extensive capillary network superficially located in the nasal mucosa and is moistened by the mucus bilayer. Most inspired particles are trapped in the mucus layer over the nasal, pharyngeal, laryngeal, and tracheal epithelium. High air velocity in nasal cavity coupled with marked air turbulence around nasal turbinates results in impaction of larger airborne particles (>30 µm) onto the mucus bilayer lining the nasal mucosa. Particles greater than 10 µm diameter are mostly removed before reaching the bronchial tree (Baskerville, 1981). Particles trapped in the mucus are handled by the mucociliary clearance mechanism. The rhythmic beating of cilia in the bronchi and bronchioli results in a mucus flow towards the pharynx of about 4–15 mm/min (Done, 1988). Like the mucus from the nasal cavity, mucus from the trachea and bronchi is delivered to the pharyngeal cavity and subsequently swallowed. Particles less than 5 µm in diameter are able to reach the alveoli. The primary defense against particulate matter in the alveolus is the alveolar macrophage. The macrophages remove foreign material (non-pathogenic particles, microbes and pathogenic microorganisms) that escapes the mucociliary defense mechanism. Several other cell types can be recovered from the broncho-alveolar region (Table 1). Besides this primary innate

immunity, a secondary specialized defense – also known as the adaptive immunity – is present at the level of the respiratory tract. This adaptive immunity consists of the humoral immunity and the cell-mediated immunity (CMI). The humoral immune response produces specific antibodies crucial in the respiratory immune defense, whereas the CMI is based on antibody-independent components, such as cytotoxic T cells, natural killer cells, activated macrophages and cells mediating antibody-dependent cytotoxicity. The CMI response is not only particularly important in viral infections, but is also assumed to play an important role in *M. hyopneumoniae* infection (Lowe *et al*., 2005; Fort *et al.*, 2008; Maes, 2010).

Table 1. Broncho-alveolar cell differentiation (mean ± SD) in high health and conventional pigs (10 weeks of age) using broncho-alveolar lavage fluid (BALF) (Judge *et al*., 2013).

	High health pigs*	**Conventional pigs†**	
BALF recovery		**Research facility**	**Farm**
Total cell count (10^4/ml)	163 ± 73	171 ± 36	199 ± 59
Alveolar macrophages (%)	85 ± 6	90 ± 3	88 ± 4
Polymorphonuclear (%)	7 ± 5	2 ± 1	4 ± 3
Lymphocytes (%)	8 ± 3	8 ± 2	8 ± 2
Eosinophils (%)	0	0	0

* High-health pigs were weaned from a swine farm with minimal disease.
† Low-health pigs were weaned from a commercial farm with a 70% prevalence of macroscopic pneumonia lesions at slaughter. Low-health pigs were raised in a research facility or a farm.

3. PORCINE RESPIRATORY DISEASE COMPLEX (PRDC)

Porcine Respiratory Disease Complex (PRDC) is a multifactorial and complex disease caused by a combination of infectious pathogens, environmental stress factors, differences in production systems, and various management practices (Opriessnig *et al*., 2011a). This complex comprises several bacterial, viral and parasitic pathogens, including *M. hyopneumoniae*, Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), Porcine Circo Virus type 2 (PCV-2), Swine Influenza Virus (SIV), Porcine Cyto Megalo Virus (PCMV), Porcine Respiratory Corona Virus (PRCV), *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*), *Haemophilus parasuis* (*H. parasuis*) and *Ascaris suum* (*A. suum*). A comprehensive review on the complex interactions

between these different respiratory pathogens involved in PRDC has been written by Opriessnig *et al*. (2011a).

In recent decades, several dual infections under experimental conditions, involving *M. hyopneumoniae*, have been performed. In a first part, dual infections involving *M. hyopneumoniae* and viral agents will be discussed, followed by a second part on dual infections involving bacterial agents. Finally, interactions between *M. hyopneumoniae* and parasites will be discussed.

3.1.Dual infections involving *M. hyopneumoniae* and viral pathogens

Early studies on the interaction between *M. hyopneumoniae* and PRRSV concluded that in weaned piglets, PRRSV did not exacerbate *M. hyopneumoniae* infection (Van Alstine *et al*., 1996). However, other studies indicated that *M. hyopneumoniae* potentiates the severity induced by PRRSV, and vaccination against *M. hyopneumoniae* alone is also able to decrease PRRSV viraemia and PRRSV-induced lung lesions in dually infected pigs (Thacker *et al*., 1999; Park *et al*., 2014; Chae, 2016). An earlier study by Thacker *et al*. (2000) revealed that both *M. hyopneumoniae* bacterin and PRRSV vaccine decreased the severity of clinical respiratory disease. Vaccination with a *M. hyopneumoniae* bacterin decreased the potentiation of PRRSV-induced pneumonia observed in the dually infected pigs (Thacker *et al*., 2000; Silin *et al*., 2001). PRRSV vaccine alone did not decrease the potentiation of PRRSV pneumonia by *M. hyopneumoniae* (Thacker *et al*., 2000). Vaccination with modified live PRRSV vaccine prior to *M. hyopneumoniae* vaccination did not interfere with vaccine efficacy or immune responses towards the latter vaccine (Boettcher *et al*., 2002). *Mycoplasma hyopneumoniae* vaccination was also effective in decreasing growth losses in spite of concurrent PRRSV infection (Moreau *et al*., 2004). In practice, the *M. hyopneumoniae* infection status should be evaluated before the use of PRRSV vaccine to control PRRVS infection in farms. Control of *M. hyopneumoniae* infection by vaccination may positively affect the full induction of immunity by a PRRSV vaccine in farms suffering from concurrent PRRSV and *M. hyopneumoniae* infection (Chae, 2016). On the other hand, PRRSV vaccine strains and pathogenic parent strains have been demonstrated to be able to induce regulatory T-cells in pigs infected with *M. hyopneumoniae*, resulting in more severe respiratory disease (LeRoith *et al*., 2011). An Italian study focusing on extent of cranio-ventral lung lesions and their risk factors revealed that the main risk factor associated with the reduction of the percentage of cranio-ventral

pulmonary consolidation was vaccination of weaners at the age of 3-5 weeks with a live modified PRRSV vaccine (Merialdi *et al*., 2012).

Co-infection of *M. hyopneumoniae* with PCV-2 has been shown to increase the severity of both PCV-2 and *M. hyopneumoniae* associated lesions (Opriessnig *et al*., 2004), mainly due to the presence of the PCV-2 antigen and major alterations in the cytokine mRNA expression profiles (Zhang *et al*., 2011). Another study in boars co-infected with PCV-2 and *M. hyopneumoniae* demonstrated a significant higher PCV-2 load in blood, resulting in more severe disease and increased viral shedding (Opriessnig *et al*., 2011b). In a sequential infection model, *M. hyopneumoniae* potentiated the severity of PCV-2 associated lung and lymphoid lesions, and increased the incidence of Porcine Circo Virus disease (PCVD) in pigs that were first inoculated with *M. hyopneumoniae* and then inoculated 2 weeks later with PCV-2 (Chae, 2016; Opriessnig *et al*., 2004). However, concurrent infection with *M. hyopneumoniae* and PCV-2 did not result in potentiation of clinical signs and lesions attributed to either infections (Sibila *et al*., 2012). In dually infected pigs, *M. hyopneumoniae* vaccination alone did not reduce PCV-2 viraemie or PCV-2-associated lung and lymphoid lesions (Seo *et al*., 2014; Chae, 2016).

Presence of other PRDC pathogens, such as SIV, result in more severe lung lesions following dual infection as compared to infection with *M. hyopneumoniae* alone (Yazawa *et al*., 2004). Initial infection with *M. hyopneumoniae* before SIV increased the clinical signs and pathogenesis of SIV H1N1, whereas no significant effect was observed for SIV H1N2 (Deblanc *et al*., 2012). Further research revealed that pre-infection of *M. hyopneumoniae* induces oxidative stress that subsequently increases the severity of infection with SIV H1N1 subtype. There was a prolonged hyperthermia, stronger reduction in mean daily weight gain and earlier viral shedding (Deblanc *et al*., 2013). These host responses could additionally be influenced by feed, which is in accordance with other research, indicating that feed restriction positively affects recovery of SIV – *M. hyopneumoniae*-dually infected pigs (Le Floc'h *et al*., 2014).

3.2.Dual infections involving *M. hyopneumoniae* and bacterial pathogens

Park *et al*. (2016b) demonstrated that *M. hyopneumoniae* increases the L-fucose composition enhancing the adherence of *P. multocida* type A, another pathogen involved in PRDC, to the bronchial and bronchiolar epithelial cells. Presence of *A. pleuropneumoniae* type 9 may be

clinically unnoticed in a pig herd, though pre-infection of these pigs with *M. hyopneumoniae* may potentiate the *A. pleuropneumoniae* infection (Marois *et al*., 2009).

Another study, exploring relations between lung lesions and bacteriology in herds with high pleurisy scores revealed that no single infectious cause of pleuritis could be found. Although *M. hyopneumoniae* has no direct relation with pleurisy lesions, the presence of *M. hyopneumoniae* could help other pathogens such as *A. pleuropneumoniae* or *P. multocida* to develop pleurisy lesions (Jirawattanapong *et al*., 2010). Infection with *M. hyopneumoniae* has been shown a predisposing factor in the development of *H. parasuis* pleuropneumonia (Yagihashi *et al*., 1984).

3.3.Dual infections involving *M. hyopneumoniae* and parasites

Concurrent exposure of pigs to *M. hyopneumoniae* and *A. suum* did not seem to exacerbate the severity of lung lesions (Zimmerman *et al*., 1973). However, Flesja and Ulvesaeter (1980a; 1980b) reported that the extent of pneumonia was associated with the presence of liver lesions due to ascarid migration. This is mainly related to life cycle of the *A. suum* parasite in the pig, involving a migration through the intestinal wall, the liver and the lungs back to the intestine, in order to evolve from the L3 larval stage to its final adult stage. Moreover, *A. suum* infection negatively affects the immune response to a *M. hyopneumoniae* vaccination and subsequent challenge. The group suffering from gastro-intestinal helminth infection had a higher mean percentage of lung pathology and the variation was higher than in the other groups (Steenhard *et al*., 2009).

4. M. HYOPNEUMONIAE – ETIOLOGY

Mycoplasma hyopneumoniae, the etiologic agent of porcine enzootic pneumonia, is present in most swine farms worldwide, causing respiratory disease due to pneumonia in growing pigs and breeding animals. *Mycoplasma hyopneumoniae* infection is, however, not only associated with conventional pig production, but has also been detected in wild boar populations (Sibila *et al*., 2010; Chiari *et al*., 2014). In the wild boar, *M. hyopneumoniae* is able to cause enzootic pneumonia microscopic lesions, although with a subclinical impact (Sibila *et al*., 2010).

Mycoplasmas are the smallest and simplest free-living organisms implicated in a variety of plant and animal diseases. Small genomes, as those of mycoplasmas, are characterized by

progressive gene loss from a Gram-positive ancestor. Moreover, insertion of mobile DNA elements is presumed to be impossible since only the essential genes have been maintained (Rocha and Blanchard, 2002).

Genome sequencing of *M. hyopneumoniae* strain 232 revealed several proteases, such as aminopeptidases or serine proteases, which might serve as virulence factors. Moreover, *M. hyopneumoniae* contains few genes with tandem repeat sequences that could be involved in phase switching or antigenic variation (Minion *et al*., 2004). Phase switching is an important mechanism of evolution and adaptation of prokaryocytes which enables them to switch the expression of important genes in an ON/OFF mode. This enables the pathogen to vary its antigen expression on the cell surface, which enables the pathogen to escape from or modulate host immune responses (Minion *et al*., 2004). Analysing the complete genome sequences of two strains of *M. hyopneumoniae* – a pathogenic strain 7448 and a non-pathogenic J-strain – revealed that strain-specific regions, including integrative and conjugal elements, genome rearrangements and alterations in adhesin sequences were potentially related to pathogenicity (Vasconcelos *et al*., 2005). Although these species possess reduced genomes, mobile elements in the genome are maintained, possibly as a mechanism for genetic variability production (Loreto *et al*., 2007). Another array-based genomic comparative hybridization analysis revealed that genetic variation could be detected in all analysed field strains but across different loci, suggesting that variation occurs throughout the genome (Madsen *et al*., 2007). *Mycoplasma hyopneumoniae* has a genome with 716 coding sequences, of which 418 are homologous to proteins with known functions (de Fonsêca *et al*., 2012), whereas 42% are annotated as hypothetical proteins. Further comparative genomic analysis revealed a total of 234 proteins involved in *M. hyopneumoniae* metabolic pathways from which 21 proteins could serve as a vaccine candidate (Damte *et al*., 2013). Analysis of the membrane phospholipid and fatty acid compositions of *M. hyopneumoniae* using thin-layer and gas chromatography revealed a predominant composition of diphosphatidyl-glycerol. The percentage of C16-C18 fatty acids comprised 79% of the total fatty acids, of which oleic and palmitic acid were the major fatty acids (Hwang *et al*., 1986).

Mycoplasma hyopneumoniae has been shown to respond to environmental stressors such as heat shock (Madsen *et al*., 2006a), iron deprivation (Madsen *et al*., 2006b) and oxidative compounds (Schafer *et al*., 2007). Following exposure to hydrogen peroxide, *M. hyopneumoniae* significantly downregulates important glycolytic pathway genes and gene transcription proteins,

as well as a protein known to activate oxidative stressor cascades in neutrophils (Schafer *et al*., 2007). In response to norepinephrine, a stress associated hormone in the pig, *M. hyopneumoniae* appears to upregulate its protein expression while downregulating its general metabolism (Oneal *et al*., 2008).

5. M. HYOPNEUMONIAE - PATHOGENESIS

The first step of infection with *M. hyopneumoniae* is adhesion along the entire length of the cilia of the ciliated epithelium of the respiratory tract (trachea, bronchi and bronchioles), although in the initial stage adhesion mostly occurs at the top of the cilia (Blanchard *et al*., 1992). Specific interaction between *M. hyopneumoniae* proteins, small secreted cleavage products, and several molecules, such as aconitase, lamin A/C and peroxiredoxin, from the porcine respiratory ciliated cells occurs during adhesion (Li *et al*., 2010). This adhesion adversely affects the function of the ciliated epithelial cells of the respiratory tract (Jenkins *et al*., 2006).

Another study demonstrated that pathogenic *M. hyopneumoniae* activates receptors coupled to G_i and G_o, which in turn activate a phospholipase C pathway, releasing Ca^{2+} from the endoplasmatic reticulum of the porcine ciliated tracheal cells (Park *et al*., 2002). These different interactions result in the induction of ciliostasis and loss of cilia (Debey and Ross, 1994; Irigoyen *et al*., 1998), which might be enabled by the mycoplasmal proteins penetrating into the ciliated cells, thereby interfering with metabolic pathways or other critical cellular processes of the ciliated cells (Li *et al*., 2010).

Colonisation of the ciliated respiratory epithelial cells with *M. hyopneumoniae* is a pre-requisite for the development of mycoplasmal pneumonia (Kwon *et al*., 2002; Chae, 2011). Using *in situ* hybridization, the organism has been detected on the luminal surface of porcine bronchi and bronchioles between 7 and 28 days post-infection (Kwon and Chae, 1999; Kwon *et al*., 2002). Associated with a positive hybridization signal in the airways, an affected bronchus or bronchiole also exhibited peri-bronchiolar lymphoid cuffing (Kwon *et al*., 2002). Further research showed that *M. hyopneumoniae* DNA could also be detected at the level of the alveolar and interstitial macrophages and type I pneumocytes of infected pigs from 14 to 35 days post-infection (Kwon *et al*., 2002). The pathogen could even be detected in internal organs such as liver, spleen and kidney of experimentally infected and contact pigs (Kwon *et al*., 2002; Le Carrou *et al*., 2006; Marois *et*

al., 2007; Marchioro *et al*., 2014b), although the clinical relevance of these finding still needs further research. Previous isolation of *M. hyopneumoniae* from lymph nodes of experimentally challenged pigs suggested that dissemination of *M. hyopneumoniae* to distal tissues could occur by way of the lymphatic system (Le Carrou *et al*., 2006).

Following initial steps of *M. hyopneumoniae* infection, increased production of mucin, both membrane-bound and secreted mucin, has been demonstrated (Kim *et al*., 2012) in Goblet cells lining the pulmonary bronchioles (Debey *et al*., 1992) and the bronchial sub-mucosal glands (Jones *et al*., 1974). The increased mucin production may result from direct stimulation of the epithelium by the *M. hyopneumoniae* infection or may arise indirectly following *M. hyopneumoniae*-induced ciliostasis (Kim *et al*., 2012). Moreover, lipid-associated membrane proteins from *M. hyopneumoniae* have been shown to induce apoptosis in lung epithelial cells through activation of several pathways (Ni *et al*., 2015), finally contributing to cell damage and pathology at the level of the respiratory tract. Microscopic lesions were characterized by hyperplasia of the peribronchial lymphoid tissue and mild neutrophilic infiltrates in the alveoli. Electron microscopy showed patchy areas with loss of cilia and presence of leukocytes and mycoplasmas in the bronchi (Irigoyen *et al*., 1998). Inducing a second challenge of pigs already suffering lesions of *M. hyopneumoniae* did not produce a new episode of clinical signs nor did it enhance morphological changes, suggesting that those pigs had become *M. hyopneumoniae* carriers (Irigoyen *et al*., 1998). Further morphologic and immunohistochemical study of the bronchus-associated lymphatic tissue (BALT) following *M. hyopneumoniae* infection revealed catarrhal broncho-interstitial pneumonia, with infiltration of inflammatory cells in the lamina propria of bronchi, bronchioles and alveolar septa. Hyperplasia of the mononuclear cells in the BALT areas was the most significant histological change (Sarradell *et al*., 2003).

A high variation in virulence exists between different *M. hyopneumoniae* strains isolated in the field. Using an infection model, significant differences between isolates were found for respiratory disease score, lung lesion score, histopathology, immunofluorescence and serology. Based on these parameters, isolates could be divided into three virulence groups: low, moderately and highly virulent strains (Vicca *et al*., 2003). Typically, a 5000 bp RAPD fragment was associated with the highly and moderately virulent strains, where it was absent in low virulent strains (Vicca *et al*., 2003). Transmission of *M. hyopneumoniae* in nursery pigs, expressed as adjusted reproduction ratio (R_n), was assessed using a transmission experiment with a low and

highly virulent *M. hyopneumoniae* strain using two seeder piglets, intra-tracheally inoculated with *M. hyopneumoniae*, and six contact piglets during a 6-week nursery period. The R_n-values of the highly and low virulent strains were estimated to be 1.47 (0.68-5.38) and 0.85 (0.33-3.39), respectively (Meyns *et al*., 2004). Depending on the virulence of the strains, one infected piglet will infect on average one pen mate during the nursery period (Meyns *et al*., 2004). The difference in virulence between highly and low virulent strains is associated with a faster *in vitro* growth, a higher capacity to multiply in the lungs and the induction of a more severe inflammation process by the highly virulent strains (Meyns *et al*., 2007). To assess the potential impact of *M. hyopneumoniae* vaccination on transmission during the nursery period, a ratio of three seeder piglets and seven contact piglets were used. The R_n-value in the vaccinated group was 2.38 (1.07-7.53), while in the non-vaccinated group, a R_n-value of 3.51 (1.51-9.34) was observed. Therefore, it was concluded that *M. hyopneumoniae* vaccination only numerically reduced the transmission of *M. hyopneumoniae* and conventional bacterins could not prevent the establishment of *M. hyopneumoniae* infection in the lungs (Meyns *et al*., 2006). Subsequent research on *M. hyopneumoniae* transmission in vaccinated animals under field conditions revealed lower percentages of *M. hyopneumoniae*-positive piglets at weaning (14 *vs.* 36%) and at the end of nursery (31 *vs.* 64%) between the vaccinated and non-vaccinated groups (Villarreal *et al*., 2011a).

Infiltration of macrophages and *M. hyopneumoniae* replication in the lungs are both reduced in vaccinated pigs, though the highly virulent strain is inhibited more than the low virulent strain (Vranckx *et al*., 2012b). Further evaluation of vaccination effect against experimental infection with highly or low virulent strains revealed a significant reduction in clinical symptoms, macroscopic and microscopic lung lesions in pigs infected with a highly virulent strain (Villarreal *et al*., 2011b), and the effect was more pronounced at 4 weeks as compared to 8 weeks post-infection. Another study evaluated the effect of an initial infection with a low virulent *M. hyopneumoniae* isolate on a subsequent infection with a highly virulent *M. hyopneumoniae* isolate. Pigs inoculated with low virulent isolates of *M. hyopneumoniae* were not protected against a subsequent infection with a highly virulent isolate 4 weeks later and may even develop more severe disease signs upon the second infection (Villarreal *et al*., 2009).

6. M. HYOPNEUMONIAE – EPIDEMIOLOGY

6.1.Role of wild boar

M. hyopneumoniae infection is not only associated with conventional pig production, but has also been detected using an antibody enzyme-linked immunosorbent assay (ELISA) in wild boar populations (Chiari *et al*., 2014). Using genotyping, phylogenetic relatedness between strains detected in wild boar and domestic pigs could be demonstrated (Kuhnert *et al*., 2011). In Switzerland, enzootic pneumonia due to *M. hyopneumoniae* has been successfully eradicated, however, sporadic outbreaks are observed with no obvious source (Stärk *et al*., 2007). Besides the possibility of outbreaks due to persisting *M. hyopneumoniae* strains within the pig population (Le Carrou *et al*., 2006), suspicion arises that wild boar might introduce *M. hyopneumoniae* into swine herds (Kuhnert and Overesch, 2014). Using molecular epidemiology, including multilocus sequence typing (MLST) genotyping, strains involved in the outbreak could only be found in geographically linked wild boar lungs after an outbreak. This leads to the conclusion that wild boar might rather be a recipient than a transmitter (Kuhnert and Overesch, 2014). Another study based on ELISA results in wild boar populations confirmed infection of piglets in early stages of their life (Chiari *et al*., 2014).

6.2.Early infections

Piglets are born free of *M. hyopneumoniae*, as *in utero* transmission has not been documented, and first exposure events occur during the lactation period, when piglets are in contact with dams shedding the microorganism (Calsamiglia and Pijoan, 2000; Nathues *et al*., 2013a). A higher percentage of gilts and younger sows were shown to shed *M. hyopneumoniae* as compared to older sows (Calsamiglia and Pijoan, 2000). Indeed, after farrowing, younger sows and their off-spring showed the highest percentage (50%) of nested PCR (nPCR) *M. hyopneumoniae*-positive samples (Tamiozzo *et al*., 2011).

Sibila *et al*. (2007a) demonstrated early infection with an increasing prevalence from 1.5% *M. hyopneumoniae*-positive piglets at 1 week of age to 3.8% at 3 weeks of age. Moreover, *M. hyopneumoniae* infection was not only detected in nasal cavities of naturally infected suckling piglets, but also in the lower respiratory airways (Sibila *et al*., 2007a). Using broncho-alveolar

lavage fluid (BALF), Moorkamp *et al*. (2009) detected 12.3% *M. hyopneumoniae*-positive suckling piglets and 10.6% *M. hyopneumoniae*-positive weaned piglets. In a German study, 3.9% *M. hyopneumoniae*-positive piglets were detected using nasal swabs at weaning (Nathues *et al*., 2013a). A European study on detection and risk factors associated with *M. hyopneumoniae* in suckling pigs in herds with respiratory problems revealed an average percentage of *M. hyopneumoniae*-positive piglets of 10.7% (CI 7.4-14.2) and at least one piglet tested positive on 68% of the herds. In 32% of the herds, more than 10% of the piglets tested positive (Villarreal *et al*., 2010). It can therefore be concluded that *M. hyopneumoniae* is widespread in 3-week old piglets across different European countries. Transmission of *M. hyopneumoniae* has been shown to occur more consistently within a farm, in contrast to *A. pleuropneumoniae* type 2, which varies more between batches within a farm (Vigre *et al*., 2004). A complete overview of early detection of *M. hyopneumoniae* is given in Table 2.

6.3.Infection levels after weaning

Following *M. hyopneumoniae* infection, the first antibody response was detectable 4-5 weeks post-infection and was stronger in piglets infected at the age of 2 weeks than at the age of 8 weeks (Strasser *et al*., 1992). In both clinically and subclinically *M. hyopneumoniae*-infected herds, seroconversion occurred between 15 and 18 weeks of age. In clinically infected herds, the percentage of *M. hyopneumoniae*-seropositive animals increased from 8% at 9 weeks of age to 52% at 18 weeks of age, whereas in subclinically infected herds, the seroprevalence was lower with 2% at 9 weeks of age and 24% at 18 weeks of age. The percentage of nPCR positive piglets at 6 weeks of age was 16% and 0% in clinically and subclinically infected herds, respectively, demonstrating that piglets become infected earlier in life in clinically infected herds (Vicca *et al*., 2002).

Direct transmission of *M. hyopneumoniae* between pigs is the main transmission route and the lack of a cell wall compromises its resilience outside the host (Browne *et al*., 2017). Pigs in direct contact with seropositive gilts were 7 times more likely to seroconvert than those with only indirect contact (Morris *et al*., 1995). Therefore, personnel and fomites are another major epidemiological risk in the transmission of *M. hyopneumoniae* from an infected herd into a naive herd (Pitkin *et al*., 2011). However, using a standard hygiene protocol before entering an

uninfected farm, it was shown that the risk of transmission of the pathogen could be controlled (Batista *et al*., 2004; Pitkin *et al*., 2011).

Table 2. Studies of *M. hyopneumoniae* prevalence at various ages (expressed as % *M. hyopneumoniae*-positive piglets) using different sampling techniques.

Piglet age (weeks)	**Prevalence (%) *M. hyopneumoniae***	**Sampling technique**	**Reference**
3	7.7-9.6	NS	Calsamiglia and Pijoan, 2000
3	2.6-13.2	NS	Ruiz *et al*., 2003
1-3	0.5-5.5	NS	Sibila *et al*., 2007a
6-9	2.0-9.0	NS	Sibila *et al*., 2007a
3	0.0-51.3	NS	Fano *et al*., 2007
3	10.6	BALF	Moorkamp *et al*., 2009
6	12.3	BALF	Moorkamp *et al*., 2009
2	2.0	Lung	Nathues *et al*., 2010
4-10	9.3	Lung	Nathues *et al*., 2010
3	10.7	NS	Villarreal *et al*., 2010
4	14.1	TBS	Fablet *et al*., 2012b
2.5-3	3.9	NS	Nathues *et al*., 2013a

NS, nasal swab; BALF, broncho-alveolar lavage fluid; TBS, tracheo-bronchial swab

To protect piglets from early infection, the influence of maternally derived antibodies (MDA) has been studied in relation to the timing of *M. hyopneumoniae* infection. The transfer of MDA to *M. hyopneumoniae*, originating from colostrum consumption following birth, is variable and does not directly relate to the amount of serum antibodies in the dam (Wallgren *et al*., 1998). The median half-life of *M. hyopneumoniae* antibodies was 15.8 days. The persistence of passively acquired *M. hyopneumoniae* antibodies was related to the initial antibody concentration in the piglet, with an estimate of 30, 45 and 63 days for piglets with an initial antibody concentration classified as low, medium and high (Morris *et al*., 1994). This level influences the duration of MDA presence in the piglets, but overall, piglets are seronegative by the age of 9 weeks (Wallgren *et al*., 1998). Kinetics of MDA in piglets born in farms endemically infected with *M. hyopneumoniae* revealed that prevalence was 80% at 1 week of age, decreasing to 45-60% at 4 weeks of age (groβe Beilage *et al*., 2005a). Sow vaccination against *M. hyopneumoniae* did not reduce the prevalence of *M. hyopneumoniae* in nasal swabs (Ruiz *et al*., 2003; Sibila *et al*., 2008).

Nevertheless, piglets born from vaccinated sows had a reduced prevalence of *M. hyopneumoniae* as demonstrated by nPCR (Ruiz *et al*., 2003) and consequently had a significant lower mean lung lesion score as compared to piglets from non-vaccinated sows (Sibila *et al*., 2008).

This hypothesis could be confirmed in a longitudinal study of respiratory infection patterns of breeding sows in France, where antibodies against *M. hyopneumoniae* were recovered from more than 55% of the sows, whereas *M. hyopneumoniae* was only detected in 2.4% of the sows. Interestingly, in herds with the lowest number of *M. hyopneumoniae*-seropositive sows, *M. hyopneumoniae* carriage was not detected at any sampling time (Fablet *et al*., 2011). Nevertheless, *M. hyopneumoniae* has been detected from the upper respiratory tract of seropositive sows, indicating that circulating antibodies against *M. hyopneumoniae* do not prevent colonization of the airways (Sibila *et al*., 2007a; Sibila *et al*., 2008). Even older sows were detected with *M. hyopneumoniae*, suggesting they still have the potential to spread the bacteria to other susceptible animals, including their offspring (Fablet *et al*., 2011). Moreover, sows and piglets showed a higher concentration of antibodies with increasing number of farrowings, which may be due to vaccination and repeated exposure of animals to *M. hyopneumoniae* under field conditions (Tamiozzo *et al*., 2011).

A recent study on *M. hyopneumoniae* detection in naturally infected gilts revealed that bacterial detection in gilts started at 110 days of age and a significant increase occurred at 140 days of age. From 140 to 230 days of age, the *M. hyopneumoniae* prevalence remained above 20%, decreasing thereafter (Takeuti *et al*., 2017b). Although *M. hyopneumoniae* detection did not reach 0% at any sampling after 110 days of age, *M. hyopneumoniae* was not detected in piglets before weaning. The *M. hyopneumoniae* detection pattern showed that in natural infections, gilts were positive for *M. hyopneumoniae* for one to three months, although occasionally long-term detection may occur. Moreover, the lack of *M. hyopneumoniae* detection throughout the study in 18.2% of the gilts indicates the existence of *M. hyopneumoniae*-negative subpopulations within positive herds (Takeuti *et al*., 2017b). Looking into more detail to the infection dynamics of *M. hyopneumoniae* in self-replacement gilts, it became apparent that circulation of *M. hyopneumoniae* varied among farms, even under similar production and management conditions (Takeuti *et al*., 2017a). In addition, molecular variability of *M. hyopneumoniae* detected within farms suggests that in cases of minimal replacement gilt introduction, bacterial diversity may be farm-specific (Takeuti *et al*., 2017a).

Under some production systems, acclimatisation of gilts to *M. hyopneumoniae* using seeder pigs has been implemented. The optimal seeder-to-naive gilt ratio, however, is essential for a reproducible and consistent result. The optimal seeder-to-naive gilt ratio in a 4-week period for successful natural exposure to *M. hyopneumoniae* revealed to be 6 seeders in a group of 10 gilts (6:4 ratio). Application of lower ratios resulted in much lower success rates, ranging from 28% (3:7) to 80% (5:5) (Roos *et al*., 2016).

From an epidemiological point of view, the question remains whether a farm is infected with only one or rather more than one *M. hyopneumoniae* strain. Based on protein variability determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), no clear evidence could be found that more than one *M. hyopneumoniae* isolate circulates within a herd at a specific time point (Calus *et al*., 2007). Another study using variable number tandem repeats (VNTR) analysis in 3 Belgian herds revealed differences in diversity and persistence of *M. hyopneumoniae* strains between herds. Whereas in one of the herd, one *M. hyopneumoniae* strain with limited clonality persisted, in another herd, there was evidence of simultaneous infection with two strains in half of the sampled pigs (Vranckx *et al*., 2011). In a subsequent longitudinal study, it was shown that within a herd, one distinct strain – persisting for at least 12 weeks in the same animals – could be detected, although clonal variants could also be identified in two herds (Vranckx *et al*., 2012a). This observation was confirmed using MLST on a wide variety of samples from different farms and geographic regions. Farms recognized to be affected by enzootic pneumonia were always associated with one single type of farm-specific *M. hyopneumoniae* clone, which differed in most cases from farm to farm (Mayor *et al*., 2007; Mayor *et al*., 2008). However, farms in close geographic or operational contact showed identical clones (Mayor *et al*., 2008). However, a more recent study using random amplification of polymorphic DNA (RAPD) and VNTR analyses demonstrated that more than one strain of *M. hyopneumoniae* might be present in a pig herd and even in a single pig, suggesting high heterogeneity between isolates of the same epidemiological source (Nathues *et al*., 2011; Vranckx *et al*., 2012a; Michiels *et al*., 2017). Recently, Fano *et al*. (2018) demonstrated that genetic variability of *M. hyopneumoniae* within swine production flows was minimal over time and flow-specific. Therefore, evolutionary pressure associated with farm management practices may not have a significant effect on genetic variability. In addition, variants across all production stages appeared to originate from vertical transmission.

7. *M. HYOPNEUMONIAE* – RISK FACTORS

Risk factors for infection with *M. hyopneumoniae* are categorized into environmental, biological and management factors. Several environmental risk factors are identified such as meteorologic conditions related to airborne transmission of the pathogen, such as moisture, UV-exposure, wind and landscape elements. Other environmental risk factors are related to housing and stable climate conditions predisposing to airway irritations, such as ammonia levels, dust concentration and particulate matter. Biological risk factors include immunosuppressive agents and contaminants. Management risk factors are focused on biosecurity, gilt introduction protocols, weaning age, farm structure and batch management practices.

7.1.*M. hyopneumoniae* – environmental risk factors

7.1.1. Meteorologic conditions

In a Spanish study (Segalés *et al*., 2012), the influence of climatological parameters on *M. hyopneumoniae* dynamics was explored. This study revealed the higher the precipitation rate, the higher the probability of being *M. hyopneumoniae* nPCR-positive on nasal swabs, whereas the lower the temperature, the higher the probability of being *M. hyopneumoniae* seropositive. From a seasonal perspective, animals born in autumn and reaching slaughter in spring had the highest probability of being infected by *M. hyopneumoniae* and the highest probability of being *M. hyopneumoniae* seropositive (Segalés *et al*., 2012).

Bio-aerosols, containing *M. hyopneumoniae* and PRRSV, are capable of spreading pathogens between herds via airborne route. Conditions common to both pathogens include cool temperature and specific wind direction, and more specifically low sunlight levels, low wind velocity in combination with rising humidity and air pressure (Dee *et al*., 2010). Evidence has shown long distance airborne transport of *M. hyopneumoniae* as far as 4.7 km from the initial source herd (Dee *et al*., 2009). Others have shown airborne transport of both *M. hyopneumoniae* and PRRSV, with pathogens remaining infectious, over longer distances up to 9.1 km (Otake *et al*., 2010). Airborne spread of both *M. hyopneumoniae* and PRRSV could be reduced using an air filtration system. All types of filters, including mechanical, antimicrobial and electrostatic filters, are equally effective at preventing aerosol transmission of both pathogens (Dee *et al*., 2011).

Interestingly, a study assessing risk factors and incidence of re-infections with *M. hyopneumoniae* in farms located in respiratory-disease-free regions of Switzerland identified large mixed breeding-finishing farms, a re-infected neighbor and a parking site for pig transport vehicles close to the farm as risk factors with a significant impact (Hege *et al*., 2002). These risk factors are mainly related to the effect of airborne transmission with *M. hyopneumoniae*.

7.1.2. Housing and stable climate conditions

Ammonia is known to have a negative impact on the mucosae of the respiratory tract. However, increased ammonia levels had no significant effect on disease development following inoculation with *M. hyopneumoniae* in combination with *P. multocida* (Andreasen *et al*., 2000a). More recently, Michiels *et al*. (2015) investigated the effect of ammonia and particulate matter on performance parameters of finishing pigs. Increasing NH_3 concentrations resulted in higher odds of pleurisy lesions and a higher number of nPCR positive nasal swabs for *M. hyopneumoniae*.

Mycoplasma hyopneumoniae has been shown to survive for several days (up to 8 days) on dry surfaces and may therefore have the potential to infect pigs by indirect transmission (Browne *et al*., 2017). The survival on dust is of particular interest. Dust can affect the health by causing irritation to the respiratory tract (Donham and Leininger, 1984). Increased levels of dust in the pig environment are associated with poor hygiene and management practices, *e.g*. when stocking density is high or ventilation is poor. These conditions have been shown to exacerbate respiratory disease (Stärk, 1999; Stärk, 2000). More precisely particulate matter < 10 µm (PM_{10}) had a significant impact on the respiratory health of finishing pigs with higher odds of pneumonia lesions and more severe pneumonia lesions in the second half of the finishing period. Throughout the study, average levels of PM_{10} were approximately 2250 µg/m³ with variation (minimum/maximum) between 1667 µg/m³ and 4412 µg/m³ (Michiels *et al*., 2015).

Moreover, *M. hyopneumoniae* could also be detected from air samples of pig farm rooms using PCR (Stärk *et al*., 1998). Using a nPCR, highly specific and 10^4 times more specific than a one-step PCR, air samples collected from pig houses where respiratory problems were present could be analysed. Therefore, air was samples using polyethersulfone membranes (pore size, 0.2 µm) mounted in filter holders. Filters were subsequently processed by dissolution and direct extraction of DNA for PCR analysis. The chance of successful detection of *M. hyopneumoniae*

was increased if air was sampled at several locations within a room and at a lower air humidity (Stärk *et al*., 1998). Therefore, air sampling PCR results could serve as a tool to assess the spread of *M. hyopneumoniae* by bio-aerosols and infection dynamics within and between swine herds (Damte *et al*., 2014).

7.2.*M. hyopneumoniae* – biological risk factors

Concurrent pathogens, such as PRRSV, PCV-2 and SIV, have a significant impact on the local immunity of the respiratory tract against *M. hyopneumoniae*. The interaction between *M. hyopneumoniae* and other respiratory pathogens has been discussed previously (cfr. 3. PRDC).

Other contaminants, such as mycotoxins, may also interact with the severity of a *M. hyopneumoniae* infection. Presence of fumonisin B resulted in an increased severity of lung lesions based on computed tomography following a *M. hyopneumoniae* infection (Pósa *et al*., 2009). However, Michiels *et al*. (2018) could not demonstrate that pigs fed with feed contaminated with DON (1800 µg/kg) increased the severity of an experimental *M. hyopneumoniae* infection.

7.3.*M. hyopneumoniae* – management risk factors

Pigs infected with *M. hyopneumoniae* can be incubatory as well as convalescent carriers of the pathogen. These convalescent carriers can remain infectious up to 254 days before total clearance of the *M. hyopneumoniae* infection (Pieters *et al*., 2009). Therefore, introduction of gilts has been identified as an important risk factor for *M. hyopneumoniae* infection into the herd and transmission of the pathogen to the offspring during the first lactation. An effective gilt acclimatization program should ideally aim to reduce *M. hyopneumoniae* shedding during gestation resulting in decreased pre-weaning colonization prevalence in piglets of first parity sows and potential respiratory problems in fatteners (Pieters & Fano, 2016).

A recent European survey focusing on gilt replacement status and acclimatization strategies revealed that 87.2% of the farmers were aware of the health status of the gilts on arrival and 57.5% introduced *M. hyopneumoniae*-positive replacements (Garza-Moreno *et al*., 2017). Acclimatization processes were applied in 77.6% of the respondents, of which *M. hyopneumoniae* vaccination was the most popular one (58.2%), followed by a combination of vaccination and

exposure to slaughter sows (21.3%). Acclimatization status was verified in approximately 50% of the herds that had initial knowledge of their status and subsequently performed predetermined acclimatization strategies. Taking into account that the assessment of acclimatization efficacy could help in optimizing replacement gilt introduction into the breeding herd, it seems these practices for *M. hyopneumoniae* are still poorly developed under European conditions (Garza-Moreno *et al*., 2017). Previously, other studies had already identified inappropriate gilt acclimatization (Moorkamp *et al*., 2009; Nathues *et al*., 2014) and frequent purchase of pigs (Maes *et al*., 2000; Meyns *et al*., 2011) as risk factors for *M. hyopneumoniae* detection in piglets at weaning, higher seroprevalence or increased prevalence of pneumonia in fattening pigs.

Another major risk for sow seropositivity was the lack of an acclimatization period for replacement boars (odds ratio (OR) 2.10) (groβe Beilage *et al*., 2009). Boars might be more important *M. hyopneumoniae* carriers and transfer of *M. hyopneumoniae* within a farm than sows (He *et al*., 2011), since a higher seroprevalence of *M. hyopneumoniae* was detected in boars (68.8%) as compared to sows (54.5%).

A study focusing at intra-farm risk factors for *M. hyopneumoniae* colonization at weaning demonstrated that the proportion of *M. hyopneumoniae*-positive piglets was correlated with the proportion of positive dams in the weaning group (Pieters *et al*., 2014), clearly demonstrating the influence of the sow in the sow-to-piglet colonization process.

A German prevalence study identified herd-specific risk factors for *M. hyopneumoniae* infections at weaning age, such as high number of purchased gilts ($n > 120$) per year, number of farrowing pens per compartment higher than 16 and week-interval between farrowing batches (Nathues *et al*., 2013a). Other risk factors were associated with contact between animals of different ages during restocking of the compartment, whereas an increased number of weaned piglets per sow per year was less likely to test positive for *M. hyopneumoniae* (Nathues *et al*., 2014).

Farm structure and organization, such as the number of production sites and separation between age categories, can also have a significant impact on the prevalence and detection of *M. hyopneumoniae*. According to Moorkamp *et al*. (2009), detection of *M. hyopneumoniae* in young piglets was mainly associated with one- and two-site production systems. On the other hand, Giacomini *et al*. (2016) demonstrated that in one-site farms, *M. hyopneumoniae* infection showed earlier, whereas in two- and three-site farms, the infection occurred later in life but the infection

spread happened faster, suggesting that contact between animals of different age favours the *M. hyopneumoniae* transmission. Moreover, the infection rate was higher in older animals and *M. hyopneumoniae* prevalence was higher in one- and two-site production systems as compared to three-site systems (Giacomini *et al.*, 2016).

A cross-sectional study in 125 farrow-to-finish farms in France revealed that pneumonia (69.3%) and pleuritis (15%) were the most frequent lesions (Fablet *et al.*, 2012a). *Mycoplasma hyopneumoniae*, PRRSV and SIV H1N1 were the major respiratory pathogens involved in pneumonia-like lesions, even though PCV-2 could also play a role. Respiratory disease could be significantly reduced by implementing specific measures including appropriate management practices to control these pathogens (Fablet *et al.*, 2012b). A strategic plan to control *M. hyopneumoniae* infection in herds should include an evaluation of sow herd management, since the risk for a sow to be *M. hyopneumoniae*-seropositive was increased in herds with two- and three-site production (OR 1.50), when piglets were not vaccinated against *M. hyopneumoniae* (OR 1.81), in herds with a 2-week batch management system (BMS; OR 1.84) and in herds without AI/AO management in the farrowing rooms (OR 1.37) (groβe Beilage *et al.*, 2009).

BMS have been developed to reduce the infection transmission from one batch to the next through an increased time interval (Mekerke and Leneveu, 2006). Based on these assumptions, it is clear that pigs produced in a continuous production system showed 99% *M. hyopneumoniae*-seropositive at slaughter, whereas pigs produced under age-segregated conditions only showed 30% seropositivity (Holmgren *et al.*, 1999). Other management factors such as AI/AO have been demonstrated favorable in reducing lung lesions and improving growth (Diekman *et al.*, 1999). Another study confirmed that EP-like lesions were affected by type of farm ventilation, presence of respiratory symptoms during the fattening period and the level of *M. hyopneumoniae* and SIV H1N2 herd prevalence (Fraile *et al.*, 2010).

Segregated weaning systems have been promoted to decrease the transmission of several respiratory pathogens among different age categories within the same herd. A study on serological patterns of several respiratory pathogens (*A. pleuropneumoniae*, *M. hyopneumoniae*, *P. multocida* and *S. suis*) confirmed that seroconversion for *M. hyopneumoniae* only occurred during the late rearing period or even not at all in segregated rearing systems. This demonstrated potential to prevent or delay infections with *M. hyopneumoniae* through specific on-farm management measures in order to reduce or eliminate transmission between different age categories or animal

groups (Wallgren *et al*., 2016). On the contrary, in farrow-to-finish farms, sero-epidemiology demonstrated that the critical period for transmission of *M. hyopneumoniae* is around the beginning of the finishing period, when pigs have low antibody concentrations (Leon *et al*., 2001).

Other farm management practices such as pig density and piglet vaccination strategies have also been demonstrated of major importance on *M. hyopneumoniae* seroprevalence and presence of macroscopic and microscopic lung lesions (Maes *et al*., 2001b; Meyns *et al*., 2011). A cross-sectional study of risk factors associated with pulmonary lesions in pigs at slaughter in Belgium revealed that seroprevalence of *M. hyopneumoniae* was positively associated with the number of pigs per pen (Meyns *et al*., 2011). A cross-sectional epidemiological study in 150 Belgian farrow-to-finish herds on non-infectious factors revealed that high frequency of purchasing gilts and slaughter in winter months (January-February) were related to increased prevalence and severity of pneumonia at slaughter (Maes *et al*., 2001b). Moreover, pigs raised in pens with slatted floors were at higher risk of more severe microscopic lesions, *i.e.* lymphohistiocytic infiltration. The impact of vaccination against *M. hyopneumoniae* will be discussed later (chapter 10.2. *M. hyopneumoniae* vaccination).

8. *M. HYOPNEUMONIAE* – DIAGNOSIS

8.1. Diagnosis

8.1.1. Non-specific diagnosis

8.1.1.1. Clinical symptoms

Infections with *M. hyopneumoniae* may occur in two disease kinetics, namely epidemic and endemic. Most *M. hyopneumoniae* infections have a subclinical course without major clinical symptoms such as coughing. Depending on the initial health status of the farm towards *M. hyopneumoniae*, the disease is first introduced into a previously *M. hyopneumoniae*-negative population which is called epidemic – although this is not necessarily associated with major clinical symptoms –, or the disease exists already for a longer period on the farm and remains circulating among different age categories within the farm which is called endemic.

An epidemic outbreak of *M. hyopneumoniae* is rather uncommon and occurs when an initially naive herd is infected. The outbreak may be characterized by a 100% morbidity and

coughing, acute respiratory distress, pyrexia and eventually death may occur (Thacker and Minion, 2012). Typically, the infection transitions into an endemic pattern over a period of 2-5 months.

Under endemic conditions, pigs suffer an intermittent dry non-productive cough with a variable intensity for weeks to months (Sibila *et al*., 2009). The onset of clinical signs is insidious, initially affecting a few animals and slowly spreading throughout the compartment or age category. In most cases, one typical age group is affected and coughing remains for at least 3 weeks (Fano *et al*., 2005a), although in some cases it remains for the entire finishing period. However, if multiple age categories suffer the same symptoms, other PRDC pathogens may be involved in the complex, such as PRRSV, PCV-2 or SIV (Nathues *et al*., 2012). Environmental conditions such as poor air quality due to particulate matter or ammonia (Michiels *et al*., 2015) may aggravate clinical signs (Maes *et al*., 1996).

8.1.1.2.Impact on performance

Effect of *M. hyopneumoniae* infection on pig performance and productivity has frequently been evaluated, though the conclusions are not always straight forward due to differences in study set-up, especially related to experimental or practical conditions and several interfering environmental factors.

It has been shown that coughing was not a good indicator for severity of pneumonia. Moreover, increasing severity of pneumonia was negatively correlated with performance during the finishing period (Straw *et al*., 1990). In a study exposing *M. hyopneumoniae*-infected pigs to different environmental constraints, a reduced weight gain could be observed during the early coughing period, although overall weight gain at slaughter was not affected (Clark *et al*., 1993). In contrast, Holmgren *et al*. (1999) observed that pigs suffering acute enzootic pneumonia due to *M. hyopneumoniae* at slaughter and pigs that seroconverted to *M. hyopneumoniae* during the late rearing period showed a lower growth rate as compared to pigs with a more chronic form of enzootic pneumonia at slaughter or pigs that seroconverted to *M. hyopneumoniae* during early rearing or not at all. This is in accordance with Martínez *et al*. (2009) who demonstrated that high *M. hyopneumoniae* seroprevalences were negatively associated with FCR.

Non-complicated *M. hyopneumoniae* infections acquired late during fattening were associated with a reduced weight gain, which was estimated at least 60 g/day (Rautiainen *et al*.,

2000). In contrast, in chronically infected swine herds no effect on average daily gain could be demonstrated (Andreasen *et al.*, 2001), which may be due to the subclinical character of the infection or to the increased compensatory growth in the period following the infection. A subsequent meta-analysis comparing the effect of *M. hyopneumoniae* vaccines on daily weight gain concluded that vaccine type, vaccination schedule nor housing system were significantly associated with average daily weight gain (Jensen *et al.*, 2002). There was however a significant increase in daily weight gain of 20-22 g between *M. hyopneumoniae*-vaccinated and non-vaccinated pigs (Jensen *et al.*, 2002), indicating that vaccination might have a positive contribution on the pig performance. More recently, early vaccination against *M. hyopneumoniae* at 1 week of age demonstrated improved daily weight gain and slaughter weight, reduced load of *M. hyopneumoniae* in nasal swabs and reduced clinical signs as compared to vaccination around weaning (3 weeks of age) when subsequently challenged at 6 weeks of age (Kim *et al.*, 2011). Similar results were obtained by Wilson *et al.* (2012), concluding that early vaccination against *M. hyopneumoniae* resulted in reduced weight losses during infection and improved average daily weight gain.

8.1.1.3.Lung lesions

8.1.1.3.1. Macroscopic lung lesions

This basic structure of the lung is frequently used in lung scoring systems (Figure 3), assessing the extent of lung lesions due to the interaction with several respiratory pathogens (Garcia-Morante *et al.*, 2016).

Over the past five decades, several different lung scoring systems have been developed. They are mainly based on a two-dimensional approach (Goodwin *et al.*, 1969; Hannan *et al.*, 1982; Madec and Kobisch, 1982; Straw *et al.*, 1986; Sibila *et al.*, 2014), although some use a three-dimensional method including lung lobe weight in their assessment (Morrison *et al.*, 1985; Christensen *et al.*, 1999; Ph. Eur, 2013). An example of a two-dimensional lung scoring system frequently used under experimental conditions (Hannan *et al.*, 1982) is given in Figure 3.

Other lung scoring systems are more suitable under field conditions and have wide spread use to assess lung lesions at slaughter to evaluate the impact of certain preventive on-farm vaccination programs (Figure 4) (Goodwin *et al.*, 1969; Straw *et al.*, 1986).

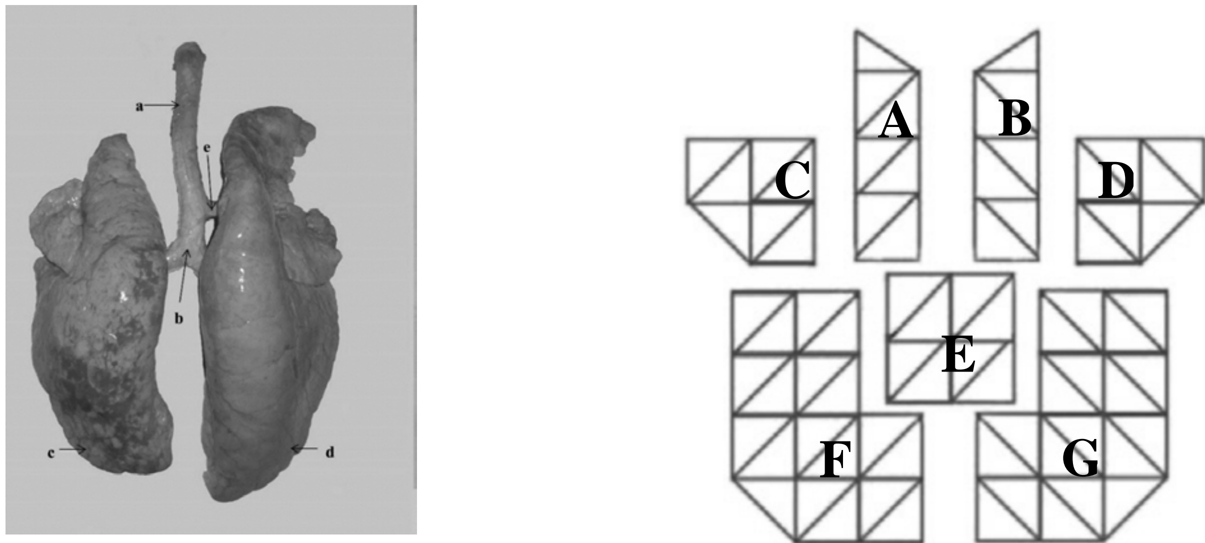

Figure 3. Schematic diagram of the lung lobes (Hannan *et al*., 1982; Garcia-Morante *et al*., 2016) used for lung scoring. (A) left cranial lobe, (B) right cranial lobe, (C) left middle lobe, (D) right middle lobe, (E) accessory lobe, (F) left caudal lobe and (F) right caudal lobe.

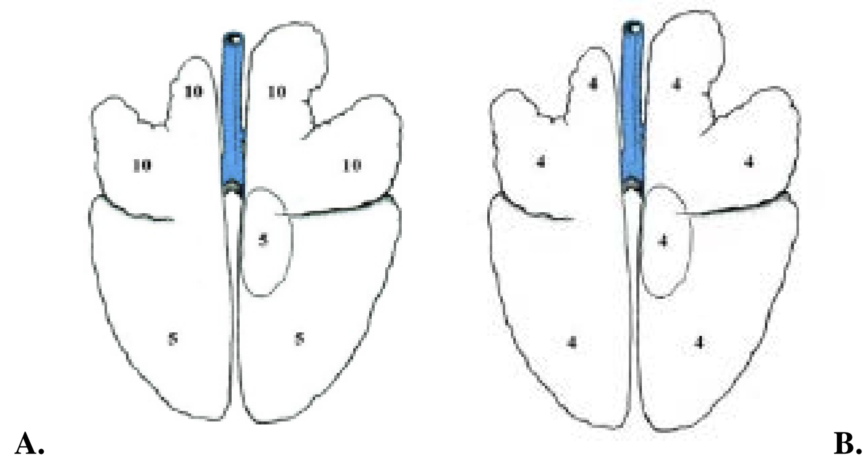

Figure 4. Example of two lung lesion scoring systems applied under field conditions to evaluate the impact of preventive on-farm vaccination programs. **A.** Two-dimensional lung lesions score by Goodwin *et al*. (1969) with quantification of the affected area from 0-10 or 0-5 depending on the lobe and summed to a maximum score of 55. **B.** Two-dimensional lung lesion score by Straw *et al*. (1986) with quantification of the affected area by means of 4 points from 0 (no lesions) over 1 (< 25% affected), 2 (25-49% affected), 3 (50-74% affected) and finally 4 (≥ 75% affected) and summed to a maximum score of 28.

Gross lesions due to *M. hyopneumoniae* infection typically consist of purple to grey rubbery areas of pulmonary consolidation, mainly located bilaterally in the apical, intermediate, accessory and cranial parts of the diaphragmatic lobes (Maes *et al*., 2008) which are frequently named cranioventral pulmonary consolidation (Garcia-Morante *et al*., 2016). The affected lungs have an increased firmness, a failure to collapse and marked edema. In uncomplicated cases, the lesions affect a small portion of the lungs and on the cut surface, the parenchyma is relatively uniform in color and catarrhal exudate is present in the airways (Thacker and Minion, 2012). In contrast, lungs affected by other respiratory pathogens have larger areas affected, are more firm and heavy, and have on cut surface gray-to-white exudate-distended alveoli. Moreover, mucopurulent exudate is present in the airways (Thacker and Minion, 2012).

The assessment of respiratory disease within a pig herd by lung lesion scoring at necropsy or abattoir inspection is frequently used to estimate the incidence of enzootic pneumonia due to *M. hyopneumoniae*. To provide a reliable measure of prevalence and severity of pneumonia at herd level, the lungs of at least 30 animals should be examined (Davies *et al*., 1995). Prevalence of lung lesions was higher the later the pigs seroconverted (Rautiainen *et al*., 2000), however the extension of the lung lesions tended to be higher among pigs that seroconverted earlier during the rearing period. A comprehensive review on the differences between lung lesions scoring systems for the assessment of *M. hyopneumoniae* has recently been published (Garcia-Morante *et al*., 2016).

8.1.1.3.2. Microscopic lung lesions

Since both clinical signs and macroscopic lesions are suggestive though not conclusive (Register *et al*., 2012; Kureljušić *et al*., 2016), further confirmation of *M. hyopneumoniae* infection can be made by microscopic lesions. Microscopic lesions are, however, not pathognomonic either and can be subacute to chronic depending on the stage of infection the pigs were in at the moment of slaughter or death. At microscopic level, *M. hyopneumoniae* produces a well-differentiated broncho-interstitial pneumonia including a peri-vascular and peri-bronchiolar lymphoplasmacytic hyperplasia, pneumocyte type II hyperplasia and edema fluids in the alveolar spaces with neutrophils, macrophages and plasmacells (Blanchard *et al*., 1992). In more chronic lesions, lymphocytic cuffs are more prominent and contain lymphoid nodules (Sibila *et al*., 2007b) with

an increased number of Goblet cells and hyperplasia of the submucosal glands of the bronchi (Thacker and Minion, 2012).

8.1.2. Specific diagnosis

8.1.2.1. Host-response to the pathogen: serology

Serological tests are commonly used to monitor the health status of pig herds. Detection of antibodies to *M. hyopneumoniae* is currently mainly performed by ELISA. In the past, other tests such as the complement fixation test (CFT) were used (Sibila *et al*., 2009). ELISA is a rapid, inexpensive and easily automated method providing useful information on the presence/absence of maternally derived or acquired antibodies, as well as on the time required for animals to seroconvert (Sibila *et al*., 2009).

Different types of ELISA tests are used under field conditions to detect *M. hyopneumoniae* antibodies (Table 4). Besides the blocking ELISA, there are several indirect ELISA tests available on the market.

Table 4. Different types of ELISA tests for detection of *M. hyopneumoniae* antibodies under field conditions.

Type of ELISA test	Commercial name	Company
Blocking ELISA	*M. hyopneumoniae* ELISA Thermo-Fisher	Oxoid ELISA
Indirect ELISA	Idexx *M. hyo* Ab test	Idexx Laboratories
	ID Screen® *M. hyopneumoniae* Indirect ELISA	ID.Vet
	BioChek *M. hyopneumoniae* ELISA	BioChek
	CIVTEST® SUIS *M. hyo*	Hipra Laboratories
	Tween-20 ELISA	-

Alternatively, surface plasmon resonance (SPR) systems could be used in serodiagnosis of *M. hyopneumoniae*. SPR-based systems are sensitive to changes in the thickness or refractive index of biomaterials at the interface between a thin gold film and an ambient medium. A recombinant 30 kDa fragment of the P97 adhesin, a protein chip based on SPR, demonstrated to be a highly specific and sensitive label-free method. Moreover, comparison with conventional ELISA

revealed a strong positive correlation (r = 0.898) between both detection techniques (Kim *et al*., 2006).

8.1.2.1.1. Serological tests on blood

Comparison of three serum antibody ELISAs for *M. hyopneumoniae* reported low sensitivity for all ELISA tests, especially in vaccinated animals and animals less than 21 days post-infection (Erlandson *et al*., 2005). Assays were inefficient at detecting serum antibodies in the early stage of *M. hyopneumoniae* infection and therefore, obtained results should be interpreted with care. Erlandson *et al*. (2005) suggested that a combination of tests to increase sensitivity may be valuable for the diagnosis of *M. hyopneumoniae* infection. This is in agreement with Levonen *et al*. (1999), who concluded that two commercial ELISAs, a direct ELISA with Tween-20 solubilized crude antigen and a competitive or blocking ELISA with monoclonal antibody reagents, were complementary in confirming potential doubtful reactions occurring in *M. hyopneumoniae* serology.

These observations were confirmed by Ameri-Mahabadi *et al*. (2005), comparing different ELISA kits on their performance depending on the day post-infection and application in experimental and field conditions. Overall, agreements between evaluated ELISA tests varied between 80.3 and 96.7% (Ameri-Mahabadi *et al*., 2005). Further research demonstrated that discrepancies in ELISA test results for *M. hyopneumoniae* could subsequently be confirmed using a Western blot immunoassay using the top-4 antigen bands of *M. hyopneumoniae* (Ameri *et al*., 2006). Another recent study showed that all tested ELISAs detected serum antibodies to *M. hyopneumoniae* between 21 and 28 days post-challenge. However, in the early stage of detection (21 days post-challenge) only between 12 and 25% of the animals tested ELISA-positive, increasing to just 35 to 48% at 28 days post-challenge (Pieters *et al*., 2017).

Similar results were obtained by Sørensen *et al*. (1997) under field conditions, showing that the mean time to onset of coughing following a *M. hyopneumoniae* infection was 13 days, whereas the mean delay between onset of coughing and seroconversion as measured by ELISA was 9 days (Sørensen *et al*., 1997b). Therefore, interpretation of serological results is difficult because several weeks must usually pass for serological reactions to become apparent (Nathues *et al*., 2006). Following statistical analysis on the number of samples needed to detect *M.*

hyopneumoniae, a compromise between epidemiological necessity and financially acceptable sample size was set at 10 samples per age group for a reliable detection of *M. hyopneumoniae* using serology (Nathues *et al*., 2006).

However, utility of antibody profiling can be hindered by: variation in ELISA results depending on the test used (Ameri-Mahabadi *et al*., 2005; Erlandson *et al*, 2005); the inability of serology to differentiate natural infection from vaccination; lack of correlation between different measures of antibody titre; variations in the detection of antibodies to different strains of *M. hyopneumoniae* (Sibila *et al*., 2009) and significant variability in the time taken by animals to seroconvert (Sørensen *et al*., 1997b; Pieters *et al*., 2017).

The inability of serology to differentiate natural infection from vaccination remains indeed a challenging issue under field conditions. Recently, an ELISA based on epitope-specific peptides as solid phase antigen allowed classification of the field samples and an eventual detection of a flourishing *M. hyopneumoniae* infection even in vaccinated herds (Meens *et al*., 2010).

An ELISA, developed based on monoclonal antibodies against a 74 kDa epitope (Oxoid ELISA, previously DAKO ELISA) of *M. hyopneumoniae* and compared to IHA, revealed an earlier detection and less cross-reactions with *M. flocculare* than the IHA (Feld *et al*., 1992). Later on, an improved ELISA using a 43 kDa purified protein from the non-pathogenic *M. hyopneumoniae* strain J demonstrated significantly higher ELISA ratio's in infected pigs and an earlier seroconversion in naturally-infected and experimentally-infected pigs than an earlier described ELISA by Sheldrake and Romalis (1992) (Djordjevic *et al*., 1994). Alternatively, a purified recombinant P46 protein, an early and species-specific immunogenic protein of *M. hyopneumoniae* (Mori *et al*., 1988), encoding a putative lipoprotein used for arginine (Futo *et al*., 1995a), was used in an ELISA for the detection of antibodies against *M. hyopneumoniae* in swine serum from 2 weeks post-infection onwards (Futo *et al*., 1995b).

L-lactate dehydrogenase (LDH, P36), a predominant immunogenic protein (Stipkovits *et al*., 1991), can be used for a *M. hyopneumoniae* ELISA (Frey *et al*., 1994), due to lack of cross-reactivity with related LDH in other mycoplasma species (Haldimann *et al*., 1993). Using this assay, *M. hyopneumoniae* LDH antibodies evolved in two subsequent phases, an early weak anti-LDH response between 5-10 weeks post-infection when clinical signs and lung lesions occurred, followed by a second strong raise from the 12th week post-infection onwards when disease signs and the infectious agent disappeared (Frey *et al*., 1994). Using a more advanced immunoblot assay,

LDH (P36) and another 29kDA partial fragment of an ABC transporter adenosine triphosphate (ATP)-binding protein detected anti-*M. hyopneumoniae* antibodies in infected pigs (Subramaniam *et al*., 2000).

Using monoclonal antibodies and recombinant P46 antigen of *M. hyopneumoniae* expressed in *E. coli*, an ELISA was developed and evaluated (Okada *et al*., 2005) under field conditions. Results showed that up to 80% of all slaughter pigs were seropositive and gradual decrease in ELISA positive samples was observed in sows with increasing parity (Okada *et al*., 2005). Recombinant P36 protein in an indirect ELISA for the detection of serum antibodies of convalescent pigs did not show any correlation with clinical and pathological findings (Caron *et al*., 2000b). Another recombinant protein P65, expressed in *E. coli* and used to produce a specific monoclonal antibody in a blocking ELISA had similar specificity and sensitivity as a commercial ELISA (Idexx; Liu *et al*., 2016).

Seroconversion under natural conditions is slower than in experimental studies (Sibila *et al*., 2009). Nevertheless, Sørensen *et al*. (1997) reported seroconversion as early as 8 days post-infection in a proportion of pigs infected by aerosol, although the other animals required 5 additional weeks to seroconvert. In contrast, other studies (Sheldrake *et al*., 1990; Kobisch *et al*., 1993) only detected antibodies 2-4 weeks after intra-tracheal infection with *M. hyopneumoniae*.

The delay in seroconversion associated with *M. hyopneumoniae* infection may be due to the pathogenesis, especially the attachment of the pathogen to the ciliated epithelial cells of the lower respiratory tract, thereby not invading the host's tissues as other pathogens (*i.e.* PRRSV) tend to do (Sibila *et al*., 2009). Therefore, the interval between infection and antigen presentation to the host tends to be longer, thus implying the seroconversion delay. Recently, Pieters *et al*. (2017) indeed confirmed a major delay between infection and initially detectable seroconversion using different commercial ELISA tests.

8.1.2.1.2. Serological tests on other fluids: BALF and OF

Recently, research has emphasized on detection of local sIgA response against *M. hyopneumoniae* in BALF. Comparing local and systemic immune responses in pigs vaccinated against *M. hyopneumoniae*, significant differences were detected in local sIgA levels in BALF (Martelli *et al*., 2014). Another comparative monitoring of specific serum IgG and respiratory

mucosal sIgA responses against three *M. hyopneumoniae* antigens, P97R1, P46 and P36 during 133 days post-infection concluded that mucosal sIgA, as detected in BALF, developed rapidly but was less sustained, whereas systemic IgG response was delayed in onset but more persistent in time (Feng *et al*., 2014). Based on the kinetics of both local and systemic immune response following *M. hyopneumoniae* infection, the P97R1-based ELISA elicited a higher specificity as compared to a commercial *M. hyopneumoniae* ELISA, particularly during the early phase of infection (Feng *et al*., 2014).

Another innovation in serological *M. hyopneumoniae* detection recently introduced is pen-based oral fluid (OF), which even might be used for a broader surveillance of respiratory pathogens (Gomes Neto *et al*., 2014). In these pen-based OF, specific *M. hyopneumoniae* IgA response was detected at 15 days post-infection whereas serum IgG response was only detected at 46 days post-infection (Gomes Neto *et al*., 2014). Therefore, *M. hyopneumoniae* IgA detection through OF sampling might be a promising alternative for early serological detection of *M. hyopneumoniae* infection.

8.1.2.2.Pathogen or parts of the pathogen

8.1.2.2.1. Isolation

The traditional reference standard for the demonstration of *M. hyopneumoniae* in lung tissue is bacteriological culture. However, culture of *M. hyopneumoniae* is difficult and impractical due to the very slow growth (4-8 weeks) (Friis, 1975). Therefore, only a handful of specialized laboratories worldwide are able to work with this fastidious pathogen. A medium for isolation and growth of *M. hyopneumoniae* was first described by Friis (1975). This medium, still commonly used, grows *M. hyopneumoniae* slowly, which results in a low cell yield. In bacteriology, the number of viable bacteria is often estimated by determining the number of colony forming units (CFU) (Calus *et al*., 2010). Due to the fact that growing *M. hyopneumoniae* colonies is difficult and requires several weeks of incubation, the CFU technique strongly underestimates the number of living cells (Calus *et al*., 2010). Therefore, other non-agar based techniques such as the CCU (color changing units) and ATP assay were developed for the assessment of freshly grown *M. hyopneumoniae* cultures (Stemke & Robertson, 1990; Calus *et al*., 2010). A comparative study

between both techniques revealed that the ATP assay was faster, more accurate and more time, work and cost effective than the CCU assay (Calus *et al.*, 2010).

8.1.2.2.2. Detection of M. hyopneumoniae antigen

Immunohistochemistry (IHC) and immunofluorescence assay (IFA) can specifically detect *M. hyopneumoniae* in lung tissue sections or smears of respiratory tract mucus. One major drawback of these techniques is, however, that a diagnosis can only be performed post-mortem (Sibila *et al.*, 2009). Additionally, only a small sample of lung tissue is tested using these techniques, increasing the risk of a false-negative result if airway is not included in the sample (Cai *et al.*, 2007). However, tissue for IHC or IFA is not randomly selected from the lung, but chosen at the edge of a lesion typically containing both healthy tissue as well as lesion tissue, thereby increasing the chances of detecting *M. hyopneumoniae*. Monoclonal antibodies to specific *M. hyopneumoniae* external proteins, such as P45 and P65, can be used for detection of *M. hyopneumoniae* by an indirect IFA and indirect immunoperoxidase assay in respectively frozen and formalin-fixed paraffin-embedded lung tissue sections (Cheikh Saad Bouh *et al.*, 2003), indicating that proper tissue preparation and an adapted test to the specific tissue charactistics is a necessity. Another group developed species-specific monoclonal antibodies to P36 that permitted effective detection of *M. hyopneumoniae* in frozen lung tissue samples by indirect IFA (Caron *et al.*, 2000).

A comparative study between *M. hyopneumoniae* detection in tracheo-bronchiolar washings (TBL) through an IFA and PCR demonstrated a good correlation between both test methods. However, in some cases *M. hyopneumoniae* could only be detected using PCR, a more sensitive test method to detect the pathogen (Blanchard *et al.*, 1996). This observation was confirmed in another study demonstrating superiority of PCR above a fluorescent antibody test (Cai *et al.*, 2007), where most of the PCR-positive and fluorescent antibody-negative samples had lesions compatible with *M. hyopneumoniae* infection.

8.1.2.2.3. Molecular detection methods

8.1.2.2.3.1.*In situ* hybridization

Colonisation of the ciliated respiratory epithelial cells with *M. hyopneumoniae* is a pre-requisite for the development of mycoplasmal pneumonia (Kwon *et al.*, 2002; Chae, 2011). *In situ* hybridization (ISH) has been used to detect *M. hyopneumoniae* on the luminal surface of porcine bronchi and bronchioles between 7 and 28 days post-infection (Kwon and Chae, 1999; Kwon *et al.*, 2002). This technique uses a digoxigenin-labelled specific probe targeting a repetitive sequence of the *M. hyopneumoniae* genome. Alternatively, a fluorescent oligonucleotide probe targeting 16S ribosomal DNA has been used for more species-specific detection of *M. hyopneumoniae*, *M. hyosynoviae* or *M. hyorhinis* (Boye *et al.*, 2001). Moreover, the different ISH techniques have proven to be applicable under both experimental (Boye *et al.*, 2001; Kwon *et al.*, 2002) and field conditions (Kwon and Chae, 1999). Previously, Abiven *et al.* (1992) compared a specifically constructed DNA probe and the conventionally used IFA for *M. hyopneumoniae* detection in lung tissue and demonstrated only a 63% agreement between both tests. Others have applied ISH for the detection of *M. hyopneumoniae* DNA in formalin-fixed, paraffin-embedded lung tissue which allows identification of lesions and *M. hyopneumoniae* DNA in the same section (Ha *et al.*, 2005). Major disadvantage of the ISH technique is the requirement for post-mortem samples and the time-consuming procedure, which is prohibitive for many diagnostic laboratories and for rapid diagnosis under field conditions (Ha *et al.*, 2005; Sibila *et al.*, 2009).

8.1.2.2.3.2.Polymerase Chain Reaction

Several PCR techniques for *M. hyopneumoniae* DNA detection in different sample types have been described throughout the last three decades (Sibila *et al.*, 2009). A comprehensive overview is given in Table 5 (adapted from Sibila *et al.*, 2009).

PCR samples can originate both from live and dead pigs through necropsy or derived from slaughterhouse samplings. However, lung samples of PRDC-negative pigs may become contaminated during slaughter mainly by the slaughterhouse environment and scalding water (Marois *et al.*, 2008). Histological examinations revealed that during scalding, contaminated water could reach the trachea and lungs of the pigs. Therefore, checks conducted at slaughter for

respiratory disorders, including PCR, have to be carried out on deeper airway samples, since nasal cavities and tonsils are not suitable for this purpose (Marois *et al*., 2008).

Initial attempts to detect *M. hyopneumoniae* DNA by PCR could confirm *M. hyopneumoniae* infection with lack of cross-reactivity to other mycoplasma species (Harasawa *et al*., 1991). Subsequent detection of *M. hyopneumoniae* in lung biopsies of infected pigs was demonstrated using oligonucleotide probes complementary to the *M. hyopneumoniae* 16S rRNA (Johansson *et al*., 1992).

nPCR has been shown a valuable addition in the diagnosis of *M. hyopneumoniae* infection, especially when non-diagnostic microscopic lesions are observed (Calsamiglia *et al*., 2000). The nPCR has also been shown suitable for detection and confirmation of *M. hyopneumoniae* in different sample materials, such as nasal swabs (Calsamiglia *et al*., 1999), broncho-alveolar swabs, frozen tissue samples and formalin-fixed paraffin-embedded lung samples (Almeida *et al*., 2012). Previous comparison of PCR with ISH on formalin-fixed, paraffin-embedded tissue revealed a 100% agreement between both techniques (Ha *et al*., 2005). Two diagnostic PCR tests based on P46 membrane protein and P36 cytosolic protein showed a good sensitivity (86.6% and 100%, respectively) and specificity (96.7% and 93.3%, respectively) in detection of *M. hyopneumoniae* in tracheo-bronchial swab samples as compared to conventional necropsy findings (Caron *et al*., 2000a). Overall, PCR methods with direct detection of *M. hyopneumoniae* from lung tissue have been able to solve the problem of difficult cultivation of *M. hyopneumoniae* (Holko *et al*., 2004). Recently, Prokěs *et al*. (2012) combined ELISA on blood with nPCR on nasal swabs to confirm *M. hyopneumoniae* in conventional herds with clinical signs of enzootic pneumonia under Slovakian conditions.

Under specific field conditions, it is important to have a PCR test with both high sensitivity and high specificity, especially in farms negative to *M. hyopneumoniae*. Recent trends to combine PCR tests for improved sensitivity and specificity demonstrated a high specificity (100%) in sample material of *M. hyopneumoniae*-negative farms. Additionally, although neither of both tests had the best sensitivity, a combination of both tests detected all *M. hyopneumoniae*-positive farms (Dubosson *et al*., 2004). The same approach was performed by Strait *et al*. (2008), who developed two rt PCR assays, based on mhp165 and mhp183, that were specific and capable of detecting all of the *M. hyopneumoniae* isolates used in the study. Moreover, combination of both PCR tests as a multiplex PCR improved overall results (Strait *et al*., 2008).

Table 5. Summary of reported PCR-based techniques used to detect *M. hyopneumoniae* DNA in porcine respiratory samples (adapted from Sibila *et al*., 2009).

Reference	Type of PCR	Amplicon Gene	Amplicon Length (bp)	Threshold of detection	Clinical samples tested
Harasawa *et al*., 1991	S	Repeated unknown sequence	520	5 ng or 1000 CFU/ml	None
Artiushin *et al*., 1993	S	Unique hypothetical gene	456	1-10 pg of DNA	BALF, Lung tissue
Stemke *et al*., 1994	S	16 SrRNA	200	1000 genome	None
Mattsson *et al*., 1995	S	16 SrRNA	649	5 CFU	NS
Blanchard *et al*., 1996	S	Putative ABC transporter	1561	500 fg	TBL
Stärk *et al*., 1998	N	MHYP1-03-950 repetitive element	808	1 cell/filter	Filtered air sample
Baumeister *et al*., 1998	S	-	853	100 CFU/ml	BALF
Calsamiglia *et al*., 1999	N	16 SrRNA	352	80 cells	NS
Verdin *et al*., 2000a	N	Putative ABC transporter	706	1fg	TBL, NS
Caron *et al*., 2000	S	Intergenic sequence (P36)	948	50 pg	Lung tissue, TBL
	S	Intergenic sequence (P46)	580	0.5 ng	NS
	M	P36 and P46	948 and 580	-	-
Kurth *et al*., 2002	N	Unique hypothetical gene	240	0.5-1 fg	TBB, BALF
Dubusson *et al*., 2004	RT	MHYP1-03-950 repeated element	808	1 fg	BS
		I-414 fragment putative ABC transporter	706	1 fg	BS
Ha *et al*., 2005	N	-	184	-	Paraffin-embedded tissue
Stakenborg *et al*., 2006	M	16 SrRNA	1000	1 pg	None
Cai *et al*., 2007	S	16 SrRNA	649	0.18 CFU/g	Lung tissue
Strait *et al*., 2008	RT	Mhp165	132	5 fg/µl – 10 ng/µl	NS, BS, BALF
	RT	Mhp183	90	5 fg/µl – 10 ng/µl	NS, BS, BALF
	M	Mhp165 and Mhp183	132 and 90	2.5 fg/µl-10 ng/µl	NS, BS, BALF
Yamaguti *et al*., 2008	N	16 SrRNA	649	-	NS, TS
Marois *et al*., 2010	RT	P97 and P102	101 and 137	1.3 genome equivalents	NS, TS, TBS, lung tissue
	RT	P46	150	13 genome equivalents	NS, TS, TBS, lung tissue
Prokěs *et al*., 2012	N	16 SrRNA	660	-	NS
Almeida *et al*., 2012	N	16 SrRNA	649	-	BAS, frozen tissue, paraffin-embedded tissue

PCR technique: S, standard; N, nested; M, multiplex; RT, real-time

Clinical sample tested: BALF, broncho-alveolar lavage fluid; NS, nasal swab; TS, tonsil swab; TBL, tracheo-bronchiolar lavage; TBB, tracheo-bronchial brushes; TBS, tracheo-bronchial swabs; BS, bronchial swabs; BAS, broncho-alveolar swab

- , not given

Originally, PCR tests were predominantly performed on post-mortem material, such as lung tissue. However, extension to TBL demonstrated that *M. hyopneumoniae* could also be detected with a low detection limit (Verdin *et al*., 2000a). For further quantification of *M. hyopneumoniae* using nPCR on TBL, an internal control should be validated. The titer of *M. hyopneumoniae* in TBL ranged approximately from 10^4 to 10^6 cells per ml of clinical sample (Verdin *et al*., 2000b). Another group applied nPCR to determine the optimal sampling site for detection of *M. hyopneumoniae* in infected pigs. They included nasal, tonsillar and tracheo-bronchial swabs, lung tissue, BALF and tracheo-bronchial brush samples. Samples obtained from BALF and tracheo-bronchial sites were most predictive of infection, whereas nasal swabs and lung tissue were less reliable indicators. Therefore, enhanced sensitivity of the used nPCR was concluded unnessary if the proper sampling sites are used (Kurth *et al*., 2002). In contrast, a nPCR, optimized for routine diagnostic monitoring of *M. hyopneumoniae* infection in swine herds, demonstrated nasal or tonsil swabs were suitable samples for *M. hyopneumoniae* detection (Yamaguti *et al*., 2008). Another study confirmed that detection of *M. hyopneumoniae* DNA in lung by qPCR, in conjunction with histopathology, was valuable as an early detection test for *M. hyopneumoniae* infection (Woolley *et al*., 2012).

Recently, comparison of a rapid sensitive loop-mediated isothermal amplification (LAMP) assay based on the P36 gene with a real-time PCR (rtPCR) on clinical samples demonstrated 100% concordance between the two assays (Liu *et al*., 2015). Moreover, the LAMP assay provided a rapid reaction and was rather inexpensive to perform, since there is no need for complex instruments or PCR systems (Li *et al*., 2013; Liu *et al*., 2015).

8.1.2.2.3.3.Molecular typing techniques

Variation in the clinical course of enzootic pneumonia (Vicca *et al*., 2002) and inconsistencies in the efficacy of vaccination have raised suspicion that *M. hyopneumoniae* isolates vary in virulence. Characterization of field isolates of *M. hyopneumoniae* by SDS-PAGE and immunoblot revealed protein and antigenic variability in different antigenic proteins such as P36, P46 and P97 (Assunção *et al*., 2005a). Other research confirmed that proteomic variability within *M. hyopneumoniae* is high. Moreover, minor differences observed between *M. hyopneumoniae* isolates from the same herd might reflect the organism's ability to alter its proteomic expression

profile under field conditions (Calus *et al*., 2007). Based on protein variability determined by SDS-PAGE, no clear evidence that more than one *M. hyopneumoniae* isolate circulated within a herd at a specific time point was found (Calus *et al*., 2007).

Previously, Frey *et al*. (1992) demonstrated genetic diversity within *M. hyopneumoniae* and Artiushin and Minion (1996) subsequently showed that *M. hyopneumoniae* strains can be divided in at least six epidemiological sub-groups based on arbitrarily primed PCR analysis. Intra-specific variability within *M. hyopneumoniae* may be demonstrated through use of restriction fragment length polymorphism (RFLP) of a highly variable gene encoding P146 (Stakenborg *et al*., 2006). Other techniques such as amplification fragment length polymorphism (AFLP) and pulsed-field gel electrophoresis (PFGE) are highly reliable and discriminatory typing techniques to outline genomic diversity of *M. hyopneumoniae* isolates (Stakenborg *et al*., 2005; Stakenborg *et al*., 2006). Indeed, *M. hyopneumoniae* isolates within a herd showed closer relationship compared to isolates from different herds (Stakenborg *et al*., 2005). Phase switching or antigenic variation in *M. hyopneumoniae* is limited due to the presence of only a few genes with tandem repeat sequences (Minion *et al*., 2004). Another array-based genomic comparative hybridization analysis revealed genetic variation across different loci in all analysed field strains, suggesting that variation occurred throughout the genome (Madsen *et al*., 2007). This observation was confirmed using MLST on a wide variety of samples from different farms and geographic regions. Farms recognized to be affected by enzootic pneumonia were always associated with one single type of farm-specific *M. hyopneumoniae* clone, which differed in most cases from farm to farm (Mayor *et al*., 2007; Mayor *et al*., 2008). However, farms in close geographic or operational contact showed identical clones (Mayor *et al*., 2008).

Clonal diversity in *M. hyopneumoniae* strains present in one single pig was first demonstrated in a Belgian study using VNTR. Whereas in one of the herd, one *M. hyopneumoniae* strain with limited clonality persisted, in another herd, there was evidence of simultaneous infection with two strains in half of the sampled pigs (Vranckx *et al*., 2011). A longitudinal study within a herd demonstrated that one distinct strain – persisting for at least 12 weeks in the same animals – was detected, although clonal variants could also be identified in two herds (Vranckx *et al*., 2012a). However, a more recent study using RAPD and VNTR analyses demonstrated that more than one strain of *M. hyopneumoniae* might be present in a pig herd and even in a single pig, suggesting high heterogeneity between isolates of the same epidemiological source (Nathues *et*

al., 2011). A recent study in France using multiple-locus variable number tandem repeats analysis (MLVA) and PCR-RFLP clustering of *M. hyopneumoniae* revealed that analysed strains from abattoir pigs were distributed among three to five clusters. This distribution was regardless of severity of lesions, indicating that clusters were not specifically associated with virulence. Moreover, the analyses showed a high diversity among field isolates of *M. hyopneumoniae* with a greater homogeneity within the same herd (Charlebois *et al*., 2014) or production system (Fano *et al*., 2018).

From an epidemiological perspective, *M. hyopneumoniae* typing would facilitate the understanding of transmission of *M. hyopneumoniae* isolates within and between herds (Sibila *et al*., 2009). Recently, a simplified MLVA method, based on the number of tandem repeats of two *M. hyopneumoniae* adhesins, P97 and P146, proved to be an efficient tool for typing *M. hyopneumoniae* with a high degree of stability, repeatability and discriminatory power (Dos Santos *et al*., 2015). Analysis of genetic variability of strains circulating in the United States of America (USA), Brazil, Mexico and Spain revealed that locus P97 had 17 different types with 2-18 repeats, whereas P146 showed a higher heterogeneity with 34 different types ranging from 7 to 48 repeats (Dos Santos *et al*., 2015). Previously, P146 had been used in a nPCR to evaluate the effect of an eradication program under Argentinian field circumstances (Tamiozzo *et al*., 2013). Recently, Takeuti *et al*. (2017b) demonstrated that circulation of *M. hyopneumoniae* in self-replacement gilts varied among farms, even under similar production and management conditions. In addition, the molecular variability of *M. hyopneumoniae* detected within farms suggests that in cases of minimal replacement gilt introduction bacterial diversity may be farm-specific (Takeuti *et al*., 2017b). However, the exact implications of this variability and diversity are not fully understood. A recent study on impact of *M. hyopneumoniae* strain diversity on lung lesions in slaughter pigs clearly demonstrated that batches of slaugher pigs with different *M. hyopneumoniae* strains had a higher prevalence and severity of mycoplasma-like lung lesions at slaughter (Michiels *et al*., 2017).

However, all molecular techniques used for epidemiological investigation of the spread and persistence of *M. hyopneumoniae* strains and clonal variants within and between farms also have some limitations. These techniques do not only have a low resolution, they are also very time-consuming and therefore limited in their practical use for field investigations. Another main issue of these techniques is the high inter-laboratory variation, limiting the possibilities to compare

results between different laboratories and specific strain tracking between different farms within a swine operation or on a regional basis.

8.2.Further diagnostic considerations

8.2.1. Diagnosis of clinical disease or monitoring: selection of representative animals

As part of disease investigation or health monitoring, several questions need to be addressed before sample collection. It all starts with the selection of representative animals to solve the specific purpose of sampling. In case of a disease investigation, representative animals are pigs that suffer from the typical clinical symptoms mentioned in the anamnesis of the pig farmer, such as coughing in case of *M. hyopneumoniae*. Health monitoring, in contrast, would aim to sample a larger number of animals from all representative age categories present on the specific farm location in order to determine if the pathogen is present and if yes, in which age category or categories.

Collection of too few animals is the most common mistake. However, the additional cost of submission of more samples must be weighed against the economic cost of the disease and the importance of establishing a correct diagnosis (Gardner, 2012). Nowadays, sample calculation tools (*e.g.* http://epitools.ausvet.com.au) are available to support the decision on the number of samples to be collected for a specific purpose. Basic imput variables needed for the calculation are: total number of animals on the farm or age group involved, disease prevalence estimate, accuracy of the outcome requested, sensitivity and specificity of the test method used. Unfortunately, these tools do not take into account the economic cost of disease, which still remains to be discussed and calculated by the farm owner and his field veterinarian.

8.2.2. Appropriate sampling strategies

Diving into more detail in the appropriate sampling strategies reveals three major approaches in sampling a specific population, namely serial sampling, cross-sectional sampling and targeted sampling. All sampling strategies have a specific protocol and both their advantages and disadvantages.

8.2.2.1.Serial sampling

Serial sampling is the collection of samples from the same animals at pre-defined time points, *e.g.* blood sampling every 4 weeks from weaning until slaughter. Serial sampling is the preferential approach to establish knowledge on the incidence or kinetics of *M. hyopneumoniae* infection within different age categories in a herd. Under optimal conditions, animals that have been sampled are marked with an additional ear tag in order to identify them for the next sampling. Serial sampling is predominantly used for serological monitoring, although direct detection of *M. hyopneumoniae* in the deeper airways could also be an option under these conditions.

Advantage of serial sampling is the collection of data from the same animal over time, which allows to establish good data on infection kinetics. Sample analysis could be postponed and grouped until all samples have been collected, although in some cases immediate analysis is preferred in order to guarantee optimal sample condition upon analysis. Disadvantages of serial sampling could be the loss of ear tags or death of some of the marked animals during the course of the follow-up period or difficulties at sample collection in the slaughterhouse. Therefore, when serial sampling is the best option to gather the required on-farm infection kinetics, an abundant number of animals should be included to overcome these issues.

8.2.2.2.Cross-sectional sampling

Cross-sectional sampling is the collection of samples from several animals in different age categories at the same time point, *e.g.* blood or TBS sampling from pigs at 4-8-12-16-20 and 24 weeks of age on the same day. Cross-sectional sampling is used for detection of a difference in prevalence of *M. hyopneumoniae* between groups of piglets, which might be age groups or pigs housed under different environmental or housing conditions (Gardner, 2012). All samples from the different groups are collected at the same time point and analysed under the same conditions.

Advantage of cross-sectional sampling is that all information on *M. hyopneumoniae* infection status or serological response is collected during only one intervention. Indeed, no supplementary ear tags need to be used nor extra animals included in the sampling to compensate for losses over time. Moreover, a good picture of the actual *M. hyopneumoniae* prevalence in the different age categories is gained upon one sampling occasion. Disadvantage is the absence of

kinetic data from the same animals (no incidence information). Differences in *M. hyopneumoniae* infection status between batches might occur (Fano *et al*., 2007), which makes cross-sectional data slightly more complicated to interpret. Other researchers (Busch *et al*., 2013) also reported the effect of AI/AO management by site and a major batch effect on the infection with both *M. hyopneumoniae* and *A. pleuropneumoniae*.

8.2.2.3.Targeted sampling

If the veterinarian's goal is to detect infection, sampling does not need to be random but can be directed towards a higher prevalence group or a group with typical clinical symptoms, *e.g.* coughing, associated with *M. hyopneumoniae* (Gardner, 2012). A benefit of targeted sampling is that presence of the pathogen can often be established with fewer samples, thus limiting the analysis costs to obtain a correct and reliable diagnosis. However, in situations where the *M. hyopneumoniae* infection is subclinical and the prevalence is lower, a sample size of 30 pigs will give 95% confidence of detecting at least one positive pig in the samples if the prevalence of infection is at least 10% and the test is perfectly sensitive (Gardner, 2012). In other cases, a sample calculation tool (http://epitools.ausvet.com.au) should be applied with specific assumptions related to sampling in order to determine the optimal sample number for the specific purpose.

8.3.Type of samples

Different sampling techniques and strategies have been developed throughout the years to sample pigs in a rapid, easy and reliable manner to collect samples for subsequent *M. hyopneumoniae* detection, mostly through PCR, although in some studies (Marois *et al*., 2007) bacteriological culture has also been used. A comprehensive summary of different sampling techniques in live pigs is given in Table 6. The initial technique to sample live pigs for *M. hyopneumoniae* detection through nasal swabs was considered the least invasive approach. However, early observations elicited that *M. hyopneumoniae* could only be detected in nasal swabs from naturally infected pigs by PCR analysis during a limited period of time (Mattsson *et al*., 1995). Further studies on *M. hyopneumoniae* epidemiology in sows and their respective piglets was carried out using nasal swabs, resulting in acceptable detection levels of *M. hyopneumoniae*

(Calsamiglia and Pijoan, 2000). This resulted in more studies on *M. hyopneumoniae* colonization and prevalence in piglets during the peri-weaning period using nasal swabs (Ruiz *et al*., 2003; Sibila *et al*., 2007a; Fano *et al*., 2007; Villarreal *et al*., 2010; Nathues *et al*., 2013a).

Meanwhile, deeper sampling techniques, namely BALF, for collection of respiratory samples in live piglets had been validated (Baumeister *et al*., 1998), although further application of BALF in *M. hyopneumoniae* prevalence studies had to wait for a decade (Moorkamp *et al*., 2008; Moorkamp *et al*., 2009). Alternatively, Marois *et al*. (2007) compared 4 different sampling techniques for the recovery of *M. hyopneumoniae* in experimentally infected piglets and concluded that TBS and TBL were the most efficient samples as compared to nasal swabs and tonsillar or oropharyngeal swabs. However, caution should be taken when using TBL, since these could have an influence on subsequent lung lesion development and evaluation (Marois *et al*., 2007). Further work on these techniques was performed by Fablet *et al*. (2010) who analysed the collected samples by PCR and concluded that TBS were 3.5 times more sensitive in detecting *M. hyopneumoniae* from infected animals as compared to nasal swabs. Moreover, significantly higher amounts of *M. hyopneumoniae* DNA were recovered at the sites of TBS than in nasal cavities or at the oral-pharyngeal site (Fablet *et al*., 2010).

Recently, Pieters *et al*. (2017) compared nasal swabs, laryngeal swabs (LS), TBL and OF for early detection of *M. hyopneumoniae* in challenged piglets. The highest sensitivity of DNA detection was present in LS, whereas OF had the lowest sensitivity. Therefore, under the conditions of this study LS tested by PCR proved to be a practical and reliable diagnostic sample for *M. hyopneumoniae* detection *in vivo* during the early stage of infection. Others have, however, demonstrated that TBS had a much higher detection rate of *M. hyopneumoniae*, especially under field conditions with natural infection of gilts (Johnson *et al*., 2018). Moreover, comparing nasal swabs and TBS from an animal welfare point of view, both sampling techniques caused a comparable endocrine stress response with the advantage of a higher yield of *M. hyopneumoniae* using TBS (Weiβ *et al*., 2018).

More recently, besides these invasive sampling techniques to collect samples for *M. hyopneumoniae* detection in live pigs, another more convenient sampling method to assess infection status for respiratory pathogens in a group of live pigs has been developed and validated, namely OF. Initial reports evaluated the OF method for SIV (Goodell *et al*., 2013). Only recently, OF sampling was applied in the detection of *M. hyopneumoniae* (Cheong *et al*., 2017; Hernandez-

Garcia *et al*., 2017; Pieters *et al*., 2017). One of the major advantage of OF sampling is the broader scope of respiratory pathogens that can be evaluated in the same sample. Nevertheless, comparison of sample types and diagnostic methods for early *in vivo* detection of *M. hyopneumoniae* revealed that LS elicited the highest sensitivity with positive PCR results from 5 days onwards, whereas OF showed the lowest sensitivity (Pieters *et al*., 2017). In this respect, sampling material can also play a crucial role in the sensitivity of pathogen detection, since a recent study comparing *M. hyopneumoniae* detection in nylon-flocked and rayon-bud swabs demonstrated that absorption and detection of *M. hyopneumoniae* was significantly higher in nylon-flocked swabs, although Ct values were only slightly higher (Takeuti *et al*., 2017c).

Table 6. Sampling techniques in living pigs to collect samples for further *M. hyopneumoniae* identification using PCR.

Reference	**Sampling technique**					
	NS	**OPS / LS**	**TBS**	**TBL**	**BALF**	**OF**
Mattsson *et al.*, 1995	x					
Baumeister *et al.*, 1998					x	
Calsamiglia and Pijoan, 2000	x					
Ruiz *et al.*, 2003	x					
Sibila *et al.*, 2007a	x					
Fano *et al.*, 2007	x					
Marois *et al.*, 2007 *	x	x	x	x		
Moorkamp *et al.*, 2008					x	
Moorkamp *et al.*, 2009					x	
Fablet *et al.*, 2010	x	x	x	x		
Villarreal *et al.*, 2010	x					
Fablet *et al.*, 2012a			x			
Nathues *et al.*, 2013a	x					
Roos *et al.*, 2016		x				x
Cheong *et al.*, 2017						x
Pieters *et al.*, 2017	x	x		x		x
Hernandez-Garcia *et al.*, 2017						x

* Marois *et al.*, 2007 – analysis with bacteriological culture

Sampling technique: NS, nasal swab; OPS, oropharyngeal swab; LS, laryngeal swab; TBS, tracheo-bronchial swab; TBL, tracheo-bronchial lavage or washing; BALF, broncho-alveolar lavage fluid; OF, oral fluid; PCR, polymerase chain reaction

8.4.Test characteristics

8.4.1. Epidemiological test characteristics

Sensitivity and specificity are statistical measures of the test performance of a binary classification test within a specific population (Verloo *et al.*, 2005). Sensitivity (Se), the true positive rate, of a test measures the proportion of positives that are correctly identified as such, whereas the specificity (Sp), the true negative rate, of a test measures the proportion of negatives that are correctly identified as such. The availability of a golden standard – a perfect reference test – is crucial within this scenario (Table 7).

The positive and negative predictive values (PPV and NPV) are the proportions of positive and negative results in diagnostic tests that are true positive or true negative results. The PPV and NPV describe the performance of a diagnostic test, although they also depend on the pathogen prevalence.

Using the data by Pieters *et al.* (2017) on serological results following experimental *M. hyopneumoniae* inoculation, resulting in 100% *M. hyopneumoniae*-positive animals, we could conclude that currently available commercial ELISA tests for *M. hyopneumoniae* have a low Se at the onset of seroconversion, since only 25-40% of the *M. hyopneumoniae*-infected animals show detectable antibody titers at 21 to 28 days post-challenge.

Table 7. Contingency table with the four possible outcomes, namely true positive (TP), false positive (FP), false negative (FN) and true negative (TN) (expressed as counts).

	Golden standard – true condition	
	Disease	No disease
Test positive	TP	FP
Test negative	FN	TN

Se, Sp, PPV and NPV can be calculated as following from Table 7 (Verloo *et al.*, 2005).

Se = TP / TP + FN

Sp = TN / TN + FP

PPV = TP / TP + FP

NPV = TN / TN + FN

8.4.2. Lab-technical test characteristics: detection limit and analytical sensitivity

The detection limit is the lowest detectable level of analyte distinguishable from zero, whereas the analytical sensitivity is the slope of the calibration curve. Therefore, the analytical sensitivity indicates the capacity of the used analytical method to differentiate between two very close concentrations of the analyte, *i.e. M. hyopneumoniae.* Detection limit and analytical sensitivity are interrelated, since the higher the analytical sensitivity, the lower the detection limit (Lozano and Cantero, 1997). The limit or threshold of detection for several PCR methods has been given in Table 5 (p. 44). Depending on the type of PCR test used, the limit of detection is expressed as a number of *M. hyopneumoniae* cells or an amount of genetic material per unit of volume (Table 5).

9. *M. HYOPNEUMONIAE* – ANTIMICROBIAL TREATMENT OPTIONS

Several antimicrobial treatments are available for *M. hyopneumoniae*, although for some classes of antimicrobials *M. hyopneumoniae* has a natural resistance, due to lack of a cell wall. Therefore, penicillins and peptides have no activity on *M. hyopneumoniae,* whereas *M. hyopneumoniae* is susceptible to other classes such as sulfonamides and tetracyclins (Williams, 1978).

Due to specific treatment protocols using chlortetracyclin or other tetracyclins, an increase in the MIC values has been demonstrated, and therefore, periodic testing of pathogen sensitivity to different antimicrobials should be carried out to detect significant shifts in resistance patterns that might develop over time (Yamamoto *et al*., 1986; Inamoto *et al*., 1994).

To improve antimicrobial resistance testing, the Sensititre® broth microdilution technique was adapted to test antimicrobial susceptibility for *M. hyopneumoniae* (Tanner *et al*., 1993) by using room temperature stable antimicrobial plates. Using this technique, it was demonstrated that both tilmicosin and enrofloxacin had a low MIC in 93% of the tested isolated (Wu *et al*., 1997). Another study evaluating the *in vitro* susceptibility of recent field isolates of *M. hyopneumoniae* to valnemulin, tiamulin and enrofloxacin showed high sensitivity to all three antimicrobial agents (Hannan *et al*., 1997). *In vitro* susceptibility of 21 *M. hyopneumoniae* field isolates revealed one

isolate with acquired resistance to lincomycin, tilmicosin and tylosin, while five isolates were resistant to flumequine and enrofloxacin (Vicca *et al*., 2004).

Antimicrobial treatment does not always eliminate *M. hyopneumoniae* entirely from the body. Despite tissue penetration of marbofloxacin, particularly in the trachea and tracheal secretions, treatment with this antimicrobial did not have any influence on *M. hyopneumoniae* recovery from tracheal swabs. Moreover, a significant decrease in susceptibility level of marbofloxacin could be detected in the different *M. hyopneumoniae* clones re-isolated after antimicrobial treatment (Le Carrou *et al*., 2006).

Currently, in-feed antibiotics, such as macrolides, lincosamides and chlortetracycline are frequently used treatment options. In-feed tiamulin hydrogen fumarate has been shown to produce a clinical benefit in pigs experiencing PRDC or *M. hyopneumoniae* infection (Roberts *et al*., 2011). More recent antibiotic molecules, such as florfenicol, have been shown to decrease the clinical effects, including the extent of pneumonic lesions, of *M. hyopneumoniae* infection in swine (Ciprián *et al*., 2012). To reduce the use of antimicrobials, group treatments using in-feed or in-water medication should be omitted. Evaluation of florfenicol injection efficacy at the onset of clinical disease revealed numerical improvement of average daily weight gain, FCR and lung lesions scores following *M. hyopneumoniae* infection (del Pozo Sancristán *et al*., 2012).

10. *M. HYOPNEUMONIAE* – CONTROL MEASURES

10.1. Management and biosecurity

Biosecurity includes both external and internal biosecurity, and is defined as a series of measures implied on a farm to control or prevent entry and spread of pathogens into the farm and between different animal groups on a farm (Barceló and Marco, 1998; Laanen *et al*., 2010). Several subcategories have been defined within the external and internal biosecurity (Table 8) with their specific weight of importance (Laanen *et al*., 2010).

For *M. hyopneumoniae*, the most important external biosecurity risks are related to *M. hyopneumoniae*-infected incoming animals, riskful contacts with external transport vehicles, incoming visitors and the aerogenic spread from *M. hyopneumoniae*-infected neighboring farms (Fano *et al*., 2005b).

Table 8. Subcategories within external and internal biosecurity with their specific weighing factors for swine pathogens in general (Laanen *et al*., 2010).

External biosecurity	**Weighing factor**	**Internal biosecurity**	**Weighing factor**
Incoming animals/sperm	24	Disease management	10
Exit animals/manure/carcasses	23	Farrowing and suckling period	14
Feed/water/materials	15	Nursery period	14
Entrance control visitors	17	Fattening period	14
Rodent and bird control	11	Compartimentation, daily work organisation and material use	28
Location and environment	10	Cleaning and disinfection	20

In practice, besides the prevention of aerogenic spread of *M. hyopneumoniae*, biosecurity measures are mainly focused on prevention of new introduction through personnel and fomites, such as coveralls and boots. A 4-year research demonstrated that a 1-night downtime period is sufficient to prevent mechanical spread of both PRRSV and *M. hyopneumoniae* by personnel and fomites (Pitkin *et al*., 2011). The question, however, remains if this 1-night downtime would also help to prevent transmission of other PRDC pathogens such as SIV or PCV-2. It may be concluded from this study that basic sanitation procedures, such as hand hygiene and implementation of both boots and coveralls should be enough and therefore, the implementation of a shower protocol is not necessary (Pitkin *et al*., 2011).

Transmission of *M. hyopneumoniae* through transport vehicles can be related to the recent observations that *M. hyopneumoniae* can survive for several days (some strains up to 8 days) on dry surfaces (Browne *et al*., 2017), in particular also stainless steel. Therefore, thorough cleaning and disinfection, including a drying period, are required for transport vehicles to minimize the risk of pathogen transmission (Dee *et al*., 2004).

From an internal biosecurity point of view, the main risk for *M. hyopneumoniae* spread and circulation originates from (subclinically) infected animals that may continue excretion for up to 254 days following infection (Pieters *et al*., 2009). In some herds, nursery depopulation appeared to be an effective method to control *M. hyopneumoniae* infection. Especially in herds where seroconversion to *M. hyopneumoniae* was detected between 10 and 16 weeks of age, indicating the occurrence of a natural *M. hyopneumoniae* infection during the nursery (4-12 weeks of age), this measure showed to be quite effective. Nursery depopulation may have prevented spread of *M.*

hyopneumoniae infection from older, previously infected nursery piglets to the recently weaned piglets (Suh *et al.*, 1998). Taking this observation into account, the implementation of better separation between age groups through BMS might be an option, since the age-window between consecutive groups might range from 2 to 5 weeks in the currently used BMS in Western Europe (Mekerke and Leneveu, 2006).

The development of BMS originally occurred in France in the late 60s and was at the very start designed for very small herds (56 sow/herd), conducting a 3-week BMS with 7 consecutive batches. The transition to 3-week BMS had to be well prepared, but once stable production groups could be installed, several advantages could be identified, such as increased work efficiency, improved zootechnical results and a better general health status. One major disadvantage of the 3-week BMS was the presence of 2 different age groups within the farrowing unit, which might still lead to pathogen transmission between groups due to lack of sufficient internal biosecurity measures. Therefore, further evolution towards one batch of piglets in the farrowing unit resulted in the development of both the 4- and 5-week BMS. These BMS, which have been implemented in several European countries, *e.g.* Belgium, over the last 20 years, have however one major drawback related to weaning age. Especially the 4-week BMS implies suckling piglets to be weaned at the age of 21 days, whereas the 5-week BMS leaves the choice among weaning ages of 21, 24 or 28 days of age, depending on the farm structure and other influencing factors. Besides 3-, 4- and 5-week BMS, 2 other types of BMS have also been developed, namely the 2- and 7-week BMS. The 2-week BMS is especially interesting for larger farms (> 1000 sows), where a 4-week BMS would lead to too large farrowing groups (> 200 sows per group), which results in too intensive labor peaks upon the farrowing period. In contrast, a 7-week BMS is very exceptional and only used under very specific conditions, due to its disadvantage of the quite large interval between consecutive groups.

10.2. *M. hyopneumoniae* vaccination

Vaccination against *M. hyopneumoniae* is a future-proof option to reduce antibiotic use in pig production. A comparative study of the preventive use of tilmicosin phosphate and *M. hyopneumoniae* vaccination in a pig herd with chronic respiratory disease revealed that the average

number of curative medication days was significantly lower in *M. hyopneumoniae* vaccinated animals as compared to tilmicosin treated animals (Mateusen *et al*., 2001).

The success of vaccination under field conditions is subject to multiple influencing factors such as vaccine storage (Vangroenweghe, 2017) and administration, antigenic differences between field and vaccine strains, infection loads, influence of MDA, existence of intercurrent infections or stress at the time of vaccination (Maes, 2014). Even the use of adjuvant (Maes, 2014) and specific types of adjuvants seems to be crucial for vaccination success (Galliher-Beckley *et al*., 2015; Virginio *et al*., 2017).

The main evaluation criteria for *M. hyopneumoniae* vaccine efficacy are based on induction of humoral and cellular immunity and reduction in occurrence and severity of lung lesions following natural or experimental *M. hyopneumoniae* infection.

10.2.1. Commercial *M. hyopneumoniae* vaccines

Early attempts to develop a *M. hyopneumoniae* vaccine were based on *M. hyopneumoniae* plasma membranes, combined with an aluminium-hydroxide adjuvant, which induced a strong immunity in vaccinated animals (Kobisch *et al*., 1987; Sheldrake *et al*., 1991). Current commercial vaccines are mostly bacterins. A comprehensive overview of the most commonly used commercially available *M. hyopneumoniae* bacterin vaccines is given in Table 9 (adapted from Maes *et al*., 2017).

Evidence from the field demonstrates different efficacies of distinct vaccination strategies against *M. hyopneumoniae* (Thacker *et al*., 1998; Baccaro *et al*., 2006; Kim *et al*., 2011; Hillen *et al*., 2014; Kristensen *et al*., 2014) although no significant differences in protective efficacy between bacterins containing homologous or heterologous *M. hyopneumoniae* strains could be demonstrated (Villarreal *et al*., 2012). Intramuscularly administered commercial bacterins each elicited a different ability to stimulate the immune response both locally and systemically (Martelli *et al*., 2014). Nevertheless, discussion remains on the mechanisms that provide protection against *M. hyopneumoniae* through the use of bacterins. A recent study could not confirm that commercial bacterins induced detectable levels of antibodies in mice against *M. hyopneumoniae* antigens strongly recognized by the swine immune system. The absence of a serological response could be attributed to the lack of antigen expression in *M. hyopneumoniae* strains used in bacterin

production or due to the low expression or misfolding of antigens during vaccine preparation (Fisch *et al*., 2016).

Throughout the years, timing of vaccination has been subject to many discussions (Haesebrouck *et al*., 2004; Holyoake and Callinan, 2006; Maes *et al*., 2008). Recently, an update on the factors influencing the timing of vaccination in piglets concluded the optimum time frame to administer a *M. hyopneumoniae* vaccine to piglets is determined based on several factors, such as piglet age, immune status, concurrent infections, infectious pressure at farm level and potential stressful periods, *i.e.* transport, handling, processing and changes in housing or feeding (Pieters and Sibila, 2017). Recent studies under experimental as well as field conditions showed that vaccination 3 days before weaning (Arsenakis *et al*., 2016; Arsenakis *et al*., 2017) conferred slightly better effects, which may be explained by the fact that weaning is a stressful event that may decrease general immunity (Juul-Madsen *et al*., 2011).

Early *M. hyopneumoniae* vaccination at 1 week of age elicited a reduction in *M. hyopneumoniae* transmission during a 6-week period post-weaning (Meyns *et al*., 2006) and elicited both local and systemic immune response in pigs (Marchioro *et al*., 2013). Some commercial bacterins demonstrated efficacy in the presence of MDA for vaccination in piglets at less than 1 week of age, showing clinical protection until 25 weeks post-vaccination (Reynolds *et al*., 2009) and lower *M. hyopneumoniae* counts in their bronchi and lung tissue (Reynolds *et al*., 2009; Wilson *et al*., 2013).

Discussion on MDA transferred from sow to piglets upon colostrum intake has been ongoing for several decades. Martelli *et al*. (2006) demonstrated vaccination of piglets in the presence or absence of MDA primed their immune system for subsequent exposure to *M. hyopneumoniae* although they only seroconverted following a natural infection with *M. hyopneumoniae.* This hypothesis was confirmed by Sibila *et al*. (2007b), demonstrating that piglets vaccinated with a single or double vaccination had an earlier seroconversion upon infection with *M. hyopneumoniae* than their unvaccinated counterparts.

In contrast, Lehner *et al*. (2008a) found that hyperimmunisation of sows with a *M. hyopneumoniae* vaccine implied that piglets could only be vaccinated at 6 weeks of age. More recent research elicited MDA to *M. hyopneumoniae* through colostrum did not interfere with neonatal *M. hyopneumoniae*-specific CMI generated through early vaccination of piglets with a commercially available *M. hyopneumoniae* bacterin. Moreover, both maternally-derived and

endogenous CMI contributed to the *M. hyopneumoniae*-specific CMI in piglets vaccinated facing MDA (Bandrick *et al*., 2014). Since cross-fostering is widely used during early lactation, the effect of cross-fostering and its relation to transfer of maternal immunity to *M. hyopneumoniae* in piglets was studied. These results revealed that transfer of *M. hyopneumoniae*-specific CMI to piglets was detected only in piglets maintained to their dams for at least 12 h after birth (Bandrick *et al*., 2011).

As multiple pathogens are involved in PRDC on many farms, vaccination against several pathogens within PRDC should be considered. Combined vaccines, especially *M. hyopneumoniae* and PCV-2 (Witvliet *et al*., 2015; Park *et al*., 2016a) or PRRSV (Bourry *et al*., 2015), have recently been developed.

A specific issue in *M. hyopneumoniae* vaccinology still remains the lack of an available marker technology, which might be used to verify correct vaccination against *M. hyopneumoniae* during the early phase of life. Using peptide-KLH (Keyhole Limpet Hemocyanin) conjugate as a marker, piglets vaccinated with the conventional commercial *M. hyopneumoniae* vaccine, blended with the marker and Montanide® IMS 1313 as an adjuvant, could be detected using peptide-specific antibodies raised against the marker component to confirm their vaccination status (Walders *et al*., 2005). Since this technology is not yet commercially available in *M. hyopneumoniae* bacterins currently on the market, other practical solutions have to be developed. An alternative approach used the CMI through a delayed-type of hypersensitivity test. Therefore, *M. hyopneumoniae* antigen was intradermally injected at 14, 21 and 28 days post-vaccination with a commercial single-dose *M. hyopneumoniae* bacterin. Pigs subsequently displayed skin reactions characterized by circumscribed, often erythematous nodules. Additionally, an *in vitro* lymphocyte stimulation assay demonstrated a significant difference in IFN-γ production among different bacterins used (Seo *et al*., 2013).

Table 9. Most commonly used commercially available *M. hyopneumoniae* bacterin vaccines (2018) – Bacterin vaccines available in only one or a few countries are not included in the table (adapted after Maes *et al*., 2017).

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

a Vaccination scheme when one ml is used for each administration. No boost vaccination needed if a 2 ml dose is used the first time.

b Combination vaccine with Porcine Circovirus type 2

c Named Suvaxyn RespiFend MH in USA

d Same name is used in the USA, but Amphigen is used as adjuvant in the USA, and vaccine can be administered from day one of age onwards

e Combination vaccine with *Haemophilus parasuis* - Named Suvaxyn RespiFend MH HPS in USA

NI - no information available on the antigen or strain

10.2.2. Experimental *M. hyopneumoniae* vaccines

Besides commercially available bacterins, many other experimental vaccines have been developed over the years (Maes *et al*., 2017) from inactivated whole cell vaccines, through recombinant subunit and chimeric subunit vaccines to recombinant vector and DNA vaccines and finally live attenuated vaccines.

An innovative approach in inactivated whole cell vaccines was the use of an oral microencapsulated *M. hyopneumoniae* vaccine through a spray drying method (Lin *et al*., 2003), eliciting detectable levels of IgG against *M. hyopneumoniae* after a second vaccination.

Recombinant subunit vaccines are based on functional knowledge of specific subunits, such as adhesins, of *M. hyopneumoniae*. In the early days, using antigens not yet fully characterized, subunit vaccines already elicited immunity (Djordjevic *et al*., 1997). Later on, several vaccines based on *M. hyopneumoniae* P97 (King *et al*., 1997) and its C-terminal repeat region R1 and R2 (Conceição *et al*., 2006; Barate *et al*., 2014) or P42 (Jorge *et al*., 2014) were developed. Further innovation made use of fusion proteins of P97 with the B subunit of the heat-labile enterotoxin (LTB) of *Escherichia coli* (Conceição *et al*., 2006) or innovative oil-based adjuvants (Barate *et al*., 2014; Jorge *et al*., 2014). Combinations of chimera of several potential protective antigens derived from *M. hyopneumoniae*, such as P36, P46, NrdF, P97 or P97R1 (Chen *et al*., 2008) or even a broader variety of antigenic proteins (Simionatto *et al*., 2010) have been evaluated. Several of these proteins were specifically recognized by convalescent pig sera, indicating they are expressed during *M. hyopneumoniae* infection (Marchioro *et al*., 2012; Simionatto *et al*., 2012). Another multi-antigen chimera composed of three antigens of *M. hyopneumoniae* (R1, P42 and NrdF) and the mucosal adjuvant *E. coli* LTB subunit developed an immune response against all *M. hyopneumoniae* antigens present in the fusion in mice (Marchioro *et al*., 2014a) and in pigs (Marchioro *et al*., 2014b), although in the latter it could not confer a significant protection against *M. hyopneumoniae* infection. In contrast, selected domains of the P46, HSP70 and MnuA antigens, delivered as recombinant subunit or DNA vaccines, elicited a strong humoral and cellular immune response (Virginio *et al*., 2014).

Others have constructed a recombinant subunit vaccine containing fragments of eight multifunctional adhesins of the *M. hyopneumoniae* P97/P102 paralog family with Alhydrogel® or Montanide™ Gel01 as adjuvant (Woolley *et al*., 2014). Recently, P97R1, P46, P95 and P42 genes

were expressed in *E. coli* and subsequently tested for an immune response in a mice model (de Oliveira *et al*., 2017).

For recombinant vector vaccines, several vector types have been explored from different attenuated strains of *Salmonella typhimurium* (Fagan *et al*., 1997; Fagan *et al*., 2001; Chen *et al*., 2006a; Chen *et al*., 2006b) over *Pseudomonas* exotoxin A (Chen *et al*., 2001), *Erysipelothrix rhusiopathiae* (Shimoji *et al*., 2003; Ogawa *et al*., 2009) and *A. pleuropneumoniae* (Zou *et al*., 2011) to a replication-defective recombinant adenovirus (Okamba *et al*., 2007).

Further steps towards an alternative approach in *M. hyopneumoniae* vaccinology were taken by Chen *et al*. (2003), evaluating a DNA vaccine with plasmid pcDNA3/P42 in BALB/c mice. Furthermore, phage display techniques have been applied to map the epitopes of *M. hyopneumoniae* (Yang *et al*., 2005).

Intramuscular immunization of mice with a DNA cocktail vaccine induced a strong Th1-polarized immune response against each of the antigens (Chen *et al*., 2008) and a combination of two approaches, starting with a DNA cocktail vaccine and boosting by a protein cocktail vaccine, generated a strong Th1-polarized and humoral immune response (Chen *et al*., 2008).

In China, the need to control the spread of *M. hyopneumoniae* has prompted the development of live attenuated vaccine strains, such as the specific *M. hyopneumoniae* 168-L strain (Feng *et al*., 2010; Li *et al*., 2012) and several administration routes have been explored from intrapulmonic (Feng *et al*., 2010; Li *et al*., 2015), intranasal (Li *et al*., 2012; Li *et al*., 2015) to aerosol (Feng *et al*., 2013). Following complete genome sequencing of the pathogenic *M. hyopneumoniae* 168 strain (Liu *et al*., 2011) and comparative genomic analysis of both the pathogenic *M. hyopneumoniae* 168 strain and its high-passaged attenuated analogue, 168-L, a series of virulence-related genes and novel virulence determinants were detected (Liu *et al*., 2013). Using different adjuvant formulations, the ISCOM-matrix adjuvant in combination with attenuated *M. hyopneumoniae* strains revealed a reduced degree of ciliary loss on the respiratory tract surface (Xiong *et al*., 2014a; Xiong *et al*., 2014b).

11. *M. HYOPNEUMONIAE* - ERADICATION STRATEGIES

Strategies for *M. hyopneumoniae* elimination from swine herds have been developed and evaluated ever since the pathogen has been identified. A comprehensive overview is given in Table 10 based on a recent review by Holst *et al*. (2015).

In the early eighties, medicated early weaning (MEW) was developed to eliminate *M. hyopneumoniae* infection (Alexander *et al*., 1980) and further adapted to modified MEW with more strategic treatment days in order to reduce overall treatment costs (Dee, 1994). Besides depopulation and repopulation, the Swiss method has been the basis of all initial efforts for disease elimination (Zimmerman *et al*., 1989). This method includes partial depopulation (*i.e.* culling or removal of all animals younger than 10 months of age) and whole herd medication, along with a two-week farrowing break towards the end of the protocol. While this method has proven highly successful, its application in large herds is difficult. For these larger herds, alternative protocols such as herd closure and medication, and whole herd medication, keeping young animals at the farms and piglet production undisturbed, have been developed. The method of whole herd medication is usually applied after clinical outbreaks of the disease, and does not require the culling of animals or a break in farrowing. However, the reported success of disease elimination is lower for this protocol compared to the herd closure and medication protocol (Holst *et al*., 2015).

In Finland, a regional eradication program for *M. hyopneumoniae* has been enrolled based on the removal of young animals from the herd and subsequent medication of the breeding stock (Rautiainen *et al*., 2001). Using vaccination combined with medication, a *M. hyopneumoniae* eradication from a finishing herd without depopulation was also reported (Heinonen *et al*., 2011).

Although elimination of *M. hyopneumoniae* is becoming increasingly common in the USA, several aspects of the protocols and the potential success could be questioned (Maes *et al*., 2017). For example, concerns exist on the likelihood to eliminate *M. hyopneumoniae* in areas with a high pig and pig herd density due to potential airborne transmission between neighboring herds. Scientific information on airborne transmission of *M. hyopneumoniae* infections is largely missing due to lack of genotyping at the occasion of regional outbreaks and the very large genotypic variation between strains among and within farms. However, previous studies showed indirect evidence of airborne transmission of *M. hyopneumoniae* (Goodwin, 1985; Fano *et al*., 2005b; Dee *et al*., 2009; Otake *et al*., 2010).

Table 10. Summary of key aspects of *M. hyopneumoniae* elimination protocols most commonly used in the United States (adapted from Holst *et al*., 2015).

Elimination protocol	**Production time loss**	**Negative replacement gilts required before, during or after elimination**	**Herd vaccination**	**Sow medication**		**Piglet medication**	**Animal introductions**	**Potential for other pathogens eliminated**
				Feed or water	**Injection**			
Depopulation/ repopulation	Yes	Yes	No	No	No	No	NA	Yes
Partial depopulation*	Yes	No	No	Yes	No	No	NA	No
Herd closure and medication	Yes	Yes	Yes	Yes	Yes	Yes	Stop during elimination	Yes
Whole-herd medication°	No	No	No	No	Yes	Yes	Continue as usual	Yes

* Swiss method; ° No herd closure

NA = not applicable

CHAPTER 2 – STUDY OBJECTIVES

Early detection of *M. hyopneumoniae* remains a difficult issue. Previous studies have been focused on early detection of *M. hyopneumoniae* in young piglets (Fablet *et al*., 2012b; Fano *et al*., 2007; Moorkamp *et al*., 2008; Moorkamp *et al*., 2009; Nathues *et al*., 2013b; Ruiz *et al*., 2003; Sibila *et al*., 2007b; Villarreal *et al*., 2010) as well as older pigs (Calsamiglia & Pijoan, 2000; Fablet *et al*., 2012b). The results however were not conclusive.

Over the last two decades, many farms have switched from a 1-week BMS for sows to more-week BMS. Indeed, farms have mainly expanded to larger commercial units and sow management has evolved more efficiently to more-week BMS (Mekerke & Leneveu, 2006). More-week BMS are mainly implemented because of the positive effects on labor organisation and optimization of housing equipment, especially in the farrowing room (Mekerke & Leneveu, 2006). However, the effect of these systems towards the on-farm epidemiology of the major pathogens, more particularly pathogens involved in PRDC, remains largely unknown. In addition, in the current production systems, it has become more important to obtain a reliable estimate of the infection status and infection patterns. Therefore, a practical approach applicable for field veterinarians, using convenient sampling methods such as serology, has to be developed. This is definitely required for the implementation of proper preventive measures, such as vaccinations, to omit major losses due to respiratory disease.

Several studies have generated variable results in relation to the prevalence of *M. hyopneumoniae* around weaning age, varying from 2 to 52%, which might be explained by differences in study design and especially sample type. Serology is one of the main monitoring tools to assess the presence of *M. hyopneumoniae* in conventional farms. However, serum antibodies can be detected using commercial ELISA kits only from 21 days post-infection onwards (Pieters *et al*., 2017). In addition, the serological response is quite variable and some pigs may remain seronegative. Finally, serology is difficult to interpret in young pigs because of possible maternally derived immunity and also in vaccinated animals as no distinction can be made between antibodies upon vaccination or infection. In contrast, other techniques based on direct detection of the pathogen or parts of the pathogen in nasal swabs, LS or BALF, result in earlier confirmation of *M. hyopneumoniae* infection (Pieters *et al*., 2017).

Interestingly, the prevalence of *M. hyopneumoniae* might not only seem to be influenced by age or farm management system, but climatic conditions also have an impact on this parameter. Preliminary research has demonstrated that the period of the year (temperature and sunlight) (Dee

et al., 2010) and climatological parameters (average daily temperature and average daily rainfall) have a significant impact on the infection dynamics of *M. hyopneumoniae* (Segalés *et al*., 2012). However, the latter study was performed using nasal swabs and serology under Spanish conditions, which are quite different from the weather conditions in Belgium and The Netherlands.

Under field conditions, early detection of *M. hyopneumoniae* is in some cases crucial to confirm and continuously guarantee the health status of outgoing replacement gilt from a nucleus breeding herd. Standard monitoring can, however, not always be performed based on serology, due to the unacceptably large interval between infection, clinical signs and subsequent seroconversion (Sørensen *et a*l., 1997b), even with the currently available commercial ELISA tests (Pieters *et al*., 2017). Therefore, early detection of *M. hyopneumoniae* using TBS qPCR might in case of a recent *M. hyopneumoniae* infection and inconclusive serological results bring clarity to the *M. hyopneumoniae* health status of a SPF nucleus breeding herd.

The objectives of this thesis were to:

1. Study the impact of BMS on the kinetics of different PRDC pathogens using serology
2. Develop and validate a more performant detection technique – TBS/qPCR – for early detection of *M. hyopneumoniae* in piglets from 2 weeks of age onwards
3. Study the prevalence of *M. hyopneumoniae* under Belgian and Dutch conditions and its relation with climatic conditions using the TBS/qPCR detection technique
4. Apply the TBS/qPCR detection technique for identification of new *M. hyopneumoniae* infections in a SPF herd under field conditions

CHAPTER 3 – HEALTH ADVANTAGES OF TRANSITION TO BATCH MANAGEMENT SYSTEMS IN FARROW-TO-FINISH PIG HERDS

Adapted from:

Vangroenweghe F, Suls L, Van Driessche E, Maes D, De Graef E (2012). Health advantages of transition to batch management systems in farrow-to-finish pig herds. Veterinarni Medicina, 57, 83–91.

ABSTRACT

Sow BMS have become more popular due to advantages in labor planning, piglet batch sizes, all-in all-out (AI/AO) practices and health management. The present study investigated the potential health advantages of 10 selected farrow-to-finish pig herds before and after transition from a 1-week BMS to a 4- or 5-week BMS. Five different animal categories (gilts, sows, piglets, growers and finishers) were sampled at three time points (T0, T1 and T2) before and after transition to a 4- or 5-week BMS. Different matrices of the animals were collected: blood, nasal swabs and faeces. Several economically important diseases were monitored through serology: *Lawsonia intracellularis* (*L. intracellularis*), PRRSV, *M. hyopneumoniae*, *A. pleuropneumoniae*; and PCR-testing: *P. multocida* dermonecrotic toxin (DNT) and *Brachyspira* species, especially the major pathogenic *Brachyspira hyodysenteriae* (*B. hyodysenteriae*). Following serological analysis, the percentage of positive animals per category and sampling occasion were calculated. Health improvement based on serology was defined as the reduction in the percentage of positive animals for a specific disease in a specified animal category. All samples were negative for *P. multocida* DNT and *B. hyodysenteriae*. Little to no improvement could be observed for PRRSV. For *L. intracellularis* an improvement could be observed in piglets (71%) and growers (56%; $P < 0.05$). For both of the respiratory pathogens, *M. hyopneumoniae* and *A. pleuropneumoniae,* significant improvement was observed in finishers (34 and 24%, respectively). In growers, only *M. hyopneumoniae* showed a significant improvement (34%). In conclusion, the transition from a 1-week BMS to a 4- or 5-week BMS in the present herds resulted in a reduction of the percentage of seropositive animals for three of the monitored economically important diseases: *L. intracellularis*, *M. hyopneumoniae* and *A. pleuropneumoniae*.

INTRODUCTION

BMS are relatively well-established in Belgian pig production. Until now, the 3-week BMS, which was the first BMS introduced in Europe (Mekerke and Leneveu, 2006), has been the most widely used system. BMS have become particularly popular due to their advantages in labor planning, increased batch size of weaned piglets and strict AI/AO practices (Mekerke and Leneveu, 2006). Besides the 3-week BMS, 4- and 5-week BMS have been introduced during the last decade.

One of the major advantages between 3-week BMS and 4- and 5-week BMS is their strict separation between consecutive batches with only one batch in the farrowing house at any one time and the potential improvement in animal health, due to fact that suckling piglets can not be switched between consecutive batches within the farrowing house (Mekerke and Leneveu, 2006). However, the latter advantage has of now only been suggested, and to the authors' knowledge has not yet been investigated.

Monitoring tools to assess the health status of different animal categories at farm level are quite diverse and may vary from conventional clinical observations over herd sampling programs – including serology (Maes *et al.*, 2001a; Fraile *et al.*, 2010; Hands *et al.*, 2010; Meyns *et al.*, 2011), bacteriological culture and PCR-testing (Hands *et al.*, 2010) – to slaughterhouse checks (Fraile *et al.*, 2010; Meyns *et al.*, 2011). In many European countries, *P. multocida* producing DNT (Christensen and Mousing, 1992; Christensen *et al.*, 1994; Fablet *et al.*, 2011), *B. hyodysenteriae* (Christensen *et al.*, 1994), *L. intracellularis* (Christensen *et al.*, 1994), PRRSV (Christensen and Mousing, 1992; Christensen *et al.*, 1994), *M. hyopneumoniae* (Christensen and Mousing, 1992; Fraile *et al.*, 2010; Meyns *et al.*, 2011; Fablet *et al.*, 2011) and *A. pleuropneumoniae* (Christensen and Mousing, 1992; Fraile *et al.*, 2010; Meyns *et al.*, 2011; Fablet *et al.*, 2011), are considered to be the most important economic diseases in closed pig herds.

The DNT produced by some strains of *P. multocida* is crucial in the pathogenesis of progressive atrophic rhinitis (Chanter and Rutter, 1989; De Jong, 1992; Pijoan, 2006). Monitoring of toxigenic *P. multocida* can be performed using the PMT ELISA or the more sensitive toxA PCR test (MacInnes *et al.*, 2008). The prevalence of toxigenic *P. multocida* has been reported to be quite low (0 to 2%; Hariharan *et al.*, 2000; Jamaludin *et al.*, 2005; MacInnes *et al.*, 2008).

Brachyspira hyodysenteriae, the cause of swine dysentery, occurs in most swine-producing countries and its prevalence has recently increased in several European countries, including Belgium (Vyt *et al.*, 2007).

Proliferative enteropathy is caused by *L. intracellularis* (McOrist *et al.*, 1993). The currently available monitoring tools for the detection of *L. intracellularis* infections are serology and faecal PCR analysis (Knittel *et al.*, 1998). Seroconversion after infection with *L. intracellularis* generally occurs two to three weeks after infection, resulting in two specific infection patterns: nursery and grower infection (Hands *et al.*, 2010). Seroprevalence data revealed a high percentage (92.9 to 97.8%) of positive pigs at 20 to 23 weeks of age (Hands *et al.*, 2010).

PRRSV has been identified as the cause of late-term abortions and stillbirths in sows, in conjunction with respiratory disease with immunodepression in weaned piglets and finishers (Christianson, 1992; Collins *et al.*, 1992). Seroconversion after PRRSV infection appears within five to 14 days and antibody titres increase rapidly to a maximum around four weeks post-infection (Labarque *et al.*, 2000; Diaz *et al.*, 2005). Within-herd seroprevalence of PRRSV in conventional farrow-to-finish pig farms in Belgium varied between 73 and 100% (Lefebvre *et al.*, 2009).

Mycoplasma hyopneumoniae plays a central role in PRDC and is widespread in pig herds (Maes *et al.*, 2008). Seroconversion to *M. hyopneumoniae* may occur up to 12 weeks post-infection (Morris *et al.*, 1995; Sørensen *et al.*, 1997b). Infection may already occur in the nursery unit (Calsamiglia and Pijoan, 2000; Fano *et al.*, 2007; Sibila *et al.*, 2007a; Villarreal *et al.*, 2010) with seroconversion occurring much later (Andreasen *et al.*, 2000b). Recently, the within herd prevalence of *M. hyopneumoniae* in slaughter pigs was found to be 79% in Belgium (Meyns *et al.*, 2010), and 82% in Spain (Fraile *et al.*, 2010).

Actinobacillus pleuropneumoniae is a primary pathogen that causes an acute fibrinous to necrotic pleuropneumonia in pigs at all ages (Sebunya and Saunders, 1983; Gottschalk and Taylor, 2006). For general *A. pleuropneumoniae* monitoring purposes, the detection of apxIV antibodies in the serum is currently the most frequently used serological method. Seroconversion to *A. pleuropneumoniae* may occur up to five weeks post-infection (Sørensen *et al.*, 1997a) and occurs preferentially at 12 to 23 weeks of age (Chiers *et al.*, 2002). Recent studies in slaughter pigs showed a within-herd prevalence of *A. pleuropneumoniae* of 63% in Belgium (Meyns *et al.*, 2011), and 89% in Spain (Fraile *et al.*, 2010).

The introduction of a BMS should lead to a more structured approach in pig farm management with larger groups of piglets, less movement and mixing of piglets of different ages, and could therefore result in a better general herd health status. Until now, little has been established regarding the health advantages of a 4- or 5-week BMS.

STUDY OBJECTIVE

The objective of the present study was to follow the health status of 10 selected Belgian farrow-to-finish pig herds during their transition from a 1-week BMS to a 4- or 5-week BMS.

MATERIALS AND METHODS

Selection of pig herds

Ten Flemish farrow-to-finish pig herds were selected based on the following inclusion criteria: at least 100 breeding sows, 80% of fattening pigs remaining on site (sow herd location), no specific pathogen-free (SPF) status, willingness of the pig farmer and his official herd veterinarian to cooperate during the entire study.

Descriptive data on the number of sows, type of BMS chosen and reproductive performance (number of piglets born and weaned per litter, number of piglets weaned per year and number of reproductive cycles per year) are given in Table 1.

All pig herds had cross-bred sows, mainly based on a crossing of Large-White and Landrace (French Landrace, Danish Landrace). Sows were fed twice a day during pregnancy and three times a day in the farrowing house during lactation. General biosecurity status was moderate to low in all farms. Only two farms had specific hygienic requirements (hand hygiene, showering, farm-specific clothing), whereas the other farms only applied the basic regulatory biosecurity requirements (changing room with coverall and boots).

The vaccination strategy in sows consisted of pseudorabies vaccination, combined *Erysipelothrix rhusiopathiae* and Parvo vaccination, and PRRSV-vaccination with a live modified vaccine (strain dependent on farm: EU or US strain). Piglets were immunized against *M.*

hyopneumoniae using a commercial bacterin (one or two shot administration) during the suckling period. Vaccination schedules remained stable during the entire duration of the study.

Transition to BMS

The choice of BMS (4- or 5-week) for each individual pig farm was based on farm-specific criteria: future investment plan with potential growth of sow numbers, available housing for critical animal subpopulations (*e.g.* farrowing crates, post-weaning facilities), possibility to manage labour peaks (*e.g.* farrowing, weaning, etc.), and the desired age of piglets at weaning (4-week BMS always require weaning at three weeks of age).

Six of the selected farms started a transition to a 4-week BMS, whereas the other four changed to a 5-week BMS. Following the choice of the farm-specific BMS, a thoroughly detailed transition plan was drawn up containing the start date of transition, sow weaning and regrouping plan, gilt synchronization plan and further internal reorganization plans, if necessary.

The selection was performed at the end of 2006. The first sampling took place between March and May 2007. Subsequently, transition to the chosen BMS was performed. Generally, full transition of a farm would take 20 weeks, which is one sow production cycle. All farms were sampled again one and two years after the start of the transition.

Sampling schedule

Within each herd, five animal categories were selected for representative sampling: gilts, older sows (> 2nd parity), weaned piglets at the end of nursery (9 to 11 weeks depending on farm situation), growers (< 45 kg) and finishers (> 80 kg). A total of 10 animals per age category were randomly sampled. Three different matrices were collected from each animal: blood, nasal swabs and faeces.

Blood was collected through puncture of the jugular vein. Nasal swabs were taken through deep intranasal swabbing of both nostrils with one nasal swab (culture swab containing Amies with charcoal; Venturi Transystem® Copan, Brescia, Italy). Faecal samples were collected rectally from five individual pigs per age category. The specific sampling schedule and the number of samples are detailed in Table 2.

The three sampling times were specifically selected to provide baseline information of the herd health status (sampling before the start of transition (T0)), and to provide information during a sufficiently long period after the transition (one (T1) and two years (T2) after the start of transition). Animal samples were selectively analysed for several disease pathogens depending on the specific animal category and the disease relevance for the concerned age group or animal category. Different important pathogens were monitored: *L. intracellularis*, PRRSV, *M. hyopneumoniae*, *A. pleuropneumoniae*, *P. multocida* DNT and *Brachyspira* species, with *B. hyodysenteriae* as the major pathogen.

Table 1. Descriptive information at the start of the study on number of sows, type of BMS that was used during the study and reproductive performance (live born and weaned piglets/litter, weaned piglets per sow per year) of 10 selected Belgian farrow-to-finish pig farms to assess the potential health advantage after transition from a 1-week BMS to a 4- or 5-week BMS.

Farm	# sows	BMS	Number of live born pigs per litter	Pigs weaned per litter	Pigs weaned per sow per year
A	130	4	11.50	10.07	23.26
B	325	4	12.10	9.81	22.17
C	110	5	11.00	9.90	21.40
D	300	4	12.20	10.70	26.23
E	130	5	10.97	9.67	21.65
F	200	4	10.30	9.18	21.65
G	200	5	11.89	10.46	23.68
H	500	4	11.86	9.79	23.50
I	240	4	12.21	10.60	25.20
J	140	5	12.61	11.11	26.90

Sample analysis and interpretation of obtained results

The samples were transported under cooled (4 °C) conditions to the diagnostic laboratory (Animal Health Care Flanders, Torhout, Belgium). Upon arrival in the laboratory (one to three h

after sampling), samples were immediately dispatched to specific analytical units for further processing.

Nasal swabs were plated out on non-selective Columbia blood agar plates and incubated at 37 °C for 24 h. Following incubation, plates with specific *P. multocida* colonies were further processed for DNT PCR analysis. First, positive cultures were lysed through heating (90 °C, 10 min). Following subsequent DNA extraction, detection of the chromosomal toxA gene was performed using a commercial PCR test (Bactotype PCR amplication kit; Labor Diagnostik Leipzig, Leipzig, Germany), according to the manufacturer's instructions.

Faecal material from individual animals was pooled into a pool of five animals and subsequently DNA was extracted using a bead-based extraction method (Cador MagAttract; Indical Bioscience GmbH, Leipzig, Germany). The procedure includes multiple washing and purification steps in order to clear potential polymerase inhibitors. The isolated DNA was subsequently amplified using the Adiavet® Brachy (Adiagene, Saint-Brieuc, France) PCR kit according to the instructions of the manufacturer. Briefly, the PCR is a multiplex PCR for different *Brachyspira* species and their specific signals are detected in separate fluorescence channels. The multiplex PCR also includes an internal positive control in order to check for the correct PCR reaction and to detect potential false negative results. The final results of the PCR test were negative or positive with different options: *B. hyodysenteriae*, *B. pilosicoli*, *B. innocens-intermedia* and *Brachyspira* species (*i.e. Brachyspira murdochii*). The sensitivity of the test was one copy of *Brachyspira* DNA according to the manufacturer's specifications.

Blood samples were incubated at room temperature for 12 h and following clotting, the supernatant serum was collected and distributed in individual vials (1 ml) for preservation through freezing (–80 °C) until analysis. Analysis of *L. intracellularis* was performed using the Bioscreen Enterisol® Ileitis kit. *Mycoplasma hyopneumoniae,* PRRSV, and *A. pleuropneumoniae* were analysed using the Idexx HerdChek testkit: M. hyopn. Ab, PRRSV and App – ApxIV ELISA, respectively. Serological interpretation was based on the manufacturer's cut-off values and test interpretation criteria. Non-interpretable (NI) results were designated as negative (Hands *et al.*, 2010), and the percentage of positive samples per sampling time point and animal category were calculated.

Table 2. Sampling schedule for all three sampling timepoints (T0 – before transition, T1 – one year after transition and T2 – two years after transition), detailing the matrices (blood/serum, nasal swabs and faecal material), animal categories (gilts, older sows, piglets, growers and finishers) pathogens (*L. intracellularis*, PRRSV, *M. hyopneumoniae*, *A. pleuropneumoniae*, *P. multocida*, *Brachyspira* spp.), specific analytical tests and the number of samples collected.

Sample type	Animal category	Pathogen that was monitored*	Analytical test	# of samples
Blood/serum	Gilts	*L. intracellularis*	ELISA	10
		PRRSV		
		M. hyopneumoniae		
	Sows (> 2 litters)	*M. hyopneumoniae*		10
	Piglets (10 wk)	*L. intracellularis*		10
		PRRSV		
		A. pleuropneumoniae		
	Growers (14wk)	*L. intracellularis*		10
		PRRSV		
		M. hyopneumoniae		
		A. pleuropneumoniae		
	Finishers (> 80 kg)	*L. intracellularis*		10
		PRRSV		
		M. hyopneumoniae		
		A. pleuropneumoniae		
Nasal swabs	Piglets (6 wk)	DNT *P. multocida*	PCR culture	12
	Piglets (10 wk)	DNT *P. multocida*		12
	Growers (14wk)	DNT *P. multocida*		12
Fecal material	Growers (14wk)	*Brachyspira* spp.	PCR faeces	5
	Finishers (20wk)	*Brachyspira* spp.		5

*Disease abbreviations: *L. intracellularis* = *Lawsonia intracellularis*, *M. hyopneumoniae* = *Mycoplasma hyopneumoniae*, *A. pleuropneumoniae* = *Actinobacillus pleuropneumoniae*, PRRSV = Porcine Reproductive and Respiratory Syndrome Virus, DNT *P. multocida* = dermonecrotic toxine of *Pasteurella multocida*

Statistical analysis

Statistical differences ($P < 0.05$) between time points T0–T1 and T0–T2 were analysed using repeated measures ANOVA (SPSS Statistics v.18; IBM®). Differences between BMS at every time point (T0, T1 and T2) were analysed using a two-sided *t*-test assuming unequal variances. Differences were considered significant when *P*-values were lower than 0.05 (two-sided test).

RESULTS

Descriptive data of study population

Ten farrow-to-finish pig herds with 130 to 500 sows were included in the trial. Based on the characteristics of individual farms and future plans, six pig herds changed to a 4-week BMS, whereas four herds chose a 5-week BMS.

PCR test results

All nasal swabs tested negative for *P. multocida* DNT and no clinical signs of atrophic rhinitis were present on these herds at any of the sampling time points. All faecal samples in growers and finishers were negative for *B. hyodysenteriae*. Nevertheless, other *Brachyspira* species, such as *B. innocens-intermedia* and *B. murdochii* were detected, both before and after the transition.

Serological results

The results of the serological screening are presented in Table 3. For *L. intracellularis*, a reduction in the percentage of positive animals in both weaned piglets and growers could be observed at T2, although this difference was only significant ($P < 0.05$) in the growers. Furthermore, in growers, the impact of a transition to a 5-week BMS had a greater positive impact on the serological reduction in *L. intracellularis* at both T1 (73%) and T2 (95%). At T1, a reduction could be observed in growers and finishers, but the improvement in finishers did not persist up to

two years after transition onset. This was mainly due to a large and significant ($P < 0.05$) difference between the BMS, with finishers in the 5-week BMS showing a consistent decrease (39%) at T2. In gilts, no improvement in *L. intracellularis* infection status was observed.

For PRRSV, no significant reduction in the percentage of positive animals could be observed, except for the gilts, which had a slight improvement (5-6%), both at T1 and T2. At T1, weaners also showed a decreased prevalence (–18%), which, did not persist throughout the entire study.

The prevalence of *M. hyopneumoniae* decreased slightly in gilts (–5 and –12%) between T0–T1 and T0–T2. A significant ($P < 0.05$) reduction could be observed in both growers (–65 and –34%) and finishers (–28 and –34%). A marked difference in the impact of BMS was observed. In growers, the 5-week BMS resulted in a greater reduction (61% at T2), whereas in finishers, the 4-week BMS showed better results (39% at both T1 and T2). Only older sows showed an increase in the number of positive animals throughout the study.

Actinobacillus pleuropneumoniae prevalence showed an overall decrease at T1 (–22 to –35%). However, this decrease (–24%) remained constant only in finishers, whereas in weaners and growers, there was a slightly increased prevalence, which was mainly due to the 4-week BMS. In the 5-week BMS, both growers and finishers showed a very consistent significant ($P < 0.05$) reduction (46 and 58%) at both T1 and T2.

Discussion

The present study assessed the potential health advantage of a transition from a conventional 1-week BMS to a 4- or 5-week BMS in 10 Flemish farrow-to-finish pig herds. The results showed that for some of the monitored pathogens, such as *L. intracellularis*, *M. hyopneumoniae* and *A. pleuropneumoniae*, an improvement in health status was observed after the change in management system. Moreover, the 5-week BMS showed more consistent improvement over time as compared to the 4-week BMS. This may be due to the longer interval between batches and the reduction in number of batches present at farm level (Chouet *et al.*, 2003).

Table 3. Serological results (expressed as % positive animals per disease and animal category with [95% confidence interval (CI)]) of 10 Flemish farrow to finish pig herds at the different sampling times (T0 – before transition, T1 – one year after transition and T2 – two years after transition to a 4- or 5-week BMS) and the difference (expressed in % improvement or decline) as compared to T0. Significant differences ($P < 0.05$) between T0–T1 and T0–T2 are indicated with an asterix (*).

Disease	Animal category	T0		T1		T2		% (T1-T0)	% (T2-T0)
		%	95% CI	%	95% CI	%	95% CI		
Lawsonia intracellularis	Gilts	76	[61.2-90.8]	86	[69.2-100.0]	81	[65.2-96.8]	+13	+7
	Piglets	7	[5.7-8.3]	9	[7.3-10.7]	2	[1.7-2.3]	+28	-71
	Growers 45 kg	43	[34.7-51.3]	22	[17.8-26.2]	19	[15.4-22.6]	-49*	-56*
	Finishers > 85 kg	50	[40.3-59.7]	61	[48.3-71.7]	51	[41.1-60.9]	-20	+2
PRRSV	Gilts	88	[70.9-100.0]	82	[66.0-98.0]	83	[66.8-99.2]	-6	-5
	Piglets	55	[44.3-65.7]	45	[36.3-53.7]	61	[49.1-72.9]	-18	+11
	Growers 45 kg	71	[57.2-84.8]	83	[65.8-98.2]	72	[58.0-86.0]	+16*	+1
	Finishers > 85 kg	80	[64.4-95.6]	84	[67.6-100.0]	89	[71.7-100.0]	+5	+11
Mycoplasma hyopneumoniae	Gilts	56	[45.1-66.9]	53	[42.7-6.3]	49	[39.5-48.5]	-5	-12
	Sows (> 2nd parity)	16	[13.0-19.0]	23	[18.6-27.4]	36	[29.0-43.0]	+43	+125*
	Growers 45 kg	49	[39.5-48.5]	17	[13.8-20.2]	32	[25.8-38.2]	-65*	-34*
	Finishers > 85 kg	70	[56.4-83.6]	50	[40.3-59.7]	46	[37.1-54.9]	-28*	-34*
Actinobacillus pleuropneumoniae	Piglets	57	[45.9-68.1]	44	[35.5-52.5]	65	[52.4-67.6]	-22	+14
	Growers 45 kg	34	[27.4-40.6]	22	[17.6-26.4]	35	[28.2-41.8]	-35*	+2
	Finishers > 85 kg	67	[54.0-80.0]	52	[41.9-62.1]	51	[41.1-60.9]	-22*	-24*

Toxigenic *P. multocida* was absent in all herds and all collected samples. This is in accordance with previous studies, showing a very low prevalence of this pathogen (Jamaludin *et al.*, 2005; MacInnes *et al.*, 2008). Besides the absence of diagnostic detection of the toxA gene of *P. multocida*, there were no clinical signs which could indicate problems with progressive atrophic rhinitis in the herds.

Although *B. hyodysenteriae* prevalence is increasing in Belgium (Vyt *et al.*, 2007), the pathogen was not detected. Some minor pathogenic *Brachyspira*, including *B. innocens-intermedia* and *B. murdochii*, could be detected, although no clinical signs possibly associated with these pathogens were observed throughout the entire study period. Transition to a BMS did not have any impact on the presence nor prevalence of these *Brachyspira* species.

For *L. intracellularis*, all farms were seropositive and the average percentage of positive animals was between 43% in growers and 76% in young gilts, which is much lower than the 93 to 98% reported in 20 to 23 week old finishers by Hands *et al.* (2010) in Great Britain and Ireland. From the present study, it can be concluded that in the studied herds, the infection pattern described by Hands *et al.* (2010) was a grower infection, since seroconversion only occurred after the nursery period. After transition to a BMS however, a clear reduction in the percentage of positive animals could be observed in the weaners and growers. Less mixing of piglets and more discipline in cleaning and disinfection have been shown to have an impact on the environmental infection pressure (Chouet *et al.*, 2003).

In the present study, PRRSV was monitored in gilts, piglets, growers and fattening pigs. The high within-herd prevalence (55–88%) at the start of study was in accordance with a recent seroprevalence study in Belgium (73–100%; Lefebvre *et al.*, 2009). Little to no improvement in animal prevalence could be observed following transition to a BMS. These results can be explained by the lack of additional biosecurity measures to reduce PRRSV circulation within the herds. Moreover, in several herds, severe outbreaks of PRRSV – especially in piglets and finishers – were seen during the study period. In contrast, one specific herd (herd C) had a comparable low prevalence of PRRSV in piglets, growers and finishers at T0 and T2, which indicates that specific farm-associated management practices and internal biosecurity measures have a significant impact on the control or spread of PRRSV.

For *M. hyopneumoniae*, increased prevalence could be observed in gilts as compared to older sows throughout the entire study. This is in accordance with observations by Calsamiglia and Pijoan (2000), showing a higher number of young sows having *M. hyopneumoniae* carrier status. Transition to a BMS had little to no impact on the prevalence of *M. hyopneumoniae* in gilts (–5 to 6%) and older sows (+43 to 125%), whereas in growers and finishers, a decrease in animal prevalence (–34%) could be observed at

T2. Management factors and housing conditions have a significant impact on the spread and control of *M. hyopneumoniae* (Maes *et al.* 2008).

Sows and gilts were not monitored for *A. pleuropneumoniae* as previous studies have shown that most farms are endemically infected (Chiers *et al.*, 2002). In contrast with Chiers *et al.* (2002), the seroprevalence of *A. pleuropneumoniae* at the end of nursery (9 to 11 weeks of age) was rather high. This may be related to high levels of MDA. In growers, a lower animal prevalence was present followed by seroconversion in the finishers, which is well in accordance with the seroconversion observed by Chiers *et al.* (2002) in 16-week old pigs.

In the present study, only 10 farrow-to-finish pig farms were selected for the monitoring of the transition to a 4- or 5-week BMS and no control farms which remained unchanged in their management systems were included. Although the herds were not randomly selected, they are considered, based on the herd characteristics, to be representative for other pig herds in Belgium. Also, it is not known which management factors associated with the transition to a BMS have caused the observed improvements for some pathogens. To answer these questions, further studies using a different study design should be conducted.

CONCLUSIONS

In conclusion, the transition of pig herds to a BMS seems to postpone or slow down the seroconversion of pigs for *L. intracellularis*, *M. hyopneumoniae* and *A. pleuropneumoniae*. This allows the farmer the possibility of vaccinating the pigs before they come in contact with the pathogen and it also allows more time for the pigs to build up immunity before infection.

The differences observed between the 4- and 5-week BMS for several pathogens (*L. intracellularis*, *M. hyopneumoniae* and *A. pleuropneumoniae*) may confirm this hypothesis. Therefore, these results indicate that the transition from a 1-week BMS to a 4- or 5-week BMS could have protective effects with regard to several economically important pathogens.

CHAPTER 4 – ASSESSMENT OF LITTER PREVALENCE OF *MYCOPLASMA HYOPNEUMONIAE* IN PRE-WEANED PIGLETS UTILIZING AN ANTE-MORTEM TRACHEO-BRONCHIAL MUCUS COLLECTION TECHNIQUE AND A REAL-TIME POLYMERASE CHAIN REACTION ASSAY

Adapted from:

Vangroenweghe F, Karriker L, Main R, Christianson E, Marsteller T, Hammen K, Bates J, Thomas P, Ellingson J, Harmon K, Abate S, Crawford K (2015). Assessment of litter prevalence of *Mycoplasma hyopneumoniae* in pre-weaned piglets utilizing an ante-mortem tracheo-bronchial mucus collection technique and a real-time polymerase chain reaction assay. Journal of Veterinary Diagnostic Investigation, 27, 606-610. doi: 10.1177/1040638715595062.

Abstract

The swine industry currently lacks validated ante-mortem methods of detecting baseline herd prevalence of *M. hyopneumoniae*. The focus of our study was to evaluate alternative ante-mortem detection techniques and to determine baseline litter prevalence in pre-weaned pig populations utilizing the selected technique and a qPCR assay. Preliminary data were analysed on weaned piglets with evidence of respiratory disease ($n = 32$). Five sample types (ante-mortem nasal swab, TBS, post-mortem deep airway swab, BALF, and lung tissue) were collected from each pig. Individual samples were tested for *M. hyopneumoniae* using qPCR. Compared to nasal swabs, TBS demonstrated higher test sensitivity ($P < 0.0001$). TBS was collected from apparently healthy pre-weaned piglets (n = 1,759; sow farms 1–3) and pre-weaned piglets exhibiting signs of respiratory disease (n = 32; sow farm 4), ranging in age from 12 to 25 days. Samples from sow farms 1–3 were pooled into 2 groups of 5 per litter (n = 360 pools from 180 litters), and qPCR was utilised to analyse each pool. A qPCR-positive result, Ct value < 37, from either pool designated the litter positive for *M. hyopneumoniae*. Two out of 180 litters revealed a positive result (1.1%). Individual qPCR assays were run on the samples collected from sow farm 4. Five out of 32 samples revealed a positive result (15.6%). TBS in combination with qPCR is a sensitive ante-mortem sampling technique that can be used to estimate the prevalence of *M. hyopneumoniae* in pre-weaned pigs, thus providing insight into the infection dynamics across the entire farrow-to-finish process.

INTRODUCTION

Mycoplasma hyopneumoniae, the causative agent for porcine enzootic pneumonia and a key component in PRDC, remains a major threat to the world's swine industry (Maes *et al.*, 2008; Sibila *et al.*, 2009). As an example, *M. hyopneumoniae* is considered to be one of the top sources of economic losses in United States' swine production (Dos Santos *et al.*, 2015). Although vaccination protocols are widely implemented in swine herds, mycoplasmal pneumonia remains a threat to swine production (Reynolds *et al.*, 2009; del Pozo Sancristán *et al.*, 2014). However, it is apparent that not all herds are affected equally, and the difference has been linked to the prevalence of *M. hyopneumoniae* in weaned pig populations (Fano *et al.*, 2007). Research suggests that the main risk factor for the development of disease is vertical transmission from sow to piglet during lactation and that the severity of the disease at slaughter may be predicted by the initial prevalence at weaning (Fano *et al.*, 2007). A 2013 study (Simionatto *et al.*, 2013) suggested vaccination as the most cost effective control measure for enzootic pneumonia. However, previous work (Thacker *et al.*, 2000; Park *et al.*, 2014) indicated that a concurrent PRRSV infection, either natural or from a modified live vaccine, reduces the efficacy of a *M. hyopneumoniae* vaccine. Because PRRSV is endemic in the United States, such data support the necessity for effective antimicrobial treatment options.

There is research to demonstrate that antimicrobial therapy is an effective method of control of clinical signs associated with enzootic pneumonia (Clark *et al.*, 1998). Studies suggested reducing prevalence in the breeding herds which in turn may reduce spread of disease to piglets (Maes *et al.*, 2008). However, ante-mortem estimation of herd prevalence and the ability to obtain herd-specific isolates for sensitivity testing is required to establish baseline data to support future research. In the recent past, several studies comparing ante-mortem diagnostic techniques to detect *M. hyopneumoniae* have been published (Marois *et al.*, 2007; Moorkamp *et al.*, 2008; Fablet *et al.*, 2010). Comparison of lung tissue and BALF of *M. hyopneumoniae*–affected piglets revealed a higher sensitivity for lung tissue (Moorkamp *et al.*, 2008). In another study, 4 different ante-mortem sampling techniques, namely TBS, BALF, nasal swabs, and tonsil scrapings were compared for their ability to detect *M. hyopneumoniae*–infected piglets using qPCR (Fablet *et al.*, 2010). The study revealed a 3.5 times higher sensitivity for TBS samples as compared to the other sample types (Fablet *et al.*, 2010). However, these studies have each focused on individual analysis

of collected samples, which can be quite expensive for screening large swine operations with low *M. hyopneumoniae* prevalences.

STUDY OBJECTIVES

In the present study, 2 diagnostic investigations are reported. The first investigation aims to identify a suitable protocol for both sample collection and diagnostic testing for *M. hyopneumoniae*, whereas the second investigation subsequently applies the selected protocol to 4 sow farms across the State of Iowa to assess litter prevalence of *M. hyopneumoniae* in the pre-weaned piglet population.

MATERIALS AND METHODS

Study 1 – Protocol for sample testing and diagnostic testing for *M. hyopneumoniae*

Study population and TBS sampling protocol

Thirty-two pre-weaned pigs, from a commercial sow farm in Iowa, showing clinical signs of respiratory disease were submitted for diagnostic evaluation at Iowa State University Veterinary Diagnostic Laboratory (VDL ISU, Ames, IA, USA). The TBS method was utilised in our study as previously described (Marois *et al.*, 2007; Fablet *et al.*, 2010) with some modifications (Vangroenweghe *et al.*, 2015b). Briefly, TBS samples were obtained following restraint of the piglets with a nose snare, and subsequent use of a mouth opener. The aspiration tube used (CH12 x 50 cm; Medinorm, Spiesen-Elversberg, Germany) was inserted through the mouth and glottis down to the tracheo-bronchial bifurcation where mucus was collected through gentle swab movement. Nasal swabs were collected, and TBS was performed ante-mortem, while deep airway swabs (down to the tracheo-bronchial split), BALF, and lung tissue, from the cranial lung lobes, were collected post-mortem from each pig. All samples were stored on ice after collection until analysis (VDL ISU, Ames, IA, USA).

PCR analysis

In an effort to identify the optimal sample site and DNA extraction method for detection of *M. hyopneumoniae*, a qPCR assay, using 3 separate extraction methods (viral, total nucleic acid [TNA], and high volume), was run on each sample (32 pigs × 5 samples × 3 extraction methods), for a total of 480 qPCR reactions. The viral and high volume extractions were performed using an RNA/DNA nucleic acid commercial extraction kit (MagMAX Pathogen RNA/DNA extraction kit, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendations. The third extraction was a total nucleic acid extraction that was performed using a different commercial kit (MagMAX Total Nucleic Acid Isolation kit, Life Technologies, Carlsbad, CA, USA) following the manufacturer's recommended protocol for OF. Each extraction method was carried out on a particle processor (Kingfisher 96 magnetic particle processor, Thermo Fisher Scientific, Waltham, MA, USA) using the processor program specific for each extraction method (viral RNA and TNA extractions: program 4462359-dw-50; high-volume extraction: program 4462359-dw-hv; www.lifetechnologies.com).

Following extraction, the nucleic acid templates were used in a qPCR (Applied Biosystems 7500 Fast real time PCR system, Life Technologies, Carlsbad, CA, USA) reaction according to manufacturer's recommendations (VetMAX–Plus qPCR master mix and VetMAX *M. hyopneumoniae* reagents, Life Technologies, Carlsbad, CA, USA).

Sample pooling approach

Based on the initial results, pooling potential was assessed for the TBS samples. Nine individual samples, all of which had originally tested positive, were pooled at a 1:5 and a 1:10 dilution, where 1 known positive sample was pooled with 4 or 9 known negative samples, respectively. The pooled Ct values using the 3 extraction methods were compared with the original individual Ct values.

Study 2 – Assess litter prevalence of *M. hyopneumoniae* in the pre-weaned piglet population in the State of Iowa (USA)

Inclusion criteria

For the second investigation, minimum inclusion criteria were as follows: no use of any antibiotics (based on veterinary prescription documents) effective against *Mycoplasma* species in either piglets or sows for at least 3 weeks prior to sampling, and the presence of at least 20 gilt litters, 20 litters from parity 2 or parity 3 sows, and 20 litters from sows currently in their fourth parity or greater on the farm in a single weaning group.

TBS sampling protocol and sample pooling

Lactating sows from each of the non-clinical case farms (sow farms 1–3) were assigned a group number based on their current parity (gilts, group 1; parity 2 or 3 sows, group 2; and fourth parity or above sows, group 3). Up to 10 piglets from 20 litters per group from the 3 sow farms were sampled using the TBS technique for a possible total of 600 piglets per farm sampled (10 piglets × 20 litters × 3 groups). In our study, samples were pooled in groups of 5 per litter so that all litters had 2 pools of samples and the litter was designated the experimental unit. Ten piglets were sampled at random if >10 piglets per litter were present. If <10 piglets per litter were present, all piglets in the litter were sampled, and the first 5 samples in the litter were pooled together and the remaining samples were then pooled. If >20 eligible litters per group were present on the farm, litters were sampled at random. All sampled piglets were between 12 and 25 days of age. From sow farm 4, 32 individual piglets exhibiting signs of respiratory disease were chosen for sample collection with the TBS technique between 13 and 20 days of age. All samples were tested individually.

PCR analysis

Each sample or sample pool was analysed using a validated *M. hyopneumoniae* mhp183 real-time PCR ((VDL ISU, Ames, IA, USA) (Strait *et al.*, 2008). Briefly, nucleic acid was extracted from TBS using a high-volume extraction technique as described above. For the pooled

samples, 150 µl from each sample was collected from the individual tube and pooled with the 4 other samples (or the remaining samples) from the same litter. The results of both PCR tests were recorded for each litter (n = 180) from sow farms 1–3. If either pooled PCR identified a positive sample, the entire litter was considered positive for *M. hyopneumoniae.* The results of the individual PCR tests from sow farm 4 were also recorded for each piglet (n = 32).

Statistical analysis

Statistical analysis was completed using commercial Software (JMP10, SAS Institute, Cary, NC, USA). A linear mixed model was conducted with the Ct values as the response variable, the method of PCR extraction and sample collection technique as the fixed effects, and the individual pig selected as the random effect. Pairwise comparisons were performed among methods of PCR extraction and among sample collection techniques.

RESULTS

The results from the first diagnostic investigation are summarized in Table 1. Nasal swabs gave the lowest frequency (6.3% positive), whereas the TBS samples provided the highest frequency with 59.3% of the pigs being qPCR positive.

Results showed that sample collection method also had a significant effect on the Ct value ($P < 0.0001$; Table 2). Pairwise comparisons revealed the markedly lower sensitivity of using nasal swabs and that the predicted least squares mean Ct value for the TBS technique was lowest compared with the other 4 sample collection methods (Table 2). Based on these observations, TBS was chosen for the field protocol. Using all extractions, 17 piglets (53.1%) with a negative nasal swab tested positive utilizing the TBS sample (Table 1).

The linear mixed model also showed that extraction method had an overall effect on the Ct value ($P < 0.01$). The interaction between extraction technique and sample collection method was not significant ($P > 0.05$). Pairwise comparisons of extraction technique revealed a significant difference between the Ct values of the viral (Ct = 32.9) and TNA (Ct = 29.5) extraction methods ($P < 0.05$), whereas pairwise comparisons between viral and high-volume (Ct = 32.1) extraction

methods and high-volume and TNA extraction methods were not statistically significant ($P > 0.05$).

Table 1. Percent *M. hyopneumoniae* polymerase chain reaction (PCR) positive by extraction method and sample type (diagnostic investigation #1).*

	Sample type				
	Live animals		Dead animals		
Extraction method	**Nasal swab**	**TBS**	**Lung**	**Deep airway swab**	**BALF**
Viral	6.25%	59.3%	46.9%	46.9%	46.9%
	(2/32)	(19/32)†	(15/32)†	(15/32)†	(15/32)†
TNA	6.25%	59.3%	46.9%	46.9%	59.3%
	(2/32)	(19/32)†	(15/32)†	(15/32)†	(19/32)†
High volume	6.25%	59.3%	50.0%	46.9%	46.9%
	(2/32)	(19/32)†	(16/32)†	(15/32)†	(15/32)†
Total (sample type)	6.25%	59.3%	47.9%	46.9%	51.0%
	(9/96)	(57/96)	(46/96)	(45/96)	(49/96)

* PCR positive = threshold cycle (Ct) < 37; PCR negative = Ct ≥ 37; TBS = tracheo-bronchial swab; BALF = broncho-alveolar lavage fluid; TNA = total nucleic acid.

† Samples positive in different sample types.

Table 2. Predicted least squares mean of threshold cycle (Ct) values for 5 sample collection techniques for *M. hyopneumoniae*.*

Sample collection technique	Type	Predicted least squares mean Ct values†
Nasal swab	Ante-mortem	37.6^{A}
TBS	Ante-mortem	29.0^{B}
Lung	Post-mortem	32.5^{C}
Deep airway swab	Post-mortem	$30.2^{D,B}$
BALF	Post-mortem	$32.1^{B,C}$

* BALF = broncho-alveolar lavage fluid; TBS = tracheo-bronchial swab.

† Values not sharing a letter are significantly different ($P < 0.05$).

The results of the pooling statistics indicated that when pooling the samples at a 1:5 and 1:10 dilution, the overall categorical outcome of the Ct value (as positive or negative) changed the least times using the high-volume extraction method. Based on a Ct cut-off value of 37, the highest sensitivity was obtained using the 1:5 dilution step. Based on all of these results, high-volume extraction and the prevalence of *M. hyopneumoniae* in young pigs with 1:5 dilution were decided as the preferential approach for the field study.

The *M. hyopneumoniae* qPCR results from the pooled litters from sow farms 1–3 are shown in Table 3. Of the 180 litters sampled, 2 litters (1.1%; 95% confidence interval: 0%, +2.62%) returned positive qPCR results. Of the 32 piglets sampled from sow farm 4, 5 individuals (15.6%) returned positive qPCR results (data not shown).

Table 3. Percent *M. hyopneumoniae* positive by pooled real-time polymerase chain reaction (qPCR) assays by group and sow farm.*

	% qPCR+				
Sow farm	**Group 1**	**Group 2**	**Group 3**	**Total pools sampled**	**Total pigs sampled**
1	0/40 = 0.0%	1/40 = 2.5%	0/40 = 0.0%	120	590
2	0/40 = 0.0%	0/40 = 0.0%	1/40 = 2.5%	120	591
3	0/40 = 0.0%	0/40 = 0.0%	0/40 = 0.0%	120	578
Total	0.00%	0.83%	0.83%	360	1,759

* qPCR positive = threshold cycle (Ct) < 37; qPCR negative = Ct ≥ 37; group 1 = gilts; group 2 = parity 2 or parity 3 sows; group 3 = parity 4 or greater sows.

DISCUSSION

Understanding the prevalence of *M. hyopneumoniae* in the swine population is important information when considering management of herd outbreaks and routine herd health monitoring. Nasal swabs have been the method of sample collection in the past for ante-mortem *M. hyopneumoniae* detection in a herd (Calsamiglia *et al.*, 1999). However, there have been several studies (Kurth *et al.*, 2002; Sibila *et al.*, 2007b; Marois *et al.*, 2009) reporting the use of more sensitive sampling techniques for detecting *M. hyopneumoniae* by PCR. These sampling methods,

although more sensitive, are conducted post-mortem. Other reports (Verdin *et al.*, 2000a; Moorkamp *et al.*, 2008; Fablet *et al.*, 2010) evaluating ante-mortem testing in combination with PCR have indicated that the ideal sampling sites are located in the lower respiratory tract of pigs, thus samples targeting the trachea and bronchi are more likely to yield *M. hyopneumoniae* organisms. A previous study (Fablet *et al.*, 2010) reported a technique that combines both ante-mortem testing capabilities with increased sensitivity. TBS proved to be 3.5 times more sensitive than nasal swabbing (Fablet *et al.*, 2010). To the authors' knowledge, no studies in the research literature can be found applying this novel sampling technique to assess prevalence of *M. hyopneumoniae* in United States' swine herds.

The results of both diagnostic investigations presented in this research suggest that TBS is the most sensitive ante-mortem test available to assess true prevalence of *M. hyopneumoniae* in the pig population. The overall litter prevalence in the non-clinical pre-weaned pig population from our study was low (1.1%) and in agreement with a 2013 study (Nathues *et al.*, 2013a) that reported a prevalence of 3.6% in the weaned pig population. However, the 2013 study (Nathues *et al.*, 2013a) assessed the prevalence at the piglet level, whereas in our study, prevalence was determined at litter level. A 2015 study was conducted using this TBS technique in Benelux pig herds without specific respiratory disease (Vangroenweghe *et al.*, 2015b). The study showed a higher *M. hyopneumoniae* prevalence of at least 7.1% at 3–5 weeks of age, increasing to 10.9% at 6–11 weeks of age (Vangroenweghe *et al.*, 2015b). A potential explanation for this high prevalence might be that piglets in the youngest group were between 21 and 35 days of age, whereas in our study, the age at sampling was between 12 and 25 days of age. Moreover, in our study piglets were selected from large sow herds operating in a multiple site production system, where all weaned piglets are immediately removed from the site to remote post-weaning facilities. This might have an impact on the overall *M. hyopneumoniae* infection pressure in the sow herds involved. Although it is suggested that *M. hyopneumoniae* can be isolated in pigs as early as 1 week old, it is shown that prevalence increases with age, peaking at around the late nursery to early finishing stage (Vangroenweghe *et al.*, 2015b). Moreover, seasonal variations (Vangroenweghe *et al.*, 2015b) and batch-to-batch variations (Fano *et al.*, 2007) also have a significant impact on the prevalence of *M. hyopneumoniae* in early post-weaning piglets. Therefore, it is possible that the piglets sampled in our study were truly negative for *M. hyopneumoniae*. Instead of targeting piglets at the age of weaning, it may be more fitting to assess the prevalence of *M. hyopneumoniae* in nursery-age

piglets. In this scenario, it is likely that the combination of recent stressful events (weaning, movement, commingling) may initiate higher bacterial loads in the herd.

The low disease prevalence reported in our study is consistent, to a degree, with previous research (Fano *et al.*, 2007; Sibila *et al.*, 2007b; Nathues *et al.*, 2013a). Using nasal swabs, the prevalence of *M. hyopneumoniae* in the weaned pig population was found to be low with quite a large batch-to-batch variation (Fano *et al.*, 2007). A previous study (Nathues *et al.*, 2013a) reported a *M. hyopneumoniae* prevalence of 3.6% in the weaned pig population utilizing nasal swabs for sample collection and nPCR for detection in clinically affected herds, while a 2007 study (Sibila *et al.*, 2007b) reported a prevalence of 3.8% in 3-week-old piglets from sows in a herd with enzootic pneumonia issues. However, the study design of these studies was different from the current study, as all samples were collected in *M. hyopneumoniae* clinical herds, whereas in our study, piglets were sampled from non-clinical farms. Another report (Vicca *et al.*, 2002), using nasal swabs and nPCR, found a disease prevalence of 16% in 6-week-old pigs clinically infected with enzootic pneumonia. This value agrees with the prevalence estimate from a clinically affected sow farm (Dos Santos *et al.*, 2015). The increase in prevalence between the non-clinical and clinical sow farms suggest that this TBS technique may be most judiciously applied to diseased herds to detect changes in the prevalence of *M. hyopneumoniae* that might have relevance for predicting performance of off-spring or timing application of interventions.

CONCLUSIONS

TBS in combination with qPCR is a sensitive ante-mortem sampling technique that can be used to estimate the prevalence of *M. hyopneumoniae* in pre-weaned pigs, thus providing insight into the infection dynamics across the entire farrow-to-finish process.

CHAPTER 5 – *MYCOPLASMA HYOPNEUMONIAE* INFECTIONS IN PERI-WEANED AND POST-WEANED PIGS IN BELGIUM AND THE NETHERLANDS: PREVALENCE AND ASSOCIATIONS WITH CLIMATIC CONDITIONS

Adapted from:

Vangroenweghe F, Labarque G, Piepers S, Strutzberg-Minder K, Maes D (2015). *Mycoplasma hyopneumoniae* infections in peri-weaned and post-weaned pigs in Belgium and The Netherlands: prevalence and associations with climatic conditions. Veterinary Journal 205, 93-97.

ABSTRACT

Mycoplasma hyopneumoniae is an important pathogen in modern intensive pig farming in Europe. The objectives of the present study were (1) to use the TBS technique to obtain data on the distribution of *M. hyopneumoniae* infections in recently weaned pigs in Belgium and The Netherlands, and (2) to look for associations between infection prevalence and specific climatic conditions. One hundred and seventy-six pig herds were randomly selected and 30 piglets sampled on each farm: 18 at 3–5 weeks of age and 12 at 6–11 weeks. Mucus collected from the tracheo-bronchial bifurcation and suspended in saline was subjected to PCR analysis for *M. hyopneumoniae.* In 27% of herds (*n* = 44) at least one piglet tested positive for *M. hyopneumoniae* at 3–5 weeks of age, and 29% (*n* = 47) at 6–11 weeks of age. The individual animal prevalence at the two ages was 7.1% and 10.9%, respectively. The probability of 3–5 week old piglets being *M. hyopneumoniae*-positive was negatively associated with the precipitation rate (odds ratio [OR] = 0.971) during the week preceding the sampling. In the older post-weaning group, the odds of being *M. hyopneumoniae*-positive at piglet level were significantly affected by season (OR of detection during autumn compared to summer 20.9). Thus, under Belgian and Dutch field conditions, piglets may be infected with *M. hyopneumoniae* very early in life, with prevalence increasing further during the post-weaning period.

INTRODUCTION

Mycoplasma hyopneumoniae, one of the main pathogens associated with enzootic pneumonia and PRDC, is an important pathogen in modern intensive pig farming in Europe (Maes *et al.*, 2008; Sibila *et al.*, 2009).

Economic losses associated with mycoplasmal infections are related to a chronic, non-productive cough, reduced growth rate, poorer feed conversion, increased medication use and a higher susceptibility to secondary pathogens such as *P. multocida* and *A. pleuropneumoniae* (Sibila *et al.*, 2009).

Diagnosis of mycoplasmal infections can be undertaken using a variety of approaches (Sibila *et al.*, 2009), including clinical signs, slaughterhouse checks of affected lungs (Fraile *et al.*, 2010; Meyns *et al.*, 2011), serological examination of relevant animal groups (Fraile *et al.*, 2010; Meyns *et al.*, 2011), direct identification of the pathogen through bacteriological culture (Marois *et al.*, 2007) or PCR techniques (Calsamiglia *et al.*, 1999; Marois *et al.*, 2010).

Various sampling sites have been used, including nasal swabs (Calsamiglia and Pijoan, 2000; Fano *et al.*, 2007; Villarreal *et al.*, 2010), tonsil scrapings (Fablet *et al.*, 2010) and BALF (Meyns *et al.*, 2004; Meyns *et al.*, 2006; Fablet *et al.*, 2010; Villarreal *et al.*, 2011b; Vranckx *et al.*, 2012a).

Recently, the TBS technique has been developed and validated for use in pigs (Fablet *et al.*, 2010). Numerous studies, using various sampling sites, have shown that suckling piglets can be infected by their dam (Calsamiglia and Pijoan, 2000; Fano *et al.*, 2007; Sibila *et al.*, 2007a; Nathues *et al.*, 2010; Villarreal *et al.*, 2010; Fablet *et al.*, 2012b; Segales *et al.*, 2012) and that further spread of infection occurs after weaning (Meyns *et al.*, 2004; Meyns *et al.*, 2006; Villarreal *et al.*, 2011b).

In Belgium and The Netherlands, limited data are available on the prevalence of *M. hyopneumoniae* around the time of weaning and during the immediate post-weaning period. As part of a larger study, Villarreal *et al.* (2010) studied *M. hyopneumoniae* prevalence in six herds from Belgium and six from The Netherlands, with coughing in grower-finisher pigs (a clinical sign associated with *M. hyopneumoniae*). In Belgium 4/6 herds and 3.3% of tested piglets were positive whereas in The Netherlands the figures were 5/6 herds and 7.8% of piglets. Vranckx *et al.* (2012a) studied four pig herds with known respiratory problems due to early infection (before week 15)

with *M. hyopneumoniae*. In these herds the percentage of pigs testing positive on qPCR increased from 35% at 6 weeks to 96% at 26 weeks of age.

In enzootic pneumonia studies, the risk of infection with *M. hyopneumoniae* has been shown to be associated with several risk factors, such as distance to non-SPF herds, herd size, density of pig population in the specific area (Stärk *et al.*, 1992), specific season for start of growing cycle, presence of breeding stock in the herd, lack of a *M. hyopneumoniae* vaccination program (Ostanello *et al.*, 2007), period of the year (temperature and sunlight) (Dee *et al.*, 2010) and climatological parameters (average daily temperature and average daily rainfall) (Segalés *et al.*, 2012).

A recent study has shown that rainfall and temperature may have a significant impact on *M. hyopneumoniae* infection dynamics (Segalés *et al.*, 2012). In that investigation, the weekly precipitation rate had a positive association (odds ratio [OR] = 1.31) with the probability of being *M. hyopneumoniae*-positive (nPCR on nasal swabs) at a pig level, while weekly temperature had a negative association (OR = 0.89) with the probability of being *M. hyopneumoniae*-negative (Segalés *et al.*, 2012).

STUDY OBJECTIVES

The present study was designed to investigate the association between the prevalence of early *M. hyopneumoniae* infections (as detected using TBS) in Belgian and Dutch pig herds (without clinical respiratory disease) and climatic conditions across the year.

MATERIALS AND METHODS

Selection of study herds

The study was conducted in Belgium and The Netherlands between April 2011 and March 2012. Closed pig herds were randomly selected through local veterinary practices in both countries. The inclusion criteria of the study herds were at least 200 sows in the herd, preferably two age groups in nursery pigs (3–5 weeks and 6–11 weeks of age) in each herd, no specific clinical signs of respiratory disease (such as coughing or sneezing) observed for at least 4 months before sampling in all age categories (peri-weaned, post-weaned and fattening pigs where present) and

no use of antimicrobials active against *M. hyopneumoniae* in piglets < 3 weeks of age or during the post-weaning period.

BMS were used in many of the herds, varying from 2-week to 5-week BMS. Each herd operated one specific BMS with the week number (2–3–4–5) indicating the interval between weaning groups. Vaccination status against *M. hyopneumoniae* was not taken into account as a selection criterion. However, in many herds (80% in Belgium, 35% in The Netherlands) piglets were vaccinated between 1 and 3 weeks of age using a *M. hyopneumoniae* bacterin. In total, 176 closed pig herds were included in the study, consisting of 73 Belgian and 103 Dutch herds. No differences in farm criteria were detected between both countries. The timing of sampling of each farm across the year is given in Table 1.

Table 1. Distribution of herds during subsequent seasons of the year, and between Belgium and The Netherlands. S1, winter; S2, spring; S3, summer and S4, autumn.

Season	**Number of herds tested**		**Total**
	Belgium	**The Netherlands**	
S1	14	33	47
S2	21	27	48
S3	6	19	25
S4	31	25	56
Total	*73*	*103*	*176*

A standard sampling protocol of 30 pigs per herd was applied (based on 18 in the peri-weaning period at 3–5 weeks of age, and 12 in the later post-weaning period; *i.e.* 6–11 weeks of age). In 13 farms, piglets of 3–5 weeks of age were not available for sampling, whereas in 14 other farms, piglets of 6–11 weeks of age were not available, both due to the specific BMS or multi-site production. The piglets within each herd were selected randomly from as many different pens in the nursery as possible. Sampling was always performed by the same trained veterinarian.

TBS sampling procedure

TBS samples were obtained following restraint of the piglets with a nose snare, and subsequent use of a mouth opener. The aspiration tube used (CH12 × 50 cm, Medinorm) was inserted through the mouth and glottis down to the tracheo-bronchial bifurcation where mucus was collected through gentle swab movement.

The tip of the swab was collected in a sterile 10 mL polystyrene tube (MLS), mixed with 1 mL sterile saline and kept at 3–5 °C until analysis within 48 h of sampling.

Analysis of TBS

The material collected by TBS was processed in a *M. hyopneumoniae* mhp183 real-time-PCR (Strait *et al.*, 2008). Briefly, nucleic acid was extracted from TBS using an RNA/DNA isolation kit (MagMAX Pathogen RNA/DNA Kit, Life Technologies) and an automated nucleic acid isolation processor (MagMAX Express 96 processor, Life Technologies) based on magnetic bead technology.

One millilitre of TBS was centrifuged for 5 min at 16,000 *g*, the pellet suspended in 400 µL lysis buffer, and 400 µL of the suspension was used as the sample. If no pellet was observable, 300 µL of the TBS was used as the sample. Bead mix and lysis/binding solution were added and the mix transferred onto a 96-well plate in the processor. Nucleic acid isolation was performed according to the manufacturer's instructions.

The PCR results were reported as negative or positive for the presence of *M. hyopneumoniae*. The detection limit for *M. hyopneumoniae* reported by Strait *et al.* (2008) was from 10 ng/µL to 2.5 fg/µL. The detection limit for the PCR was validated for TBS spiked with dilutions of *M. hyopneumoniae* strain J (ATCC 25934) of at least 5 fg/µL.

Data categorization for seasonality

In order to assess associations between climatic parameters and *M. hyopneumoniae* infection, herds were categorized into four groups based on the sampling season. Sampling was undertaken at the following rates in each season: S1 (winter), 47 herds, 1422 piglets; S2 (spring),

48 herds, 1334 piglets; S3 (summer), 25 herds, 723 piglets; and S4 (autumn), 56 herds, 1809 piglets.

Climatic data

Meteorological information with air temperatures including minima and maxima (°C; T, Tmax and Tmin, respectively), relative humidity (percentage; RH), rainfall (L/m^2; P), wind direction (°; WD) and wind speed (m/s; WS) were recorded daily from April 2011 to March 2012 based on data from the local meteorological institute (KNMI, Koninklijk Nederlands Meteorologisch Instituut) collected at a recording location (Valkenswaard, The Netherlands) central for the entire sampling area in both countries. In addition, a rolling average of the data from the last 1, 4 and 10 weeks before the day of sampling was calculated for all sampling days throughout the study period.

Piglet and farm prevalences and within-herd prevalence

Piglet and herd *M. hyopneumoniae* prevalences were calculated both overall for the entire study period and per season (S1–S4). A farm was considered *M. hyopneumoniae*-positive if at least one of the piglets sampled in the specified age category was positive for *M. hyopneumoniae*. The within-herd prevalence was calculated as the percentage of *M. hyopneumoniae*-positive herds per *M. hyopneumoniae* prevalence category, with categories assigned as follows: 0%, 1–10%, 11–20%, etc.

Statistical analysis

Logistic mixed regression models using first-order penalized quasi-likelihood algorithms were developed in MlWiN 2.02 (Centre of Multilevel Modeling). Herd was included as a random effect to correct for clustering of piglets within a herd. Initially, univariable associations were tested between the binary outcome variables: (1) presence of *M. hyopneumoniae* at the piglet level at 3–5 weeks (0 = negative for *M. hyopneumoniae* at 3–5 weeks; 1 = positive for *M. hyopneumoniae* at 3–5 weeks) and (2) presence of *M. hyopneumoniae* at the piglet level at 6–11

weeks (0 = negative for *M. hyopneumoniae* at 6–11 weeks; 1 = positive for *M. hyopneumoniae* at 6–11 weeks), and the independent variables season or the different climate parameters measured on the day of sampling (T, Tmin, Tmax, WD, WS, RH, and P), over the last week (w) (T_1w, Tmax_1w, Tmin_1w, WD_1w, WS_1w, RH_1w, and P_1w), the last 4 weeks (T_4w, Tmax_4w, Tmin_4w, WD_4w,WS_4w, RH_4w, and P_4w) and the last 10 weeks (T_10w, Tmax_10w, Tmin_10w, WD_10w, WS_10w, RH_10w, and P_10w) before sampling.

Statistical significance in this step was assessed at $P < 0.20$. Spearman correlation coefficients were calculated for the significant independent variables to avoid multicollinearity. If two independent variables had a correlation coefficient ≥ 0.6, only one was selected for further analysis based on biological relevance. In the third step, separate multivariate models were fitted for the two dependent variables.

Results

Seasonal variation in *M. hyopneumoniae* prevalence at piglet and farm level

Over all of the farms we sampled, the prevalence of *M. hyopneumoniae* in piglets at 3–5 weeks of age was 7.1% (95% confidence interval [CI] 6.2–8.0%) while at 6–11 weeks of age it was 10.9% (95% CI 9.6–12.2%). On the *M. hyopneumoniae*-positive farms, the prevalence at the piglet level of *M. hyopneumoniae* increased from 26.3% (95% CI 13.3–39.3%) at 3–5 weeks of age to 36.8% (95% CI 23.0–50.6%) at 6–11 weeks of age.

In 3–5 weeks old piglets, the highest piglet level prevalence of 10.0% (95% CI 7.6–12.4%) was observed in S2 (spring), followed by S1 (winter) at 8.4% (95% CI 6.5–10.0%), S4 (autumn) at 6.2% (95% CI 4.8–7.5%) and S3 (summer) at 2.3% (95% CI 0.7–3.7%) (Figure 1). These differences were not statistically significant. In contrast there was a significant effect of season in the older group ($P = 0.05$). In S3 (summer), the prevalence in the older piglets was only 1.6% (95% CI 0.3–2.9%), compared with S4 (autumn) when 15.5% (95% CI 12.6–18.4%) of piglets tested positive (Figure 1).

The overall percentage of *M. hyopneumoniae*-positive herds was almost the same for the weanling piglets (27.0%; 95% CI 20.8 – 33.8%) and the older post-weaning piglets (29.0%; 95% CI 22.4 – 35.6%) (Figure 2). The percentage of *M. hyopneumoniae*-positive herds with weanling piglets followed the same trends as the individual piglet prevalence, resulting in the highest

percentage in S2 (spring; 34.9%; 95% CI 20.6–49.1%) and the lowest percentage in S3 (summer; 17.4%; 95% CI 1.9–32.9%). In the older post-weaning piglets, the percentage of *M. hyopneumoniae*-positive herds was highest in S4 (autumn; 42.6%; 95% CI 28.4–56.6%), followed by S2 (spring; 31.9%; 95% CI 18.6–45.2%). The lowest percentage of *M. hyopneumoniae*-positive herds was again observed during S3 (summer; 12.0%; 95% CI −0.7–24.7%).

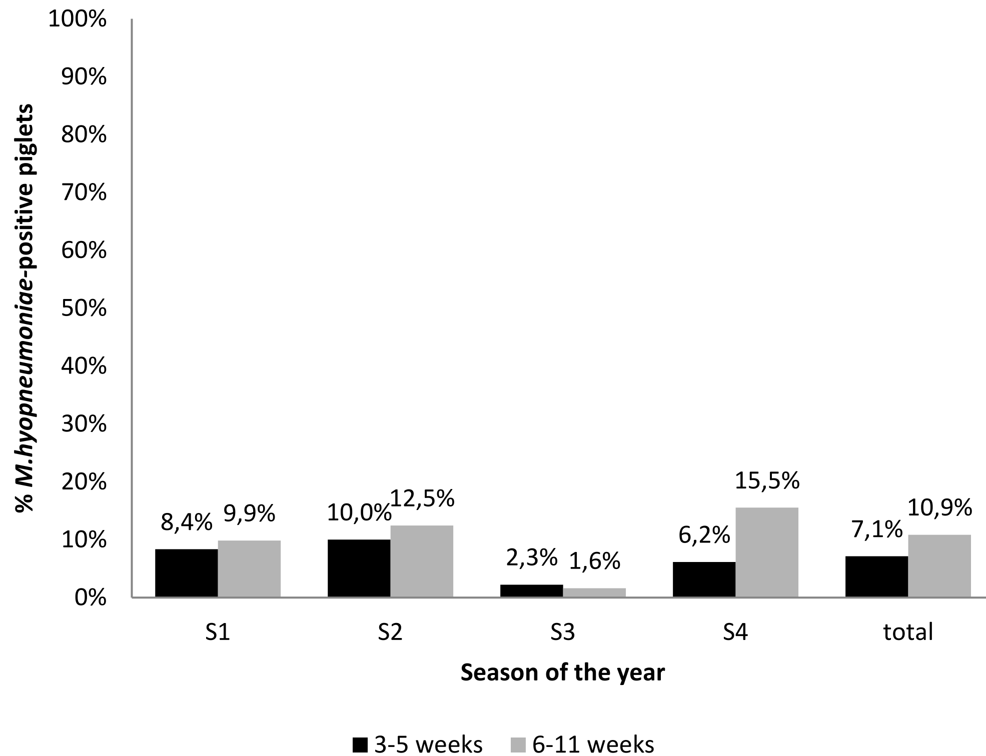

Figure 1. Bar chart showing the percentage of *M. hyopneumoniae*-positive piglets at 3–5 weeks of age (black bars) and 6–11 weeks of age (grey bars) in Belgium and The Netherlands during subsequent seasons throughout the year. S1, winter; S2, spring; S3, summer; and S4, autumn.

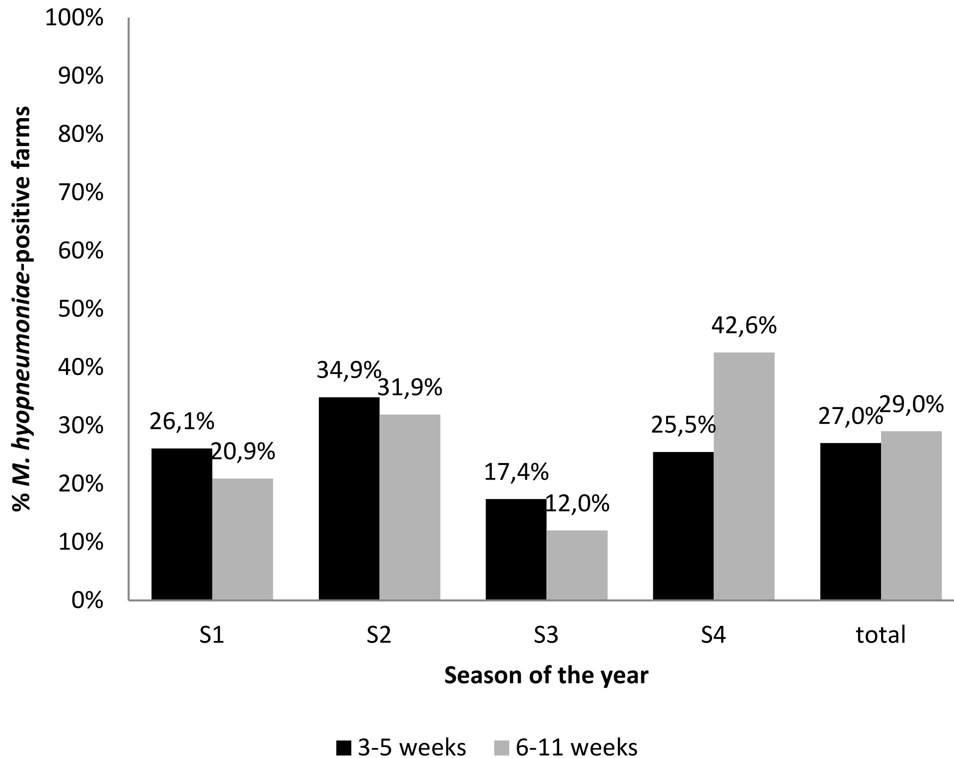

Figure 2. Bar chart showing the percentage of *M. hyopneumoniae*-positive herds at 3–5 weeks of age (black bars) and 6–11 weeks of age (grey bars) in Belgium and The Netherlands during subsequent seasons throughout the year. S1, winter; S2, spring; S3, summer; and S4, autumn.

The distribution of herds with different categories of within-herd *M. hyopneumoniae* prevalence is given in Figure 3. At 3–5 weeks of age, 59.1% of the *M. hyopneumoniae*-positive herds had a within-herd prevalence of *M. hyopneumoniae* between 1 and 20%. At 6–11 weeks of age, the pattern was more scattered with 40.4% of the *M. hyopneumoniae*-positive herds having a within-herd prevalence of *M. hyopneumoniae* between 1% and 20% and 25.5% having a within-herd prevalence of *M. hyopneumoniae* of > 50% (Figure 3).

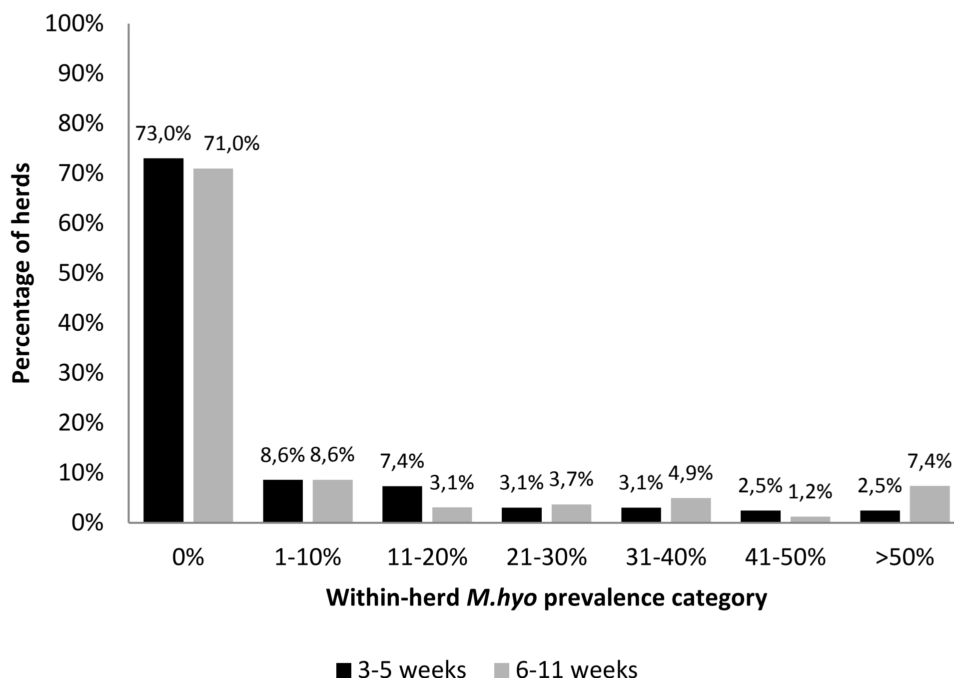

Figure 3. Bar chart showing the within-herd *M. hyopneumoniae* prevalence of 176 Belgian and Dutch pig herds sampled at 3–5 weeks of age (black bars) and 6–11 weeks of age (grey bars).

Impact of climatic factors on test positivity of *M. hyopneumoniae*

A moderate correlation was observed between rainfall and relative humidity ($r = 0.270$; $P < 0.001$), and relative humidity and ambient temperature ($r = -0.372$; $P < 0.001$). Rainfall was not correlated with ambient temperature ($r = 0.002$; $P = 0.983$). The probability for 3–5 week old piglets being *M. hyopneumoniae*-positive was negatively associated with rainfall (β = -0.029; OR = 0.971; $P = 0.02$) during the week preceding the sampling. In 6–11 week old piglets, the odds of being *M. hyopneumoniae*-positive at the piglet level were significantly affected by season with a 20.9 × higher odds of *M. hyopneumoniae* detection in S4 than S3 ($P < 0.01$). The average outdoor relative humidity over the 4 weeks preceding sampling (β = 0.032; OR = 1.032; $P < 0.05$) and the minimum average outdoor temperature during the week preceding sampling (β = 0.015; OR = 1.014; $P < 0.01$) were also positively associated with the probability that piglets of 6–11 weeks old would be *M. hyopneumoniae*-positive.

DISCUSSION

In the present study, the prevalence of *M. hyopneumoniae*-positive piglets around weaning (3–5 weeks of age) was in the same range as has been reported by many other recent studies (Calsamiglia and Pijoan, 2000; Fano *et al.*, 2007; Sibila *et al.*, 2007a; Nathues *et al.*, 2010; Villarreal *et al.*, 2010; Fablet *et al.*, 2012b). Nevertheless, when compared to Villarreal *et al.* (2010), on a herd level, a significantly lower percentage of *M. hyopneumoniae*-positive herds was detected around weaning age (3–4 weeks of age), which may be due to differences in study design, principally the inclusion criteria and the sampling procedure. Villarreal *et al.* (2010) only included pig herds with a recent history of coughing in grower-finisher pigs, whereas our study herds had no indicative clinical signs of respiratory disease in peri-weaned or post-weaned piglets, or in fattening pigs.

In a recent French study which evaluated data from pigs from 125 herds (Fablet *et al.*, 2012b), it was found that 33.6% of the farms had at least one *M. hyopneumoniae* PCR-positive piglet at 4 weeks of age increasing to 39.2% at 10 weeks of age, slightly higher than the prevalences reported in the present study (27.0 and 29.0% at 3–5 and 6–11 weeks of age, respectively). The within-herd prevalence was also higher, 14.1% and 16.1% at 4 and 10 weeks of age, respectively, compared to the 7.1% and 10.9% at 3–5 and 6–11 weeks of age in the present study. Again these differences are likely to be due to differences in prevalence of respiratory disease, with ~2/3 of the herds in the French study having respiratory disease detected at slaughter.

In our study, the difference in *M. hyopneumoniae* prevalence between piglets at 3–5 weeks of age and the subsequent group of 6–11 weeks of age can be explained by an age effect and a batch-to-batch variation. Also, Fano *et al.* (2007) showed an important disparity between-batch prevalence (0 to 51.3%), which was observed when different batches were sampled over a year. However, in our study, different piglet batches in the same herd were sampled concurrently, in contrast to Fano *et al.* (2007) who sampled subsequent batches at weaning over the entire year. Nevertheless, during most seasons (winter, spring and autumn), an increase in *M. hyopneumoniae* test-positive piglets between 3–5 weeks of age and 6–11 weeks of age was observed, which is in agreement with transmission results obtained in previous studies (Meyns *et al.*, 2004; Meyns *et al.*, 2006; Villarreal *et al.*, 2011b), which show a clear increase in the percentage of *M. hyopneumoniae*-positive piglets between young weanling piglets and those of older weaner pigs.

The effect of weather on *M. hyopneumoniae* prevalence in peri-weaned and post-weaned piglets was partly in accordance with the findings of Segalés *et al.* (2012), who showed a positive relation, at the piglet level, between *M. hyopneumoniae*-positive nasal swabs and rainfall and between *M. hyopneumoniae*-positive serology and weekly temperature. In our study, a significant negative association with rainfall was observed for piglets around weaning (3–5 weeks of age), whereas in older weaners (6–11 weeks of age), season of the year, outdoor relative humidity and minimum outdoor temperature were positively associated with the probability of being *M. hyopneumoniae*-positive at the piglet level.

Overall, the odds of being *M. hyopneumoniae*-positive at piglet level were highest during autumn (S4) and lowest during summer (S3), which, in Belgium and The Netherlands, coincides with the high rainfall season and the highest ambient temperatures with the lowest relative humidity, respectively. In previous studies, a higher frequency and transmission of respiratory problems occurred during cold wet seasons or lower environmental temperatures (Goodwin, 1985; Dee *et al.*, 2010; Otake *et al.*, 2010), with more favourable conditions for the survival of *M. hyopneumoniae*. *Mycoplasma hyopneumoniae* is known to be very sensitive to UV-light and dry environments under summer conditions (Jorsal and Thomsen, 1988), whereas survival for at least 31 days in water at temperatures of 2–7 °C is possible (Goodwin, 1985). These associations with weather conditions explain the seasonal differences in *M. hyopneumoniae* that we found.

In our study, batch-to-batch variations could be observed, consistent with the findings of other studies (Fano *et al.*, 2007; Sibila *et al.*, 2007b). Therefore, predicting the presence of early infection may be impossible. Nevertheless, some studies have shown a significant relationship between prevalence of early *M. hyopneumoniae* infections in piglets and subsequent prevalence of lung lesions at slaughter (Fano *et al.*, 2007; Sibila *et al.*, 2007b), although this is not always the case (Vranckx *et al.*, 2012a).

CONCLUSIONS

On Belgian and Dutch rearing farms, this study showed that piglets may become infected by *M. hyopneumoniae* very early in their life, with up to 10% of animals being infected at 3–5 weeks of age. Further spread of *M. hyopneumoniae* during the post-weaning period was also apparent, as demonstrated by higher prevalences (up to 15.5%) at 6–11 weeks of age.

Significant associations of *M. hyopneumoniae* prevalence with several climatic conditions, such as rainfall, outdoor relative humidity and minimum outdoor temperature could be observed. Overall, the odds of being *M. hyopneumoniae*-positive at piglet level in Belgium and The Netherlands were highest during autumn and lowest during summer.

CHAPTER 6 – SHORT COMMUNICATION: CONFIRMATION OF *MYCOPLASMA HYOPNEUMONIAE* IN A BREEDING HERD THROUGH TRACHEO-BRONCHIAL SWAB SAMPLING AND PCR

Adapted from:

Vangroenweghe F, Willems E, Thas O, Maes D (2018). Short communication: confirmation of *Mycoplasma hyopneumoniae* in a breeding herd through tracheo-bronchial swab sampling and PCR. Veterinary Record, 183, 1-3. doi: 10.1136/vr.104712.

ABSTRACT

Mycoplasma hyopneumoniae occurs worldwide and causes major economic losses to the pig industry. Dedicated programs to monitor for freedom of *M. hyopneumoniae* were developed within breeding companies delivering high health breeding animals, using serology (ELISA) as the preferential approach to screen for *M. hyopneumoniae.* A cluster of two high health breeding farms in Eastern-Europe were shown positive for *M. hyopneumoniae* using the conventional ELISA serology, confirmation analysis with another ELISA test did not exclude potential infection. Throughout the monitoring period, no coughing, typical lung lesions at necropsy or at slaughter could be detected which could confirm the *M. hyopneumoniae* health status. Since suspected *M. hyopneumoniae* ELISA results have an enormous economic impact on further nucleus breeding herd activities, it is essential to confirm the *M. hyopneumoniae* health status. Using PCR testing of TBS, samples confirmed the presence of *M. hyopneumoniae* in the herd that was serologically negative or doubtful for *M. hyopneumoniae* and without typical lesions and clinical signs of *M. hyopneumoniae* infection. Pigs (n = 186) of different ages (4, 8, 22 and 26 weeks of age) and breeding gilts (27, 31 and 35 weeks of age) and sows were sampled using TBS and subsequent qPCR analysis. TBS samples demonstrated no early detection at 4-8 weeks of age, however older rearing gilts were found at least partially (10-25%) positive. This positive status extended to gilts present in the sow herd with 100% positivity from 27 weeks of age onwards. Detection of genetic material of the pathogen present in the herd through *in vivo* sampling using TBS and subsequent qPCR analysis is a valuable confirmation tool in herds with suspected presence of *M. hyopneumoniae*, although clinical symptoms and lung lesions could not be detected. Future sampling programs certifying high health breeding herds free of *M. hyopneumoniae* should apply this *in vivo* sampling technique coupled to PCR detection to confirm their health status related to *M. hyopneumoniae.*

INTRODUCTION

Mycoplasma hyopneumoniae occurs worldwide and causes major economic losses to the pig industry. Affected pigs show chronic coughing, are more susceptible to other respiratory infections and have reduced performance (Maes *et al*., 2008). On endemically infected farms, piglets may become infected with *M. hyopneumoniae* during the suckling period and pigs can be tested positive from weaning onwards (Villareal *et al*., 2011; Fablet *et al*., 2012a; Vangroenweghe *et al*., 2015a).

Dedicated programs to monitor for freedom of *M. hyopneumoniae* were developed within breeding companies delivering high health breeding animals. Serology is the preferential approach in order to screen for *M. hyopneumoniae*, using an ELISA test (Sørensen *et al*. 1997; Andreasen *et al*., 2000b; Reynolds *et al*., 2009; Meyns *et al*., 2011). In case of positive serology, further decisions on farm health status and related consequences are based on additional confirmation tests. Currently used ELISA tests have variability in time to seroconvert following natural infection (Sibila *et al*., 2009), variation in ELISA results and low test sensitivity (35-65% depending on the test used; Erlandson *et al*., 2005), difficult interpretation in young animals because of possible MDA and variations in the detection of antibodies to different *M. hyopneumoniae* strains (Strait *et al*., 2004). When SPF breeding pig herds become infected with *M. hyopneumoniae*, the interval to emergence of clinical symptoms is a matter of concern, because transfer of subclinically infected breeding animals to customers constitutes a potential risk of spreading the pathogen (Thomsen *et al*., 1992).

Clinical diagnosis of enzootic pneumonia can be verified by serological analysis (Sørensen *et al*., 1997b). However, in SPF programs, herd prevalence of *M. hyopneumoniae* infections is often low and the positive herd predictive value of a serological result decreases progressively with the decreasing herd prevalence (Sørensen *et al*., 1992). In the Danish SPF program, final verification of herd infection with *M. hyopneumoniae* is consequently performed by demonstration of the presence of the pathogen (Sørensen *et al*., 1997b). Bacteriological isolation of *M. hyopneumoniae* from affected lungs is considered the ‘gold standard’ diagnostic technique but *M. hyopneumoniae* culture requires specialized isolation medium, is laborious, time-consuming (isolation from field samples requires 4–8 weeks) and can frequently become overgrown (Sibila *et al*., 2009). Other techniques (IHC, IFA) specifically detect *M. hyopneumoniae* in lung tissue

sections or smears, but have the drawback that diagnosis can only be made post-mortem. Moreover, only a small piece of lung tissue is tested when using these techniques, increasing the risk of a false-negative result (Sibila *et al.*, 2007a). Therefore, other innovative sampling techniques, such as TBS sampling (Fablet *et al.*, 2010; Vangroenweghe *et al.*, 2015a, Vangroenweghe *et al.*, 2015b) have been introduced in combination with qPCR testing for *M. hyopneumoniae* to reliably detect the pathogen.

STUDY OBJECTIVE

The objective of the current case report is to show the added value of TBS sampling in the confirmation of a suspect *M. hyopneumoniae*-seropositive situation.

MATERIALS AND METHODS

Case description

A cluster of two high health breeding farms in Eastern-Europe (2000-sow herd, farm A; 3600-gilt rearing facility, farm B) had been negative for *M. hyopneumoniae* for 10 years using a standard serological monitoring schedule (2x/year; 25 samples per farm). Besides freedom of *M. hyopneumoniae*, the farms were also negative for *P. multocida* DNT+, *Sarcoptes scabiei* var. *suis*, *Hematopinus suis*, *B. hyodysenteriae*, PRRSV and *A. pleuropneumoniae*. At weaning, piglets from farm A are moved to farm B, gilts produced at farm B are re-introduced in farm A at 26 weeks of age.

The farms were shown positive (2015; farm A, 59/183; farm B, 19/116) for *M. hyopneumoniae* using the conventional ELISA serology (Idexx HerdChek Mhyo ELISA, indirect ELISA; Idexx Laboratories). Looking for confirmation with a second ELISA test (BioChek *Mhyo* Ab kit; BioChek), however, samples initially showed negative serology, but in a later phase confirmed the positive serology (farm A, 39/59; farm B, 11/19). However, throughout the entire monitoring period, no coughing was observed and neither lung lesions typical for *M. hyopneumoniae* at necropsy nor at slaughter could be detected. Evolution of the % of positive animals per sampling date and farm (A and B) are given in Figure 1.

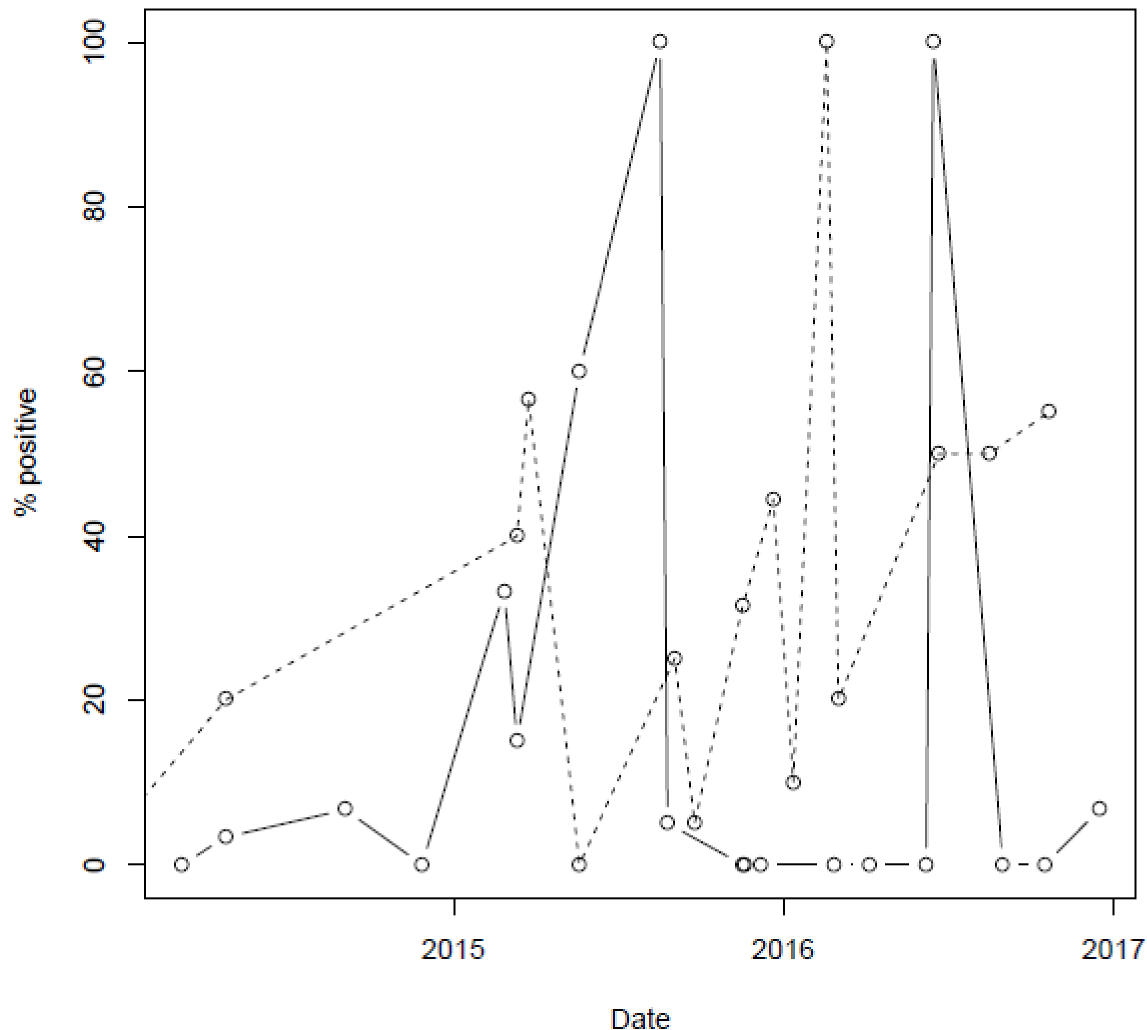

Figure 1. Evolution of % of positive animals (based on S/P-ratio > 0.40) in regular monitoring ELISA test (IDEXX HerdChek Mhyo ELISA; IDEXX Laboratories) in both herd A (sow herd, dotted line, $n = 30$ per time point) and herd B (gilt rearing herd, straight line, $n = 20$ per time point).

Under practical field conditions, positive ELISA results only, without other clinical indications of the presence of a *M. hyopneumoniae* infection on the farm, remain difficult to accept by the farmer as the consequences create a significant economic impact on the nucleus breeding herd activities. Therefore, the detection of genetic material of the pathogen ensures a greater degree of specificity and PCR is increasingly considered the diagnostic test of choice for samples obtained *in vivo* or at necropsy (Strait *et al*., 2008).

Sampling for determination of freedom of disease

Statistical calculation to assess the minimal sample number to show 'freedom of disease' in the cluster was performed (http://epitools.ausvet.com.au) with the assumption of maximum 2% prevalence for *M. hyopneumoniae.* Based on this calculation, 186 samples were collected from pigs of different ages: 4, 8, 22 and 26 weeks, gilts (27, 31 and 35 weeks) and sows within the cluster.

Diagnostic approach with TBS

Tracheo-bronchial sampling was performed as previously described (Vangroenweghe *et al*., 2015a; Vangroenweghe *et al*., 2015b). Briefly, TBS samples were obtained following restraint of the piglets with a nose snare, and subsequent use of a mouth opener. The aspiration tube used (CH12 × 50 cm; Medinorm) was inserted through the mouth and glottis down to the tracheo-bronchial bifurcation where mucus was collected through gentle swab movement. The tip of the swab was collected in a sterile 10 mL polystyrene tube (MLS), mixed with 1 mL sterile saline and kept at 3–5 °C until analysis within 48 h of sampling.

Analysis of TBS

The material collected by TBS was processed in a *M. hyopneumoniae* mhp183 real-time-PCR (Strait *et al*., 2008). Briefly, nucleic acid was extracted from TBS using an RNA/DNA isolation kit (MagMAX Pathogen RNA/DNA Kit; Life Technologies) and an automated nucleic acid isolation processor (MagMAX Express 96 processor; Life Technologies) based on magnetic bead technology. One millilitre of TBS was centrifuged for 5 min at 16,000 *g*, the pellet suspended in 400 µL lysis buffer, and 400 µL of the suspension was used as the sample. If no pellet was observable, 300 µL of the TBS was used as the sample. Bead mix and lysis/binding solution were added and the mix transferred onto a 96-well plate in the processor. Nucleic acid isolation was performed according to the manufacturer's instructions. The PCR results were reported as negative or positive for the presence of *M. hyopneumoniae*. The detection limit for *M. hyopneumoniae*

reported (Strait *et al*., 2008) was from 10 ng/µL to 2.5 fg/µL. The detection limit for the PCR was validated for TBS spiked with dilutions of *M. hyopneumoniae* strain J (ATCC 25934) of at least 5 fg/µL.

Statistical analysis

To determine potential association between animals positive for *M. hyopneumoniae* serology in farm A and B, logistic regression was used for a range of different lag times . The strongest association was selected by means of the Akaike's Information Criterion (AIC), and the resulting parameter estimates and *P*-values were reported. To account for model selection (selecting model with smallest AIC) a bootstrap procedure was implemented in which the outcome variable (positive serology in B) was resampled to mimic the null hypothesis. In each bootstrap run (1000 runs) the model selection procedure was applied and the parameter estimate of the optimal model was retained. The set of 1000 parameter estimates served as the null distribution.

Results

The results of the AIC demonstrated a significant positive association between serologically *M. hyopneumoniae*-positive animals (% positive) in farm A and farm B 30 days earlier (OR = 20.51, 95% CI [1.79; 246.06]; $P = 0.0147$). Collected TBS samples were negative until 8 weeks of age, but older gilts before (22 and 26 weeks of age) and after (27,31 and 35 weeks of age) introduction in the breeding herd were shown *M. hyopneumoniae*-positive (Figure 2).

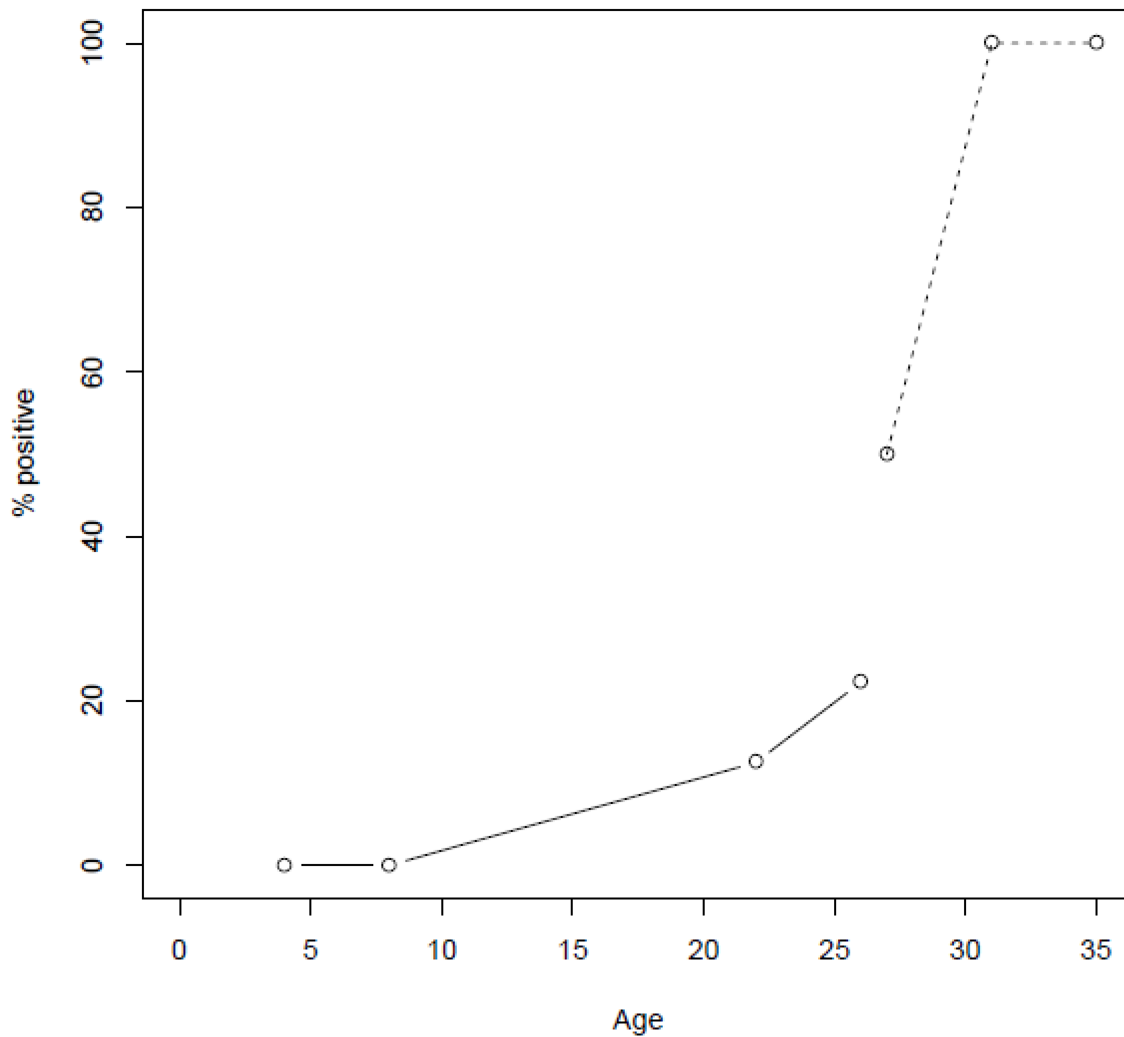

Figure 2. Tracheo-bronchial swab (TBS) sampling results of different age categories at the gilt rearing herd (4-8-22-26 weeks of age; straight line) and older gilts (27-31-35 weeks of age; dotted line) after introduction into the sow herd. The figure shows the percentage of *M. hyopneumoniae*-positive TBS samples using a qPCR test for *M. hyopneumoniae*.

Discussion

The current case report demonstrated that diagnosis of a *M. hyopneumoniae* infection at low prevalence is difficult using the standard monitoring tools (Sørensen *et al*. 1997; Maes *et al*., 2008; Meyns *et al*., 2011). Therefore, collection of TBS samples to confirm the *M. hyopneumoniae* health status of the breeding herd was essential to take crucial decisions concerning health

management within the breeding company. Analysis of the *M. hyopneumoniae* infection kinetics based on serology showed a clear positive association between serological results in both farms in relation to the introduction of gilts into the sow herd. TBS samples demonstrated no early infection at 4-8 weeks of age, however, older rearing gilts were found at least partially (10-25%) positive, in contrast to the large variation observed with serology (0-60%) depending on the time of sampling. This positive status extended to the gilts present in the sow herd with 100% positivity from 27 weeks onwards.

From a diagnostic point of view, however, TBS sampling and subsequent qPCR detection of *M. hyopneumoniae* should not be used as a first line diagnostic tool for standard monitoring purposes. Other more convenient and cheaper sampling techniques, such as blood sampling and ELISA testing, are more appropriate as a first line diagnostic approach. This routine sampling protocol should be secondarily supported by other relevant diagnostic sampling, such as necropsy of dead or euthanized animals and/or samples collected upon slaughterhouse lung lesion checks. Only when these different diagnostic components are inconclusive to confirm the presence of *M. hyopneumoniae* in the herd, a TBS/qPCR detection method should be applied to definitely confirm the *M. hyopneumoniae* status of the herd.

Confirmation of the positive *M. hyopneumoniae* status resulted in a subsequent discussion on *M. hyopneumoniae* elimination plans and schedules which were feasible and suitable for the specific farm (Rautiainen *et al*., 2001; Heinonen *et al*., 2011).

CONCLUSIONS

In conclusion, qPCR testing of TBS samples confirmed the presence of *M. hyopneumoniae* in a herd that was serologically negative or suspect for *M. hyopneumoniae* and without typical lesions and clinical signs of *M. hyopneumoniae* infection, and allowed the farmer to implement proper health management practices to control or eliminate *M. hyopneumoniae* from the breeding herd.

CHAPTER 7 – CASE REPORT: USE OF TRACHEO-BRONCHIAL SWAB qPCR TESTING TO CONFIRM *MYCOPLASMA HYOPNEUMONIAE* SEROPOSITIVITY IN A SPF BREEDING HERD

Adapted from:

Vangroenweghe F, Willems E, Malášek J, Thas O, Maes D (2018). Use of tracheo-bronchial swab qPCR testing to confirm *Mycoplasma hyopneumoniae* seropositivity in a SPF breeding herd. Porcine Health Management, 3, 24-30. doi: 10.1186/s40813-018-0088-3.

ABSTRACT

A dedicated program to monitor for freedom of several economically important diseases is present within most of the breeding companies that currently deliver high health breeding animals to their customers. Serology is therefore the preferential approach in order to screen for most of these diseases, including *M. hyopneumoniae*. However, in case of positive serology, further decisions on farm health status and the related consequences should be based on additional confirmation tests. The current case report demonstrates that TBS sampling is a suitable alternative to confirm a suspect *M. hyopneumoniae*-seropositive situation. A Central-European SPF herd was shown positive (90% positive, 10% suspect; *n* = 10) for *M. hyopneumoniae* using the conventional ELISA serology (Idexx HerdChek Mhyo ELISA; Idexx Laboratories) and a second ELISA test (IDEIA™ *Mycoplasma hyopneumoniae* EIA kit; Oxoid – Thermo Scientific) did not exclude potential *M. hyopneumoniae* infection (10% positive, 70% suspect; *n* = 10). Further follow-up remained inconclusive on both tests. Throughout the entire monitoring period of 6 months, no coughing, necropsy lesions or lesions at slaughter could be detected which could confirm the *M. hyopneumoniae* health status. TBS sampling was used to confirm the health status for *M. hyopneumoniae*. In total, 162 samples were collected at different ages (*n* = 18 per age category): piglets at 3-6-9-12 and 15 wks of age, rearing gilts at 18-21-24 and 27 weeks of age. Collected TBS samples were negative for *M. hyopneumoniae* until 15 wks of age, but rearing gilts were highly *M. hyopneumoniae*-positive from 18 wks onwards with 87-100% *M. hyopneumoniae*-positive animals and qPCR Ct values between 25 and 33. This case report shows that collection of TBS samples to confirm the *M. hyopneumoniae* infection status of a breeding herd was able to provide additional information – namely *M. hyopneumoniae* infection limited to the gilt rearing section only – to serology in order to make crucial decisions concerning health management and eradication strategies within the breeding herd.

INTRODUCTION

Mycoplasma hyopneumoniae, the primary pathogen of enzootic pneumonia, occurs worldwide and causes major economic losses to the pig industry. The pathogen adheres to and damages the ciliated epithelium of the respiratory tract. Affected pigs usually show chronic coughing, are more susceptible to other respiratory infections and have a reduced performance (Maes *et al*., 2008). Moreover, *M. hyopneumoniae* plays a key role in PRDC through interactions with several other respiratory pathogens.

Piglets can become infected with *M. hyopneumoniae* during the suckling period and many studies have shown *M. hyopneumoniae*-positive animals from weaning onwards (Fano *et al*., 2007; Sibila *et al*., 2007a; Villarreal *et al.,* 2010; Fablet *et al*., 2012a; Vangroenweghe *et al*., 2015a; Vangroenweghe *et al*., 2015b). Moreover, once infected with *M. hyopneumoniae*, animals can excrete the pathogen over a long period of time, with total clearance lasting until 254 days post-infection (Pieters *et al*., 2009). This implies that infected gilts could carry *M. hyopneumoniae* well across their first pregnancy into their first lactation cycle, infecting their offspring with *M. hyopneumoniae* in early life (Pieters & Fano, 2016).

Therefore, dedicated programs to monitor for freedom of *M. hyopneumoniae* have been developed within breeding companies that currently deliver high health breeding animals to their customers. Serology using ELISA is the preferential approach in order to screen for *M. hyopneumoniae* (Morris *et al*., 1995; Sørensen *et al*., 1997b; Andreasen *et al*., 2000b; Martelli *et al*., 2006; Reynolds *et al*., 2009; Meyns *et al*., 2011). In case of positive serology, further decisions on farm health status and the related consequences should be based on additional confirmation tests. Clinical diagnosis of enzootic pneumonia can be verified by serological analysis (Sørensen *et al*., 1997b). However, in SPF programs, the herd prevalence of *M. hyopneumoniae* infections is often low and the positive herd predictive value of a serological result decreases progressively with the decreasing herd prevalence (Sørensen *et al*., 1992). Moreover, ELISA testing of sera from naturally infected pigs does not detect early-stage infection prior to seroconversion (Sørensen *et al*., 1993; Sitjar *et al*., 1996), and infection and vaccination responses are indistinguishable. Under field conditions, the mean time to onset of coughing following a *M. hyopneumoniae* infection was 13 days, whereas the mean time between onset of coughing and seroconversion as measured by ELISA was 9 days (Sørensen *et al*., 1997b). Recent research has shown that currently used ELISA

tests only start showing a seroconversion from 21 days post-infection onwards (Pieters *et al*., 2017). The percentage of animals seroconverting in the early stages of *M. hyopneumoniae* infection using one of the commercially available *M. hyopneumoniae* ELISAs remains relatively low (16-22% at 21 days and 35-45% at 28 days post-infection) (Pieters *et al*., 2017). This implies that a large number of samples is needed to reliably detect the presence of *M. hyopneumoniae* within the monitored herd. In the Danish SPF program, the final verification of herd infection with *M. hyopneumoniae* is consequently performed by demonstration of the agent (Sørensen *et al*., 1997b). A recent comparative study on diagnostic sampling approach for *M. hyopneumoniae* detection showed that LS were a reliable option to establish early detection of *M. hyopneumoniae*, followed by BALF and nasal swabs (Pieters *et al*., 2017). Other innovative sampling techniques, such as TBS sampling (Fablet *et al*., 2010; Vangroenweghe *et al*., 2015a; Vangroenweghe *et al*., 2015b) have been introduced in combination with PCR detection of *M. hyopneumoniae* to reliably detect the pathogen of infected animals.

STUDY OBJECTIVE

The objective of the current case report is to show that TBS sampling is a suitable method to confirm a suspect *M. hyopneumoniae*-seropositive situation.

MATERIALS AND METHODS

Case description

A high health breeding farm in Central Eastern Europe (220-sow herd) had been negative for *M. hyopneumoniae* for more than 20 years using a standard serological monitoring schedule (3x/year; 25 samples per time point) with a commercially available *M. hyopneumoniae* ELISA test (Idexx HerdChek Mhyo ELISA, indirect ELISA; Idexx Laboratories). Besides freedom of *M. hyopneumoniae*, the farm was also negative for *P. multocida* DNT+, *Sarcoptes scabiei* var. *suis*, *B. hyodysenteriae*, PRRSV and *A. pleuropneumoniae*.

External biosecurity is at the highest level, with no entrance to visitors and strict shower protocols for all farm personnel upon entrance of the farm. Internal biosecurity is also well

established with boot hygiene (washing and disinfection) between production groups (sows, piglets, rearing gilts), clean disinfection baths at entrance of each individual compartment and no movement of other materials (cleaning equipment, pig handling materials, etc.) between production groups.

The sow farm is run on a 3-week BMS with 7 groups of 32 sows each. Productive sows, weaned piglets and rearing gilts are housed in separate buildings on the premises. Successive batches of weaned piglets from 4 weeks until 16 weeks of age are housed in separate nursery compartments with strict AI/AO strategies. From 16 weeks of age onwards, rearing gilts are housed in a larger barn that is not managed according to AI/AO strategies.

Standard serological monitoring for *M. hyopneumoniae*

At gilt delivery (27 weeks of age), regular serological sampling to assess *M. hyopneumoniae* status was performed throughout the last decade, repeatedly confirming the *M. hyopneumoniae*-negative status. The farm first tested positive for *M. hyopneumoniae* using the first ELISA serology (Idexx HerdChek Mhyo ELISA, indirect ELISA, Tween 20 extract of *M. hyopneumoniae*, Se 30-39%, Sp 100% (Erlandson *et al*., 2005); Idexx Laboratories) in March 2017. Additional monitoring one month later (Idexx) confirmed the *M. hyopneumoniae* positivity and therefore, a second ELISA test (IDEIA™ *Mycoplasma hyopneumoniae* EIA kit, competitive or blocking ELISA, monoclonal antibodies against 74 kDa protein, Se 77.5%, Sp 99.3% (Levonen *et al*., 1999); Oxoid – Thermo Scientific) was performed, demonstrating a clear evolution towards lower *M. hyopneumoniae*-positivity in both ELISA tests used. This decreasing trend did however not persist in the fourth sampling showing again a gradual increase in *M. hyopneumoniae* titers. The number of positive samples obtained with both ELISA tests on each sampling date are given in Table 1.

Table 1. *Mycoplasma hyopneumoniae* standard ELISA (Idexx HerdChek Mhyo ELISA, indirect ELISA; Idexx Laboratories; S/P-ratio, sample to positive ratio) monitoring results from March 2017 onwards. Results of the second ELISA test (IDEIA™ *Mycoplasma hyopneumoniae*; Oxoid – Thermo Scientific; PI, percentage of inhibition) are also given. Table demonstrates total number of samples and number of samples with negative (Idexx, $S/P < 0.30$; IDEIA™, $PI \geq 65\%$), suspect (Idexx, $0.30 \leq S/P \leq 0.40$; IDEIA™, $50\% \geq PI > 65\%$) or positive (Idexx, $S/P > 0.40$; IDEIA™, $PI < 50\%$) *M. hyopneumoniae* ELISA results. Mean titers (± SEM) for Idexx ELISA (expressed as S/P-ratio) and IDEIA™ ELISA (expressed as PI) are given per sampling time point.

Date	**Analytical test**	**Analytical result**			**Results (mean±SEM)**	**Total sample number**
		Negative	**Suspect**	**Positive**		
20.3.2017	Idexx	0	1	9	0.86±0.15	10
	IDEIA™	2	7	1	59.7±7.7%	10
21.4.2017	Idexx	11	7	7	0.43±0.08	25
	IDEIA™	17	4	4	65.4±14.3%	25
25.4.2017	Idexx	18	0	4	0.17±0.04	22
	IDEIA™	4	0	0	90.3±15.0%	4
9.8.2017	Idexx	11	1	8	0.36±0.06	20
	IDEIA™	2	0	2	58.0±11.8%	4

Monitoring of clinical signs and lung lesions

Moreover, throughout the entire monitoring period, no coughing, lung lesions at necropsy or at slaughter could be detected.

Epidemiological information: *M. hyopneumoniae* in neighboring farms and wind direction

Several other swine farms, some belonging to the same production group, are located within a range of 2-3 km from the described SPF farm. Farm VS, a fattening unit directly related to the SPF source farm, is located at 1.35 km air distance in western direction and Farm VH, an unrelated 35-sow herd, is located at 2.25 km air distance in south-south-eastern direction. Epidemiological information on disease state, including *M. hyopneumoniae*, is actively exchanged among these different farms. Under the local conditions, wind direction is most often from a western direction with high wind speed (source: http://oze.tzb-info.cz/vetrna-energie/9800-vetrne-podminky-v-ceske-republice-ve-vysce-10-m-nad-povrchem-ii). During the last quarter of 2017, both farms (VS, fattening unit and VH, sow unit) were detected *M. hyopneumoniae*-positive on serological monitoring using the conventional ELISA test (Idexx HerdChek Mhyo ELISA, indirect ELISA; Idexx Laboratories).

Diagnostic approach with TBS

Tracheo-bronchial sampling was performed as previously described (Vangroenweghe et al., 2015a; Vangroenweghe et al., 2015b). Briefly, TBS samples were obtained following restraint of the piglets with a nose snare, and subsequent use of a mouth opener. The aspiration tube used (CH12 × 50 cm; Medinorm) was inserted through the mouth and glottis down to the tracheo-bronchial bifurcation where mucus was collected through gentle swab movement. The tip of the swab was collected in a sterile 10 mL polystyrene tube (MLS), mixed with 1 mL sterile saline and kept at 3–5 °C until analysis within 48 h of sampling.

Analysis of TBS

The material collected by TBS was processed in a *M. hyopneumoniae* mhp183 real-time-PCR (Strait *et al*., 2008). Nucleic acid was extracted from TBS using an RNA/DNA isolation kit (MagMAX Pathogen RNA/DNA Kit; Life Technologies) and an automated nucleic acid isolation processor (MagMAX Express 96 processor; Life Technologies) based on magnetic bead technology. One millilitre of TBS was centrifuged for 5 min at 16,000 *g*, the pellet suspended in 400 µL lysis buffer, and 400 µL of the suspension was used as the sample. If no pellet was observable, 300 µL of the TBS was used as the sample. Bead mix and lysis/binding solution were added and the mix transferred onto a 96-well plate in the processor. Nucleic acid isolation was performed according to the manufacturer's instructions. The PCR results were reported as negative ($Ct \geq 37$) or positive ($Ct < 37$) for the presence of *M. hyopneumoniae* based on a Ct threshold value. The detection limit range for *M. hyopneumoniae* reported (Strait *et al*., 2008) was from 10 ng/µL to 2.5 fg/µL. The detection limit for the PCR was validated for TBS spiked with dilutions of *M. hyopneumoniae* strain J (ATCC 25934) of at least 5 fg/µL.

Sampling for determination of freedom of disease

The minimal number of samples needed to show 'freedom of disease' in the farm was calculated (http://epitools.ausvet.com.au), using FreeCalc – sample size calculation for freedom testing with imperfect tests using the modified hypergeometric distribution for exact hypothesis testing, with the assumption that maximum 2.5% of the animals (n = 6000) present on the farm were positive for *M. hyopneumoniae,* assuming test sensitivity and specificity of the TBS/qPCR combination of 75 and 100%, respectively (Strait *et al*., 2008; Fablet *et al*., 2010). Based on this calculation, a total of 162 samples were collected at different ages (n = 18 per age category): piglets at 3-6-9-12 and 15 weeks of age and rearing gilts at 18-21-24 and 27 weeks of age.

Statistical analysis for TBS positivity and Ct value

For the assessment of the overall effect of sampling date on the probability of a positive qPCR result, a Pearson chi-squared test was used. Pairwise comparisons between sampling dates

was also performed with Pearson chi-squared tests, with *P*-values adjusted with the Holm procedure. All per-comparison *P*-values were computed by referring to the permutation null distribution of the test statistics. The latter were approximated based on 2000 random permutations. The overall effect of age ($n = 9$ age groups) on the average Ct values was assessed by means of an F-test in a one-way ANOVA. Post-hoc multiple comparisons of means were performed with Tukey's method. The overall effect of age ($n = 9$ age groups) on the probability of a positive qPCR result was assessed by means of the Pearson chi-squared test. Multiple pairwise comparisons were also done with Pearson chi-squared tests, with *P*-values adjusted with Holm's procedue.

All overall tests were performed at the 5% level of significance. Multiple comparison tests were performed simultaneously at the 5% familywise error rate (FWER) level. All data analyses were performed with the R statistical software version 3.4.3. (R Core Team, 2007).

RESULTS

During lactation and nursery phase (from 3 until 15 weeks of age), no *M. hyopneumoniae* could be detected using qPCR testing. However, once the animals entered the rearing barn located at the same site, *M. hyopneumoniae* was clearly present. Among the gilts from 18 until 27 weeks of age, the percentage of positive animals significantly increased ($P < 0.05$) to 87-100% in the oldest age group (Figure 1). PCR Ct values initially decreased with age, starting at 29 in animals of 18 weeks of age, to 25 in animals of 21 weeks of age; and subsequently increased up to 30 and 33 in animals of 24 and 27 weeks of age, respectively (Figure 1). Ct values were significantly different ($P < 0.05$) from 37, the cut-off value for qPCR test positivity.

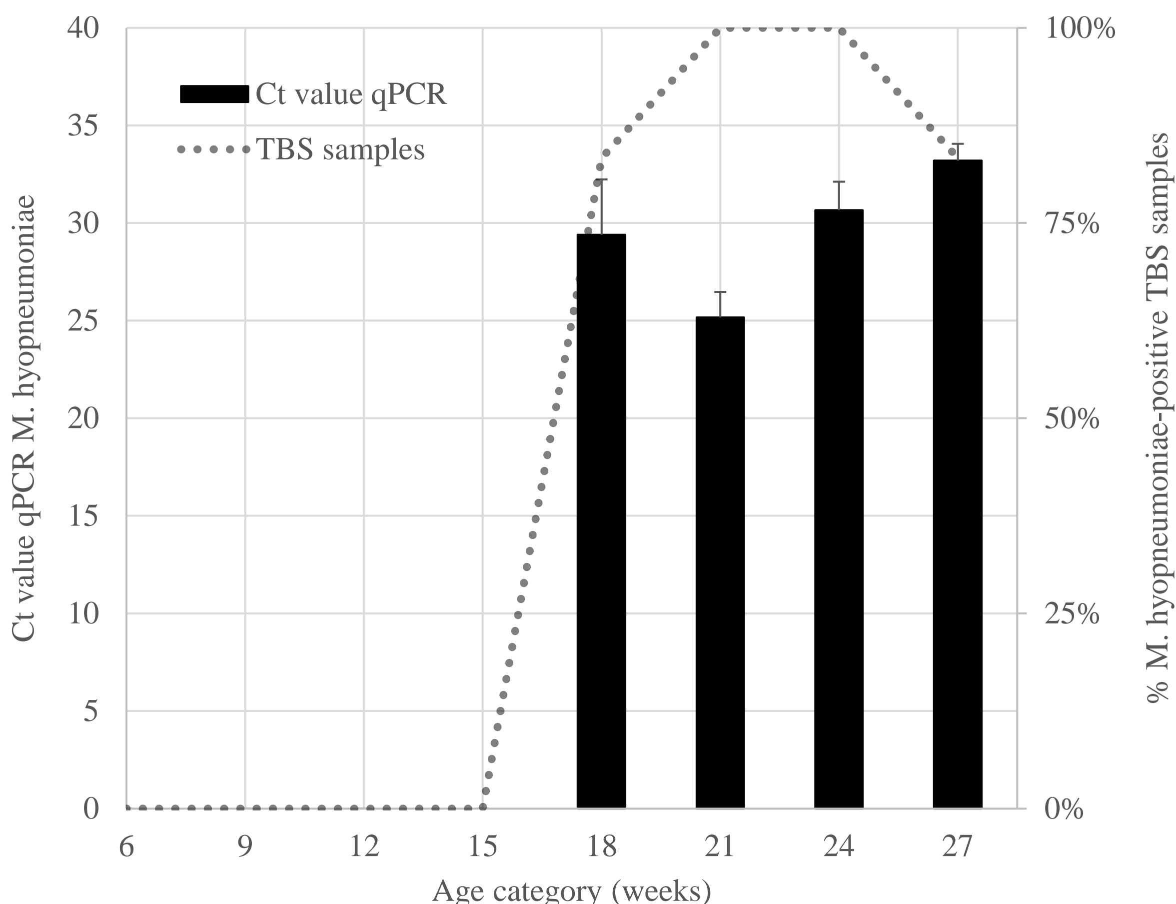

Figure 1. Tracheo-bronchial swab (TBS) sampling results of different age categories (n=18 animals per age category) at the Central-European SPF herd. Piglets of 3-6-9-12 and 15 weeks and rearing gilt of 18-21-24 and 27 weeks of age were sampled. The qPCR *M. hyopneumoniae*-positive animals per age category were expressed as percentage (dashed grey line). The Ct values of those qPCR *M. hyopneumoniae*-positive TBS samples (Ct < 37) were expressed as means (± SEM; black bars).

Discussion

The current case report demonstrated that diagnosis of a *M. hyopneumoniae* infection can be difficult using the standard monitoring tools, such as clinical observation, serology (Sørensen *et al*., 1997b) and slaughterhouse checks for typical lung lesions (Maes *et al*., 2008). It was also shown that qPCR testing on TBS samples confirmed the *M. hyopneumoniae* health status of a

breeding herd. This allowed the farmer to make decisions concerning health management within the breeding company.

Interestingly, the collected TBS samples only showed *M. hyopneumoniae*-positive results from 18 weeks onwards, which is the age category that enters the gilt rearing barn. Until now, it is unclear how the *M. hyopneumoniae* infection has entered the farm. Generally, the major source of *M. hyopneumoniae* introduction into a farm is considered newly arrived subclinically *M. hyopneumoniae*-infected gilts, especially since *M. hyopneumoniae*-infected animals can excrete the pathogen for up to 254 days following initial infection (Pieters *et al*., 2009). However, this nucleus herd has not received any live breeding animals for many years.

A second hypothesis is that the *M. hyopneumoniae* entered the farm by airborne transmission from infected neighboring farms. Under favorable climatic conditions, aerogenous spread of *M. hyopneumoniae* between neighboring herds over varying distances has been demonstrated (Stärk *et al*., 1998; Dee *et al*., 2009; Otake *et al*. 2010). Indeed, the farm in the reported case had neighboring swine herds within a 1.5 to 3-km range. Moreover, epidemiological information shared among these neighboring farms clearly indicated that a break in *M. hyopneumoniae* health status had also happened during this year based on the results of their regular serological monitoring. Unfortunately, we do not have details on which of these swine farms first broke with its *M. hyopneumoniae* health status, although based on prevailing wind direction, farm VS which is located west from the SPF nucleus herd could be a good candidate.

Another source of potential introduction might be through a *M. hyopneumoniae*-infected transport vehicle. Especially under winter conditions, cleaning and disinfection of transport vehicles is not always easy to perform. Recently, *M. hyopneumoniae* survival on stainless steel at 4°C was demonstrated for at least 2 days (maximum 8 days for some strains) (Browne *et al*., 2017). This would imply that the regular procedure for SPF pig-free downtime of 48 hours for transport vehicles cannot always guarantee a 100% *M. hyopneumoniae*-free vehicle. Analysis of the *M. hyopneumoniae* infection kinetics on the farm revealed that the *M. hyopneumoniae* infection was first detected within the gilt rearing barn. This is also the location from which gilts are selected and loaded for external transport. Therefore, the probability of a biosecurity breach at the gilt loading point seems possible, since external biosecurity at all other levels has always been at the very high level. Moreover, once the *M. hyopneumoniae* infection entered this section of the farm, *M. hyopneumoniae* could easily further spread within the age groups from 18 until 27 weeks, which

are not managed under strict AI/AO conditions. The reason why no coughing nor typical lung lesions were present is not clear. It may be due to the rather good housing conditions, *e.g.* air quality and level of dust (Stärk, 1999; Stärk, 2000) and/or the fact that a low-virulent strain was circulating (Vicca *et al*., 2003).

Although a break in biosecurity seems unlikely, it should be considered as another option for *M. hyopneumoniae* introduction into the farm. A 4-year research demonstrated that a 1-night downtime period is sufficient to prevent mechanical spread of both PRRSV and *M. hyopneumoniae* by personnel and fomites (Pitkin *et al*., 2011). Moreover, that study concluded that basic sanitation procedures, such as hand hygiene and the use of both boots and coveralls should be enough to prevent mechanical spread and therefore, the implementation of a shower protocol is even not necessary (Pitkin *et al*., 2011). In the present breeding farm, besides regular personnel, showering in on every entry, no visitors were admitted, except for the TBS sampling needed to confirm the *M. hyopneumoniae* infection status.

Further diagnostics is ongoing to confirm the *M. hyopneumoniae* infection status of the sow population, since TBS could not detect *M. hyopneumoniae*-positive piglets till 15 weeks of age. Based on the results of this monitoring, a specified eradication plan will be designed to result in a renewed *M. hyopneumoniae*-negative gilt outflow to the end customers.

CONCLUSIONS

The present case report showed that for several months, *M. hyopneumoniae* infection in a SPF herd may occur without clinical symptoms and typical lung lesions. In addition, serological testing may be difficult to interpret, and qPCR testing on TBS may be needed to establish a conclusive diagnosis.

CHAPTER 8 – GENERAL DISCUSSION

The general discussion will focus on the different steps in *M. hyopneumoniae* diagnostics we have taken throughout the study of early detection of *M. hyopneumoniae*. First, we will discuss *M. hyopneumoniae* serology and the benefits of BMS. Subsequently, TBS will be discussed and compared to other live pig sampling techniques. The results obtained under US conditions and the seasonal effects observed in Belgium and The Netherlands will be further elaborated. Finally, the implication of *M. hyopneumoniae* detection in two SPF herds will be discussed in order to come to a final conclusion. The general discussion will close with some practical implications and options for future research. A general overview is given in Table 1.

Table 1. General overview of the different research chapters with the tests being used, the study aims and a summary of the most important findings.

Chapter	Tests	Study aim	Summary of findings
3	ELISA	✓ Effect of transition from 1-week to 4- or 5-week BMS on prevalence of swine pathogens	✓ Transition to 4- or 5-week BMS results in health bonus for: ✓ *M. hyopneumoniae* ✓ *A. pleuropneumoniae* ✓ *L. intracellularis*
4	TBS/qPCR	✓ Validation of TBS/qPCR compared to other sampling methods ✓ Determine *M. hyopneumoniae* prevalence under US conditions	✓ TBS results in the best recovery of *M. hyopneumoniae* in live animals ✓ Weaned piglets under US conditions have a very low *M. hyopneumoniae* prevalence (1.1%)
5	TBS/qPCR	✓ Prevalence of *M. hyopneumoniae* in Belgium and The Netherlands ✓ Impact of climatic factors	✓ 7.1% *M. hyopneumoniae*-positive piglets at 3-5 weeks of age, rising to 10.9% at 6-11 weeks of age ✓ Highest *M. hyopneumoniae* prevalence during autumn ✓ *M. hyopneumoniae* prevalence influenced by climatic factors
6	TBS/qPCR	✓ Confirm *M. hyopneumoniae* presence in a 2-site SPF-facility with suspect serological results	✓ *M. hyopneumoniae* presence confirmed in : ✓ Gilts (27-31 and 35 weeks of age) in sow herd ✓ Gilt rearing gilt facilities at 22 and 26 weeks of age
7	TBS/qPCR	✓ Confirm *M. hyopneumoniae* presence in a SPF-herd with suspect serological results	✓ *M. hyopneumoniae* presence confirmed in : ✓ Gilt rearing unit from 18 weeks of age onwards

The diagnostic approach towards *M. hyopneumoniae*, a major pathogen in PRDC with a large economic impact on profitability of modern pig production, remains a difficult issue under practical conditions (Sibila *et al.*, 2009; Maes *et al.*, 2017; Pieters *et al.*, 2017). Throughout the years, a broad variety of diagnostic methods have been developed and evaluated for diagnosis of *M. hyopneumoniae*. These diagnostic methods range from assessment of clinical signs of coughing and coughing index (Morris *et al.*, 1995; Maes *et al.*, 1999; Thacker *et al.*, 2000; Vicca *et al.*, 2003; Meyns *et al.*, 2006; Marois *et al.*, 2007), macroscopic and microscopic lesions at necropsy, including immunohistochemical (Sarradell *et al.*, 2003; Rodriguez *et al.*, 2004) and immunofluorescent identification in lung tissue samples (Kobisch *et al.*, 1978; Piffer and Ross, 1985). Additionally, different lung scoring systems at slaughter have been developed (Garcia-Morante *et al.*, 2016). Laboratory diagnostics comprise serology from complement fixation tests to ELISA tests based on several specific adhesion factors (Strait *et al.*, 2004; Ameri-Mahabadi *et al.*, 2005; Erlandson *et al.*, 2005) and molecular identification of the pathogen in several sample types, such as lung tissue, nasal swabs (Calsamiglia and Pijoan, 2000; Sibila *et al.*, 2007a; Nathues *et al.*, 2013a), LS, BALF (Kurth *et al.*, 2002; Marois *et al.*, 2008) and TBS (Marois *et al.*, 2008; Fablet *et al.*, 2010).

However, under field conditions and for standard monitoring purposes, swine veterinarians and routine diagnostic laboratories have limited their approach mostly to clinical signs, macroscopic and microscopic evaluation upon necropsy, including lung lesion scoring at slaughter and serological monitoring. Clinical signs and lung lesions can only give a tentative diagnosis, which needs further confirmation with laboratory tests. Recently, the use of mobile systems (SOMO; SoundTalks NV, Leuven, Belgium) for cough recording at barn level with subsequent analysis of coughing patterns have been developed and validated under field conditions (Maes *et al.*, 2017). This potential tool might support early *M. hyopneumoniae* diagnosis, although it still remains difficult for such tools to specifically differentiate coughing by *M. hyopneumoniae* from other major pathogens involved in PRDC, such as SIV, PRRSV and *A. pleuropneumoniae*. Therefore, even with a positive indication on clinical *M. hyopneumoniae*-indicative coughing, confirmation through pathogen identification remains crucial for further implementation of treatment or preventive measures.

The early detection of *M. hyopneumoniae* through necropsy or lung lesion scoring systems at slaughter also remains under discussion. The lesions, namely purple to grey consolidated areas

affecting predominantly the apical and middle lobes and eventually the cranial part of the diaphragmatic lobes, identified in both necropsy or slaughterhouse assessment are suggestive, but not pathognomonic for *M. hyopneumoniae* infection (Maes *et al.*, 2017). Other pathogens such as SIV or *P. multocida* should be considered as most probable differential diagnoses (Sibila *et al.*, 2009). Moreover, since animals are dead or slaughtered at the moment of the diagnosis, the definition of 'early' detection can not really be applied and curative or preventive measures will only have an effect on the next batches of animals within the same production system.

For several decades, therefore, serological tests – more specifically ELISA – have been used to detect and monitor *M. hyopneumoniae* at herd level. Early studies have shown a serious delay between the initial clinical signs of coughing and the first detection of *M. hyopneumoniae* using ELISA of about 22 days (Sørensen *et al.*, 1997). This delay in seroconversion has recently been reconfirmed for two commercially available ELISA tests, demonstrating a minimum interval of 21 days before the first incomplete seroconversion could be detected in both ELISA tests (Pieters *et al.*, 2017). Moreover, substantial differences exist between different ELISA tests towards their reactivity following initial seroconversion (Erlandson *et al.*, 2005; Pieters *et al.*, 2017) in naive animals. Therefore, serological monitoring can neither be considered as a tool for early *M. hyopneumoniae* detection under field conditions. Nevertheless, for routine farm monitoring and follow-up on *M. hyopneumoniae* infection pattern changes due to adaptations of management, such as a transition from a 1-week to a 4-week or 5-week BMS, serological tests have shown their value (Chapter 3). Under these circumstances, details on exact infection time point or age have no particular importance and focus is mainly on overall health status in relation to *M. hyopneumoniae*. Therefore, the most important questions to be addressed are: 1/ are piglets from gilts more susceptible to be infected with *M. hyopneumoniae*, 2/ is the herd conducted in a 1-, 2- or 3-site production, and 3/ is there a regional effect concerning swine herd density. In Chapter 3, several age categories were sampled at fixed time points and long-term evolution in *M. hyopneumoniae* infection pressure was assessed. Under these conditions, the observed delay of at least 21 days in seroconversion with the currently available commercial ELISA tests has no practical importance, since a more general picture of the *M. hyopneumoniae* kinetics on the farms is generated. The study results clearly showed a significant impact of chosen farm BMS on the outcome of the *M. hyopneumoniae* health status, especially during the fattening period. The implementation of BMS has several health advantages on both intestinal (*L. intracellularis*) and

respiratory (*M. hyopneumoniae*, *A. pleuropneumoniae*) diseases mainly due to some major structural adaptations in farm and animal management. As already mentioned, the development of BMS originates in France more than 50 years ago. At the start, the system was designed for very small herds run in a 3-week BMS with 7 batches and an inventory of 56 sows at herd level. Later on, other variations were developed such as the 2-, 4-, 5- and 7-week BMS. The fixed interval of 2-3-4 or 5 weeks between batches implies no possibilities for overlay of runt and small piglets between consecutive farrowing batches during the lactation period – especially in 4- and 5-week BMS with only one single farrowing room - limiting the spread of different pathogens between groups. Moreover, under practical conditions, BMS results in a larger number of animals per batch and more strict AI/AO-rules to be implemented for compartments within the nursery and fattening units. This results in a more structured approach for the caretaker to visit the different compartments or units according to age category and therefore, omit deliberate or undeliberate transmission of several pathogens through fomites such as boots, coveralls and other materials. Our results also indicated that newly introduced gilts and reproductive sows in all parities were highly *M. hyopneumoniae*-seropositive, which is in accordance with earlier studies (Calsamiglia and Pijoan, 2000; Sibila *et al.*, 2007a; Lehner *et al.*, 2008b). Taking into account that newly introduced gilts with an active *M. hyopneumoniae* infection can excrete the pathogen for up to 254 days post-infection, it is quite important to know their status at introduction. Infection in replacement gilts during quarantine/adaptation or even following introduction into the herd could potentially compromise the *M. hyopneumoniae* infection status of young piglets, since these might already be infected during the lactation period (Pieters & Fano, 2016).

Taking these considerations into account, a reliable and early detection of *M. hyopneumoniae* in living animals under field conditions is urgently needed in order to be able to confirm an upcoming infection as early as possible. This is crucial under modern pig farming conditions to efficiently apply curative and/or preventive measures to omit further spread within the farm. Especially lactating gilts with an active *M. hyopneumoniae* infection present in the farrowing room imply a major risk for further transmission of *M. hyopneumoniae* to their offspring, resulting in a fairly high number of *M. hyopneumoniae*-positive piglets at weaning (Calsamiglia and Pijoan, 2000; Ruiz *et al.*, 2003; Fano *et al.*, 2007; Sibila *et al.*, 2007a; Moorkamp *et al.*, 2009; Fablet *et al.*, 2010; Nathues *et al.*, 2010; Villarreal *et al.*, 2010; Nathues *et al.*, 2013a). However, until recently, most *M. hyopneumoniae* prevalence studies in piglets and sows have been

conducted using the most easily applicable sampling technique, namely nasal swabs (Calsamiglia and Pijoan, 2000; Ruiz *et al.*, 2003; Fano *et al.*, 2007; Sibila *et al.*, 2007a; Villarreal *et al.*, 2010; Nathues *et al.*, 2013a). A study comparing different sampling techniques for *M. hyopneumoniae* recovery in piglets has demonstrated that nasal swabs have 3.89 times less sensitivity in recovering *M. hyopneumoniae* from infected piglets as compared to the TBS technique, with LS and BALF in an intermediate position (1.39 and 1.09 times less sensivitity compared to TBS, respectively) (Fablet *et al.*, 2010). However, the study was conducted with only 60 piglets inoculated with *M. hyopneumoniae* under experimental conditions, which might differ from *M. hyopneumoniae* infection under field conditions towards infection dose, strain type and housing conditions. Nevertheless, based on these experimental results (Fablet *et al.*, 2010), *M. hyopneumoniae* prevalence results might be underestimated when using nasal swabs under field conditions.

In order to further elaborate on the TBS sampling technique, a first study (Chapter 4) was conducted to evaluate the performance of TBS in comparison to nasal swabs and post-mortem deep airway swabs, BALF and lung tissue samples. Comparison of 3 extraction methods on these samples demonstrated consistent results in all three methods for TBS, whereas other sampling techniques such as nasal swabs had a limited recovery rate (6.25%). All three post-mortem sampling techniques – BALF, lung tissue samples and deep airway swabs – had a good recovery, though as already stated earlier, for future monitoring purposes, sampling techniques in live animals should be preferred. Our study (Chapter 4) also confirmed the results of Fablet *et al.* (2010) concerning BALF, being a little less sensitive for the recovery of *M. hyopneumoniae* than TBS, although in our study BALF was conducted only post-mortem. However, to our opinion, BALF sampling technique has several disadvantages for live piglet sampling as compared to TBS, being the potential need to sedate the animal before the procedure, the use of a more rigid catheter and the variability in the volume of fluid recovered from the lavage in different piglets, which might subsequently interfere with the detection limit of the qPCR used to detect *M. hyopneumoniae* in these samples. Looking into more detail into the level of recovery of *M. hyopneumoniae* (based on the Ct values in the qPCR) for the live piglet sampling techniques, TBS samples resulted in the lowest Ct values, whereas nasal swabs had a significantly lower yield in the qPCR (Chapter 4). Only deep airway swab samples revealed a comparable recovery to TBS with lung tissue samples and BALF obtaining intermediate results. Though, these samples – except for BALF – can only

be taken in dead animals and are therefore not suitable for the intended purpose of live piglet monitoring.

The second part of this study (Chapter 4) evaluated the *M. hyopneumoniae* prevalence in suckling piglets between 12-25 days of days under US field conditions. The low *M. hyopneumoniae* prevalence observed in our study is consistent, to a certain degree, with previous studies (Fano *et al.*, 2007; Sibila *et al.*, 2007a; Nathues *et al.*, 2013a). Fano *et al.* (2007) demonstrated a low prevalence of *M. hyopneumoniae* in weaned piglets at 3 weeks of age with a quite large batch-to-batch variation. Both other studies reported *M. hyopneumoniae* prevalences of 3.6% to 3.8% using nasal swabs (Sibila *et al.*, 2007a; Nathues *et al.*, 2013a). However, the study design of these studies was different from our study, as all samples were collected in herds clinically affected with *M. hyopneumoniae*, whereas in our study, piglets were sampled in 3 randomly selected non-clinical farms. Therefore, even using a more performant sampling technique such as TBS, lower *M. hyopneumoniae* prevalence levels were obtained (Chapter 4), indicating a very low presence of *M. hyopneumoniae* in suckling piglets during the second part of lactation.

The risk of *M. hyopneumoniae* infection in pigs is not solely based on direct or indirect contact with potentially *M. hyopneumoniae*-infected animals, but it is also related to the presence of several other risk factors. These risk factors might be the distance to non-SPF herds, herd size, density of the pig population in a certain area (Stärk *et al.*, 1992), environmental factors such as dust, ammonia, carbon dioxide (Donham, 1991), dust particle size (Michiels *et al.*, 2015), stocking density and poor ventilation (Stärk, 1999; Stärk, 2000), presence of infected breeding stock in the herd, lack of a *M. hyopneumoniae* vaccination programme (Ostanello *et al.*, 2007), period of the year or season, especially temperature and sunlight (Dee *et al.*, 2010) and climatic parameters such as average daily temperature and average daily rainfall (Segalés *et al.*, 2012). Under Spanish conditions, rainfall and temperature had a significant impact on *M. hyopneumoniae* infection dynamics (Segalés *et al.*, 2012). However, climatic conditions largely differ between Spain and the conditions in Belgium and The Netherlands. Therefore, the association between early detection of *M. hyopneumoniae* in peri-weaned and post-weaned piglets and local climatic conditions was investigated across the year (Chapter 5). From the perspective of *M. hyopneumoniae*-positive piglets, the average *M. hyopneumoniae* prevalence of 7.1% in peri-weaned piglets (3-5 weeks of age) was comparable to other studies within the same age category (Calsamiglia and Pijoan, 2000;

Ruiz *et al.*, 2003; Sibila *et al.*, 2007a). In post-weaned piglets, the 10.9% prevalence of *M. hyopneumoniae* was in the same range as a limited number of studies that also sampled into this age category (Sibila *et al.*, 2007a; Moorkamp *et al.*, 2009), although Fablet *et al.* (2012) revealed a slightly higher prevalence (16.1%) in 10-week old piglets using the same live piglet TBS sampling technique. Our study (Chapter 5) also revealed some interesting seasonal *M. hyopneumoniae* prevalence data, demonstrating the highest percentage of *M. hyopneumoniae*-positive piglets during spring, whereas under summer conditions – in our region characterized by higher temperatures, little precipitation and many hours of sunlight – *M. hyopneumoniae* seems hardly detectable. Based on previous observations by Dee *et al.* (2010), this observation can indeed be confirmed, because the pathogen is highly sensitive to temperature and sunlight, both negatively affecting *M. hyopneumoniae* survival. The study (Chapter 5) under Belgian and Dutch conditions generally detected higher percentages of *M. hyopneumoniae*-positive piglets as compared to our previous study under US conditions (Chapter 4), which might be explained by the different age of sampled animals and large differences in farm and management structure between both regions. Whereas in Belgium and The Netherlands most sow farms keep their piglets on-site at least until 10-11 weeks of age, the sow farms included in the US study had no weaned piglets at the sow location. This might influence the overall *M. hyopneumoniae* infection pressure for the specific age categories present at the production site.

Besides the seasonal effects observed in our study, the effect of climatic parameters on the detected *M. hyopneumoniae* prevalence in peri-weaned and post-weaned piglets was in accordance with the findings by Segalés *et al.* (2012), who also demonstrated a positive relation at piglet level between *M. hyopneumoniae*-positive nasal swabs and rainfall, and between *M. hyopneumoniae*-positive serology and weekly temperature. In our study (Chapter 5), a significant negative association with rainfall was observed for peri-weaned piglets, whereas for post-weaned piglets, autumn, outdoor relative humidity and minimum outdoor temperature were positively associated with the presence of *M. hyopneumoniae* at piglet level. *Mycoplasma hyopneumoniae* infection in piglets around weaning has previously been associated with subsequent prevalence of *M. hyopneumoniae*-associated lung lesions at slaughter (Fano *et al.*, 2007; Sibila *et al.*, 2007b), although this is not always the case (Vranckx *et al.*, 2012a). Nevertheless, the ability to detect *M. hyopneumoniae* in live piglets at an early stage using TBS might help to better control the

subsequent spread of *M. hyopneumoniae* within the batch through strategic treatment or improved management, including preventive measures such as early vaccination against *M. hyopneumoniae*.

Monitoring of the farm health status using serology has been discussed previously, but, in some cases, intensive monitoring schedules are also applied to certify SPF herds from several economically important diseases, such as PRRSV and *M. hyopneumoniae*. Based on specific sampling schedules and preset assumptions of required certainty to detect a pathogen when present, SPF herds are monitored on a regular basis (3-4x per year).

However, taking into account the previously cited limitations of serological tests (Sørensen *et al.*, 1997; Pieters *et al.*, 2017), serious doubts could be made towards reliability of these monitoring schedules to detect a recent introduction of *M. hyopneumoniae* into the herd through airborne spread (Dee *et al.*, 2009; Dee *et al.*, 2010; Otake *et al.*, 2010) or mechanical vectors such as transport vehicles or contaminated visitors (Pitkin *et al*., 2011). Both case reports (Chapter 6-7) clearly demonstrate the aforementioned issues of discrepancy between different ELISA tests used for standard *M. hyopneumoniae* monitoring throughout the world. For nucleus breeding herds with an SPF status, it is of crucial importance to continuously guarantee the health status of all animals leaving the farm and therefore, no doubts in relation to *M. hyopneumoniae* infection status can be tolerated. Therefore, a more performant technique of live pig sampling with reliable early *M. hyopneumoniae* detection was used to clarify the doubtful and suspect status concerning potential presence of *M. hyopneumoniae* (Chapter 6-7). In both cases, suspect serological results obtained with the first screening ELISA could not be consistently confirmed using a second ELISA test. Moreover, both cases were complicated due to the absence of clinical signs of coughing or lung lesions at slaughter. The pathogen could never be identified from any lung tissue sample throughout the interval between the first positive ELISA tests and the final confirmation of its presence using TBS/qPCR. It can be concluded that TBS sampling of live piglets or pigs is the preferable method to obtain an early detection of *M. hyopneumoniae*. Therefore, future standard monitoring protocols on *M. hyopneumoniae*-free SPF herds should include, besides a broad serological screening, a dedicated TBS screening in order to cover the gap between a new potential *M. hyopneumoniae* infection and the detectable seroconversion which is at least 21 days (Pieters *et al.*, 2017) for most of the currently used commercial ELISA tests for *M. hyopneumoniae*. TBS can indeed play a crucial role in completing the entire clinical picture of a respiratory problem due

to *M. hyopneumoniae*, besided the use of other first and second line diagnostics such as serology, necropsy and lung lesion scoring.

Taking all these aspects into account, it is clear that an early and reliable diagnosis of *M. hyopneumoniae* infection under practical conditions in modern pig production has a major economic impact within our national and even broader international context. First, early diagnosis of *M. hyopneumoniae* in a batch of coughing fattening pigs may results in a rapid and efficient antimicrobial treatment with a lower amount of antibiotics used and less pathology resulting in growth retardation and prolonged days to slaughter. Secondly, our ability to already detect piglets infected with *M. hyopneumoniae* at weaning may help the field veterinarian and the pig farmer to make a correct decision on the age of piglet vaccination against *M. hyopneumoniae*. Thirdly, application of TBS sampling combined with qPCR detection within standard monitoring programs for SPF breeding herds would guarantee an earlier detection of newly introduced *M. hyopneumoniae* infection as compared to the currently used serological monitoring schedules. This could in turn limit economic damage within the SPF breeding supply chain to customers receiving certified *M. hyopneumoniae*-free replacement gilts.

Conclusions

- Positive effects of sow BMS are mainly related to the prolonged interval between consecutive production groups, limiting piglet overlay to later batches and thus reducing infection spread within the farm.
- Serological detection of *M. hyopneumoniae* is suitable for long-term health monitoring in *M. hyopneumoniae*-positive farms and to evaluate management adaptations that might impact the on-farm *M. hyopneumoniae* kinetics and infection pressure.
- When early and reliable detection of *M. hyopneumoniae* is required in animals that might suffer interference with presence of MDA against *M. hyopneumoniae* (piglets of 3-5 weeks of age), a more sensitive and performant live sampling technique is required, such as TBS sampling.
- In order to assess the potential application of TBS as compared to other live sampling techniques such as nasal swabs and BALF, comparison of the three techniques revealed superiority of TBS as to recovery of *M. hyopneumoniae*-positive animals and number of pathogens (as indicated by qPCR Ct values). Under US conditions, peri-weaned piglets

(12-25 days) had a low prevalence (1.1%) of *M. hyopneumoniae*, mainly due to the absence of specific clinical signs and the typical US sow herd management.

- Therefore, we further applied TBS to study the impact of climatic factors on the prevalence of *M. hyopneumoniae* in peri-weaned and post-weaned piglets, which revealed seasonal influences and specific climatic effects related to temperature and humidity. At piglet level, an average of 7.1% of the peri-weaned piglets were infected, whereas in post-weaned piglets 10.9% were detected *M. hyopneumoniae*-positive. Autumn, outdoor relative humidity and minimum outdoor temperature were positively associated with the presence of *M. hyopneumoniae* at piglet level.
- TBS was applied to confirm *M. hyopneumoniae* infection status of SPF herds with suspect serological results and proved to be a reliable and sensitive technique to recover *M. hyopneumoniae* from previously naive animals that were recently infected, resulting in confusing, inconclusive serological results.

Practical implications and future research

- The evolution in BMS systems is ongoing, not only towards the specific interval between two consecutive batches, but also concerning age at weaning. From a regulatory point of view, piglets can not be weaned under the age of 21 days in Europe, which might happen in some specific BMS systems. Therefore, further research to assess the most ideal BMS system for optimal farm health would be interesting comparing not only 2-3-4-5 weeks BMS but also their combinations with weaning at 21-24-27 days of age.
- *Mycoplasma hyopneumoniae* diagnosis should be further elaborated, especially towards the optimization of sampling protocols for specific field conditions, such as early detection of the pathogen, certification of freedom of disease or other diagnostic approaches. Nowadays, it becomes more and more important to have a rapid and reliable diagnosis and therefore PCR tests should be available wherever possible and needed. Recent evolutions towards on-farm test applications (LAMP and helicase-dependent amplification technology (Buchan & Ledeboer, 2014) and on-site PCR kits) should be further developed and validated in order to have immediate results. This would help to advice an efficient treatment, which could result in less antibiotic use as compared to treatment of animals already suffering from *M. hyopneumoniae* in a more chronic stage of the disease.

- In the near future, technology already applied in clinical and food microbiology will become readily available for use in veterinary diagnostic laboratories. Besides automation in the form of sample-to-result instrumentation for qPCR assay, which reduces labor and limits the risk for contamination during manipulation, multiplex tests are now available that enable single specimens to be interrogated for the presence of multiple pathogens associated with various clinical syndromes. Digital PCR and next-generation sequencing will push the landscape of molecular diagnostics further, allowing for analysis of complex, polymicrobial specimens and enabling accurate quantification of organisms present at very low levels (< 0.01% of the microbial consortium) in a specimen (Buchan & Ledeboer, 2014). Another promising technique is Matrix-Assisted Laser Desorption Ionization-Time to Flight MS (MALDI-TOF), which enables the identification of bacteria and other microorganisms by non-fragmenting or 'soft ionization' techniques (Buchan & Ledeboer, 2014). If these technologies become available for *M. hyopneumoniae* diagnostics, faster, more accurate diagnosis can be performed, especially if combined with a sampling technique that provides a high yield of pathogenic material, such as TBS.
- TBS combined with improved isolation methods – such as automation of primary processing and plating, coupled with initial culture examination aided by high-resolution optics (Buchan & Ledeboer, 2014) – could help to improve collection of field strains to further monitor antimicrobial sensitivity (MycoPath; Klein *et al*., 2017) which in turn would assist in reducing ineffective treatments against *M. hyopneumoniae* in case of antimicrobial resistance.
- *Mycoplasma hyopneumoniae* epidemiology remains a challenging domain of research, which still undergoes interesting new evolutions, especially within the field of early gilt exposure to *M. hyopneumoniae* in order to prevent late excretion during lactation that might affect the piglets' *M. hyopneumoniae* infection status. Recently, several studies have been performed to evaluate the optimal transmission of *M. hyopneumoniae* from infected to naive gilts through direct contact (Roos *et al.*, 2016; Yeske, 2018) or using more challenging techniques such as intra-tracheal inoculation (Yeske, 2018) or even aerosol applications of lung homogenate (Nickel *et al*., 2018; Yeske, 2018).
- In these cases, transmission success of *M. hyopneumoniae* is crucial for future stability of on-farm *M. hyopneumoniae* infection status (Figure 1) and should therefore be checked

using an early detection, such as TBS sampling. Interestingly, the above stated inoculation methods are still under development and evaluation concerning the inoculum dose, the number of subsequent exposure events and the efficacy to transmit *M. hyopneumoniae* to 100% of the animals exposed. Therefore, new experiments should be designed to determine the minimal exposure in order to obtain colonization of the respiratory tract. For these purpose, TBS could be applied as an early detection tool.

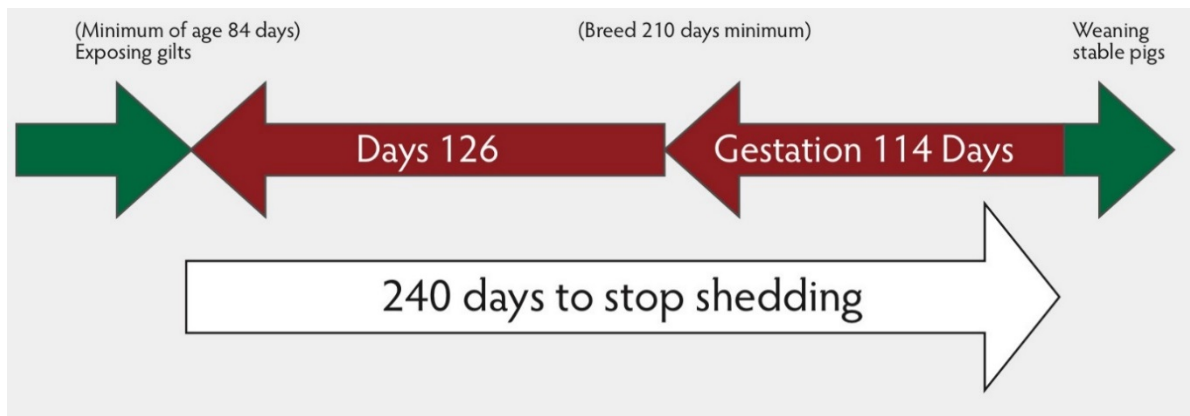

Figure 1. Gilt *M. hyopneumoniae* exposure timeline (adapted from Yeske, 2016).

- Besides the deliberate or accidental exposure of gilts to *M. hyopneumoniae*, checking the actual *M. hyopneumoniae* status is also of major importance. A recent survey on *M. hyopneumoniae* gilt introduction in conventional farms revealed major room for improvement, especially towards increasing the knowledge on the *M. hyopneumoniae* status of replacement gilts at the moment of arrival into the quarantine/adaptation facilities (Garza-Moreno *et al.*, 2017). Again, early *M. hyopneumoniae* detection is crucial in order to adapt the preventive measures to the current health status at gilt delivery. In case of *M. hyopneumoniae*-positive gilts, curative treatment might be considered, whereas in case of a *M. hyopneumoniae*-negative status, thorough vaccination with a *M. hyopneumoniae* vaccine might be considered to rapidly boost the gilt's immunity before exposure at introduction into the existing conventional *M. hyopneumoniae*-positive sow population (Garza-Moreno *et al.*, 2018).
- Finally, as piglet infection status for *M. hyopneumoniae* at weaning is a leading indicator towards the percentage of lung lesions at slaughter (Fano *et al.*, 2007), it still remains of

major importance to monitor their *M. hyopneumoniae* infection status on a regular basis. Especially since it has clearly been demonstrated that between-batch variability might be high and unpredictable based on the previous batch (Fano *et al.*, 2007). Within these monitoring schedules focused on early detection of *M. hyopneumoniae*, long before seroconversion may occur, TBS could play a predominant role in the near future.

REFERENCES

Abiven P, Blanchard B, Saillard C, Kobisch M, Bove JM (1992). A specific DNA probe for detecting *Mycoplasma hyopneumoniae* in experimentally infected pigs. Molecular and Cellular Probes 6, 423-429.

Alexander TJL, Thornton K, Boon G, Lysons RJ, Gush A (1980). Medicated early weaning to obtain pigs free from pathogens endemic in the herd of origin. Veterinary Record 106, 114-119.

Almeida PR, Andrade CP, Almeida LL, Oliveira LGS, Castro LA, Zlotowski P, da Silva SC, Driemeier D (2012). Nested-PCR for the detection of *Mycoplasma hyopneumoniae* in bronchial alveolar swabs, frozen tissues and formalin-fixed paraffin-embedded swine lung samples: comparative evaluation with immunohistochemical findings and histological features. Pesquisa Veterinária Brasileira 32, 715-720.

Ameri-Mahabadi M, Zhou E-M, Hsu WH (2005). Comparison of two swine *Mycoplasma hyopneumoniae* enzyme-linked immunosorbent assays for detection of antibodies from vaccinated pigs and field serum samples. Journal of Veterinary Diagnostic Investigation 17, 61-64.

Ameri M, Zhou E-M, Hsu WH (2006). Western blot immunoassay as a confirmatory test for the presence of anti-*Mycoplasma hyopneumoniae* antibodies in swine serum. Journal of Veterinary Diagnostic Investigation 18, 198-201.

Andreasen M, Bækbo P, Nielsen JP (2000a). Lack of effect of aerial ammonia on atrophic rhinitis and pneumonia induced by *Mycoplasma hyopneumoniae* and toxigenic *Pasteurella multocida*. Journal of Veterinary Medicine B 47, 161-171.

Andreasen M, Nielsen JP, Bækbo P, Willeberg P, Botner A (2000b). A longitudinal study of serological patterns of respiratory infections in nine infected Danish swine herds. Preventive Veterinary Medicine 45, 221-235.

Andreasen M, Mousing J, Krogsgaard Thomsen L (2001). No overall relationship between average daily weight gain and the serological response to *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* in eight chronically infected Danish swine herds. Preventive Veterinary Medicine 49, 19-28.

Arsenakis I, Panzavolta L, Michiels A, Del Pozo Sancristán R, Boyen F, Haesebrouck F, Maes D (2016). Efficacy of *Mycoplasma hyopneumoniae* vaccination before and at weaning against experimental challenge infection in pigs. BMC Veterinary Research 12, 63-69.

Arsenakis I, Michiels A, del Pozo Sancristán R, Boyen F, Haesebrouck F, Maes D (2017). *Mycoplasma hyopneumoniae* vaccination at or shortly before weaning under field conditions: a randomized efficacy trial. Veterinary Record 181, 19-25.

Artiushin S, Stipkovits L, Minion FC (1993). Development of polymerase chain reaction primers to detect *Mycoplasma hyopneumoniae*. Molecular and Cellular Probes 7, 381-385.

Artiushin S, Minion FC (1996). Arbitrarily primed PCR analysis of *Mycoplasma hyopneumoniae* field isolates demonstrates genetic heterogeneity. International Journal of Systematic Bacteriology 46, 324-328.

Assunção P, de la Fe C, Ramirez AS, Gonzáles Llamazares OR, Poveda JB (2005). Protein and antigenic variability among *Mycoplasma hyopneumoniae* strains by SDS-PAGE and immunoblot. Veterinary Research Communications 29, 563-574.

Baccaro MR, Hirose F, Umehara O, Gonçalves LCB, Doto DS, Paixão R, Shinya LT, Moreno AM (2006). Comparative efficacy of two single-dose bacterins in the control of *Mycoplasma hyopneumoniae* in swine raised under commercial conditions in Brazil. Veterinary Journal 172, 526-531.

Bandrick M, Pieters M, Pijoan C, Molitor TW (2008). Passive transfer of maternal *Mycoplasma hyopneumoniae*-specific cellular immunity to piglets. Clinical and Vaccine Immunology 15, 540-543.

Bandrick M, Pieters M, Pijoan C, Baidoo SK, Molitro TW (2011). Effect of cross-fostering on transfer of maternal immunity to *Mycoplasma hyopneumoniae* to piglets. Veterinary Record 168, 100-105.

Bandrick M, Theis K, Molitor TW (2014). Maternal immunity enhances *Mycoplasma hyopneumoniae* vaccination induced cell-mediated immune responses in piglets. BMC Veterinary Research 10, 124-134.

Barate AK, Cho Y, Truong QL, Hahn T-W (2014). Immunogenicity of IMS 1113 plus soluble subunit and chimeric proteins containing *Mycoplasma hyopneumoniae* P97 C-terminal repeat regions. FEMS Microbiology 352, 213-220.

Barcelo J, Marco E (1998). On farm biosecurity. In: Proceedings of the 15th International Pig Veterinary Society Congress. Birmingham, England. p.129-133.

Baskerville A (1981). Pneumonia in pigs: a review. New-Zealand Veterinary Journal 29, 216-218.

Batista L, Pijoan C, Ruiz A, Utrera V, Dee S (2004). Assessment of transmission of *Mycoplasma hyopneumoniae* by personnel. Journal of Swine Health and Production 12, 75-77.

Baumeister AK, Runge M, Ganter M, Feenstra AA, Delbeck F, Kirchhoff H (1998). Detection of *Mycoplasma hyopneumoniae* in broncho-alveolar lavage fluids of pigs by PCR. Journal of Clinical Microbiology 36, 1984-1988.

Blanchard B, Vena MM, Cavalier A, Le Lannic J, Gouranton J, Kobisch M (1992). Electron microscopic observation of the respiratory tract of SPF piglets inoculated with *Mycoplasma hyopneumoniae*. Veterinary Microbiology 30, 329-341.

Blanchard B, Kobisch M, Bové JM, Saillard C (1996). Polymerase chain reaction for *Mycoplasma hyopneumoniae* detection in tracheobronchiolar washings in pigs. Molecular and Cellular Probes 10, 15-22.

Boettcher TB, Thacker BJ, Halbur PG, Waters WR, Nutsch R, Thacker EL (2002). Vaccine efficacy and immune response to *Mycoplasma hyopneumoniae* challenge in pigs vaccinated against porcine reproductive and respiratory syndrome virus and *M hyopneumoniae*. Journal of Swine Health and Production 10, 259-264.

Bourry O, Fablet C, Simon G, Marois-Créhan C (2015). Efficacy of combined vaccination against *Mycoplasma hyopneumoniae* and porcine reproductive and respiratory syndrome virus in dually infected pigs. Veterinary Microbiology 180, 230-236.

Boye M, Jensen TK, Ahrens P, Hagedorn-Olsen T, Friis NF (2001). *In situ* hybridization for identification and differentiation of *Mycoplasma hyopneumoniae*, *Mycoplasma hyosynoviae* and *Mycoplasma hyorhinis* in formalin-fixed porcine tissue sections. APMIS 109, 656-664.

Browne C, Loeffler A, Holt HR, Chang YM, Lloyd DH, Nevel A (2017). Low temperature and dust favor in vitro survival of *Mycoplasma hyopneumoniae*: time to revisit indirect transmission in pig housing. Letters in Applied Microbiology 64, 2-7.

Busch ME, Steinmetz H, Jensen T (2013). The effect of all-in all-out management by site on infection with *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* in finishers. Proceedings European Symposium on Porcine Health Management. Edinburgh, United Kingdom. p. 59.

Cai HY, van Dreumel T, McEwen B, Hornby G, Bell-Rogers P, McRaild P, Josephson G, Maxie G (2007). Application and field validation of a PCR assay for the detection of *Mycoplasma hyopneumoniae* from swine lung tissue samples. Journal of Veterinary Diagnostic Investigation 19, 91-95.

Calsamiglia M, Pijoan C, Trigo A (1999). Application of a nested polymerase chain reaction assay to detect *Mycoplasma hyopneumoniae* from nasal swabs. Journal of Veterinary Diagnostic Investigations 11, 246-251.

Calsamiglia M, Collins JE, Pijoan C (2000). Correlation between the presence of enzootic pneumonia lesions and detection of *Mycoplasma hyopneumoniae* in bronchial swabs by PCR. Veterinary Microbiology 76, 299-303.

Calsamiglia M, Pijoan C (2000). Colonisation state and colostral immunity to *Mycoplasma hyopneumoniae* of different parity sows. Veterinary Record 146, 530-532.

Calus D, Baele M, Meyns T, de Kruif A, Butaye P, Decostere A, Haesebrouck F, Maes D (2007). Protein variability among *Mycoplasma hyopneumoniae* isolates. Veterinary Microbiology 120, 284-291.

Calus D, Maes D, Vranckx K, Villarreal I, Pasmans F, Haesebrouck F (2010). Validation of ATP luminometry for rapid and accurate titration of *Mycoplasma hyopneumoniae* in Friis medium and a comparison with the color changing units assay. Journal of Microbiological Methods 83, 335-340.

Caron J, Ouardani M, Dea S (2000a). Diagnosis and differentiation of *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis* infections in pigs by PCR amplification of the p36 and p46 genes. Journal of Clinical Microbiology 38, 1390-1396.

Caron J, Sawyer N, Ben Abdel Moumen B, Cheikh Saad Bouh K, Dea S (2000b). Species-specific monoclonal antibodies to *Escherichia coli*-expressed p36 cytosolic protein of *Mycoplasma hyopneumoniae*. Clinical and Diagnostic Laboratory Immunology 7, 528-535.

Chae C (2011). Vaccinating pigs against *Mycoplasma hyopneumoniae* infection: failure to prevent transmission. Veterinary Journal 188, 7-8.

Chae C (2016). Porcine respiratory disease complex: interaction of vaccination and porcine circovirus type 2, porcine reproductive and respiratory syndrome virus, and *Mycoplasma hyopneumoniae*. Veterinary Journal 212, 1-6.

Chanter N, Rutter JM (1989). Pasteurellosis in pigs and the determinants of virulence of toxigenic Pasteurella multocida. In: Adam C, Rutter JM (eds.). Pasteurella and Pasteurellosis. Academic Press, London, UK. 161-195.

Charlebois A, Créhan CM, Hélie P, Gagnon CA, Gottschalk M, Archambault M (2014). Genetic diversity of *Mycoplasma hyopneumoniae* isolates of abattoir pigs. Veterinary Microbiology 168, 348-356.

Cheikh Saad Bouh K, Shareck F, Dea S (2003). Monoclonal antibodies to *Escherichia coli*-expressed P46 and P65 membranous proteins for specific immunodetection of *Mycoplasma hyopneumoniae* in lungs of infected pigs. Clinical and Diagnostic Laboratory Immunology 10, 459-468.

Chen J-R, Liao C-W, Mao SJT, Weng C-N (2001). A recombinant chimera composed of repeat region RR1 of *Mycoplasma hyopneumoniae* adhesion with *Pseudomonas* exotoxin: in vivo evaluation of specific IgG response in mice and pigs. Veterinary Microbiology 80, 347-357.

Chen Y-L, Wang S-N, Yang W-J, Chen Y-J, Lin H-H, Shiuan D (2003). Expression and immunogenicity of *Mycoplasma hyopneumoniae* heat shock protein antigen P42 by DNA vaccination. Infection and Immunity 71, 1155-1160.

Chen AY, Fry SR, Forbes-Faulkner J, Daggard G, Mukkur TKS (2006a). Evaluation of the immunogenicity of the P97R1 adhesin of *Mycoplasma hyopneumoniae* as a mucosal vaccine in mice. Journal of Medical Microbiology 55, 923-929.

Chen AY, Fry SR, Forbes-Faulkner J, Daggard GE, Mukkur TK (2006b). Comparative immunogenicity of *M. hyopneumoniae* NrdF encoded in different expression systems delivered orally via attenuated *S. typhimurium* aroA in mice. Veterinary Microbiology 114, 252-259.

Chen AY, Fry SR, Daggard GE, Mukkur TKS (2008). Evaluation of immune response to recombinant potential protective antigens of *Mycoplasma hyopneumoniae* delivered as cocktail DNA and/or recombinant protein vaccine in mice. Vaccine 26, 4372-4378.

Cheong Y, Oh C, Lee K, Cho KH (2017). Survey of porcine respiratory disease complex-associated pathogens among commercial pig farms in Korea via oral fluid method. Journal of Veterinary Science 18, 283-289.

Chiari M, Ferrari N, Zanoni M, Alborali L (2014). *Mycoplasma hyopneumoniae* temporal trends of infection and pathological effects in wild boar populations. European Journal of Wildlife Research 60, 187-192.

Chiers K, Donne E, Van Overbeke I, Ducatelle R, Haesebrouck F (2002). *Actinobacillus pleuropneumoniae* infections in closed swine herds: infection patterns and serological profiles. Veterinary Microbiology 85, 343-352.

Chouet S, Pietro C, Mieli L, Veenhuizen MF, McOrist S (2003). Some patterns of exposure to *Lawsonia intracellularis* infection on European pig farms. Veterinary Record 152, 14-17.

Christensen G, Mousing J (1992). Respiratory system. In: Leman AD, Straw BE, Mengeling WL, D'Allaire S, Taylor DJ (eds.). Diseases of Swine. Iowa State University Press, Ames, IA. 138-162.

Christensen J, Ellegaard B, Kirkegaard Petersen B, Willeberg P, Mousing J (1994). Pig health and production surveillance in Denmark: sampling design, data recording and measures of disease frequency. Preventive Veterinary Medicine 20, 47-61.

Christensen G, Sørensen V, Mousing J (1999). Diseases of respiratory system. In: Straw BE, D'Allaire S, Mengeling W, Taylor DJ, eds. Diseases of Swine. Iowa University Press, Ames, IA. p. 913-940.

Christianson WT (1992). Stillbirths, mummies, abortions, and early embryonic death. The Veterinary Clinics of North American – Food Animal Practice 8, 623-639.

Ciprián A, Palacios JM, Quintanar D, Batista L, Colmenares G, Cruz T, Romero A, Schitzlein W, Mendoza S (2012). Florfenicol feed supplemented decrease the clinical effects of *Mycoplasma hyopneumoniae* experimental infection in swine in Mexico. Research in Veterinary Science 92, 191-196.

Clark LK, Armstrong CH, Scheidt AB, Van Alstine WG (1993). The effect of *Mycoplasma hyopneumoniae* infection on growth in pigs with or without environmental constraints. Journal of Swine Health and Production 1, 10-14.

Clark LK, Wu CW, Van Alstine WG, Knox KE (1998). Evaluation of the effectiveness of a macrolide antibiotic on reduction of respiratory pathogens in 12-day and 21-day weaned pigs. Journal of Swine Health Production 6, 257-262.

Collins JE, Benfield DA, Christianson WT, Harris L, Hennings JC, Shaw DP, Goyal SM, McCullough S, Morrisson RB, Joo HS, Gorcyca D, Chladek D (1992). Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. Journal of Veterinary Diagnostic Investigations 4, 117-126.

Conceição FR, Moreira AN, Dellagostin OA (2006). A recombinant chimera composed of R1 repeat region of *Mycoplasma hyopneumoniae* P97 adhesin with *Escherichia coli* heat-labile enterotoxin B subunit elicits immune response in mice. Vaccine 24, 5734-5743.

Damte D, Suh J-W, Lee S-J, Yohannes SB, Hossain MA, Park S-C (2013). Putative drug and vaccine target protein identification using comparative genomic analysis of KEGG annotated metabolic pathways of *Mycoplasma hyopneumoniae*. Genomics 102, 47-56.

Damte D, Yohanes SB, Hossain MA, Lee S-J, Rhee M-H, Kim Y-H, Park S-C (2014). Detection of naturally aerolized *Mycoplasma hyopneumoniae* from the air of selected swine farms. Aerobiologica 30, 205-209.

Davies PR, Bahnson PB, Grass JJ, Marsh WE, Dial GD (1995). Comparison of methods for measurement of enzootic pneumonia lesions in pigs. American Journal of Veterinary Research 56, 9-14.

de Fonsêca MM, Zaha A, Cafferena ER, Vasconcelos ATR (2012). Structure-based functional inference of hypothetical proteins from *Mycoplasma hyopneumoniae*. Journal of Molecular Modelling 18, 1917-1925.

De Jong MF (1992). (Progressive) atrophic rhinitis. In: Leman AD, Straw BE, Mengeling WL, D'Allaire S, Taylor DJ (eds.). Diseases of Swine. Iowa State University Press, Ames, IA. p. 414-435.

de Oliveira NR, Jorge S, Gomes CK, Rizzi C, Pacce VD, Collares TF, Monte LG, Dellagostin OA (2017). A novel chimeric protein composed of recombinant *Mycoplasma hyopneumoniae* antigens as a vaccine candidate evaluated in mice. Veterinary Microbiology 201, 146-153.

Debey MC, Jacobson CD, Ross RF (1992). Histochemical and morphological changes in the porcine airway epithelial cells in response to infection with *Mycoplasma hyopneumoniae*. American Journal of Veterinary Research 53, 1705-1710.

Debey MC, Ross RF (1994). Ciliostatis and loss of cilia induced by *Mycoplasma hyopneumoniae* in porcine tracheal organ cultures. Infection and Immunity 62, 5312-5318.

Deblanc C, Gorin S, Quéguiner S, Gautier-Bouchardon AV, Ferré S, Amenna N, Cariolet R, Simon G (2012). Pre-infection of pigs with *Mycoplasma hyopneumoniae* modifies outcomes of infection with European swine influenza virus of H1N1, but not H1N2 subtype. Veterinary Microbiology 157, 96-105.

Deblanc C, Robert F, Pinard T, Gorin S, Quéguiner S, Gautier-Bouchardon AV, Ferré S, Garraud JM, Cariolet R, Brack M, Simon G (2013). Pre-infection of pigs with *Mycoplasma hyopneumoniae* induces oxidative stress

that influences outcomes of a subsequent infection with a swine influenza virus of H1N1 subtype. Veterinary Microbiology 162, 643-651.

Dee SA (1994). Apparent prevention of *Mycoplasma hyopneumoniae* infection in growing pigs with a low-cost modified medicated early-weaning program. Journal of Swine Health and Production 2, 7-12.

Dee S, Deen J, Burns D, Douthit G, Pijoan C (2004). An assessment of sanitation protocols for commercial transport vehicles contaminated with porcine reproductive and respiratory syndrome virus. Canadian Journal of Veterinary Research 68, 208-214.

Dee S, Otake S, Oliveira S, Deen J (2009). Evidence of long distance airborne transport of porcine reproductive and respiratory syndrome virus and *Mycoplasma hyopneumoniae*. Veterinary Research 40, 39-52.

Dee S, Otake S, Deen J (2010). Use of a production region model to assess the efficacy of various air filtration systems for preventing airborne transmission of porcine reproductive and respiratory syndrome virus and *Mycoplasma hyopneumoniae*: results from a 2-year study. Virus Research 154, 177-184.

Dee S, Pitkin A, Otake S, Deen J (2011). A four-year summary of air filtration system efficacy for preventing airborne spread of porcine reproductive and respiratory syndrome virus and *Mycoplasma hyopneumoniae*. Journal of Swine Health and Production 19, 292-294.

del Pozo Sancristán R, Thiry J, Vranckx K, López Rodríguez A, Chiers K, Haesebrouck F, Thomas E, Maes D (2012). Efficacy of florfenicol injection in the treatment of *Mycoplasma hyopneumoniae* induced respiratory disease in pigs. Veterinary Journal 194, 420-422.

del Pozo Sacristán R, Sierens A, Marchioro S, Vangroenweghe F, Jourquin J, Labarque G, Haesebrouck F, Maes D (2014). Efficacy of early *Mycoplasma hyopneumoniae* vaccination against mixed respiratory disease in older fattening pigs. Veterinary Record 174, 197-203.

Diaz I, Darwich L, Pappaterra G, Pujols J, Mateu E (2005). Immune responses of pigs after experimental infection with a European strain of Porcine Reproductive and Respiratory Syndrome virus. Journal of Genetic Virology 86, 1943-1951.

Diekman MA, Scheidt AB, Grant AL, Kelly DT, Sutton AL, Martin TG, Cline TR (1999). Effect of vaccination against *Mycoplasma hyopneumoniae* on health, growth, and pubertal status of gilts exposed to moderate ammonia concentrations in all-in-all-out versus continuous-flow systems. Journal of Swine Health and Production 7, 55-61.

Djordjevic SP, Eamens GJ, Romalis LF, Saunders MM (1994). An improved enzyme linked immunosorbent assay (ELISA) for the detection of porcine serum antibodies against *Mycoplasma hyopneumoniae*. Veterinary Microbiology 39, 261-274.

Djordjevic SP, Eamens GJ, Romalis LF, Nicholls PJ, Taylor V, Chin J (1997). Serum and mucosal antibody responses and protection in pigs vaccinated against *Mycoplasma hyopneumoniae* with vaccines containing a denatured membrane antigen pool and adjuvant. Austrian Veterinary Journal 75, 504-511.

Done SH (1988). Some aspects of respiratory defence with special reference to immunity. Pig Veterinary Society Proceedings 20, 31-60.

Donham KJ, Leininger JR (1984). Animal studies of potential chonic lung disease of workers in swine confinement buildings. American Journal of Veterinary Research 45, 926-931.

Donham KJ (1991). Association of environmental air contaminants with disease and productivity in swine. American Journal of Veterinary Research 52, 1723-1730.

Dos Santos LF, Sreevatsan S, Torremorell M, Moreira M, Sibila M, Pieters M (2015). Genotype distribution of *Mycoplasma hyopneumoniae* in swine herds from different geographical regions. Veterinary Microbiology 175, 374-381.

Dubosson CR, Conzelmann C, Miserez R, Boerlin P, Frey J, Zimmermann W, Häni H, Kuhnert P (2004). Development of two real-time PCR assays for the detection of *Mycoplasma hyopneumoniae* in clinical samples. Veterinary Microbiology 102, 55-65.

Erlandson KR, Evans RB, Thacker BJ, Wegner MW, Thacker EL (2005). Evaluation of three serum antibody enzyme-linked immunosorbent assays for *Mycoplasma hyopneumoniae*. Journal of Swine Health and Production 13, 198-203.

Fablet C, Marois C, Kobisch M, Madec F, Rose N (2010). Estimation of the sensitivity of four sampling methods for *Mycoplasma hyopneumoniae* detection in live pigs using a Bayesian approach. Veterinary Microbiology 143, 238-245.

Fablet C, Marois C, Kuntz-Simon G, Rose N, Dorenlor V, Eono F, Eveno E, Jolly JP, De Devendec L, Tocqueville V, Quéguiner S, Gorin S, Kobisch M, Madec F (2011). Longitudinal study of respiratory infection patterns of breedings sows in five farrow-to-finish herds. Veterinary Microbiology 147, 329-339.

Fablet C, Marois C, Dorenlor V, Eono F, Eveno E, Jolly JP, Le Devendec L, Kobisch M, Madec F, Rose N (2012a). Bacterial pathogens associated with lung lesions in slaughter pigs from 125 herds. Research in Veterinary Science 93, 627-630.

Fablet C, Marois-Créhan C, Simon G, Grasland B, Jestin A, Kobisch M, Madec F, Rose N (2012b). Infectious agents associated with respiratory diseases in 125 farrow-to-finish herds: a cross-sectional study. Veterinary Microbiology 157, 152-163.

Fagan PK, Djordjevic SP, Chin J, Eamens GJ, Walker MJ (1997). Oral immunization of mice with attenuated *Salmonella typhimurium aroA* expressing a recombinant *Mycoplasma hyopneumoniae* antigen (NrdF). Infection and Immunity 65, 2502-2507.

Fagan PK, Walker MJ, Chin J, Eamens GJ, Djordjevic SP (2001). Oral immunization of swine with attenuated *Salmonella typhimurium a roA* SL3261 expressing a recombinant antigen of *Mycoplasma hyopneumoniae* (NrdF) primes the immune system for a NrdF specific secretory IgA response in the lungs. Microbial Pathogenesis 30, 101-110.

Fano E, Pijoan C, Dee S (2005a). Dynamics and persistence of *Mycoplasma hyopneumoniae* infection in pigs. Canadian Journal of Veterinary Research 69, 223-228.

Fano E, Pijoan C, Dee S (2005b). Evaluation of the aerosol transmission of a mixed infection of *Mycoplasma hyopneumoniae* and porcine reproductive and respiratory syndrome virus. Veterinary Record 157, 105-108.

Fano E, Pijoan C, Dee S, Deen J (2007). Effect of *Mycoplasma hyopneumoniae* colonization at weaning on disease severity in growing pigs. Canadian Journal of Veterinary Research 71, 195-200.

Fano E, Anderson A, Sponheim A, Dalquist L, Pieters M (2018). Minimal *Mycoplasma hyopneumoniae* genetic variability within production flows. In: Proceedings of 9th European Symposium on Porcine Health Management. Barcelona, Spain. p. 96.

Feld NC, Qvist P, Ahrens P, Friis NF, Meyling A (1992). A monoclonal blocking ELISA detecting serum antibodies to *Mycoplasma hyopneumoniae*. Veterinary Microbiology 30, 35-46.

Feng Z-X, Shao G-Q, Liu M-J, Wang H-Y, Gan Y, Wu X-S (2010). Development and validation of a sIgA-ELISA for the detection of *Mycoplasma hyopneumoniae* infection. Veterinary Microbiology 143, 410-416.

Feng Z-X, Bai Y, Yao J-T, Pharr GT, Wan X-F, Xiao S-B, Chi L-C, Gan Y, Wang H-Y, Wei Y-N, Liu M-J, Xiong Q-Y, Bai F-F, Li B, Wu X-S, Shao G-Q (2014). Use of serological and mucosal immune responses to *Mycoplasma hyopneumoniae* antigens P97R1, P46 and P36 in the diagnosis of infection. Veterinary Journal 202, 128-133.

Fisch A, Beutinger Marchioro S, Klazer Gomes C, Galli V, Rodriguez de Oliveira N, Simionatto S, Dellagostin OA, Mendonça M, Nunes Moreira A, Conceição FR (2016). Commercial bacterins did not induce detectable levels of antibodies in mice against *Mycoplasma hyopneumoniae* antigens strongly recognized by swine immune system. Trials in Vaccinology 5, 32-37.

Flesja K, Ulvesaeter H (1980a). Pathological lesions in swine at slaughter. Acta Veterinaria Scandinavica 74, 1-22.

Flesja KI, Ulvesaeter HO (1980b). Pathological lesions in swine at slaughter. III. Inter-relationship between pathological lesions, and between pathological lesions and 1) carcass quality and 2) carcass weight. Acta Veterinaria Scandinavica 74, 1-22.

Fort M, Fernandes LT, Nofrarias M, Díaz I, Sibila M, Pujols J, Mateu E, Segalés J (2009). Development of cell-mediated immunity to porcine circovirus type 2 (PCV2) in caesarean-derived, colostrum-deprived piglets. Veterinary Immunology and Immunopathology 129, 101-107.

Fraile L, Alegre A, López-Jiménez R, Nofrarías M, Segalés J (2010). Risk factors associated with pleuritic and cranio-ventral pulmonary consolidation in slaughter-age pigs. Veterinary Journal 184, 326-333.

Frey J, Haldimann A, Nicolet J (1992). Chromosomal heterogeneity of variuous *Mycoplasma hyopneumoniae* field strains. International Journal of Systematic Bacteriology 42, 27-280.

Frey J, Haldimann A, Kobisch M, Nicolet J (1994). Immune response against the L-lactate dehydrogenase of *Mycoplasma hyopneumoniae* in enzootic pneumonia of swine. Microbial Pathogenesis 17, 313-322.

Friis NF (1975). Some recommendations concerning primary isolation of *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare*: a survey. Nordic Veterinary Medicine 27, 337-339.

Futo S, Seto Y, Mitsuse S, Mori Y, Suzuki T, Kawai K (1995a). Molecular cloning of a 46-kilodalton surface antigen (P46) gene from *Mycoplasma hyopneumoniae*: direct evidence of CGG codon usage for arginine. Journal of Bacteriology 177, 1915-1917.

Futo S, Seto Y, Okada M, Sato S, Suzuki T, Kawai K, Imada Y, Mori Y (1995b). Recombinant 46-kilodalton surface antigen (P46) of *Mycoplasma hyopneumoniae* expressed in *Escherichia coli* can be used for early specific

diagnosis of mycoplasmal pneumonia of swine by enzyme-linked immunosorbent assay. Journal of Clincal Microbiology 33, 680-683.

Galliher-Beckley A, Pappan LK, Madera R, Burakova Y, Waters A, Nickles M, Li X, Nietfeld J, Schlup JR, Zhong Q, McVey S, Dritz SS, Shi J (2015). Characterization of a novel oil-in-water emulsion adjuvant for swine influenza virus and *Mycoplasma hyopneumoniae* vaccines. Vaccine 33, 2903-2908.

Garcia-Morante B, Segalés J, Fraile L, Pérez de Rozas A, Maiti H, Coll T, Sibila M (2016). Assessment of *Mycoplasma hyopneumoniae*-induced pneumonia using different lung lesions scoring systems: a comparative review. Journal of Comparative Pathology 154, 125-134.

Gardner IA (2012). Analysis and use of diagnostic data. In: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, eds. Diseases of Swine, 10th edition. Wiley-Blackwell, A John Wiley & Sons, Inc., Chichester, West Sussex, UK. p. 94-105.

Garza-Moreno L, Segalés J, Pieters M, Romagosa A, Sibila M (2017). Survey on *Mycoplasma hyopneumoniae* gilt acclimatization practices in Europe. Porcine Health Management 3, 21-28.

Garza-Moreno L, Segalés J, Pieters M, Romagosa A, Sibila M (2018). Acclimation strategies in gilts to control *Mycoplasma hyopneumoniae* infection. Veterinary Microbiology 219, 23-29.

Giacomini E, Ferrari N, Pitozzi A, Remistani M, Giardiello D, Maes D, Alborali GL (2016). Dynamics of *Mycoplasma hyopneumoniae* seroconversion and infection in pigs in the three main production systems. Veterinary Research Communications 40, 81-88.

Gomes Neto JC, Strait EL, Raymond M, Ramirez A, Minion FC (2014). Antibody responses of swine following infection with *Mycoplasma hyopneumoniae*, *M. hyorhinis*, *M. hyosynoviae* and *M. flocculare*. Veterinary Microbiology 174, 163-171.

Goodell CK, Prickett J, Kittawornrat A, Zhou F, Rauh R, Nelson W, O'Connell C, Burrell A, Wang C, Yoon KJ, Zimmerman JJ (2013). Probability of detecting influenza A virus subtypes H1N1 and H3N2 in individual pig nasal swabs and pen-based oral fluid specimens over time. Veterinary Microbiology 166, 450-460.

Goodwin RF, Hodgson RG, Whittlestone P, Woodhams RL (1969). Some experiments relating to artificial immunity in enzootic pneumonia of pigs. Journal of Hygiene (London) 67, 465-476.

Goodwin RF (1985). Apparent reinfection of enzootic-pneumonia-free pig herds: Search for possible causes. Veterinary Record 116, 690-694.

Gottschalk M, Taylor DJ (2006). *Actinobacillus pleuropneumoniae*. In: Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ (eds.). Diseases of Swine. Iowa State University Press, Ames, IA. 563-576.

groβe Beilage E, Schreiber A, Pabst T (2005). Diagnostik der Enzootischen Pneumonie in Schweineherder nach Impfung gegen *Mycoplasma hyopneumoniae*. Teil 1: Seroreaktionen von Schweinen auf verschiedene Impfschemata. Tierärztliche Praxis 33, 239-235.

groβe Beilage E, Rohde N, Krieter J (2009). Seroprevalence and risk factors associated with seropositivity in sows from 67 herds in north-west Germany infected with *Mycoplasma hyopneumoniae*. Preventive Veterinary Medicine 88, 255-263.

Ha S-K, Choi C, Kim O, Song H-C, Lim E-S, Kim S-H, Kwang K-K, Chae C (2005). Development of nested polymerase chain reaction for the detection of *Mycoplasma hyopneumoniae* in formalin-fixed paraffin-embedded lung tissue. Astralian Veterinary Journal 83, 442-444.

Haesebrouck F, Pasmans F, Chiers K, Maes D, Ducatelle R, Decostere A (2004). Efficacy of vaccines against bacterial diseases in swine: what can we expect? Veterinary Microbiology 100, 255-268.

Haldimann A, Nicolet J, Frey J (1993). DNA sequence determination and biochemical analysis of the immunogenic protein P36, the lactate dehydrogenase (LDH) of *Mycoplasma hyopneumoniae*. Journal of General Microbiology 139, 317-323.

Hands I, McOrist S, Blunt K, Lawrence K (2010). Current infection patterns of porcine proliferative enteropathy in Great Britain and the Republic of Ireland. Veterinary Record 167, 343-344.

Hannan PC, Bhogal BS, Fish JP (1982). Tylosine tartrate and tiamutilin effects on experimental piglet pneumonia induced with pneumonic pig lung homogenate containing mycoplasmas, bacteria and viruses. Research in Veterinary Science 33, 76-88.

Hannan PCT, Windsor HM, Ripley PH (1997). In vitro susceptibilities of recent field isolates of *Mycplasma hyopneumoniae* and *Mycoplasma hyosynoviae* to valnemulin (Econor®), tiamulin and enrofloxacin and the in vitro development of resistance to certain antimicrobial agents in *Mycoplasma hyopneumoniae*. Research in Veterinary Science 63, 157-160.

Harasawa R, Koshimizu K, Takeda O, Uemori T, Asada K, Kato I (1991). Detection of *Mycoplasma hyopneumoniae* DNA by the polymerase chain reaction. Molecular and Cellular Probes 5, 103-109.

Hariharan H, Cepica A, Qian B, Heaney S, Hurnik D (2000). Toxigenic and drug resistance properties of porcine *Pasteurella multocida* isolates from Prince Edward Island. Canadian Veterinary Journal 41, 798-792.

He Y, Xu MJ, Zhou DH, Zou FC, Lin RQ, Yin CC, He XH, Liang R, Zhu XQ (2011). Seroprevalence of *Mycoplasma hyopneumoniae* in subtropical southern China. Tropical Animal Health and Production 43, 695-698.

Hege R, Zimmermann W, Scheidegger R, Stärk KDC (2002). Incidence of reinfections with *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* in pig farms located in respiratory-disease-free regions in Switzerland. Acta Veterinaria Scandinavica 43, 145-156.

Heinonen M, Laurila T, Vidgren G, Levonen K (2011). Eradication of *Mycoplasma hyopneumoniae* from a swine finishing herd without total depopulation. The Veterinary Journal 188, 110-114.

Hernandez-Garcia J, Robben N, Magnée D, Eley T, Dennis I, Kayes SM, Thomson JR, Tucker AW (2017). The use of oral fluids to monitor key pathogens in porcine respiratory disease complex. Porcine Health Management 5, 3-7.

Hillen S, von Berg S, Köhler K, Reinacher M, Willems H, Reiner G (2014). Occurrence and severity of lung lesions in slaughter pigs vaccinated against *Mycoplasma hyopneumoniae* with different strategies. Preventive Veterinary Medicine 113, 580-588.

Holko I, Urbanova J, Holkova T, Kmet V (2004). Diagnostics of main bacterial agents in porcine respiratory diseases complex (PRDC) using PCR detection of *Mycoplasma hyopneumoniae*. Veterinarni Medicina 49, 35-41.

Holmgren N, Lundeheim N, Wallgren P (1999). Infections with *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* in fattening pigs. Influence of piglet production systems and influence on production parameters. Journal of Veterinary Medicine B 46, 535-544.

Holst S, Yeske P, Pieters M (2015). Elimination of *Mycoplasma hyopneumoniae* from breed-to wean farms: a review of current protocols with emphasis on herd closure and medication. Journal of Swine Health and Production 23, 321-330.

Holyoake PK, Callinan PL (2006). How effective is *Mycoplasma hyopneumoniae* vaccination in pigs less than three weeks of age? Journal of Swine Health and Production 14, 189-195.

Hwang F, Wen D-C, Wu Y-W, Li Y-Z, Dong Q-H, Wang S-M (1986). Studies on the phospholipid composition of pathogenic cell membranes of *Mycoplasma hyopneumoniae*. FEBS Letters 195, 323-326.

Inamoto T, Takahashi H, Yamamoto K, Nakai Y, Ogimoto K (1994). Antibiotic susceptibility of *Mycoplasma hyopneumoniae* isolated from swine. Journal of Veterinary Medical Science 56, 393-394.

Irigoyen LF, Van Alstine W, Turek J, Clark LK (1998). Ultrastructural observation of the airways of recoveree and susceptible pigs after inoculation with *Mycoplasma hyopneumoniae*. Pesquisa Veterinária Brasileira 18, 1-7.

Jamaludin R, Blackall PJ, Hansen MF, Humphrey S, Styles M (2005). Phenotypic and genotypic characterization of *Pasteurella multocida* isolated from pigs at slaughter in New Zealand. New Zealand Veterinary Journal 53, 203-207.

Jenkins C, Wilton JL, Minion FC, Falconer L, Walker MJ, Djordjevic SP (2006). Two domains within the *Mycoplasma hyopneumoniae* cilium adhesin bind heparin. Infection and Immunity 74, 481-487.

Jensen CS, Ersbøll AK, Nielsen JP (2002). A meta-analysis comparing the effect of vaccines against *Mycoplasma hyopneumoniae* on daily weight gain in pigs. Preventive Veterinary Medicine 54, 265-278.

Jirawattanapong P, Stocke-Zurwieden N, van Leengoed L, Wisselink H, Raymakers R, Cruijsen T, van der Peet-Schwering C, Nielen M, van Nes A (2010). Pleuritis in slaughter pigs: relations between lung lesions and bacteriology in 10 herds with high pleuritis. Research in Veterinary Science 88, 11-15.

Johansson K-E, Mattsson JG, Jacobsson K, Fernandez C, Bergström K, Bölske G, Wallgren P, Göbel UB (1992). Specificity of oligonucleotide probes complementary to evolutionarily variable reions of 16S rRNA from *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis*. Research in Veterinary Science 52, 195-204.

Johnson C, Farkas A, Cano JP, Clavijo MJ (2018). What happens when *M. hyopneumoniae* enters a herd? Longitudinal assessment of *M. hyopneumoniae* natural infection in gilts. In: Proceedings of 9th European Symposium on Porcine Health Management. Barcelona, Spain. p. 82.

Jones R, Baskerville A, Reid L (1974). Histochemical identification of glycoproteins in pig bronchial epithelium: (a) normal and (b) hypertrophied from enzootic pneumonia. Journal of Pathology 116, 1-11.

Jorge S, de Oliveira NR, Machioro SB, Fisch A, Gomes CK, Hartleben CP, Conceição FR, Dellagostin OA (2014). The *Mycoplasma hyopneumoniae* recombinant heat shock protein P42 induces an immune response in pigs under field conditions. Comparative Immunology, Microbiology and Infectious Diseases 37, 229-236.

Jorsal SE, Thomsen BL (1988). A Cox regression analysis of risk factors related to *Mycoplasma suipneumoniae* reinfection in Danish SPF-herds. Acta Veterinaria Scandinavica 84, 436-438.

Judge EP, Lynne Hughes JM, Egan JJ, Maguire M, Molloy EL, O'Dea S (2014). Anatomy and bronchoscopy of the porcine lung: a model for translational respiratory medicine. American Journal of Respiratory Cell and Molecular Biology 51, 334-343.

Juul-Madsen HR, Jensen KH, Nielsen J, Damgaard BM (2010). Ontogeny and characterization of blood leukocyte subsets and serum proteins in piglets before and after weaning. Veterinary Immunology and Immunopathology 133, 95-108.

Kim TJ, Cho HS, Park NY, Lee JI (2006). Serodiagnostic comparison between two methods, ELISA and surface plasmon resonance for the detection of antibody titres of *Mycoplasma hyopneumoniae*. Journal of Veterinary Medicine B 53, 87-90.

Kim D, Kim CH, Han K, Seo HW, Oh Y, Park C, Kang I, Chae C (2011). Comparative efficacy of commercial *Mycoplasma hyopneumoniae* and porcine circovirus 2 (PCV2) vaccines in pigs experimentally infected with *M. hyopneumoniae* and PCV2. Vaccine 29, 3206-3212.

Kim CH, Oh Y, Han K, Seo HW, Seo HW, Kim D, Park C, Kang I, Chae C (2012). Expression of secreted and membrane-bound mucins in the airways of piglets experimentally infected with *Mycoplasma hyopneumoniae*. Veterinary Journal 192, 120-127.

King KW, Faulds DH, Rosey EL, Yancey RJ Jr (1997). Characterization of the gene encoding Mhp1 from *Mycoplasma hyopneumoniae* and examination of Mhp1's vaccine potential. Vaccine 15, 25-35.

Klein U, de Jong A, Moyaert H, El Garth F, Leon R, Richard-Mazet A, Rose M, Maes D, Pridmore A, Thomson JR, Ayling RD (2017). Antimicrobial susceptibility monitoring of *Mycoplasma hyopneumoniae* and *Mycoplasma bovis* isolated in Europe. Veterinary Microbiology 204, 188-193.

Knittel JP, Jordan D, Schwartz K, Janke B, Roof M, McOrist S, Harris D (1998). Evaluation of antemortem polymerase chain reaction and serological methods for detection of *Lawsonia intracellularis*-exposed pigs. American Journal of Veterinary Research 59, 722-726.

Kobisch M, Tillon J, Vannier P, Magneur S, Morvan P (1978). Pneumonie enzootique à Mycoplasma suipneumoniae chez le porc: diagnostic rapide et recherches d'anticorps. Recueil de Médecine Vétérinaire 154, 847-852.

Kobisch M, Quillien L, Tillon JP, Wróblewski H (1987). The *Mycoplasma hyopneumoniae* plasma membrane as a vaccine against porcine enzootic pneumonia. Annales Institut Pasteur – Immunology 138, 693-705.

Kobisch M, Blanchard B, Le Potier MF (1993). *Mycoplasma hyopneumoniae* infection in pigs: duration of the disease and resistance to infection. Veterinary Research 24, 67-77.

Kristensen CS, Vinther J, Svensmark B, Bækbo P (2014). A field evaluation of two vaccines against *Mycoplasma hyopneumoniae* infection in pigs. Acta Veterinaria Scandinavica 56, 24-30.

Kuhnert P, Overesch G, Belloy L (2011). Genotyping of *Mycoplasma hyopneumoniae* in wild boar lung samples. Veterinary Microbiology 152, 191-195.

Kuhnert P, Overesch G (2014). Molecular epidemiology of *Mycoplasma hyopneumoniae* from outbreaks of enzootic pneumonia in domestic pigs and the role of wild boar. Veterinary Microbiology 174, 261-266.

Kureljušić B, Weissenbacher-Lang C, Nedorost N, Stixenberger D, Weissenböck H (2016). Association between *Pneumocystis* spp. and co-infections with *Bordetella bronchiseptica*, *Mycoplasma hyopneumoniae* and *Pasteurella multocida* in Austrian pigs with pneumonia. The Veterinary Journal 207, 177-179.

Kurth KT, Hsu T, Snook ER, Thacker EL, Thacker BJ, Minion FC (2002). Use of a *Mycoplasma hyopneumoniae* nested polymerase chain reaction test to determine the optimal sampling sites in swine. Journal of Veterinary Diagnostic Investigation 14, 463-469.

Kwon D, Chae C (1999). Detection and localization of *Mycoplasma hyopneumoniae* DNA in lungs from naturally infected pigs by *in situ* hybridization using a digoxigenin-labeled probe. Veterinary Pathology 36, 308-313.

Kwon D, Choi C, Chae C (2002). Chonologic localization of *Mycoplasma hyopneumoniae* in experimentally infected pigs. Veterinary Pathology 39, 584-587.

Laanen M, Beek J, Ribbens S, Vangroenweghe F, Maes D, Dewulf J (2010). Bioveiligheid op varkensbedrijven: ontwikkeling van een online scoresysteem en de resultaten van de eerste 99 deelnemende bedrijven. Vlaams Diergeneeskundig Tijdschrift 79, 302-306.

Labarque GG, Nauwynck HJ, Van Reeth K, Pensaert M (2000). Effect of cellular changes and onset of humoral immunity on the replication of porcine reproductive and respiratory syndrome virus in the lungs of pigs. Journal of Genetic Virology 81, 1327-1334.

Le Carrou J, Laurentie M, Kobisch M, Gautier-Bouchardon AV (2006). Persistence of *Mycoplasma hyopneumoniae* in experimentally infected pigs after marbofloxain treatment and detection of mutations in the *parC* gene. Antimicrobial Agents and Chemotherapy 50, 1959-1966.

Le Floc'h N, Deblanc C, Cariolet R, Gautier-Bouchardon AV, Merlot E, Simon G (2014). Effect of feed restriction on performance and postprandial nutrient metabolism in pigs co-infected with *Mycoplasma hyopneumoniae* and swine influenza virus. PLoS ONE 9:e104605. doi: 10.1371/journal.pone.0104605.

Lefebvre DJ, Van Reeth K, Vangroenweghe F, Maes D, Van Driessche E, Laitat M, Nauwynck HJ (2009). Serosurvey for viruses associated with reproductive failure in newly introduced gilts and in multiparous sows in Belgian sow herds. Flemish Veterinary Journal 78, 429-435.

Lehner S, Meemken D, Nathues H, grosse Beilage E (2008a). Effectivität von Impfungen gegen *Mycoplasma hyopneumoniae* bei Schweinen von geimpften resp. nicht geimpften Sauen. Tierärztliche Praxis 36, 399-406.

Lehner S, Nathues H, grosse Beilage E (2008b). Untersuchungen an Sauen zur Kinetik von Antikörpern gegen *Mycoplasma hyopneumoniae* um den Geburtszeitpunkt. Tierärztliche Praxis 36, 298-302.

Leon EA, Madec F, Taylor NM, Kobisch M (2001). Seroepidemiology of *Mycoplasma hyopneumoniae* in pigs from farrow-to-finish farms. Veterinary Microbiology 78, 331-341.

LeRoith T, Hammond S, Todd SM, Ni Y, Cecere T, Pelzer KD (2011). A modified live PRRSV vaccine and the pathogenic parent strain induce regulatory T cells in pigs naturally infected with *Mycoplasma hyopneumoniae*. Veterinary Immunology and Immunopathology 140, 312-316.

Levonen K, Sihvo E, Veijalainen P (1999). Comparison of two commercial enzyme-linked immunosorbent assays for the detection of antibodies against *Mycoplasma hyopneumoniae* and correlation with herd status. Journal of Veterinary Diagnostic Investigation 11, 547-549.

Li J, Minion FC, Peterson AC, Jiang F, Yang S, Guo P, Li J, Wu W (2013). Loop-mediated isothermal amplication for rapid and convenient detection of *Mycoplasma hyopneumoniae*. World Journal of Microbiology and Biotechnology 29, 607-616.

Li P, Li Y, Shao G, Yu Q, Yang Q (2015). Comparison of immune responses to intranasal and intrapulmonary vaccinations with the attenuated *Mycoplasma hyopneumoniae* 168 strain in pigs. Journal of Veterinary Medical Science 77, 519-525.

Li Y-Z, Ho Y-P, Chen S-T, Shiuan D (2010). Proteomic analysis of the interactions between *Mycoplasma hyopneumoniae* and porcine tracheal ciliated cells. Applied Biochemistry and Biotechnology 160, 2248-2255.

Li Y, Li P, Wang X, Yu Q, Yang Q (2012). Co-administration of attenuated *Mycoplasma hyopneumoniae* 168 strains with bacterial DNA enhances the local and systemic immune response after intranasal vaccination in pigs. Vaccine 30, 2153-2158.

Lin JH, Weng CN, Liao CW, Yeh KS, Pan MJ (2003). Protective effects of oral microencapsulated *Mycoplasma hyopneumoniae* vaccine prepared by co-spray drying method. Journal of Veterinary Medical Science 65, 69-74.

Liu W, Feng Z, Fang L, Zhou Z, Li Q, Li S, Luo R, Wang L, Chen H, Shao G, Xiao S (2011). Complete genome sequence of *Mycoplasma hyopneumoniae* strains 168. Journal of Bacteriology 193, 1016-1017.

Liu W, Xiao S, Li M, Guo S, Li S, Luo R, Feng Z, Li B, Zhou Z, Shao G, Chen H, Fang L (2013). Comparative genomic analyses of *Mycoplasma hyopneumoniae* pathogenic 168 strain and its high-passaged attenuated strain. BMC Genomics 14, 80-92.

Liu MJ, Du GM, Bai FF, Wu YZ, Xiong QY, Feng ZX, Li B, Shao GQ (2015). A rapid and sensitive loop-mediated isothermal amplification procedure (LAMP) for *Mycoplasma hyopneumoniae* detection based on the *p36* gene. Genetics and Molecular Research 14, 4677-4686.

Liu M, Du G, Zhang Y, Wu Y, Wang H, Li B, Bai Y, Feng Z, Xiong Q, Bai F, Browning GF, Shao G (2016). Development of a blocking ELISA for detection of *Mycoplasma hyopneumoniae* infection based on a monoclonal antibody against protein P65. Journal of Veterinary Medical Science 78, 1319-1322.

Loreto ELS, Ortiz MF, Porta JIR (2007). Insertion sequences as variability generators in the *Mycoplasma hyopneumoniae* and *M. synoviae* genomes. Genetics and Molecular Biology 30, 283-289.

Lowe JE, Husmann R, Firkins LD, Zuckermann FA, Goldberg TL (2005). Correlation of cell-mediated immunity against porcine reproductive and respiratory syndrome virus with protection against reproductive failure in sows during outbreaks of porcine reproductive and respiratory syndrome in commercial herds. Journal of American Veterinary Medical Association 226, 1707-1711.

Lozano D, Cantero M (2016). Difference between analytical sensitivity and detection limit. American Journal of Clinical Pathology 107, 619.

MacInnes JI, Gottschalk M, Lone AG, Metcalf DS, Ojha S, Rosendal T, Watson SB, Friendship RM (2008). Prevalence of *Actinobacillus pleuropneumoniae*, *Actinobacillus suis*, *Haemophilus parasuis*, *Pasteurella*

multocida, and *Streptococcus suis* in representative Ontario swine herds. Canadian Journal of Veterinary Research 72, 242-248.

Madec F, Kobisch M (1982). Bilan lesionnel des poumons de porcs charcutiers à l'abbatoir. Journées de la Recherche Porcine en France 14, 405-412.

Madsen ML, Nettleton D, Tacker EL, Edwards R, Minion FC (2006). Transcriptional profiling of *Mycoplasma hyopneumoniae* during heat shock using microarrays. Infection and Immunity 74, 160-166.

Madsen ML, Oneal MJ, Gardner SW, Strait EL, Nettleton D, Tacker EL, Minion FC (2007). Array-based genomic comparative hybridization analysis of field strains of *Mycoplasma hyopneumoniae*. Journal of Bacteriology 189, 7977-7982.

Maes D, Verdonck M, Deluyker H, de Kruif A (1996). Enzootic pneumonia in pigs. Veterinary Quarterly 18, 104-109.

Maes D, Deluyker H, Verdonck M, Castryck F, Miry C, Vrijens B, de Kruif A (1999). Risk indicators for the seroprevalence of *Mycoplasma hyopneumoniae*, porcine influenza viruses and Aujeszky's disease virus in slaughter pigs from fattening pig herds. Journal of Veterinary Medicine B 46, 341-352.

Maes D, Deluyker H, Verdonck M, Castryck F, Miry C, Vrijens B, de Kruif A (2000). Risk indicators for the seroprevalences of *Mycoplasma hyopneumoniae*, porcine influenza viruses and Aujeszky's disease virus in slaughter pigs from farrow-to-finish pig herds in Belgium. Veterinary Research 31, 313-327.

Maes D, Chiers K, Haesebrouck F, Laevens H, Verdonck M, de Kruif A (2001a). Herd factors associated with the seroprevalence of *Actinobacillus pleuropneumoniae* serovars 2, 3 and 9 in slaughter pigs from farrow-to-finish herds. Veterinary Research 32, 409-419.

Maes D, Deluyker H, Verdonck M, Castryck F, Miry C, Vrijens B, Ducatelle R, de Kruif A (2001b). Non-infectious herd factors associated with macroscopic and microscopic lung lesions in slaughter pigs from farrow-to-finish pig herds. Veterinary Record 148, 41-46.

Maes D, Segalés J, Meyns T, Sibila M, Pieters M, Haesebrouck F (2008). Review: Control of Mycoplasma hyopneumoniae infections in pigs. Veterinary Microbiology 126, 297-309.

Maes D (2010). *Mycoplasma hyopneumoniae* infections in pigs: update on epidemiology and control. Proceedings of International Pig Veterinary Society. Vancouver, Canada. 18-21 July. p. 30-35.

Maes D (2014). Vaccination against *Mycoplasma hyopneumoniae* infection in pigs: room for improvement. Veterinary Journal 200, 214-215.

Maes D, Sibila M, Kuhnert P, Segalés J, Haesebrouck F, Pieters M (2017). Update on *Mycoplasma hyopneumoniae* infections in pigs: knowledge gaps for improved disease control. Transboundary and Emerging Diseases 62, 1-15. doi: 10.1111/tbed.12677.

Marchioro SB, Simionatto S, Galli V, Conceição FR, Brum CB, Fisch A, Gomes CK, Dellagostin OA (2012). Production and characterization of recombinant transmembrane proteins from *Mycoplasma hyopneumoniae*. Veterinary Microbiology 155, 44-52.

Marchioro SB, Maes D, Flahou B, Pasmans F, Del Pozo Sancristán R, Vranckx K, Melkebeek V, Cox E, Wuyts N, Haesbrouck F (2013). Local and systemic immune responses in pigs intramuscularly injected with an inactivated *Mycoplasma hyopneumoniae* vaccine. 31, 1305-1311.

Marchioro SB, Fisch A, Gomes CK, Jorge S, Galli V, Haesebrouck F, Maes D, Dellagostin O, Conceição FR (2014a). Local and systemic immune responses induced by a recombinant chimeric protein containing *Mycoplasma hyopneumoniae* antigens fused to the B subunit of *Escherichia coli* heat-labile enterotoxin LTB. Veterinary Microbiology 173, 166-171.

Marchioro SB, Del Pozo Sancristán R, Michiels A, Haesebrouck F, Conceição FR, Dellagostin OA, Maes D (2014b). Immune response of chimaeric protein vaccine containing *Mycoplasma hyopneumoniae* antigens and LTB against experimental *M. hyopneumoniae* infection in pigs. Vaccine 32, 4689-4694.

Marois C, Le Carrou J, Kobisch M, Gautier-Bouchardon AV (2007). Isolation of *Mycoplasma hyopneumoniae* from different sampling sites in experimentally infected and contact SPF pigs. Veterinary Microbiology 120, 96-104.

Marois C, Cariolet R, Morvan H, Kobisch M (2008). Transmission of pathogenic respiratory bacteria to specific pathogen free pigs at slaughter. Veterinary Microbiology 129, 325-332.

Marois C, Gottschalk M, Morvan H, Fablet C, Madec F, Kobisch M (2009). Experimental infection of SPF pigs with *Actinobacillus pleuropneumoniae* serotype 9 alone or in association with *Mycoplasma hyopneumoniae*. Veterinary Microbiology 135, 283-291.

Marois C, Dory D, Fablet C, Madec F, Kobish M (2010). Development of a quantitative Real-Time TaqMan PCR assay for determination of the minimal dose of *Mycoplasma hyopneumoniae* strain 116 required to induce pneumonia in SPF pigs. Journal of Applied Microbiology 108, 1523-1533.

Martelli P, Terreni M, Guazetti S, Cavirani S (2006). Antibody response to *Mycoplasma hyopneumoniae* infection in vaccinated pigs with or without maternal antibodies induced by sow vaccination. Journal of Veterinary Medicine B 53, 229-233.

Martelli P, Saleri R, Cavalli V, De Angelis E, Ferrari L, Benetti M, Ferrarini G, Merialdi G, Borghetti P (2014). Systemic and local immune response in pigs intradermally and intramuscularly injected with inactivated *Mycoplasma hyopneumoniae* vaccines. Veterinary Microbiology 168, 357-364.

Martínez J, Peris B, Gómez EA, Corpa JM (2009). The relationship between infectious and non-infectious herd factors with pneumonia at slaughter and productive parameters in fattening pigs. Veterinary Journal 179, 240-246.

Mateusen B, Maes D, Hoflack G, Verdonck M, de Kruif A (2001). A comparative study of the preventive use of tilmicosin phosphate (Pulmotil premix®) and *Mycoplasma hyopneumoniae* vaccination in a pig herd with chronic respiratory disease. Journal of Veterinary Medicine B 48, 733-741.

Mattsson JG, Bergström K, Wallgren P, Johansson K-E (1995). Detection of *Mycoplasma hyopneumoniae* in nose swabs from pigs by in vitro amplication of the 16S rRNA gene. Journal of Clinical Microbiology 33, 893-897.

Mayor D, Zeeh F, Frey J, Kuhnert P (2007). Diversity of *Mycoplasma hyopneumoniae* in pig farms revealed by direct molecular typing of clinical material. Veterinary Research 38, 391-398.

Mayor D, Jores J, Korczak MB, Kuhnert P (2008). Multilocus sequence typing (MLST) of *Mycoplasma hyopneumoniae*: a diverse pathogen with limited clonality. Veterinary Microbiology 127, 63-72.

McOrist S, Jasni S, Mackie RA, MacIntyre N, Neef N, Lawson GH (1993). Reproduction of proliferative enteropathy with pure cultures of ileal symbiont intracellularis. Infection and Immunity 61, 4286-4292.

Meens J, Bolotin V, Frank R, Böhmer J, Gerlach G-F (2010). Characterization of a highly immunogenic *Mycoplasma hyopneumoniae* lipoprotein Mhp366 identified by peptide-spot array. Veterinary Microbiology 142, 293-302.

Mekerke B, Leneveu P (2006). Modifications de conduit de bandes et impact sur la situation sanitaire: analyse de quelques examples. Proceedings Association Française de Medecine Veterinaire Porcine, 7–8 December, Toulouse, France. p. 49-64.

Merialdi G, Dottory M, Bonilauri P, Luppi A, Gozio S, Spaggiari, Martelli P (2012). Survey of pleuritic and pulmonary lesions in pigs at abattoir with a focus on the extent of the condition and herd risk factors. Veterinary Journal 193, 234-239.

Meyns T, Maes D, Dewulf J, Vicca J, Haesebrouck F, de Kruif A (2004). Quantification of the spread of *Mycoplasma hyopneumoniae* in nursery pigs using transmission experiments. Preventive Veterinary Medicine 66, 265-275.

Meyns T, Dewulf J, de Kruif A, Calus D, Haesebrouck F, Maes D (2006). Comparison of transmission of *Mycoplasma hyopneumoniae* in vaccinated and non-vaccinated populations. Vaccine 24, 7081-7086.

Meyns T, Maes D, Calus D, Ribbens S, Dewulf J, Chiers K, de Kruif A, Cox E, Decostere A, Haesebrouck F (2007). Interactions of highly and low virulent *Mycoplasma hyopneumoniae* isolates with the respiratory tract of pigs. Veterinary Microbiology 120, 87-95.

Meyns T, Van Steelant J, Rolly E, Dewulf J, Haesebrouck F, Maes D (2011). A cross-sectional study of risk factors associated with pulmonary lesions in pigs at slaughter. Veterinary Journal 187, 388-392.

Michiels A, Piepers S, Ulens T, Van Ransbeeck N, del Pozo Sancristán R, Sierens A, Haesebrouck F, Demeyer P, Maes D (2015). Impact of particulate matter and ammonia on average daily weight gain, mortality and lung lesions in pigs. Preventive Veterinary Medicine 121, 99-107.

Michiels A, Vranckx K, Piepers S, del Pozo Sancristán R, Arsenakis I, Boyen F, Haesebrouck F, Maes D (2017). Impact of diversity of *Mycoplasma hyopneumoniae* strains on lung lesions in slaughter pigs. Veterinary Research 48, 2-15.

Michiels A, Arsenakis I, Matthijs A, Boyen F, Haesaert G, Audenaert K, Eeckhout M, Croubels S, Haesebrouck F, Maes D (2018). Clinical impact of deoxynivalenol on the severity of an experimental *Mycoplasma hyopneumoniae* infection in pigs. BMC Veterinary Research submitted.

Minion FC, Lefkowitz EJ, Madsen ML, Cleary BJ, Swartzell SM, Mahairas GG (2004). The genome sequence of *Mycoplasma hyopneumoniae* strain 232, the agent of swine mycoplasmosis. Journal of Bacteriology 186, 7123-7133.

Moorkamp L, Nathues H, Spergser J, Tegeler R, grosse Beilage E (2008). Detection of respiratory pathogens in porcine lung tissue and lavage fluid. Veterinary Journal 175, 273-275.

Moorkamp L, Hewicker-Trautwein M, groβe Beilage E (2009). Occurrence of *Mycoplasma hyopneumoniae* in coughing piglets (3-6 weeks of age) from 50 herds with a history of endemic respiratory disease. Transboundary and Emerging Disease 56, 54-56.

Moreau IA, Miller GY, Bahnson PB (2004). Effects of *Mycoplasma hyopneumoniae* vaccine on pigs naturally infected with *Mycoplasma hyopneumoniae* and porcine reproductive and respiratory syndrome virus. Vaccine 22, 2328-2333.

Mori Y, Hamaoka T, Sato S, Takeuchi S (1988). Immunoblotting analysis of antibody response in swine experimentally inoculated with *Mycoplasma hyopneumoniae*. Veterinary Immunology and Immunopathology 19, 239-250.

Morris CR, Gardner IA, Hietala SK, Carpenter TE, Anderson RJ, Parker KM (1994). Persistence of passively acquired antibodies to *Mycoplasma hyopneumoniae* in a swine herd. Preventive Veterinary Medicine 21, 29-41.

Morris CR, Gardner IA, Hietala SK, Carpenter TE, Anderson RJ, Parker KM (1995). Seroepidemiologic study of natural transmission of *Mycoplasma hyopneumoniae* in a swine herd. Preventive Veterinary Medicine 21, 323-337.

Morrison RB, Pijoan C, Hilley HD, Rapp V (1985). Microorganisms associated with pneumonia in slaughter weight swine. Canadian Journal of Comparative Medicine 49, 129-137.

Nathues H, Strutzberg-Minder K, Kreienbrock L, groβe Beilage E (2006). Chances and limits of serological testing in pig herds in case of *Mycoplasma hyopneumoniae* infection. Deutsche Tierärztliche Wochenschrift 113, 448-452.

Nathues H, Kubiak R, Tegeler R, groβe Beilage E (2010). Occurrence of *Mycoplasma hyopneumoniae* infections in suckling and nursery pigs in a region of high pig density. Veterinary Record 166, 194-198.

Nathues H, groβe Beilage E, Kreienbrock L, Rosengarten R, Spergser J (2011). RAPD and VNTR analyses demonstrate genotypic heterogeneity of *Mycoplasma hyopneumoniae* isolates from pigs housed in a region with high pig density. Veterinary Microbiology 152, 338-345.

Nathues H. Spergser J, Rosengarten R, Kreienbrock L, Groβe Beilage E (2012). Value of the clinical examination in diagnosing enzootic pneumonia in fattening pigs. Veterinary Journal 193, 443-447.

Nathues H, Woeste H, Doehring S, Fahrion AS, Doherr MG, groβe Beilage E (2013a). Herd specific risk factors for *Mycoplasma hyopneumoniae* infections in suckling pigs at the age of weaning. Acta Veterinaria Scandinavica 55, 30-42.

Nathues H, Doehring S, Woeste H, Fahrion AS, Doherr MG, Grosse Beilage E (2013b). Individual risk factors for *Mycoplasma hyopneumoniae* infections in suckling pigs at the age of weaning. Acta Veterinaria Scandinavica 55, 44-55. doi: 10.1186/1751-0147-55-44.

Nathues H, Chang YM, Wieland B, Rechter G, Spergser J, Rosengarten R, Kreienbrock L, groβe Beilage E (2014). Herd-level risk factors for the seropositivity to *Mycoplasma hyopneumoniae* and the occurrence of enzootic pneumonia among fattening pigs in areas of endemic infection and high pig density. Transboundary and Emerging Diseases 61, 316-328.

Ni B, Bai FF, Wei Y, Liu MJ, Feng ZX, Xiong QY, Hua LZ, Shao GQ (2015). Apoptosis induced by lipid-associated membrane proteins from *Mycoplasma hyopneumoniae* in a porcine lung epithelial cell line with the involvement of caspase 3 and the MAPK pathway. Genetics and Molecular Research 14, 11429-11443.

Nickel M, Toohill E, Lehman J (2018). Use of a hurricane fogger for *Mycoplasma hyopneumoniae* inoculation in nursery age gilts. In: Proceedings of 49th Annual Meeting of American Association of Swine Veterinarians. San Diego, CA. p. 97-98.

Ogawa Y, Oishi E, Muneta Y, Sano A, Hikono H, Shibahara T, Yagi Y, Shimoji Y (2009). Oral vaccination against mycoplasmal pneumonia of swine using a live *Erysipelothrix rhusiopathiae* vaccine strain as a vector. Vaccine 27, 4543-4550.

Okada M, Asai T, Futo S, Mori Y, Mukai T, Yazawa S, Uto T, Shibata I, Sato S (2005). Serological diagnosis of enzootic pneumonia of swine by a double-sandwich enzyme-linked immunosorbent assay using a monoclonal antibody and recombinant antigen (P46) of *Mycoplasma hyopneumoniae*. Veterinary Microbiology 105, 251-259.

Okamba FR, Moreau E, Cheikh Saad Bouh K, Gagnon CA, Massie B, Arella M (2007). Immune responses induced by replication-defective adenovirus expressing the C-terminal portion of the *Mycoplasma hyopneumoiae* P97 adhesin. Clinical and Vaccine Immunology 14, 767-774.

Oneal MJ, Schafer ER, Madsen ML, Minion FC (2008). Global transcriptional analysis of *Mycoplasma hyopneumoniae* following exposure to norepinephrine. Micriobiology 154, 2581-2588.

Opriessnig T, Thacker EL, Yu S, Fenaux M, Meng X-J, Halbur PG (2004). Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by dual infection with *Mycoplasma hyopneumoniae* and porcine circovirus type 2. Veterinary Pathology 41, 624-640.

Opriessnig T, Giménez-Lirola LG, Halbur PG (2011a). Polymicrobial respiratory disease in pigs. Animal Health Research Reviews 12, 133-148.

Opriessnig T, Madson DM, Schalk S, Brockmeier S, Shen HG, Beach NM, Meng XJ, Baker RB, Zanella EL, Halbur PG (2011b). Porcine circovirus type 2 (PCV2) vaccination is effective in reducing disease and PCV2 shedding in semen of boars concurrently infected with PCV2 and *Mycoplasma hyopneumoniae*. Theriogeniology 76, 351-360.

Ostanello F, Dottori M, Gusmara C, Leotti G, Sala V (2007). Pneumoniae disease assessment using a slaughterhouse lung-scoring method. Journal of Veterinary Medicine A 54, 70-75.

Otake S, Dee S, Corzo C, Oliveira S, Deen J (2010). Long-distance airborne transport of infectious PRRSV and *Mycoplasma hyopneumoniae* from a swine population infected with multiple viral variants. Veterinary Microbiology 145, 198-208.

Park S-C, Yibchok-Anun S, Cheng H, Young TF, Thacker EL, Minion FC, Ross RF, Hsu WH (2002). *Mycoplasma hyopneumoniae* increases intracellular calcium release in porcine ciliated tracheal cells. Infection and Immunity 70, 2502-2506.

Park S-J, Seo HW, Park C, Chae C (2014). Interaction between single-dose *Mycoplasma hyopneumoniae* and porcine reproductive and respiratory syndrome virus vaccines on dually infected pigs. Research in Veterinary Science 96, 516-522.

Park C, Jeong J, Choi K, Chae C (2016). Efficacy of a new bivalent vaccine of porcine circovirus type 2 and *Mycoplasma hyopneumoniae* (Fostera™ PCV MH) under experimental conditions. Vaccine 34, 270-275.

Ph. Eur. (European Pharmacopoeia) (2013) Monograph 04/2013:2448. Porcine enzootic pneumonia vaccine (inactivated). Ph. Eur., 8th Edit., Strasbourg, France.

Pieters M, Pijoan C, Fano E, Dee S (2009). An assessment of the duration of *Mycoplasma hyopneumoniae* infection in an experimentally infected population of pigs. Veterinary Microbiology 134, 261-266.

Pieters M, Cline GS, Payne BJ, Prado C, Ertl JR, Rendahl AK (2014). Intra-farm risk factors for *Mycoplasma hyopneumoniae* colonization at weaning age. Veterinary Microbiology 172, 575-580.

Pieters M, Fano E (2016). *Mycoplasma hyopneumoniae* management in gilts. Veterinary Record 178, 122-123.

Pieters M, Daniels J, Rovira A (2017). Comparison of sample types and diagnostic methods for *in vivo* detection of *Mycoplasma hyopneumoniae* during early stages of infection. Veterinary Microbiology 203, 103-109.

Pieters M, Sibila M (2017). When is the best time to vaccinate piglets against *Mycoplasma hyopneumoniae?* Veterinary Record 181, 16-17.

Piffer I, Ross RF (1985). Immunofluorescence technique for detection of *Mycoplasma hyopneumoniae* in swine lungs. Pesquisa Agropecuáia Brasileira 20, 877-882.

Pijoan C (2006). Pneumonic pasteurellosis. In: Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ (eds.). Diseases of Swine. Iowa State University Press, Ames, IA. 719-726.

Pitkin A, Otake S, Dee S (2011). A one-night downtime period prevents the spread of porcine reproductive and respiratory syndrome virus and *Mycoplasma hyopneumoniae* by personnel and fomites (boots and coveralls). Journal of Swine Health and Production 19, 345-348.

Pósa R, Kovács M, Donkó T, Szabó-Fodor J, Mondok J, Bogner P, Repa I, Magyar T (2009). Effect of *Mycoplasma hyopneumoniae* and fumonisin B_1 toxin on the lung in pigs. Italian Journal of Animal Science 8, 172-174.

Prokeš M, Zendulkova D, Rosenbergová K, Treml F, Ondrejková A, Beníšek Z, Ondrejka R, Korytár L, Slepecká E, Süli J, Haladová E, Maženskŷ D (2012). Detection of *Mycoplasma hyopneumoniae* by ELISA and nested PCR from blood samples and nasal swabs from pigs in Slovakia. Acta Veterinaria Brno 81, 327-331.

R Core Team (2007). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/

Rautainen E, Virtala A-M, Wallgren P, Saloniemi H (2000). Varying effects of infections with *Mycoplasma hyopneumoniae* on the weight gain recorded in three different multisource fattening pig herds. Journal of Veterinary Medicine B 47, 461-469.

Rautiainen E, Oravainen J, Virolainen JV, Tuovinen V (2001). Regional eradication of *Mycoplasma hyopneumoniae* from pig herds and documentation of freedom of the disease. Acta Veterinaria Scandinavica 42, 355-364.

Register K, Brockmeier S, de Jong M, Pijoan C (2012). Pasteurellosis. In: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, eds. Diseases of Swine, 10th edition. Wiley-Blackwell, A John Wiley & Sons, Inc., Chichester, West Sussex, UK. p. 798-810.

Reynolds SC, St Aubin LB, Sabbadini LG, Kula J, Vogelaar J, Runnels P, Peters AR (2009). Reduced lung lesions in pigs challenged 25 weeks after the administration of a single dose of *Mycoplasma hyopneumoniae* vaccine at approximately 1 week of age. Veterinary Journal 181, 312-320.

Roberts E, Hammer JM, Lechtenberg K, Roycroft L, King S (2011). Investigation of tiamulin hydrogen fumerate in-feed antibiotic for the control of porcine respiratory disease complex that includes *Mycoplasma hyopneumoniae.* Journal of Swine Health and Production 19, 218-225.

Rocha EPC, Blanchard A (2002). Genomic repeats, genome plasticity and the dynamics of *Mycoplasma* evolution. Nucleic Acids Research 30, 2031-2042.

Rodriguez, F., Ramirez, G.A., Sarradell, J., Andrada, M., Lorenzo, H., (2004). Immunohistochemical labeling of cytokines in lung lesions of pigs naturally infected with *Mycoplasma hyopneumoniae*. Journal of Comparative Pathology 130, 306-312.

Roos LR, Fano E, Homwong N, Payne B, Pieters M (2016). A model to investigate the optimal seeder-to-naive ratio for successful natural *Mycoplasma hyopneumoniae* gilt exposure prior to entering the breeding herd. Veterinary Microbiology 184, 51-58.

Ruiz AR, Utrera V, Pijoan C (2003). Effect of *Mycoplasma hyopneumoniae* sow vaccination on piglet colonization at weaning. Journal of Swine Health and Production 11, 131-135.

Sarradell J, Andrada M, Ramírez AS, Fernández A, Gómez-Villamandos JC, Jover A, Lorenzo H, Herráez P, Rodríguez F (2003). A morphologic and immunohistochemical study of the bronchus-associated lymphoid tissue of pigs naturally infected with *Mycoplasma hyopneumoniae*. Veterinary Pathology 40, 395-404.

Sebunya TN, Saunders JR (1983). *Actinobacillus pleuropneumoniae* infections in swine: a review. Journal American Veterinary Medical Association 182, 1331-1337.

Schafer ER, Oneal MJ, Madsen ML, Minion FC (2007). Global transcriptional analysis of *Mycoplasma hyopneumoniae* following exposure to hydrogen peroxide. Microbiology 153, 3785-3790.

Segalés J, Valero O, Espinal A, Lopez-Soria S, Nofrarias M, Calsamiglia M, Sibila M (2012). Exploratory study on the influence of climatological parameters on *Mycoplasma hyopneumoniae* infection dynamics. International Journal on Biometeorology 56, 1167-1171.

Seo HW, Han K, Oh Y, Park C, Choo EJ, Kim S-H, Lee B-H, Chae C (2013). Comparison of cell-mediated immunity induced by three commercial single-dose *Mycoplasma hyopneumoniae* bacterin in pigs. Journal of Veterinary Medical Science 75, 245-247.

Seo HW, Park S-J, Park C, Chae C (2014). Interaction of porcine circovirus type 2 and *Mycoplasma hyopneumoniae* vaccines on dually infected pigs. Vaccine 32, 2480-2486.

Sheldrake RF, Gardner IA, Saunders MM, Romalis LF (1990). Serum antibody response to *Mycoplasma hyopneumoniae* measured by enzyme-linked immunosorbent assay after experimental and natural infection of pigs. Australian Veterinary Journal 67, 39-42.

Sheldrake RF, Gardner IA, Saunders MM, Romalis LF (1991). Intraperitoneal vaccination of pigs to control *Mycoplasma hyopneumoniae*. Research in Veterinary Science 51, 285-291.

Sheldrake RF, Romalis LF (1992). Evaluation of an enzyme-linked immunosorbent assay for the detection of *Mycooplasma hyopneumoniae* antibodies in porcine serum. Australian Veterinary Journal 69, 255-258.

Shimoji Y, Oishi E, Muneta Y, Nosaka H, Mori Y (2003). Vaccine efficacy of the attenuated *Erysipelothrix rhusiopathiae* YS-19 expressing a recombinant protein of *Mycoplasma hyopneumoniae* P97 adhesin against mycoplasmal pneumonia of swine. Vaccine 21, 532-537.

Sibila M, Nofrarias M, Lopez-Soria S, Segalés J, Riera P, Llopart D, Calsamiglia M (2007a). Exploratory field study on *Mycoplasma hyopneumoniae* infection in suckling pigs. Veterinary Microbiology 121, 352-356.

Sibila M, Nofrarías N, López-Soria S, Segalés J, Valero O, Espinal A, Calsamiglia M (2007b). Chronological study of *Mycoplasma hyopneumoniae* infection, seroconversion and associated lung lesions in vaccinated and non-vaccinated pigs. Veterinary Microbiology 122, 97-107.

Sibila M, Bernal R, Torrents D, Riera P, Llopart D, Calsamiglia M, Segalés J (2008). Effect of sow vaccination against *Mycoplasma hyopneumoniae* on sow and piglet colonization and seroconversion and pig lung lesions at slaughter. Veterinary Microbiology 127, 165-170.

Sibila M, Pieters M, Molitor T, Maes D, Haesebrouck F, Segalés J (2009). Current perspectives on the diagnosis and epidemiology of *Mycoplasma hyopneumoniae* infection. Veterinary Journal 181, 221-231.

Sibila M, Mentaberre G, Boadella M, Huerta E, Casas-Díaz E, Vicente J, Gortázar C, Marco I, Lavín S, Segalés J (2010). Serological, pathological and polymerase chain reaction studies on *Mycoplasma hyopneumoniae* infection in the wild boar. Veterinary Microbiology 144, 214-218.

Sibila M, Fort M, Nofrarías M, Pérez de Rozas A, Galindo-Cardiel I, Mateu E, Segalés J (2012). Simultaneous porcine circovirus type 2 and *Mycoplasma hyopneumoniae* co-inoculation does not potentiate disease in conventional pigs. Journal of Comparative Pathology 147, 285-295.

Sibila M, Aragón V, Fraile L, Segalés J (2014). Comparison of four lung scoring systems for the assessment of the pathological outcomes derived from *Actinobacillus pleuropneumoniae* experimental infections. BMC Veterinary Research 10, 165-174

Silin DS, Lyobomska OV, Wang CN (2001). *Mycoplasma hyopneumoniae* vaccination influence on porcine reproductive and respiratory syndrome virus and *Mycoplasma hyopneumoniae* co-infection. Acta Veterinaria Brno 70, 413-420.

Simionatto S, Marchioro SB, Galli V, Hartwig DD, Carlessi RM, Munari FM, Laurino JP, Conceição FR, Dellagostin OA (2010). Cloning and purification of recombinant proteins of *Mycoplasma hyopneumoniae* expressed in *Escherichia coli*. Protein Expression and Purification 69, 132-136.

Simionatto S, Marchioro SB, Galli V, Brum CB, Klein CS, Rebelatto R, Silva EF, Borstuk S, Conceição FR, Dellagostin OA (2012). Immunological characterization of *Mycoplasma hyopneumoniae* recombinant proteins. Comparative Immunology, Microbiology and Infectious Diseases 35, 209-216.

Simionatto S, Marchioro S, Maes D, Dellagostin O (2013). *Mycoplasma hyopneumoniae*: from disease to vaccine development. Veterinary Microbiology 165, 234-242.

Sitjar M, Noyes EP, Simon X, Pijoan C (1996). Relationships among seroconversion to *Mycoplasma hyopneumoniae*, lung lesions, and production parameters in pigs. Journal of Swine Health and Production 4, 273-277.

Sørensen V, Barfod K, Feld NC (1992). Evaluation of a monoclonal blocking ELISA and IHA for antibodies to *Mycoplasma hyopneumoniae* in SPF-herds. Veterinary Record 130, 488-490.

Sørensen V, Barfod K, Feld NC, Vraa-Andersen L (1993). Application of enzyme-linked immunosorbent assay for the surveillance of Mycoplasma hyopneumoniae infection in pigs. Revolutions in Science and Technology 12, 593-604.

Sørensen V, Nielsen JP, Barfod K, Schirmer AL (1997a). Inoculation of pigs with *Actinobacillus pleuropneumoniae* serotypes 1, 5b, 6, 7, 8, 10 and 12: clinical, serological and pathological observations. In: Sorensen V (ed.). Evaluation of Laboratory Diagnostic Assays for Monitoring Respiratory Infections in Pigs. [Ph.D. Thesis.] The Royal Veterinary and Agricultural University, Federation of Danish Pig Producers and Slaughterhouses, Danish Veterinary Laboratory, Copenhagen, Denmark. p. 82-93.

Sørensen V, Ahrens P, Barfod K, Feenstra AA, Feld NC, Friis NF, Bille-Hansen V, Jensen NE, Pedersen MW (1997b). *Mycoplasma hyopneumoniae* infection in pigs: duration of the disease and evaluation of four diagnostic assays. Veterinary Microbiology 54, 23-34.

Speksnijder DC, Jaarsma DA, Verheij TJ, Wagenaar JA (2015). Attitudes and perceptions of Dutch veterinarians on their role in the reduction of antimicrobial use in farm animals. Preventive Veterinary Medicine 121, 365-373.

Stakenborg T, Vicca J, Butaye P, Maes D, Peeters J, de Kruif A, Haesebrouck F (2005). The diversity of *Mycoplasma hyopneumoniae* within and between herds using pulsed-field gel electrophoresis. Veterinary Microbiology 109, 29-36.

Stakenborg T, Vicca J, Maes D, Peeters J, de Kruif A, Haesebrouck F, Butaye P (2006). Comparison of molecular techniques for the typing of *Mycoplasma hyopneumoniae* isolates. Journal of Microbiological Methods 66, 263-275.

Stärk KD (1999). The role of infectious aerosols in disease transmission in pigs. The Veterinary Journal 158, 164-181.

Stärk KD, Keller H, Eggenberger E (1992) Risk factors for the reinfection of specific pathogen-free pig breeding herds with enzootic pneumonia. Veterinary Record 131, 532-535.

Stärk KDC, Nicolet J, Frey J (1998). Detection of *Mycoplasma hyopneumoniae* by air sampling with a nested PCR assay. Applied and Environmental Microbiology 64, 543-548.

Stärk KD (2000). Epidemiological investigation of the influence of environmental risk factors on respiratory diseases in swine – a literature review. Veterinary Journal 159, 37-56.

Stärk KD, Miserez R, Siegmann S, Ochs H, Infanger P, Schmidt J (2007). A succesful national control programme for enzootic respiratory disease in pigs in Switzerland. Reviews in Science and Technology 26, 595-606.

Steenhard NR, Jungersen G, Kokotovic B, Beshah E, Dawson HD, Urban JF Jr, Roepstorff A, Thamsborg SM (2009). *Ascaris suum* infection negatively affects the response to a *Mycoplasma hyopneumoniae* vaccination and subsequent challenge infection in pigs. Vaccine 27, 5161-5169.

Stemke GW, Robertson JA (1990). The growth response of *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare* based upon ATP-dependent luminometry. Veterinary Microbiology 24, 135-142.

Stemke GW, Huang Y, Laigret F, Bové JM (1994). Cloning the ribosomal RNA operons of *Mycoplasma flocculare* and comparison with those of *Mycoplasma hyopneumoniae*. Microbiology 140, 857-860.

Stipkovits L, Nicolet J, Haldimann A, Frey J (1991). Use of antibodies against P36 protein of *Mycoplasma hyopneumoniae* for the identification of *M. hyopneumoniae* strains. Molecular and Cellular Probes 5, 451-457.

Strait EL, Erickson BZ, Thacker EL (2004). Analysis of *Mycoplasma hyopneumoniae* field isolates. In: Proceedings of 35th Annual Meeting of American Association of Swine Veterinarians. Des Moines, IA. p. 95

Strait EL, Madsen M, Minion F, Christopher-Hennings J, Dammen M, Jones K, Thacker E (2008). Real-time PCR assays to address genetic diversity among strains of *Mycoplasma hyopneumoniae*. Journal of Clinical Microbiology 46, 2491-2498.

Strasser M, Abiven P, Kobisch M, Nicolet J (1992). Immunological and pathological reactions in piglets experimentally infected with *Mycoplasma hyopneumoniae* and/or *Mycoplasma flocculare*. Veterinary Immunology and Immunopathology 31, 141-153.

Straw BE, Backstrom L, Leman AD (1986). Examination of swine at slaughter. Part II. Findings at slaughter and their significance. Compendium on Continuing Education for the Practicing Veterinarian 8, 106-112.

Straw BE, Shin SJ, Yeager AE (1990). Effect of pneumonia on growth rate and feed efficiency of minimal disease pigs exposed to *Actinobacillus pleuropneumoniae* and *Mycoplasma hyopneumoniae*. Preventive Veterinary Medicine 9, 287-294.

Subramaniam S, Frey J, Huang B, Djordjevic S, Kwang J (2000). Immunoblot assays using recombinant antigens for the detection of *Mycoplasma hyopneumoniae* antibodies. Veterinary Microbiology 75, 99-106.

Suh D, Rutten S, Dee SA, Joo HS, Pijoan C (1998). Effect of nursery depopulation on the seroprevalence of *Mycoplasma hyopneumoniae* in nursery pigs. Journal of Swine Health and Production 6, 151-155.

Takeuti KL, de Barcellos DESN, de Andrade CP, de Almeida LL (2017a). Infection dynamics and genetic variability of *Mycoplasma hyopneumoniae* in self-replacement gilts. Veterinary Microbiology 208, 18-24.

Takeuti KL, de Barcellos DESN, de Lara AC, Kunrath CF (2017b). Detection of *Mycoplasma hyopneumoniae* in naturally infected gilts over time. Veterinary Microbiology 203, 215-220.

Takeuti KL, de Barcellos DESN, Pieters M (2017c). *Mycoplasma hyopneumoniae* detection in nylon-flocked and rayon-bud swabs. Journal of Microbiological Methods 141, 118-120.

Tamiozzo P, Parada ACJ, Pelliza B, Ambrogi A (2011). Some aspects about the colonization and serological state of *Mycoplasma hyopneumoniae* vaccinated sows and their piglets by parity distribution. Archives Medicine Vétérinaire 43, 251-258.

Tamiozzo P, Lucchesi PMA, Ambrogi A (2013). Monitoring for *Mycoplasma hyopneumoniae* before and after a partial depopulation program using a typing scheme based on the polyserine repeat motif of *p146*. Journal of Swine Health and Production 21, 309-312.

Tanner AC, Erickson BZ, Ross RF (1993). Adaptation of the Sensititre® broth microdilution technique to antimicrobial susceptibility testing of *Mycoplasma hyopneumoniae*. Veterinary Microbiology 36, 301-306.

Thacker EL, Thacker BJ, Boettcher TB, Jayappa H (1998). Comparison of antibody production, lymphocyte stimulation, and protection induced by four commercial *Mycoplasma hyopneumoniae* bacterins. Journal of Swine Health and Production 6, 107-112.

Thacker EL, Halbur PG, Ross RF, Thanawongnuwech R, Thacker BJ (1999). *Mycoplasma hyopneumoniae* potentiation of porcine reproductive and respiratory syndrome virus-induced pneumonia. Journal of Clinical Microbiology 37, 620-627.

Thacker EL, Thacker BJ, Young TF, Halbur PG (2000). Effect of vaccination on the potentiation of porcine reproductive and respiratory syndrome virus (PRRSV)-induced pneumonia by *Mycoplasma hyopneumoniae*. Vaccine 18, 1244-1252.

Thacker EL, Minion FC (2012). Mycoplasmosis. In: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, eds. Diseases of Swine, 10th edition. Wiley-Blackwell, A John Wiley & Sons, Inc., Chichester, West Sussex, UK. p. 779-797.

Thomsen BL, Jorsal SE, Andersen S, Willeberg P (1992). The Cox regression model applied to risk factor analysis of infections in the breeding and multiplying herds in the Danish SPF system. Preventive Veterinary Medicine 12, 287-297.

Van Alstine WG, Stevenson GW, Kanitz CL (1996). Porcine reproductive and respiratory syndrome virus does not exacerbate *Mycoplasma hyopneumoniae* infection in young pigs. Veterinary Microbiology 49, 297-303.

Van Alstine WG (2012). Respiratory system. In: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, eds. Diseases of Swine, 10th edition. Wiley-Blackwell, A John Wiley & Sons, Inc., Chichester, West Sussex, UK. p. 348-362.

Vangroenweghe F, Karriker L, Main R, Christianson E, Marsteller T, Hammen K, Bates J, Thomas P, Ellingson J, Harmon K, Abate S, Crawford K (2015a). Assessment of litter prevalence of *Mycoplasma hyopneumoniae* in preweaned piglets utilizing an antemorten tracheobronchial mucus collection technique and real-time polymerase chain reaction assay. Journal of Veterinary Diagnostic Investigation 27, 606-610.

Vangroenweghe FACJ, Labarque GL, Piepers S, Strutzberg-Minder K, Maes D (2015b). *Mycoplasma hyopneumoniae* infections in peri-weaned and post-weaned pigs in Belgium and The Netherlands: prevalence and associations with climatic conditions. Veterinary Journal 205, 93-97.

Vangroenweghe F (2017). Good vaccination practice: it all starts with a good vaccine storage temperature. Porcine Health Management 3, 24-30.

Vasconcelos AT, Ferreira HB, Bizarro CV, Bonatto SL, Carvalho MO, Pinto PM, Almeida DF, Almeida LG, Almeida R, Alves-Filho L, Assunção EN, Azevedo VA, Bogo MR, Brigido MM, Brocchi M, Burity HA, Camargo AA, Camargo SS, Carepo MS, Carraro DM, de Mattos Cascardo JC, Castro LA, Cavalcanti G, Chemale G, Collevatti RG, Cunha CW, Dallagiovanna B, Dambrós BP, Dellagostin OA, Falcão C, Fantinatti-Garboggini F, Felipe MS, Fiorentin L, Franco GR, Freitas NS, Frías D, Grangeiro TB, Grisard EC, Guimarães CT, Hungria M, Jardim SN, Krieger MA, Laurino JP, Lima LF, Lopes MI, Loreto EL, Madeira HM, Manfio GP,

Maranhão AQ, Martinkovics CT, Medeiros SR, Moreira MA, Neiva M, Ramalho-Neto CE, Nicolás MF, Oliveira SC, Paixão RF, Pedrosa FO, Pena SD, Pereira M, Pereira-Ferrari L, Piffer I, Pinto LS, Potrich DP, Salim AC, Santos FR, Schmitt R, Schneider MP, Schrank A, Schrank IS, Schuck AF, Seuanez HN, Silva DW, Silva R, Silva SC, Soares CM, Souza KR, Souza RC, Staats CC, Steffens MB, Teixeira SM, Urmenyi TP, Vainstein MH, Zuccherato LW, Simpson AJ, Zaha A (2005). Swine and poultry pathogens: the complete genome sequences of two strains of *Mycoplasma hyopneumoniae* and a strain of *Mycoplasma synoviae*. Journal of Bacteriology 187, 5568-5577.

Verdin E, Saillard C, Labbé A, Bové JM, Kobisch M (2000a). A nested PCR assay for detection of *Mycoplasma hyopneumoniae* in tracheobronchiolar washings from pigs. Veterinary Microbiology 76, 31-40.

Verdin E, Kobisch M, Bové JM, Garnier M, Saillard C (2000b). Use of an internal control in a nested-PCR assay for *Mycoplasma hyopneumoniae* detection and quantification in tracheobronchiolar washings from pigs. Molecular and Cellular Probes 14, 365-372.

Verloo D, Dewulf J, Maes D, Mintiens K, Laevens H, Boelaert F (2005). Diagnostische testen: validatie, interpretatie en de gevolgen op de besluitvorming. Vlaams Diergeneeskundig Tijdschrift 74, 27-34.

Vicca J, Maes D, Thermote L, Peeters J, Haesebrouck F, de Kruif A (2002). Patterns of *Mycoplasma yopneumoniae* infections in Belgian farrow-to-finish pig herds with diverging disease-course. Journal of Veterinary Medicine B 49, 349-353.

Vicca J, Stakenborg T, Maes D, Butaye P, Peeters J, de Kruif A, Haesebrouck F (2003). Evaluation of virulence of *Mycoplasma hyopneumoniae* field isolates. Veterinary Microbiology 97, 177-190.

Vicca J, Stakenborg T, Maes D, Butaye P, Peeters J, de Kruif A, Haesebrouck F (2004). In vitro susceptibilities of *Mycoplasma hyopneumoniae* field isolates. Antimicrobial Agents and Chemotherapy 48, 4470-4472.

Vigre H, Dohoo IR, Stryhn H, Busch ME (2004). Intra-unit correlations in seroconversion to *Actinobacillus pleuropneumoniae* and *Mycoplasma hyopneumoniae* at different levels in Danish multi-site pig production facilities. Preventive Veterinary Medicine 63, 9-28.

Villarreal I, Maes D, Meyns T, Gebruers F, Calus D, Pasmans F, Haesebrouck F (2009). Infection with a low virulent *Mycoplasma hyopneumoniae* isolate does not protect piglets against subsequent infection with a high virulent *M. hyopneumoniae* isolate. Vaccine 27, 1875-1879.

Villarreal I, Vranckx K, Duchateau L, Pasmans F, Haesebrouck F, Jensen JC, Nanjiani IA, Maes D (2010). Early *Mycoplasma hyopneumoniae* infections in European suckling pigs in herds with respiratory problems: Detection rate and risk factors. Veterinarni Medicina 55, 318-324.

Villarreal I, Maes D, Vranckx K, Calus D, Pasmans F, Haesebrouck F (2011a). Effect of vaccination of pigs against experimental infection with high and low virulence *Mycoplasma hyopneumoniae* strains. Vaccine 29, 1731-1735.

Villarreal I, Meyns T, Dewulf J, Vranckx K, Calus D, Pasmans F, Haesebrouck F, Maes D (2011b). The effect of vaccination on the transmission of *Mycoplasma hyopneumoniae* in pigs under field conditions. Veterinary Journal 188, 48-52.

Villarreal I, Vranckx K, Calus D, Pasmans F, Haesebrouck F, Maes D (2012). Effect of challenge of pigs previously immunized with inactivated vaccines containing homologous and heterologous *Mycoplasma hyopneumoniae* strains. BCM Veterinary Research 8, 2-8.

Virginio VG, Honchoroski T, Paes JA, Schuck DC, Zaha A, Ferreira HB (2014). Immune responses elicited by *Mycoplasma hyopneumoniae* recombinant antigens and DNA constructs with potential use in vaccination against porcine enzootic pneumonia. Vaccine 32, 5832-5838.

Virginio VG, Bandeira NC, dos Anjos Leal FM, Lancellotti M, Zaha A, Ferreira HB (2017). Assessment of the adjuvant activity of mesoporous silica nanoparticles in recombinant *Mycoplasma hyopneumoniae* antigen vaccines. Heliyon 3:e00225. doi:10/1016/j.heliyon.2016.e0022

Vranckx K, Maes D, Calus D, Villarreal I, Pasmans F, Haesebrouck F (2011). Multiple-locus Variable-Number Tandem Repeat analysis is a suitable tool for differentiation of *Mycoplasma hyopneumoniae* strains without cultivation. Journal of Clinical Microbiology 49, 2020-2023.

Vranckx K, Maes D, del Pozo Sancristán R, Pasmans F, Haesebrouck F (2012a). A longitudinal study of the diversity and dynamics of *Mycoplasma hyopneumoniae* infections in pig herds. Veterinary Microbiology 156, 315-321.

Vranckx K, Maes D, Marchioro SB, Villarreal I, Chiers K, Pasmans F, Haesebrouck F (2012b). Vaccination reduces macrophage infiltration in bronchus-associated lymphoid tissue in pigs infected with a highly virulent *Mycoplasma hyopneumoniae* strain. BMC Veterinary Research 8, 24-30.

Vyt P, Heylen P, Neven M, Castryck F (2007). A practical approach to the elimination of swine dysentery (*Brachyspira hyodysenteriae*) from single-site farrow-to-finish herds: Flemish Veterinary Journal 76, 124-129.

Walders B, Raschke A, Neugebauer M, Geuther E, Bertling W, Reiser C, Buck A, Strich S, Hess J (2005). Blending of a conventional *Mycoplasma hyopneumoniae* vaccine with a positive marker: tracking of immunized pigs by peptide-specific antibodies raised to the marker component. Research in Veterinary Science 78, 135-141.

Wallgren P, Bölske G, Gustafsson S, Mattsson S, Fossum C (1998). Humoral immune response to *Mycoplasma hyopneumoniae* in sows and offspring following an outbreak of mycoplasmosis. Veterinary Microbiology 60, 193-205.

Wallgren P, Nörregård E, Molander B, Persson M, Ehlorsson C-J (2016). Serological patterns of *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, *Pasteurella multocida* and *Streptococcus suis* in pig herds affected by pleuritis. Acta Veterinaria Scandinavica 58, 71-79.

Weiβ C, Beffort L, Renken C, Froelich S, Zoels S, Ritzmann M (2018). Effect of tracheobronchial-swabbing and other diagnostic tools on the endrocrine stress response of pigs. In: Proceeding of 9th European Symposium on Porcine Health Management. Barcelona, Spain. p. 86.

Williams PP (1978). In vitro susceptibility of *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis* to fifty-one antimicrobial agents. Antimicrobial Agents and Chemotherapy 14, 210-213.

Wilson S, Van Brussel L, Saunders G, Taylor L, Zimmermann L, Heinritzi K, Ritzmann M, Banholzer E, Eddicks M (2012). Vaccination of piglets at 1 weeks of age with an inactivated *Mycoplasma hyopneumoniae* vaccine reduces lung lesions and improves average daily gain in body weight. Vaccine 30, 7625-7629.

Wilson S, Van Brussel L, Saunders G, Runnels P, Taylor L, Fredrickson D, Salt J (2013). Vaccination of piglets up to 1 weeks of age with a single-dose *Mycoplasma hyopneumoniae* vaccine induces protective immunity within 2 weeks against virulent challenge in the presence of MDA. Clinical and Vaccine Immunology 20, 720-724.

Witvliet M, Holtslag H, Nell T, Segers R, Fachinger V (2015). Efficacy and safety of a combined porcine circovirus and *Mycoplasma hyopneumoniae* vaccine in finishing pigs. Trials in Vaccinology 4, 43-49.

Woolley LK, Fell S, Gonsalves JR, Walker MJ, Djordjevic SP, Jenkins C, Eamens GJ (2012). Evaluation of clinical, histological and immunological changes and qPCR detection of *Mycoplasma hyopneumoniae* in tissues during the early stages of mycoplasmal pneumonia in pigs after experimental challenge with two field strains. Veterinary Microbiology 161, 186-195.

Woolley LK, Fell SA, Gonsalves JR, Raymond BBA, CollinS D, Kuit TA, Walker MJ, Djordjevic SP, Eamens GJ, Jenkins C (2014). Evaluation of recombinant *Mycoplasma hyopneumoniae* P97/P102 paralogs formulated with selected adjuvants as vaccines against mycoplasmal pneumonia in pigs. Vaccine 32, 4333-4341.

Wu CC, Shryrock TR, Lin TL, Veenhuizen MF (1997). Testing antimicrobial susceptibility against *Mycoplasma hyopneumoniae* in vitro. Journal of Swine Health and Production 5, 227-230.

Xiong Q, Wei Y, Feng Z, Gan Y, Liu Z, Liu M, Bai F, Shao G (2014a). Protective efficacy of a live attenuated *Mycoplasma hyopneumoniae* vaccine with an ISCOM-matrix adjuvant in pigs. Veterinary Journal 199, 268-274.

Xiong Q, Wei Y, Xie H, Feng Z, Gan Y, Wang C, Liu M, Bai F, Xie F, Shao G (2014b). Effect of different adjuvant formulations on the immunogenicity and protective effect of a live *Mycoplasma hyopneumoniae* vaccine after intramuscular inoculation. Vaccine 32, 3445-3451.

Yagihashi T, Nunoya T, Mitui T, Tajima M (1984). Effect of *Mycoplasma hyopneumoniae* infection on the development of *Haemophilus pleuropneumoniae* pneumonia in pigs. Japanese Journal of Veterinary Science 46, 705-713.

Yamaguti M, Muller EE, Piffer AI, Kich JD, Klein CS, Kuchiishi SS (2008). Detection of *Mycoplasma hyopneumoniae* by polymerase chain reaction in swine presenting respiratory problems. Brazilian Journal of Microbiology 39, 471-476.

Yamamoto K, Koshimizu K, Ogata M (1986). *In vitro* susceptibility of *Mycoplasma hyopneumoniae* to antibiotics. Japanese Journal of Veterinary Science 48, 1-5.

Yang W-J, Lai J-F, Pen K-C, Chiang H-J, Weng C-N, Shiuan D (2005). Epitope mapping in *Mycoplasma hyopneumoniae* using phage displayed peptide libraries and the immune responses of the selected phagotypes. Journal of Immunological Methods 304, 15-29.

Yazawa S, Okada M, Ono M Fujii S, Okuda Y, Shibata I, Kida H (2004). Experimental dual infection of pigs with an H1N1 swine influenza virus (A/Sw/Hok/2/81) and *Mycoplasma hyopneumoniae*. Veterinary Microbiology 98, 221-228.

Yeske P (2016). *Mycoplasma hyopneumoniae* eliminiation. In: Proceedings of 47th Annual Meeting of American Association of Swine Veterinarians. New Orleans, LA. p. 376-380.

Yeske P (2018). *Mycoplasma hyopneumoniae*: lateral transmission and gilt exposure methods. In: Proceedings of 49th Annual Meeting of American Association of Swine Veterinarians. San Diego, CA. p. 482-484.

Zhang H, Lunney JK, Baker RB, Opriessnig T (2011). Cytokine and chemokine mRNA expression profiles in tracheo-bronchial lymph nodes from pigs singularly infected or coinfected with porcine circovirus type 2 (PCV2) and *Mycoplasma hyopneumoniae* (MHYO). Veterinary Immunology and Immunopathology 140, 152-158.

Zimmerman DR, Spear ML, Switzer WP (1973). Effect of *Mycoplasma hyopneumoniae* infection, pyrantel treatment and protein nutrition on performance of pigs exposed to soil containing *Ascaris suum* ova. Journal of Animal Science 36, 894-897.

Zimmerman W, Odermatt W, Tschudi P (1989). Enzootic pneumonia (EP): the partial curing of EP-reinfected swine herds as an alternative to total cure. Schweizer Archiv fur Tielheilkunde 131, 179-191.

Zou H-Y, Liu X-J, Ma F-Y, Chen P, Zhou R, He Q-G (2011). Attentuated *Actinobacillus pleuropneumoniae* as a bacterial vector for expression of *Mycoplasma hyopneumoniae* P36 gene. Journal of Gene Medicine 13, 221-229.

SUMMARY

Chapter 1 - The introduction gives a comprehensive review of PRDC and more specifically *M. hyopneumoniae*, the etiology of enzootic pneumonia. The review covers *M. hyopneumoniae* etiology, pathogenesis with focus on epidemiology and diagnosis with focus on serology, detection of the pathogen itself and specific sampling approaches in living animals. Finally, *M. hyopneumoniae* control measures, including biosecurity measures, vaccination and eradication strategies are discussed.

Chapter 2 – Study objectives of the presented work are to evaluate the health bonus of batch management systems (BMS) for different pathogens using serology, to validate and apply a tracheo-bronchial swab (TBS) sampling technique for sampling of live pigs under field conditions in the US, Belgium and The Netherlands for prevalence of *M. hyopneumoniae*. The technique is subsequently evaluated in two SPF farms to confirm an inconclusive serological monitoring of *M. hyopneumoniae.*

Chapter 3 – BMS are frequently used to improve pig health at farm level. The impact of a transition from a 1-week BMS to a 4- or 5-week BMS was studied on the infection levels of different pathogens, such as *M. hyopneumoniae*, Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), *L. intracellularis*, *A. pleuropneumoniae*, *B. hyodysenteriae* and *P. multocida* DNT. Transition to a 4- or 5-week BMS results in a health bonus for *L. intracellularis*, *M. hyopneumoniae* and *A. pleuropneumoniae*, which results in a significant decrease of infection within different age categories. Serological follow-up of *M. hyopneumoniae* using ELISA is a useful tool to evaluate strategic interventions with a long-term impact on pig health.

Chapter 4 – Early detection of *M. hyopneumoniae* through ELISA has been shown difficult. Therefore, different sampling techniques on live and dead pigs have been validated and evaluated under US field conditions. TBS sampling demonstrated a consistently high recovery of *M. hyopneumoniae* as compared to nasal swabs and other sampling techniques such as deep airway swabs, BALF or lung tissue samples. Subsequent application of TBS in suckling piglets (12 to 25 days of age) under US field conditions revealed a very low prevalence (1.1%) of *M. hyopneumoniae* in sow farms without clinical respiratory symptoms.

Chapter 5 – Application of TBS to reveal *M. hyopneumoniae* prevalence in Belgium and The Netherlands demonstrated a higher *M. hyopneumoniae* prevalence of 7.1% in peri-weaned and 10.9% post-weaned piglets. Approximately 27-29% of the sampled farms had at least one *M. hyopneumoniae*-positive piglet in the peri-weaning or post-weaning period. Furthermore, the

association of *M. hyopneumoniae* prevalence with climatic data was studied. The probability of peri-weaned piglets being *M. hyopneumoniae*-positive was negatively associated with rainfall during the week preceding sampling. In post-weaned piglets, the average outdoor relative humidity over the 4 weeks preceding sampling and the minimum average outdoor temperature during the week preceding sampling were positively associated with *M. hyopneumoniae* positivity.

Chapter 6 & 7 – Detection of *M. hyopneumoniae* was performed using TBS and qPCR in two different SPF herds in Eastern Europe with a history of suspect *M. hyopneumoniae*-positive results in the absence of confirmatory clinical signs or necropsy lesions indicative to *M. hyopneumoniae*. In the first case (Chapter 6), *M. hyopneumoniae* was only detected in replacement gilts and reproductive sow at the sow unit, whereas piglets and young rearing gilts remained negative for *M. hyopneumoniae* in all TBS samples. The second case (Chapter 7) reports a recent outbreak with inconclusive serological results where *M. hyopneumoniae* was detected from 18 weeks of age onwards in the gilt rearing unit. In both cases, TBS could reveal the presence of *M. hyopneumoniae* whereas clinical signs and necropsy lesions were absent and serology remained inconclusive using 2 different ELISA tests on the blood samples.

SAMENVATTING

Hoofdstuk 1 – De inleiding geeft een uitgebreid overzicht van PRDC en meer specifiek *M. hyopneumoniae*, het etiologisch agens van enzootische pneumonie bij varkens. Het overzicht omvat de voornaamste karakteristieken van *M. hyopneumoniae*, de pathogenese van de infectie met focus op de epidemiologie en de diagnose met focus op serologie, detectie van de kiem zelf en de specifieke bemonsteringstechnieken bij levende dieren. Finaal worden de controlemaatregelen, inclusief bioveiligheidsmaatregelen, vaccinatie en eradicatiestrategieën, bediscussieerd.

Hoofdstuk 2 – De doelstelling van het voorgestelde werk is de gezondheidsbonus van meerwekensystemen evalueren voor verschillende pathogenen via serologie, een tracheo-bronchial swab (TBS) techniek voor bemonstering van *M. hyopneumoniae* bij levende varkens valideren en toepassen onder praktijkomstandigheden in de VS, Belgie en Nederland voor bepalen van de prevalentie van *M. hyopneumoniae*. Verder wordt de techniek op enkele SPF bedrijven geëvalueerd om een niet eenduidige serologische monitoring van *M. hyopneumoniae* uit te klaren.

Hoofdstuk 3 – Meerwekensystemen worden frequent aangewend om de gezondheid van de varkens op bedrijfsniveau te verbeteren. De impact van een overgang van een 1-wekensysteem naar een 4- of 5-wekensysteem werd geëvalueerd op de infectiedruk van verschillende pathogenen, zoals ondermeer *M. hyopneumoniae*, Porcien Reproductief en Respiratoir Syndroom Virus (PRRSV), *L. intracellularis*, *A. pleuropneumoniae*, *B. hyodysenteriae* en *P. multocida* DNT. Overgang naar een 4- of 5-wekensysteem levert een gezondheidsbonus op voor *L. intracellularis*, *M. hyopneumoniae* en *A. pleuropneumoniae*, waarbij een significant daling van de infectie optreedt in verschillende leeftijdscategoriën. Een serologische opvolging van *M. hyopneumoniae* met ELISA blijkt een nuttig instrument om strategische interventies met een langetermijnsimpact op de varkensgezondheid te evalueren.

Hoofdstuk 4 – Vroege detectie van *M. hyopneumoniae* infectie door ELISA blijkt niettemin moeilijk. Daarom werden verschillende bemonsteringstechnieken op levende en dode biggen gevalideerd en geëvalueerd onder praktijkomstandigheden in de VS. PCR onderzoek van TBS leidde consistent tot meer positieve uitslagen van *M. hyopneumoniae* in vergelijking met neusswabs en andere bemonsteringstechnieken zoals diepe luchtwegswabs, BALF longspoelvocht of longweefselmonsters. Toepassing van TBS bij zuigende biggen (12 tot 25 dagen leeftijd) onder omstandigheden in de VS leverden een zeer lage prevalentie (1.1%) van *M. hyopneumoniae* op in zeugenbedrijven zonder klinische ademhalingssymptomen.

Hoofdstuk 5 – Toepassing van TBS in België en Nederland toonde een *M. hyopneumoniae* prevalentie aan van 7.1% in pasgespeende biggen en 10.9% in het tweede deel van de batterijperiode. Ongeveer 27-29% van de besmonsterde bedrijven had minstens 1 gespeende big positief voor *M. hyopneumoniae*. Verder werd het verband tussen de *M. hyopneumoniae* prevalentie en buitenklimaatsfactoren bestudeerd. De kans op een *M. hyopneumoniae*-positief speenbig was negatief geassocieerd met neerslag in de week voorafgaand aan de bemonstering. Bij biggen in het tweede deel van de batterijperiode waren gemiddelde relatieve vochtigheid in de laatste 4 weken en de gemiddelde minimumbuitentemperatuur in de week voor bemonstering positief geassocieerd met *M. hyopneumoniae*-positieve biggen.

Hoofdstuk 6 & 7 – Detectie van *M. hyopneumoniae* via TBS/qPCR werd vervolgens uitgevoerd op twee verschillende SPF bedrijven in Oost-Europa met een geschiedenis van twijfelachtige *M. hyopneumoniae*-positieve resultaten in afwezigheid van klinische ziektetekenen of longletsels indicatief voor *M. hyopneumoniae*. In het eerste geval (Hoofdstuk 6) werd enkel *M. hyopneumoniae* gedetecteerd bij recent geïntroduceerde gelten en productieve zeugen op het zeugenbedrijf, terwijl de biggen en opfokgelten *M. hyopneumoniae*-negatief bleven in alle TBS monsters. Het tweede geval (Hoofdstuk 7) rapporteert een recente uitbraak met niet eenduidige serologische resultaten, waarbij *M. hyopneumoniae* werd gedetecteerd vanaf 18 weken leeftijd in de opfokstal voor gelten. In beide studies kon TBS de aanwezigheid van *M. hyopneumoniae* aantonen in afwezigheid van klinische ziektetekenen en autopsieletsels. De serologie bleef twijfelachtig zelfs bij gebruik van 2 verschillende ELISA testen op de sera.

DANKWOORD

Het is gelukt. Weinigen dromen ervan, nog minder doen het. Een tweede doctoraal proefschrift schrijven was initieel helemaal niet de bedoeling, maar door een gelukkige of ongelukkige – ’t is te zien hoe je het bekijkt – samenloop van omstandigheden kwam het idee om het onderzoek dat ik tijdens mijn veldwerk als Technical Consultant bij Elanco Animal Health verrichtte naar een eenvoudige en vroege diagnose voor *Mycoplasma hyopneumoniae* te bundelen. De resultaten zijn dan ook een bekroning van 7 jaar wetenschappelijke onderzoek onder veldomstandigheden, die onmogelijk zouden zijn geweest zonder de steun, hulp, goede raad en het vertrouwen van vele collega’s en betrokkenen. Ik wil dan ook iedereen die rechtstreeks of onrechtstreeks met dit werk betrokken was van harte bedanken. Namen noemen is altijd een gevaarlijk klus daar dit onvermijdelijk impliceert dat er ook mensen vergeten kunnen worden. Niettemin doe ik een poging om toch enkele mensen speciaal te bedanken.

Het verhaal van de tracheo-bronchiale swabs begon voor mij in Frankrijk, waar we onder impuls van Geoffrey Labarque in samenwerking met het ANSES een field training kregen in het nemen van TBS (voor de Nederlanders onder ons, geen Ter Beschikking Stelling, maar Tracheo-Bronchiale Swabs). Onder positieve stimulans van Christiaan – mede door specifieke vragen vanuit de markt, met name de varkensdierenartsen – heb ik deze techniek verder uitgebouwd en ontwikkeld, niet enkel voor jonge pasgespeende biggen maar voor alle diercategorieën tot en met pas-geïntroduceerde gelten. Staalnames doe je nooit alleen, daarom ook een woord van dank voor de Elanco collega’s die mij door de jaren heen bij gelegenheid in de varkensstal hebben ondersteund: Christiaan, Liesbet, Diederik en Koen NL. Uiteraard ook een oprecht woord van dank aan de vele varkensdierenartsen in Benelux, jawel zelfs Luxemburg, waarmee ik in de afgelopen 7 jaar heb samengewerkt en waarvan een aantal ondertussen na training en oefening zelf ook TBS-specialist geworden zijn. Keep the good work going! Jullie maakten het ook mogelijk om de technische ondersteuning te combineren met een voldoende aantal monsters om de studies tot een goed einde te brengen. De vele uren veldwerk hou je natuurlijk enkel vol als dit ook ten volle op support van bovenaf kan rekenen. Bedankt Eddy and thanks to Ramiro for the motivating interest you both took in TBS sampling upon our field days in Benelux.

Besides the local Elanco team, I would like to thank all my technical colleagues in Europe and worldwide who gave me the opportunity to share my expertise on TBS, *M. hyopneumoniae* and PRDC with: Thomas, Kirsten, Katerina, Primoz, Lina, Roselle, Robert (Bob), Eric (US) and Thomas (US). Additionally, I would like to thank Locke Karriker and Rodger Main from Iowa

State University – Veterinary Diagnostic Laboratory for our great cooperation and Maria Pieters from Minnesota University – Veterinary Population Medicine Department and Veterinary Diagnostic Laboratory for our great discussion and the nice field work and great time in the 'Minnesota twins' (Minneapolis – St. Paul).

Verder wil ik een speciaal woord van dank richten tot Eveline Willems voor de fijne samenwerking in het bestuderen van de problematiek van verdachte *M. hyopneumoniae* serologie zonder kliniek op twee Oost-Europese SPF-bedrijven. Bedankt voor de transparante communicatie en informatie-overdracht, waardoor de uitgebreide TBS bemonsteringen ook konden leiden tot twee interessante case reports die het sluitstuk van mijn PhD thesis vormen.

Een PhD thesis tot een goed einde brengen vereist natuurlijk ook een team van kritische en motiverende promotoren. Die heb ik gevonden in prof. Olivier Thas en prof. Dominiek Maes. Olivier, bedankt voor de urenlange discussie over de manier waarop we de data-analyse zouden aanvliegen en tot een goed einde brengen en voor de support in het 'verbeteren' van belangrijke passages in de thesis. Dominiek, een bijzonder woord van de dank voor de kritische blik op de verschillende papers die leidde tot succesvolle publicatie en in het bijzonder op de uitgebreide literatuurstudie voor de inleiding van de thesis.

Ook een woordje van dank aan mijn ouders en schoonouders voor hun begrip als ik weer eens geen tijd had om langs te komen of het slechts tot een vluchtige passage kwam.

Maar, een tweede thesis schrijven, daar moet je niet alleen een beetje gek voor zijn ... je moet ook een stevige support van het thuisfront krijgen voor de vele uren vrije tijd die je investeeert in nog maar eens wat literatuur lezen, een hoofstukje afwerken, een herwerking van een publicatie uitvoeren of data invoeren in Excel. Ellen, Victor, Arthur & Elise-Marie, bedankt voor jullie eindeloze geduld en jullie aanmoediging in deze onderneming. Straks moeten we bedenken hoe we al die uren gaan invullen, zodat ik niet in het spreekwoordelijke 'zwarte gat' val.

Aan allen, bij naam genoemd of anoniem, een welgemeend woord van dank.

CURRICULUM VITAE

Frédéric Vangroenweghe was born on January 30th 1975 in Menen (Belgium). He obtained his Degree in Veterinary Medicine (DVM) with the greatest distinction in 1999. He received the Vétoquinol Price for his dissertation on vaccination against *Actinobacillus pleuropneumoniae* in swine and the Award of the Faculty of Veterinary Medicine for best student over the 6 years of study. From September 1st 1999 onward, he worked as an assistant at the Milk Secretion and Mastitis Research Center (MMRC, Department of Physiology-Biochemistry-Biometrics, Faculty of Veterinary Medicine, Ghent University), where he received training in veterinary physiology and bovine *Escherichia coli* mastitis. He participated in the practical teaching of the 2nd and 3rd year bachelor students Veterinary Medicine. In 2004, he obtained a PhD in Veterinary Sciences (Faculty of Veterinary Medicine, Ghent University) with his thesis entitled '*Escherichia coli* mastitis in primiparous cows' under the guidance of Prof. dr. Christian Burvenich. He obtained a Master in Veterinary Public Health and Food Safety (Faculty of Veterinary Medicine, Ghent University) in 2005. Following an internship and residency, he was certified as an EVBS™ European Veterinary Specialist in Porcine Health Management (Dipl. ECPHM) in 2012. Since 2011, he works as a Senior Technical Consultant Swine Benelux with addition of UK & Republic of Ireland since 2018. He is currently the President of International Pig Veterinary Society – Belgian Branch. He is also an active member of the European Pig Producers – Belgium and the 'Vereniging voor Epidemiologie en Economie' (VEE). During his work in Elanco Animal Health, he has organized several workshops on practical TBS training worldwide. He actively participated at several national and international congresses with oral communications and poster presentations. He is author and co-authors of over 35 scientific papers in peer-reviewed national and international journals. He also contributes as a reviewer to several national and international peer-reviewed journals.

SPONSORS

Great thanks to all sponsors for support in printing this work:

Animal Science Products Inc.

BIO-X Diagnostics

Elanco Animal Health

Ghent University

Idexx Europe BV

IVD GmbH

Topigs Norsvin International BV