Universitat Autònoma de Barcelona

Moving towards an improved experimental model for reproduction of pneumonia induced by Mycoplasma hyopneumoniae

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Tesi doctoral presentada per **Beatriz García Morante** per accedir al grau de Doctora en el marc del programa de Doctorat en Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la direcció de la Dra. **Marina Sibila Vidal** i del Dr. **Joaquim Segalés Coma**.

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La Dra. **Marina Sibila Vidal**, investigadora del Centre de Recerca en Sanitat Animal, de l'Institut de Recerca i Tecnologia Agroalimentàries (CReSA-IRTA), i el Dr. **Joaquim Segalés Coma**, professor titular del Departament de Sanitat i Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona (UAB) i director del CReSA-IRTA.

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Als meus pares,

per no tallar-me mai les ales sinó fer-me sempre volar més alt

i al Pol, l'amor de la meva vida,

per acompanyar-me en aquest viatge sense deixar-me mai de la mà

PART III: GENERAL DISCUSSION AND CONCLUSIONS

LIST OF ABBREVIATIONS

ADWG	Average daily weight gain
AE	Aerosol
APE	Average prediction error
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BALF	Broncho-alveolar lavage fluid
BALT	Bronchus-associated lymphoid tissue
BIP	Broncho-interstitial pneumonia
C	Conventional
CCU	Colour changing units
CDCD	Caesarean-derived, colostrum-deprived
CFU	Colony forming units
CP	Complexity parameter
Ct	Cycle threshold
CT	Culture
CVPC	Cranio-ventral pulmonary consolidation
D	Days post-culture inoculation
DNA	Deoxyribonucleic acid
Dpi	Days post-inoculation
DsDNA	Double-stranded DNA
ELISA	Linked immunosorbent assay
EP	Enzootic pneumonia
ET	Endotracheal
G	Mean generation time
GAM	Generalized additive model
IL	Interleukin
IN	Intranasal

IT	Intratracheal
LH	Lung homogenate
Log	Logarithmic
LW/BW	Lung weight/body weight ratio
MP	Mycoplasmal pneumonia
NA	Non-applicable
NPCR	Nested PCR
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCV2	*Porcine circovirus type 2*
PRDC	Porcine respiratory disease complex
PRRSV	*Porcine reproductive and respiratory syndrome virus*
qPCR	Quantitative real time PCR
RFU	Relative fluorescence units
RLU	Relative light units
Rn	Reproduction ratio
RRNA	Ribosomal ribonucleic acid
SD	Standard deviation
SIV	*Swine influenza virus*
S/P	Sample-to-positive ratio
SPF	Specific-pathogen free
TT	Transtracheal
VIP	Variable importance
Wpi	Weeks post-inoculation

ABSTRACT

The infection with *Mycoplasma hyopneumoniae* is widespread in almost all pig producing countries. Despite its importance, there are still relevant points to be resolved; some of them concern the complex pathogenesis of the infection as well as the resultant pneumonia outcome. For instance, *M. hyopneumoniae* experimental models are a good starting point for addressing some of these unresolved matters, although wide inconsistencies are seen in reproducibility, prevalence and severity of pneumonia. Among others, the latter is a major concern in the case of veterinary medicine product efficacy compliance, especially in vaccinology, where a significant reduction in the lung lesion score is required compared to non-vaccinated pigs. Thus, a validated *M. hyopneumoniae* experimental model enhancing severe pneumonia reproduction is of interest. Therefore, the present thesis was raised to provide insights into this matter through the identification and optimization of experimental conditions playing a role in pneumonia outcome. Furthermore, evaluation of *ante-mortem* parameters that would serve to foresee such outcome was also addressed.

The first study aimed to identify determinants for the successful reproduction of pneumonia under experimental conditions. To reach this goal, a systematic review of the literature followed by a multivariable statistical analysis, named recursive partitioning, was done. The results obtained constitute the first insight into those conditions supporting pneumonia development under experimental settings. *M. hyopneumoniae* experimental models pursuing reproduction of severe pneumonia should

assess lung lesions within a study period of 8 weeks and include inoculation of *M. hyopneumoniae*-free pigs older than 3.5 weeks of age and co-infected with another swine respiratory pathogen. Besides, other two experimental conditions were further investigated individually into the following chapters. In the second study, pneumonia outcome consequence of *M. hyopneumoniae* inoculation by means of three inoculation routes (endotracheal, intranasal and aerosol) was compared. As a result, the endotracheal was the most optimal route of inoculation to induce severe pneumonia within a period of 4 weeks after inoculation. On the other side, in the third study, the growth in culture of *M. hyopneumoniae* strains differing in pathogenicity was daily followed by different techniques, which were evaluated as putative titration methodologies. Results obtained proved that real-time culture titration assays can be used to describe the *in vitro* growth of *M. hyopneumoniae* and to standardise *M. hyopneumoniae* culture protocols in the laboratory as well as inoculum production for experimental applications. The last study evaluated different humoral immune response parameters, both at local and systemic levels, as *ante-mortem* predictors of occurrence and severity of lung lesions associated with *M. hyopneumoniae*. Such an evaluation was done either in experimentally and naturally infected pigs. Overall, results indicated that humoral immune parameters may be used as pneumonia predictors in a population-based approach. Moreover, a new insight into the humoral immune response against *M. hyopneumoniae* is provided by reporting a predominant IgG2 systemic response.

RESUMEN

La infección por *Mycoplasma hyopneumoniae* está ampliamente extendida en la mayoría de países productores de porcino. A pesar de su importancia, todavía hay puntos relevantes que quedan por resolver; algunos de ellos referentes a la compleja patogénesis de la infección así como de la neumonía resultante. Aunque los modelos experimentales de *M. hyopneumoniae* son un buen punto de partida para abordar estas cuestiones, en este escenario se observa una amplia variabilidad tanto en la reproducibilidad como en la prevalencia y la gravedad de dicha neumonía. Esta variabilidad es preocupante, por ejemplo, en el caso de querer cumplir con los requisitos de eficacia de productos medicamentosos veterinarios frente al patógeno en cuestión, especialmente en el caso de las vacunas, ya que se requiere una reducción significativa de las lesiones pulmonares al compararlas con las de cerdos no vacunados. Así pues, un modelo experimental de *M. hyopneumoniae* validado y que asegure la reproducción de neumonía es de interés. De este modo, esta tesis se planteó para identificar y optimizar las condiciones experimentales que pueden jugar un papel importante en el desarrollo de neumonía. Además, en ella también se evalúan parámetros *ante-mortem* con cierto potencial para predecir la prevalencia y la gravedad de neumonía.

El primer estudio tuvo como objetivo identificar determinantes para la reproducción de neumonía en condiciones experimentales. Para lograrlo, se realizó una revisión sistemática de la literatura seguido de un análisis estadístico multivariante, denominado análisis recursivo de partición. Los

resultados proporcionados constituyen una primera visión de las condiciones que favorecen el desarrollo de la neumonía en entornos experimentales. Así, los modelos experimentales de *M. hyopneumoniae* que persiguen la reproducción de neumonía deben evaluar las lesiones pulmonares dentro de un período de estudio de 8 semanas e incluir inoculación de cerdos libres de *M. hyopneumoniae*, mayores de 3.5 semanas de edad y co-infectados con otro patógeno respiratorio porcino. Además, en los siguientes capítulos se evaluaron otras dos condiciones experimentales de forma individual. En el segundo estudio se comparó la neumonía resultante de la inoculación de *M. hyopneumoniae* mediante tres rutas distintas (endotraqueal, intranasal y aerosol). La vía endotraqueal resultó ser la mejor ruta de inoculación para inducir neumonía en un período de 4 semanas después de la inoculación. Por otra parte, en el tercer estudio se usaron diferentes técnicas para el seguimiento diario del crecimiento en cultivo de cepas de *M. hyopneumoniae* de distinto potencial patogénico. Así, dichas técnicas se evaluaron como posibles metodologías de titulación. Los resultados obtenidos demuestran que aquellas técnicas que titulan los cultivos a tiempo real se pueden utilizar para describir el crecimiento *in vitro* de *M. hyopneumoniae*. Esto ayuda a normalizar los protocolos de cultivo de este patógeno en el laboratorio, así como la producción de inóculos para aplicaciones experimentales. En el último estudio se evaluó la capacidad de diferentes parámetros de la respuesta inmunitaria humoral, tanto a nivel local como sistémico, para predecir *ante-mortem* la ocurrencia y la gravedad de las lesiones pulmonares asociadas a *M. hyopneumoniae*. En este caso, dicha evaluación se realizó tanto en cerdos infectados experimental como naturalmente. En general,

los resultados indican que los parámetros inmunológicos humorales pueden utilizarse para predecir neumonía bajo un enfoque poblacional. Este capítulo también describe una respuesta humoral sistémica frente al patógeno en la que predomina la subclase IgG2.

RESUM

La infecció per *Mycoplasma hyopneumoniae* està àmpliament estesa per la majoria de països productors de porcí. Malgrat la seva importància, encara hi ha punts rellevants que queden per resoldre; alguns d'ells referents a la complexa patogènesi de la infecció així com de la pneumònia que en resulta. Tot i que els models experimentals de *M. hyopneumoniae* són un bon punt de sortida per tractar aquestes qüestions, en aquest escenari s'observa una àmplia variabilitat tant en la reproductibilitat com en la prevalença i la gravetat de la pneumònia. Aquesta variabilitat és preocupant, per exemple, en el cas de voler complir amb els requisits d'eficàcia per a productes medicamentosos d'ús veterinari, especialment en el cas de les vacunes, ja que es demana una reducció significativa de les lesions pulmonars al comparar amb les de porcs no vacunats. Així doncs, un model experimental de *M. hyopneumoniae* validat i que asseguri la reproducció de pneumònia és d'interès. Aquesta tesi, de fet, es va plantejar per identificar i optimitzar les condicions experimentals que més poden influenciar el desenvolupament de pneumònia. A més, també s'hi avaluen paràmetres *ante-mortem* amb cert potencial per predir la prevalença i la gravetat de pneumònia.

El primer estudi tenia com a objectiu identificar determinants per a la reproducció de pneumònia en condicions experimentals. Per aconseguir-ho, es va realitzar una revisió sistemàtica de la literatura seguit d'un anàlisi estadístic multivariant, denominat anàlisi recursiu de partició. Els resultats proporcionats constitueixen una primera visió de les condicions que donen

suport al desenvolupament de pneumònia en entorns experimentals. Així, els models experimentals de *M. hyopneumoniae* que persegueixen la reproducció de pneumònia han d'avaluar les lesions pulmonars dins d'un període d'estudi de 8 setmanes i incloure inoculació de porcs lliures de *M. hyopneumoniae*, majors de 3.5 setmanes d'edat i co-infectats amb un altre patogen respiratori porcí. A més, dues condicions experimentals van ser avaluades de forma individual en els següents capítols. En el segon estudi es va comparar la pneumònia resultant de la inoculació artificial de *M. hyopneumoniae* mitjançant tres rutes diferents (endotraqueal, intranasal i aerosol). Com a resultat, la via endotraqueal va resultar ser la millor ruta d'inoculació per induir pneumònia en un període de 4 setmanes després de la inoculació. D'altra banda, en el tercer estudi es van usar diferents tècniques per al seguiment diari del creixement en cultiu de soques de *M. hyopneumoniae* de diferent patogenicitat. D'aquesta manera, aquestes tècniques es van poder avaluar com a possibles metodologies de titulació. Els resultats obtinguts demostren que aquelles tècniques que titulen els cultius a temps real es poden utilitzar per descriure el creixement *in vitro* de *M. hyopneumoniae*. Això ajuda a normalitzar els protocols de cultiu d'aquest patogen al laboratori, així com la producció d'inòculs per a aplicacions experimentals. L'últim estudi avaluà la capacitat de diferents paràmetres de la resposta immunitària humoral, tant a nivell local com sistèmic, per predir *ante-mortem* tant l'ocurrència com la gravetat de les lesions pulmonars associades a *M. hyopneumoniae*. En aquest cas, aquesta avaluació es va realitzar tant en porcs infectats experimental com naturalment. En general, els resultats indiquen que els paràmetres immunològics humorals es poden utilitzar per predir pneumònia sota un

enfocament poblacional. Aquest capítol també descriu una resposta humoral sistèmica on predomina la subclasse IgG2 enfront el patogen.

PUBLICATIONS

The results presented in this thesis have been published or submitted for publication in international scientific peer-reviewed journals:

Garcia-Morante, B., Segalés, J., Fraile, L., Pérez de Rozas, A., Maiti, H., Coll, T., Sibila M., 2016a. Assessment of *Mycoplasma hyopneumoniae*-induced pneumonia using different lung lesion scoring systems: a comparative review. J Comp Pathol. 154, 125-34.

Garcia-Morante, B., Segalés, J., López-Soria, S., Pérez de Rozas, A., Maiti, H., Coll, T., Sibila, M., 2016b. Induction of mycoplasmal pneumonia in experimentally infected pigs by means of different inoculation routes. Vet Res. 47, 54.

Garcia-Morante, B., Segalés, J., Fraile, L., Llardén, G., Coll, T., Sibila, M. Potential use of local and systemic humoral immune response parameters to forecast *Mycoplasma hyopneumoniae* associated lung lesions. Accepted in PLoS One.

Garcia-Morante, B., Segalés, J., Serrano, E., Sibila, M. Determinants for swine mycoplasmal pneumonia reproduction under experimental conditions: a systematic review and recursive partitioning analysis. Submitted for publication.

Garcia-Morante, B., Dors, A., León-Kempis, R., Pérez de Rozas, A., Segalés, J., Sibila, M. Assessment of the *in vitro* growing dynamics and kinetics of the non-pathogenic J and pathogenic 11 and 232 *Mycoplasma hyopneumoniae* strains. Submitted for publication.

PART I

General Introduction, Hypothesis and Objectives

"I a cada instant gasteu tota una vida perquè no hi ha futur que no us pertanyi" Miquel Martí i Pol

Chapter 1

General Introduction

1.1. *MYCOPLASMA HYOPNEUMONIAE* AND DISEASE

Worldwide, respiratory diseases continue causing substantial losses to the swine industry, being regarded as some of the most serious disease problems in intensive swine production systems (Van Alstine, 2012). The respiratory tract of swine is colonized by several bacteria, among which *Mycoplasma hyopneumoniae* is a recognized pathogenic species (Thacker and Minion, 2012).

M. hyopneumoniae is the primary aetiological agent of enzootic pneumonia (EP), which was first recognized as a disease in the 1950s and originally thought to be of viral origin (Lannek *et al.*, 1955). In 1965, Maré and Switzer in the United States and Goodwin and others in the United Kingdom identified a *Mycoplasma* sp. as causal agent of EP and named it *M. hyopneumoniae* and *Mycoplasma suipneumoniae*, respectively (Goodwin *et al.*, 1965; Maré and Switzer, 1965). Afterwards, *M. hyopneumoniae* and *M. suipneumoniae* were found indistinguishable and the name *M. hyopneumoniae*, being the earlier of two, was chosen (Rose *et al.*, 1979). Since then, the role of *M. hyopneumoniae* in respiratory disease in pigs has been increasingly recognized.

M. hyopneumoniae is considered a primary respiratory pathogen, thus, able to induce disease by itself, namely mycoplasmal pneumonia (MP). Notwithstanding, its role in swine respiratory disease is generally through its interaction with other pathogens, resulting in either EP or in the porcine respiratory disease complex (PRDC) (Opriessnig *et al.*, 2011;

Thacker and Minion, 2012), both considered leading causes of economic loss to swine industry throughout the world (Thacker and Minion, 2012).

1.1.1. Enzootic pneumonia

In EP, *M. hyopneumoniae* is in co-infection with other bacteria such as *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Mycoplasma hyorhinis*, *Streptococcus suis*, *Haemophilus parasuis* and/or *Bordetella bronchiseptica* (Sibila *et al.*, 2009; Thacker and Minion, 2012). Some of the previously mentioned bacteria are considered secondary or opportunistic infectious pathogens that typically need help from another co-infecting pathogen, such as *M. hyopneumoniae*, or cofactor to induce substantial lesions in the respiratory system (Opriessnig *et al.*, 2011). Although *M. hyorhinis* has been typically considered a secondary pathogen in EP (Thacker and Minion, 2012), its implication as primary pathogen in EP-like lesions has been sporadically proposed (Lin *et al.*, 2006), pointing out that *M. hyopneumoniae* is not the only mycoplasma species that can elicit such lung lesions.

1.1.2. The porcine respiratory disease complex

The term PRDC has been used to describe a clinical condition associated with mixed respiratory infections of multiple aetiologies (Hansen *et al.*, 2010; Opriessnig *et al.*, 2011). Currently, PRDC is recognised when *M. hyopneumoniae* and/or other bacteria together with certain viral pathogens play a central role as primary agents (Hansen *et al.*, 2010; Opriessnig *et al.*, 2011). Diverse studies have shown that the most commonly associated virus with PRDC are *Porcine reproductive and*

respiratory syndrome virus (PRRSV), *Swine influenza virus* (SIV) and *Porcine circovirus type 2* (PCV2) whereas *M. hyopneumoniae*, *A. pleuropneumoniae* and *P. multocida* are the most frequently involved bacteria (Choi *et al.*, 2003; Hansen *et al.*, 2010; Palzer *et al.*, 2008; Thacker, 2001). Additionally, all those bacteria potentially implicated in EP listed above could be also associated with PRDC (Sibila *et al.*, 2009). It is worth mentioning that in the majority of porcine pneumonias, a mixture of *Mycoplasma* spp., other bacteria and viruses are usually identified, indicating that PRDC is probably more prevalent in pigs than EP (Hansen *et al.*, 2010; Kim *et al.*, 2003).

1.2. CHARACTERISTICS OF *MYCOPLASMA HYOPNEUMONIAE*

1.2.1. Aetiology

Mycoplasmas are taxonomically classified as members of the class *Mollicutes* (Razin *et al.*, 1998). In fact, the majority of the known species of the aforesaid class belong to *Mycoplasma* genus (Razin *et al.*, 1998). *Mollicutes* are phenotypically distinguished from other bacteria by their small size and pleomorphism, the latter due to the lack of a cell wall (Razin *et al.*, 1998). Bacteria in this class are also recognised as the smallest self-replicating organisms, as they have a small genome size, ranging from 214, in *Mycoplasma* spp., to 2.220 Kbp in *Spiroplasma* spp. (Muto and Ushida, 2002; Razin *et al.*, 1998). The latter precludes *Mollicutes* possession of an extensive range of metabolic pathways, particularly the biosynthetic ones, present in other bacterial groups (Pollack *et al.*, 1997; Siqueira *et al.*, 2013). Therefore, they need to obtain

essential metabolites from their growth environment (Thacker and Minion, 2012). Additionally, the *Mollicutes* genome has a low G+C content, thought to result from a strong A+T biased mutation pressure that has been probably involved in their evolution from a gram-positive ancestor bacterium (Muto and Ushida, 2002; Thacker and Minion, 2012).

In particular, morphology of *M. hyopneumoniae* cells are coccoid or coccobacillary to short filamentous, with a medium diameter between 0.20 to 0.80 μm (Blanchard *et al.*, 1992; Tajima and Yagihashi, 1982). Importantly, *M. hyopneumoniae* belongs to the most sequenced porcine mycoplasmas. Up to the present, five *M. hyopneumoniae* strains (i.e. J, 232, 7448, 168 and 7422) have been sequenced (Liu *et al.*, 2013; Minion *et al.*, 2004; Siqueira *et al.*, 2013; Vasconcelos *et al.*, 2005). *M. hyopneumoniae* genome size range from 893 to 926 Kbp, depending on the strain, with a G+C content about 28% (Liu *et al.*, 2013; Minion *et al.*, 2004; Vasconcelos *et al.*, 2005). Indeed, its genome comprises one of the highest A+T contents among *Mycoplasma* species (Minion *et al.*, 2004). Based on the sequence of the 16S rRNA, *M. hyopneumoniae* is phylogenetically closely related to other porcine mycoplasmas such as *M. hyorhinis* and *Mycoplasma flocculare* (Peters *et al.*, 2008; Stemke *et al.*, 1992). Nonetheless, such close phylogenetic similarities are not observed with *Mycoplasma pulmonis* and *Mycoplasma pneumoniae*, two organisms causing similar disease in mice and humans, respectively (Munoz *et al.*, 2011; Stemke *et al.*, 1992).

1.2.2. Isolation and culturing

M. hyopneumoniae is one of the most fastidious mycoplasmas to isolate and culture (Friis, 1975). It requires a highly enriched media that can be easily overgrown by other bacteria inhabiting the pig respiratory tract, typically *M. hyorhinis*, and by any environmental organism (Friis, 1975; Thacker and Minion, 2012). The most widely used liquid medium for isolation and culture of *M. hyopneumoniae* was developed by Niels Friis (1975). Isolation is achieved from a suspension of a lung portion immediately adjacent to the lung area affected by MP in an appropriate liquid medium (Etheridge *et al.*, 1979; Friis, 1975). In general terms, *M. hyopneumoniae* does not grow on agar medium upon primary isolation, meaning that firstly a passage on broth is needed (Razin *et al.*, 1984). Moreover, *M. hyopneumoniae* grows slowly in culture compared with other porcine mycoplasmas, producing turbidity and an acid colour shift to the liquid media incubated at 37°C (Thacker and Minion, 2012) (Figure 1-1). Inoculation of solid agar medium and incubation in a 5% carbon dioxide atmosphere results in colonies after a minimum of 2 to 3 days (Thacker and Minion, 2012) (Figure 1-2). However, since growth on solid medium is considered particularly difficult, the number of viable bacteria, often estimated by the number of colony forming units (CFU), is usually considered inappropriate for this organism (Calus *et al.*, 2010; Cook *et al.*, 2016). Consequently, an alternative non-agar based method, namely colour changing units (CCU), is used as golden standard for culture titration (Calus *et al.*, 2010; Cook *et al.*, 2016). It is worth mentioning that since Friis (1975), few improvements have been made with respect to media supporting *M. hyopneumoniae* growth.

Fairly recently, traditional liquid medium ingredient concentrations, such as horse and porcine serum and yeast extract, have been optimized (Hwang *et al.*, 2010). Moreover, basis of an improved and selective culture medium for *M. hyopneumoniae* have been proposed (Cook *et al.*, 2016).

Figure 1-1. Tubes containing 10-fold serial dilutions of *M. hyopneumoniae* J type strain (ATCC®25934™) culture and an uninoculated tube (last tube) serving as control. *M. hyopneumoniae* growth produce turbidity of the medium accompanied by medium colour change from red to orange to yellow. Inoculated tubes were incubated for 7 days at 37°C in an aerobic atmosphere.

Figure 1-2. Colonies of *M. hyopneumoniae* J type strain (ATCC®25934™) on solid media. Colonies are brownish, rough, with a well-delimited margin and nipple. Inoculated agar plates were incubated for 7 days at 37°C in a 5% CO_2 atmosphere. Colonies were photographed at 40x.

1.2.3. Diversity

Since Vicca *et al*. (2003) first described significant differences in virulence among *M. hyopneumoniae* isolates, numerous molecular typing techniques have been implemented to further investigate heterogeneity of *M. hyopneumoniae*, providing new insights in the epidemiology of such pathogen (Sibila *et al*., 2009).

A high diversity of *M. hyopneumoniae* strains is recognised in the swine population (Thacker and Minion, 2012). However, some discrepancies have been identified among studies with respect of inter- and intra-herd diversity. At inter-herd level, farms being in close geographic or operative contact have been either found to be affected by the same or a limited number of variants (Mayor *et al*., 2007; Pantoja *et al*., 2016; Savic *et al*., 2010) or by various non-phylogenetically related strains (Dos Santos *et al*., 2015; Stakenborg *et al*., 2006). In regards to intra-herd diversity, both low (Charlebois *et al*., 2014; Mayor *et al*., 2008; Mayor *et*

al., 2007; Savic *et al.*, 2010; Stakenborg *et al.*, 2005; Stakenborg *et al.*, 2006; Vranckx *et al.*, 2012a) and high (Michiels *et al.*, 2017; Nathues *et al.*, 2011; Tamiozzo *et al.*, 2015; Vranckx *et al.*, 2011) variability of isolates has been described. Nevertheless, EP farm outbreaks are generally associated with a single or predominant *M. hyopneumoniae* strain (Mayor *et al.*, 2008; Mayor *et al.*, 2007; Nathues *et al.*, 2011). Moreover, a single pig may be infected with multiple strains of *M. hyopneumoniae* (Michiels *et al.*, 2017; Nathues *et al.*, 2011; Tamiozzo *et al.*, 2015; Vranckx *et al.*, 2011); lastly, three different strains in a pig have been found (Michiels *et al.*, 2017). Whether simultaneous or subsequent infections with more than one strain result in more severe lung lesions remains unclear. Although not always observed (Charlebois *et al.*, 2014), more severe lung lesions at batch (Michiels *et al.*, 2017; Vranckx *et al.*, 2011) and at individual (Villarreal *et al.*, 2009) levels have been associated with the presence of more than one *M. hyopneumoniae* isolate.

In many species, differences in pathogenicity between strains correspond to significant differences at the genomic level (Pinto *et al.*, 2009). As stated above, genetic heterogeneity of *M. hyopneumoniae* strains has been demonstrated but evidence for direct association with virulence is still pending. Some virulence genomic markers have been suggested (Charlebois *et al.*, 2014; Vicca *et al.*, 2003) but, unfortunately, their potential to distinguish between low and high virulent strains has either lacked reproducibility (Nathues *et al.*, 2011) or has not been further investigated. Data generated by sequencing and comparative analysis of three *M. hyopneumoniae* strains allowed the identification of

strain-specific regions potentially related to pathogenicity (Minion *et al.*, 2004; Vasconcelos *et al.*, 2005). The presence of an integrative conjugal element, a mobile DNA element that may be involved in genetic recombination events and pathogenicity (Pinto *et al.*, 2007), was found in 232 and 7448 *M. hyopneumoniae* pathogenic strains (Vasconcelos *et al.*, 2005). Providing support to this hypothesis, Pantoja *et al*. (2016) recently suggested herd *in situ* recombination between *M. hyopneumoniae* strains. Furthermore, comparative genomic analyses have also identified various coding DNA sequences (which indicate regions likely to encode proteins) considered candidate virulence genes (Liu *et al.*, 2013; Vasconcelos *et al.*, 2005). Lastly, DNA repeats may result in diversity of pathogenic phenotypes (Bayliss and Palmer, 2012). Although subtle differences were observed, the level of DNA repeat conservation has been recently described to be higher in a pathogenic *M. hyopneumoniae* strain compared with a non-pathogenic counterpart (Cattani *et al.*, 2016).

Differences between pathogenic determinants might not be predominantly at the genomic level. Rather, these differences may be associated with variations in expression levels of genes encoding virulence factors (Pinto *et al.*, 2009). Transcriptomic studies have been unable to identify differentially expressed genes specifically related to *M. hyopneumoniae* virulence (Madsen *et al.*, 2008), whereas proteomic studies have recently provided insights in pathogenicity differences between *M. hyopneumoniae* strains. Non-pathogenic J and pathogenic 232 strains showed at least 10 proteins with differential expression levels (Li *et al.*, 2009). Overall, the non-pathogenic strain protein

expression profile is suggestive of a non-infective proliferative lifestyle, with most of the identified proteins assigned related to metabolism. In contrast, most of the identified proteins from pathogenic strains were predicted to be related with the ability to infect pigs (Li *et al.*, 2009; Pinto *et al.*, 2009).

Despite all these efforts and the provided understandings into *M. hyopneumoniae* virulence determinants, the mechanisms underlying this bacterium's pathogenicity remain elusive and, thus, laboratory methods predicting the virulence of strains are still unavailable (Nathues *et al.*, 2011; Sibila *et al.*, 2009).

1.3. EPIDEMIOLOGY OF *MYCOPLASMA HYOPNEUMONIAE*

M. hyopneumoniae is one of the main primary respiratory pathogens of concern in countries with pigs reared under intensive conditions and confined systems (Thacker and Minion, 2012), being highly prevalent in almost all swine producing areas (Fablet *et al.*, 2012; Fraile *et al.*, 2010; Meyns *et al.*, 2011; Nathues *et al.*, 2014; Rautiainen *et al.*, 2001). Indeed, European swine herds are, generally, endemically infected with *M. hyopneumoniae*, as illustrated by antibody prevalence of 62.9% in Germany (Nathues *et al.*, 2014), 70.8% in France (Fablet *et al.*, 2012), 79% in Belgium (Meyns *et al.*, 2011) and 82% in Spain (Fraile *et al.*, 2010). Notwithstanding, this organism is not ubiquitous in all European countries, such as Denmark, Finland and Switzerland, in which eradication local programs have been and/or are currently applied

(Maes *et al.*, 2008; Rautiainen *et al.*, 2001; Stärk *et al.*, 2007). Although the highest infection levels befall during the grow-finishing period (Sibila *et al.*, 2009), infection may occur at all production phases.

M. hyopneumoniae infection in sows is common in areas of endemicity and high pig density (Calsamiglia and Pijoan, 2000; Grosse Beilage *et al.*, 2009; Sibila *et al.*, 2007a; Sibila *et al.*, 2007b). It is generally accepted that sows play an important role in maintaining the infection within a herd since infected gilts and sows can transmit *M. hyopneumoniae* to newly introduced gilts (Pieters *et al.*, 2009; Roos *et al.*, 2016) and to their offspring (Grosse Beilage *et al.*, 2009; Sibila *et al.*, 2007a). Particularly, gilts and low parity sows have been suggested to be more infectious than older parity sows (Fano *et al.*, 2007), denoting that their suckling pigs may be at a higher risk of becoming positive by vertical transmission (Calsamiglia and Pijoan, 2000; Sibila *et al.*, 2008). As supporting evidence, the risk of a herd to have *M. hyopneumoniae* infection among suckling pigs was increased when the total number of purchased gilts per year was high (Nathues *et al.*, 2013). In consequence, a satisfactory acclimation of gilts is gaining importance, since it may result in a lower likelihood of *M. hyopneumoniae* infection and of developing clinical signs of EP in fattening pigs (Nathues *et al.*, 2014; Pieters and Fano, 2016).

In herds with a history of respiratory disease, *M. hyopneumoniae* prevalence in suckling pigs may range from 0.5% to 3.8% in nasal swabs (Sibila *et al.*, 2007a; Sibila *et al.*, 2007b) and rise up to 13% in broncho-

alveolar lavage fluid (BALF) (Moorkamp *et al.*, 2009). Once *M. hyopneumoniae* infection is established in suckling piglets, transmission to littermates and later to penmates occurs (Thacker and Minion, 2012). In such situation, a progressive decrease in the percentage of seropositive suckling pigs due to maternal antibodies waned is normally followed by a slow increase in the number of seropositive animals due to infection towards the end of the nursery period (Calsamiglia *et al.*, 1999; Moorkamp *et al.*, 2009; Sibila *et al.*, 2007b). In weaned pigs, *M. hyopneumoniae* prevalence up to 60% has been reported (Calsamiglia *et al.*, 1999; Fano *et al.*, 2007; Sibila *et al.*, 2008). In a majority of herds, however, significant *M. hyopneumoniae* transmission among penmates and amplification of the infection begins at weaning, resulting in up to 100% *M. hyopneumoniae* prevalence (Fano *et al.*, 2007; Maes *et al.*, 1998) and a gradually increase of the number of seropositive animals towards the middle-end of the fattening period (Sibila *et al.*, 2007b).

Within herds, *M. hyopneumoniae* is mainly spread through direct contact from infected to susceptible pigs (Sibila *et al.*, 2009), though indirect or airborne transmission seems to also play a role in the spread of the agent within a population (Fano *et al.*, 2005; Leon *et al.*, 2001). However, nose-to-nose contact remains the most effective route of transmission during acute and chronic phases of infection (Marois *et al.*, 2007; Morris *et al.*, 1995b; Pieters *et al.*, 2010). Importantly, experimentally infected animals with *M. hyopneumoniae* can become chronic carriers of the pathogen and able to infect susceptible pigs for up to 200 days post-inoculation (dpi) (Pieters *et al.*, 2009). Actually, a single infected weaned pig was found to

infect, on average, one penmate during a nursery period of 6 weeks under experimental settings (Meyns *et al.*, 2006; Meyns *et al.*, 2004), although such reproduction ratio (*Rn*) was lower at herd level (0.56 during a 6 week nursery period) (Villarreal *et al.*, 2011). Recently, the survival of *M. hyopneumoniae* onto different surfaces commonly encountered in pig units has been evaluated (Browne *et al.*, 2017). As a result, maximum survival of *M. hyopneumoniae* outside the host was 8 days at cooler temperatures (4°C), providing a new vision of the potential within herd indirect transmission routes (Browne *et al.*, 2017).

In a naïve herd, *M. hyopneumoniae* might be introduced by airborne transmission or by purchase of subclinically infected pigs (Sibila *et al.*, 2009). The role of fomites, indeed, has not yet been unequivocally proven (Batista *et al.*, 2004). In 1985, the airborne transmission between herds was suggested for the first time (Goodwin, 1985) and, since then, it has gained more significance. Evidence of airborne spread of *M. hyopneumoniae* up to 9.2 km has been described under experimental conditions (Otake *et al.*, 2010).

1.4. PATHOGENESIS OF *MYCOPLASMA HYOPNEUMONIAE*

Mycoplasmas usually exhibit a rather strict host and tissue specificity, probably reflecting their nutritionally nature and obligate parasitic mode of life (Razin *et al.*, 1998). Thus, *M. hyopneumoniae* is a host specific pathogen that only infects pig and its pathogenesis is complex, involving long-term colonization of airway epithelium and, usually, interaction with other infectious organisms. Adhesion of *M. hyopneumoniae* to the epithelial linings of the porcine respiratory tract is a prerequisite for colonization and infection (Razin *et al.*, 1998; Thacker and Minion, 2012).

1.4.1. Adherence and colonization

Numerous studies have shown that *M. hyopneumoniae* predominantly locates between cilia and microvilli of the bronchiolar and bronchial epithelial cells, without penetration into the lung parenchyma and rarely present in alveoli (Blanchard *et al.*, 1992; DeBey and Ross, 1994; Mebus and Underdahl, 1977; Sarradell *et al.*, 2003; Tajima and Yagihashi, 1982; Underdahl *et al.*, 1980). Ultrastructural studies have also revealed the presence of a polysaccharide capsule (Tajima and Yagihashi, 1982) as well as fine fibrils radiating from *M. hyopneumoniae* cells (Blanchard *et al.*, 1992; Sarradell *et al.*, 2003) that may probably be implicated in adhesion of the organism (Li *et al.*, 2009; Tajima and Yagihashi, 1982; Zielinski and Ross, 1993).

Attachment of *M. hyopneumoniae* is associated with degenerative changes in the ciliated epithelial cells (Blanchard *et al.*, 1992; Sarradell *et al.*, 2003) (Figure 1-3). This results in a significant reduction in the efficiency of clearance by the mucociliary apparatus, which greatly contributes to the establishment and proliferation of upper respiratory commensal bacteria and/or other organisms as secondary pathogens (Thacker and Minion, 2012).

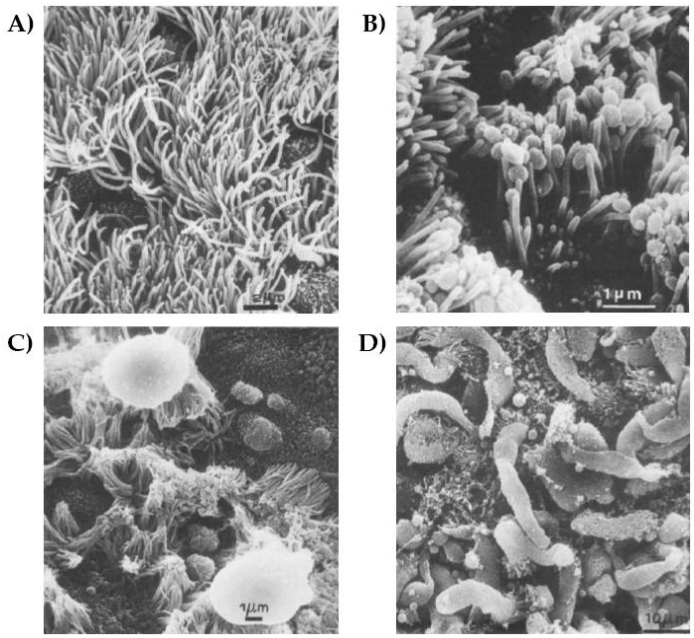


Figure 1-3. Scanning electron microscopy images of the trachea surface of an uninoculated pig and pigs experimentally inoculated with *M. hyopneumoniae*. A) Healthy trachea surface with ciliated epithelial cells and cells with microvilli, bar=2 μm. **B)** Trachea surface at 2 weeks post-inoculation (wpi). *M. hyopneumoniae* appears closely associated with the upper part of cilia, bar=1 μm. **C)** Trachea surface at 2 wpi. Epithelial cells have lost cilia and *M. hyopneumoniae* cells are associated with remaining cilia. There is mucus accumulation on microvilli, bar=1 μm. **D)** Bronchi surface at 8 wpi. An alteration of the epithelium surface is noted with free ciliated cells and mucus, bar=10 μm. Figure from Blanchard *et al*. (1992).

While the exact method of adherence is poorly understood, several *M. hyopneumoniae* surface proteins binding to target cells have been identified. Zhang *et al*. (1995) characterized the first *M. hyopneumoniae* adhesin; a 97 kDa surface protein designated P97 (Zhang *et al*., 1995). Such protein is encoded by the *mhp183* gene, located in a two-gene operon together with the *mhp182* one, which encodes P102, another cell surface-located protein (Adams *et al*., 2005; Hsu and Minion, 1998). The abovementioned genes have six paralogs within the *M. hyopneumoniae* genome (Adams *et al*., 2005) that may be differentially expressed and provide antigenic and functional variation (Deutscher *et al*., 2010; Hsu and Minion, 1998; Seymour *et al*., 2010; Wilton *et al*., 2009). Indeed, most of the currently described *M. hyopneumoniae* adhesins belong to the P97/P102 paralog family (Seymour *et al*., 2010). Furthermore, and as support of the above, P97 is not exclusively responsible for adherence of *M. hyopneumoniae*, since binding to cilia still occurs when adherence via P97 is blocked (King *et al*., 1997; Zhang *et al*., 1995).

Adhesins are targets of endoproteolytic cleavage events that generate smaller products exhibited on the *M. hyopneumoniae* cell surface (Burnett *et al*., 2006; Seymour *et al*., 2010; Wilton *et al*., 2009). These products bind epithelial cilia and extracellular matrix components such as mucin, heparin, fibronectin and plasminogen (Burnett *et al*., 2006; Deutscher *et al*., 2010; Jenkins *et al*., 2006; Seymour *et al*., 2010; Wilton *et al*., 2009). Recruitment of any of the abovementioned smaller products to the mycoplasma cell surface might provide a bridging molecule that bind a

range of extracellular matrix components, thereby increasing the potential of *M. hyopneumoniae* to colonize the host (Seymour *et al*., 2011; Seymour *et al*., 2012).

Ultrastructural studies have shown no evidence of *M. hyopneumoniae* crossing the epithelial cell barrier (Blanchard *et al*., 1992; DeBey and Ross, 1994; Mebus and Underdahl, 1977; Sarradell *et al*., 2003; Tajima and Yagihashi, 1982; Underdahl *et al*., 1980), suggesting the bacterium not being able to disseminate systemically. However, some works have isolated *M. hyopneumoniae* from pericardial fluid (Buttenschon *et al*., 1997), brain (Friis, 1974) and, later on, from lymph nodes, liver, spleen and kidneys of experimentally challenged and contact-exposed pigs (Le Carrou *et al*., 2006; Marois *et al*., 2007). Furthermore, *M. hyopneumoniae* DNA was also detected in these same abdominal tissues of pigs challenged with a high-pathogenic strain but not in those challenged with a low-pathogenic one (Woolley *et al*., 2012). These findings, to be further confirmed, indicate that *M. hyopneumoniae* might possess mechanisms for tissue invasion, migration and colonization of other tissues than respiratory sites. Remarkably, it has been suggested a possible relationship between *M. hyopneumoniae* adhesins ability to interact with plasminogen and fibronectin and its putative capacity to adhere and invade subepithelial sites (Burnett *et al*., 2006; Seymour *et al*., 2012; Wilton *et al*., 2009). The question whether mycoplasmas can enter into epithelial cells has not been easy to resolve and, although the mechanism of cell entry is still unclear, intracellular location, even for a short period, has been confirmed (Razin *et al*., 1998). Newly, *M.*

hyopneumoniae has been described to invade epithelial porcine cells and reside free within cytoplasm, thus, becoming a recently suggested intracellular pathogen (Raymond *et al*., 2016).

1.4.2. Damage mechanisms

In support to the hypothesis that adhesion is relevant for pathogenicity, it has been suggested that *M. hyopneumoniae* culture medium adaptation results in an important decrease in adherence and a subsequent reduced potential to induce lung lesions (DeBey and Ross, 1994; Tajima and Yagihashi, 1982; Young *et al*., 2000; Zhang *et al*., 1994, 1995; Zielinski and Ross, 1993). Contrarily, Calus *et al*. (2009) could not demonstrate significant differences between adherence capacities of *M. hyopneumoniae* field isolates previously classified according to virulence (Calus *et al*., 2009; Vicca *et al*., 2003). Besides discrepancies, virulence factors associated with *M. hyopneumoniae* colonization remain to be elucidated, although some insights have been gained in terms of potential damage mechanisms.

The mechanisms by which *M. hyopneumoniae* induces cilia loss was firstly associated to the intracellular free-calcium (Ca^{2+}) released as a result of *M. hyopneumoniae* attachment to the respiratory epithelium (Park *et al*., 2002; Zhang *et al*., 1994). Later on, however, this hypothesis was not corroborated and the rise of intracellular Ca^{2+} was attributed to an unidentified soluble factor (Hwang *et al*., 2006). The damage to the host cells has been also attributed to toxic by-products of mycoplasma metabolism (Razin *et al*., 1998). Actually, mycoplasmas can use glycerol

as a carbon source, thus enabling the production of the highly toxic reactive oxygen species, such as hydrogen peroxide (H_2O_2) (Bischof *et al*., 2009; Hames *et al*., 2009; Pilo *et al*., 2005). Although it is not known whether this is also applicable for *M. hyopneumoniae*, a highly conserved homologous gene to that codifying glycerol phosphate oxidase enzyme has been found in its genome (Ferrarini *et al*., 2016). In addition, proteins with nuclease (Minion *et al*., 1993; Schmidt *et al*., 2007) or protease (Minion *et al*., 2004; Vasconcelos *et al*., 2005) activity have been identified or predicted in *M. hyopneumoniae*. Nevertheless, demonstration of their potential cytotoxic effect, as described in other Mycoplasma species (Diedershagen *et al*., 2007; Ferreira and Castro, 2007), is still pending.

1.5. IMMUNE RESPONSES TO *MYCOPLASMA HYOPNEUMONIAE*

Following colonization, a series of complex *M. hyopneumoniae*-host immune system interactions take place. *M. hyopneumoniae* survival, proliferation and disease development appear to be dependent upon modulation of the innate and adaptive respiratory immune responses (Maes *et al*., 1996; Thacker, 2001). This altered immune response is generally unable to rapidly clear infection, which results in a chronic colonization of the pig airways and a prolonged pulmonary inflammatory response (Thacker and Minion, 2012). While immunological events are considered to play a prominent role in *M. hyopneumoniae* pathogenesis, the exact mechanisms by which *M. hyopneumoniae* modulates the immune system and the implications that

this might have at immunopathological level remain poorly understood (Thacker and Minion, 2012).

1.5.1. Innate immune response

Innate immunity is the first line of defence for host protection against invading pathogens. In response to *M. hyopneumoniae*, Toll-like receptors 2 and 6 expressed by pulmonary alveolar macrophages initiate intracellular NF-$_k$B and MAPK signalling pathways that culminate in the upregulation of pro-inflammatory cytokines and other compounds such as nitric oxide (NO) (Bin *et al*., 2014; Hwang *et al*., 2011; Muneta *et al*., 2003). Besides, *M. hyopneumoniae* has been shown to actively suppress the immune system of the host during early stages of infection by inhibiting macrophage-mediated phagocytosis (Bin *et al*., 2014; Caruso and Ross, 1990). Such an impaired phagocytic capacity is likely important in the reduced clearance of *M. hyopneumoniae* as well as other secondary pathogens (Caruso and Ross, 1990). In addition, nasal cavity dendritic cells in pigs experimentally infected with *M. hyopneumoniae* decreased in number and its presentation capacity was compromised (Shen *et al*., 2017).

1.5.2. Acquired cellular immune response

Tajima *et al*. (1984) firstly confirmed the role of the cellular immune system in the pathogenesis of *M. hyopneumoniae* infection by the use thymectomized pigs, which displayed decreased lesion severity in response to experimental *M. hyopneumoniae* infection (Tajima *et al*., 1984). These results suggested that a T-cell-dependent mechanism may be

important in the development of pneumonia. Helper T-cells, in fact, is the most numerous subset in the lung lymphoid infiltration of *M. hyopneumoniae* infected pigs, although cytotoxic T-cells are also present (Okada *et al.*, 2000; Sarradell *et al.*, 2003).

Increased levels of numerous cytokines (i.e. IL-1 (α and β), IL-2, IL-4, IL-6, IL-8, IL-10, IL-18, IL-12, IFN-ɣ, TNF-α and prostaglandin E2) have been detected in BALF (Asai *et al.*, 1993; Asai *et al.*, 1994; Muneta *et al.*, 2006; Muneta *et al.*, 2008; Nuntaprasert *et al.*, 2004; Thanawongnuwech *et al.*, 2004; Thanawongnuwech and Thacker, 2003; Woolley *et al.*, 2013) as well as in pulmonary tissue (Lorenzo *et al.*, 2006; Redondo *et al.*, 2009; Rodríguez *et al.*, 2007; Rodríguez *et al.*, 2004) of *M. hyopneumoniae* infected pigs. Recently, IL-10, IL-5 and IL-13 have been described to be expressed by epithelial and/or mononuclear cells of the airways of *M. hyopneumoniae* infected pigs (Rodríguez *et al.*, 2016; Shen *et al.*, 2017). The abovementioned range of expressed cytokines is associated with both a Th1 and Th2 immune responses (Hirahara and Nakayama, 2016; Spellberg and Edwards, 2001). Actually, IL-1, IL-2, IL-12, TNF-α and IFN-γ are considered Th1 (pro-inflammatory) cytokines. These cytokines cause stimulation of CD8+ cytolytic T lymphocytes, crucial for host defence against intracellular pathogens, and activate B cells to produce IgG2 antibodies. On the other hand, IL-4, IL-5, IL-10 and IL-13 are considered Th2 (anti-inflammatory) cytokines, which downregulate Th1 cytokines and favour humoral immunity and secretion of IgG1 (Crawley and Wilkie, 2003; Spellberg and Edwards, 2001). While the increased secretion of pro-inflammatory cytokines probably exacerbates

the inflammatory reaction, causing tissue injury of the lung (Redondo *et al.*, 2009; Sarradell *et al.*, 2003), anti-inflammatory cytokines negatively regulate the immune response, preventing excessive inflammation during the course of infection (Rutz and Ouyang, 2016). Nevertheless, whether there is involvement of a Th1 or Th2-biased immune response to *M. hyopneumoniae* infection in pigs and if this is related to protection remain unknown.

1.5.3. Acquired humoral immune response

The induction of systemic humoral immune response by *M. hyopneumoniae* is considered to be slow and the time elapsed from infection to seroconversion is highly variable (Sibila *et al.*, 2009). Under experimental settings, antibodies may start to be detected between 2 and 4 weeks post-inoculation (wpi) (Sibila *et al.*, 2009), although seroconversion as early as 7 to 9 dpi has been reported at individual animal level (Sheldrake *et al.*, 1990; Sørensen *et al.*, 1997). Nonetheless, 7 to 9 wpi may be needed for all *M. hyopneumoniae* inoculated pigs in an experiment to become seropositive (Fano *et al.*, 2005; Pieters *et al.*, 2009). Seroconversion under natural conditions may be probably slower than in experimental studies, taking place its onset by 4 to 5 wpi (Fano *et al.*, 2005; Sitjar *et al.*, 1996). Seroconversion delay may be partly explained by the poorly *M. hyopneumoniae* invasive nature, since this organism is mostly attached to the ciliated respiratory epithelium, which may compromise the antigen presentation process (Maes *et al.*, 1996; Sitjar *et al.*, 1996). Remarkably, no direct correlation has been found between the

induction of serum antibodies and protection against *M. hyopneumoniae* (Djordjevic *et al.*, 1997; Kobisch *et al.*, 1993; Kristensen *et al.*, 1981).

The local humoral immune response appears to be important in the pathogenesis of *M. hyopneumoniae* infection (Sarradell *et al.*, 2003). While IgA inhibits bacterial adherence to the respiratory mucosa, IgG favours phagocytosis through its opsonic activity (Walker *et al.*, 1996). Lungs from experimentally infected pigs with *M. hyopneumoniae* revealed an increased number of IgG- and IgA-producing cells from 7 to 21 dpi, but not at 28 and 35 dpi. (Redondo *et al.*, 2009; Sarradell *et al.*, 2003). In agreement, the number of IgA positive cells decreased in the nasal cavity after 28 days of *M. hyopneumoniae* inoculation (Shen *et al.*, 2017). Although locally secreted IgA after *M. hyopneumoniae* vaccination has been postulated to play a pivotal role in protection (Marchioro *et al.*, 2013; Martelli *et al.*, 2014; Thacker *et al.*, 2000), such type of antibodies elicited after *M. hyopneumoniae* challenge did not prevent lung lesion development (Djordjevic *et al.*, 1997); thus, the role of local IgA in *M. hyopneumoniae* infection is not totally clear.

1.6. CLINICAL SIGNS AND LUNG LESIONS ASSOCIATED WITH *MYCOPLASMA HYOPNEUMONIAE*

1.6.1. Clinical signs

The main clinical sign attributed to *M. hyopneumoniae* infection is a dry, non-productive cough (Sibila *et al*., 2009). However, coughing is one of the most common signs of respiratory disease and can be caused by a variety of infectious agents (Nathues *et al*., 2012; Thacker and Minion, 2012). In endemically *M. hyopneumoniae* infected herds, an insidious onset of a dry, non-productive cough, affecting particularly pigs at the finishing stage of the production cycle, is suggestive of *M. hyopneumoniae* involvement (Nathues *et al*., 2012; Sibila *et al*., 2009; Thacker and Minion, 2012). In such endemic situation, while coughing could appear throughout the growing period, subclinical infections may also be common (Sibila *et al*., 2009; Thacker and Minion, 2012). Although unusual, when *M. hyopneumoniae* is introduced to an immunologically naïve herd, the abovementioned situation is aggravated (Thacker and Minion, 2012), since animals of all ages become susceptible, morbidity rise significantly and coughing is frequently accompanied by more severe clinical signs such as acute respiratory distress, pyrexia, and, sporadically, death of animals (Thacker and Minion, 2012).

M. hyopneumoniae infection has been also associated with reduced growth performance parameters, basically, average daily weight gain (ADWG) and reduced feed efficiency. Nevertheless, information related to this aspect is controversial (Thacker and Minion, 2012). In some

studies, no detrimental effects of *M. hyopneumoniae* infection on overall growth performance under experimental (Escobar *et al.*, 2002) or natural (Scheidt *et al.*, 1990; Straw *et al.*, 1990) settings were reported, whereas opposite results were obtained by other authors (Hill *et al.*, 1992; Paisley *et al.*, 1993; Pointon *et al.*, 1985; Straw *et al.*, 1989). At the very end, swine herds are rarely infected only with *M. hyopneumoniae*, thus, mainly mixed infections occur (i.e. EP and PRDC). When other pathogens are involved, both clinical signs and affectation of growth performance parameters are likely more severe and of multifactorial origin (Thacker and Minion, 2012).

1.6.2. Macroscopic lung lesions

Grossly, MP consists of pulmonary consolidated areas, often well demarcated and more collapsed than the adjacent normal lung tissue, affecting mainly the cranio-ventral regions (i.e. apical and cardiac lobes and the cranial parts of diaphragmatic lobes) (Van Alstine, 2012). Such macroscopic pattern is referred in the present dissertation as cranio-ventral pulmonary consolidation (CVPC) (Figure 1-4).

In uncomplicated natural infections, the lesions rarely affect extended portions of the lungs. On cut surface, the parenchyma is relatively uniform in colour, and a catarrhal exudate in the airways may be observed (Thacker and Minion, 2012).

In contrast, when larger portions are affected and mucopurulent exudate within the airways as well as other pathological findings such

as pleurisy and/or interstitial oedema are observed, the implication of other respiratory pathogens is guaranteed (Thacker and Minion, 2012).

Experimentally, such lesions may appear by 1 wpi onwards and reach maximal extension and severity by 4 wpi (Kobisch *et al.*, 1993; Maes *et al.*, 1996; Sørensen *et al.*, 1997). Lesion recovery is evident by 8 wpi, although few macroscopic lesions (interlobular scarring with tissue retraction) may remain until 12 wpi (Kobisch *et al.*, 1993; Maes *et al.*, 1996; Sørensen *et al.*, 1997).

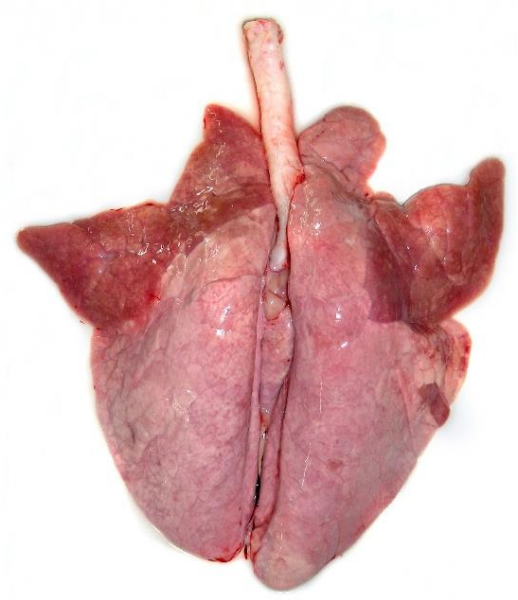


Figure 1-4. Lung from a pig experimentally inoculated with *M. hyopneumoniae* and necropsied at 28 dpi. Red to brown consolidated areas on the cranio-ventral parts of the apical and cardiac lung lobes. The right diaphragmatic lobe appears slightly affected.

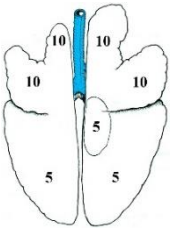
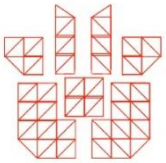
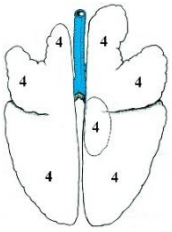
Importantly, although CVPC is suggestive of *M. hyopneumoniae* participation and it may be found referred as *M. hyopneumoniae*-like

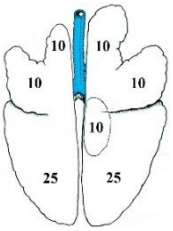
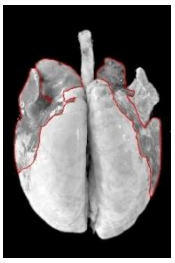
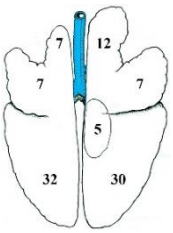
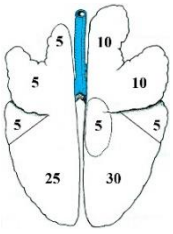
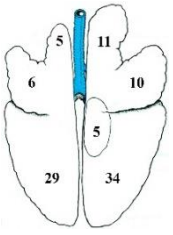
lung lesions, it is not unique of this organism, as others, particularly *P. multocida* and SIV, may cause similar gross lung lesions (Sibila *et al.*, 2009; Thacker, 2001).

1.6.3. Cranio-ventral pulmonary consolidation scoring systems

To quantify a lesion, scoring the extent of the pathological change is necessary. Different scoring systems have been described in the past years to monitor CVPC under experimental (Hannan *et al.*, 1982; Ph. Eur., 2013) as well as field and/or slaughterhouse (Christensen *et al.*, 1999; Madec and Kobisch, 1982; Morrison *et al.*, 1985; Straw *et al.*, 1986) conditions. Remarkably, most of them are still currently in place (Table 1-1). The majority of CVPC scoring systems are based on lung visual evaluation and palpation. This evaluation ascertain the extent of CVPC by means of two or three-dimensional approaches. While the formers express the extension of affected lung area, the last ones express the weight of affected tissue. In addition, scoring systems consider differently some anatomical factors such as the relative size or weight of lung lobes or the *M. hyopneumoniae* lesion pattern. Overall lesion scores may be expressed in percentages or in points. The already mentioned differences have been suggested to partly explain discrepancies in lung lesion scoring system results of different studies (Morrison *et al.*, 1985). Although few comparisons have been made between some lung scoring methods (Davies *et al.*, 1995; Morrison *et al.*, 1985; Mousing and Christensen, 1993), a current comparison between systems used for CVPC evaluation is missing and judgement of different CVPC severities reported along literature is not possible.

Table 1-1. Summary of the lung scoring systems most frequently reported in the peer-reviewed literature to evaluate the presence and extension of CVPC. Lung drawing is modified from an external source (König and Liebich, 2005).

Approach	Parameter considered	Reference	Description of the scoring system	Scoring units	Maximum total score	Scoring representation
Two-dimension (Area)	Lesion pattern	Goodwin *et al*. (1969)	Quantification of the affected area by means of 0 to 10 points or 0 to 5 points, depending on the lobe. Lung lobes points are summed to provide an overall area lung score	Points	55	
Two-dimension (Area)	Lung lobes sizes	Hannan *et al*. (1982)	Representation of the affected area in a lung diagram. The number of triangles affected per lobe is multiplied per five and divided by the number of triangles of each lung lobe. Each lobe score is summed to provide an overall area lung score	Points	35	
Two-dimension (Area)	Non applicable	Madec and Kobisch, (1982)	Quantification of the affected area by means of four points: 0) no lesion; 1) lesion affecting <25% of the lobe surface; 2) lesion affecting 25-49% of the surface; 3) lesion affecting 50-74% of the surface; 4) lesion affecting ≥75% of the surface. Points per lobe are summed to provide an overall area lung score	Points	28	

Approach	Parameter considered	Reference	Description of the scoring system	Scoring units	Maximum total score	Scoring representation
Two-dimension (Area)	Lung lobes sizes	Straw *et al*. (1986)	The percentage of each lobe affected area is multiplied by the lobe relative size and summed to provide the total area percentage of affected lung	%	100	
Two-dimension (Area)	Non applicable	Image analysis	Quantification of the affected lung area using a picture of the lung. The lesion area and the total area of the lung are delimited in the picture and analysed digitally to determine the proportion of the overall affected area	%	100	
Three-dimension (Volume)	Lung lobes weights	Morrison *et al*. (1985)	The percentage of each lobe affected area is multiplied by the lobe relative weight and summed to provide the total weight percentage of affected lung	%	100	
Three-dimension (Volume)	Lung lobes weights and *M. hyopneumoniae* lesion pattern	Christensen *et al*. (1999)	The percentage of each lobe affected area is multiplied by the lobe relative weight and summed to provide the total weight percentage of affected lung. An extra scoring is given to the cranial area of the diaphragmatic lobes	%	100	
Three-dimension (Volume)	Lung lobes weights	Ph. Eur. (2013)	The percentage of each lobe affected area is multiplied by the lobe relative weight and summed to provide the total weight percentage of affected lung	%	100	

1.6.4. Microscopic lung lesions

Microscopically, *M. hyopneumoniae* induces a broncho-interstitial pneumonia (BIP) consisting of mainly lymphocytes and macrophages infiltration in peribronchiolar, perivascular and alveolar septa (Redondo *et al.*, 2009; Sarradell *et al.*, 2003). Other findings may include hyperplasia of the epithelium of the airways and alveoli and presence of serous fluid and fluid-distended macrophages as well as fewer neutrophils, lymphocytes, and plasma cells in airways and alveolus lumen (Thacker and Minion, 2012). As the infection progress, a prominent hyperplasia of the bronchus-associated lymphoid tissue (BALT) associated with intrapulmonary airways is observed (Pabst and Binns, 1994; Sarradell *et al.*, 2003).

When other respiratory pathogens are involved, BIP rapidly evolves to a suppurative bronchopneumonia with neutrophilic and mucous exudate in alveoli and airways (Thacker and Minion, 2012). In recovering lesions, alveoli are collapsed and/or emphysematous and lymphoid nodules as well as fibrosis are commonly observed in peribronchial regions (Thacker and Minion, 2012).

The severity of BIP related to *M. hyopneumoniae* infection may be scored according to the degree of peribronchiolar and perivascular lymphohistiocytic infiltration (Figure 1-5) (Calsamiglia *et al.*, 2000; Morris *et al.*, 1995a; Opriessnig *et al.*, 2004).

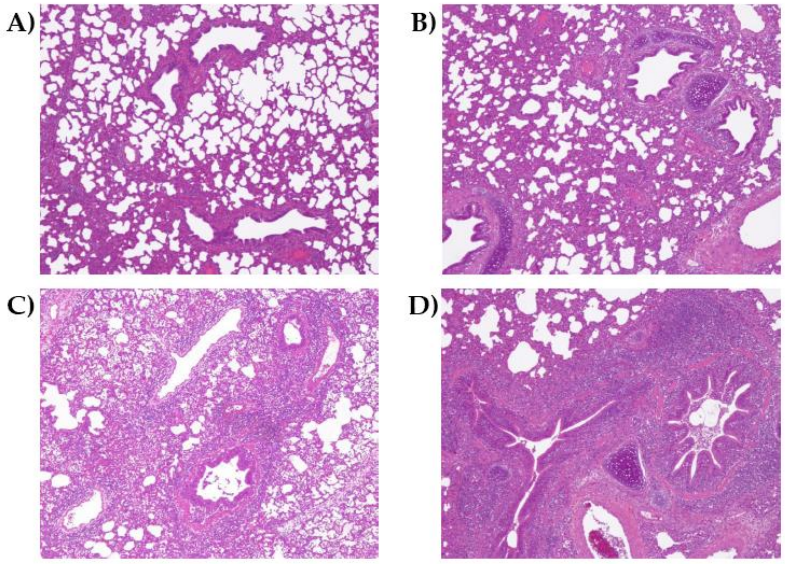


Figure 1-5. Histological sections of lungs from pigs experimentally inoculated with *M. hyopneumoniae* and sacrificed at 28 dpi. A) Limited and **B)** moderate cellular infiltrates around airways, blood vessels and alveolar septa. **C)** Mild to moderate BIP centred on bronchioles with cell exudates in the airways and alveoli spaces. **D)** Severe BIP accompanied with cellular exudates and evident peribronchial and perivascular lymphoid follicles. While **A)** and **B)** correspond to scores 1 and 2 and are non-suggestive of *M. hyopneumoniae* infection, **C)** and **D)** correspond to scores 3 and 4, respectively; suggestive of MP (Calsamiglia *et al.*, 2000).

1.7. *MYCOPLASMA HYOPNEUMONIAE* DIAGNOSIS, CONTROL AND PREVENTION

1.7.1. Diagnosis

The abovementioned clinical signs and lung lesions are the basis for a presumptive diagnosis of mycoplasmosis. However, given the lack of specificity, definitive diagnosis requires the demonstration of *M. hyopneumoniae* implication by laboratory testing (Maes *et al.*, 2008;

Thacker, 2004). Typically, isolation and culture of an organism is considered the "gold standard" for diagnosis (Thacker and Minion, 2012). However, in the case of *M. hyopneumoniae*, bacteriological culture is not used for routine diagnosis, mostly due to its fastidious *in vitro* growth. Therefore, bacterial culture is pursued under specific circumstances and failure to isolate *M. hyopneumoniae* cannot be the basis to rule out infection (Maes *et al.*, 2008; Sibila *et al.*, 2009).

MP or EP herd monitoring is normally based on enzyme linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) and abattoir surveillance methods (i.e. macroscopic lung lesion scoring) (Sibila *et al.*, 2009). Although molecular techniques enhance the detection of *M. hyopneumoniae*, antibody assessment by serological testing remains the most common method used to detect exposure to the agent (Fano *et al.*, 2012). Given that each of these diagnostic techniques has limitations, their use in combination is required to optimise diagnostic accuracy (Sibila *et al.*, 2009; Thacker and Minion, 2012). Furthermore and in combination with serology, the quantitative assessment of the onset of coughing has been proposed to support the diagnosis of EP at a group level, even substituting PCR in certain circumstances (Nathues *et al.*, 2012).

Despite serology is particularly useful for economical, rapid and high-throughput sample analyses, interpretation of results can be challenging and the following considerations need to be taken into account:

- Test sensibility: serological results may vary depending on the ELISA test used and the *M. hyopneumoniae* infection stage (Ameri-Mahabadi *et al.*, 2005; Fano *et al.*, 2012; Gomes Neto *et al.*, 2014).
- Test specificity: cross-reactivity with *M. flocculare* antibodies can occur (Bereiter *et al.*, 1990; Gomes Neto *et al.*, 2014).
- Seroconversion moment: the time by which infected animals seroconvert is highly variable (Sibila *et al.*, 2009; Thacker, 2004).
- Co-infection with other swine pathogens: increased levels of antibodies when *M. hyopneumoniae* is in co-infection with PRRSV (Thacker *et al.*, 1999), PCV2 (Opriessnig *et al.*, 2004) or SIV (Thacker *et al.*, 2001) may appear, although this is not always observed (Sibila *et al.*, 2012).
- Vaccination: serology is unable to differentiate between antibodies consequence of natural infection from those due to vaccination (Sibila *et al.*, 2009).
- Relationship with CVPC: since there is no correlation between antibody titres raised after infection and protection (Djordjevic *et al.*, 1997; Kobisch *et al.*, 1993), a possible relationship between antibody levels and lung lesions induced by *M. hyopneumoniae* has been raised (Fraile *et al.*, 2010; Merialdi *et al.*, 2012; Morris *et al.*, 1995a; Van Til *et al.*, 1991; Yagihashi *et al.*, 1993).

A number of PCR assays are available in the literature and reported to be both sensitive and specific for *M. hyopneumoniae* (Sibila *et al.*, 2009). In addition, PCR assays can be applied to various types of samples,

taken from death or alive pigs; however, lower respiratory tract samples are generally preferred. Hence, lung tissue, tracheo-bronchial swabs and BALF are recommended samples for *M. hyopneumoniae* detection (Calsamiglia and Pijoan, 2000; Fablet *et al.*, 2010; Kurth *et al.*, 2002; Makhanon *et al.*, 2012; Marois *et al.*, 2007; Strait *et al.*, 2008). Since the abovementioned samples are obtained *post-mortem*, current literature suggests laryngeal swabs as the preferred *in vivo* sample type for *M. hyopneumoniae* compared to nasal swabs and tracheo-bronchial lavage (Pieters and Rovira, 2013; Roos *et al.*, 2016).

Genome differences do exist between *M. hyopneumoniae* strains, implying a potential lack of detection of certain isolates by some PCR methods (Strait *et al.*, 2008). In consequence, genomic typing methods are gradually being implemented to differentiate *M. hyopneumoniae* isolates. Typing techniques can be divided into methods that focus on the entire *M. hyopneumoniae* genome or only on specific and well-identified DNA fragments (Sibila *et al.*, 2009). This latter group of techniques do not generally require *M. hyopneumoniae* cultivation and is, therefore, often preferred (De Castro *et al.*, 2006; Tamiozzo *et al.*, 2015; Vranckx *et al.*, 2011).

1.7.2. Control and prevention

Effective control and prevention of *M. hyopneumoniae* associated diseases are based on the optimization of management practices and housing conditions, antimicrobial medication and vaccination (Maes *et al.*, 2008). Vaccination with commercial bacterins is still considered the

most effective practice for controlling *M. hyopneumoniae* infection (Mateusen *et al.*, 2002; Thacker and Minion, 2012). Indeed, vaccination is widely extended in countries with intensive pig production systems, being in some cases applied in more than 70% of the herds (Maes *et al.*, 2008).

M. hyopneumoniae vaccination has been commonly demonstrated efficacious (Arsenakis *et al.*, 2016; Baccaro *et al.*, 2006; Tassis *et al.*, 2012; Villarreal *et al.*, 2011; Vranckx *et al.*, 2012b; Wilson *et al.*, 2012). Overall, the benefit of vaccination is based on reduced clinical signs and lung lesions, reducing performance losses and antimicrobial medication (Maes *et al.*, 2008; Simionatto *et al.*, 2013). Despite the aforesaid beneficial effects, these bacterins provide only partial protection and do not prevent the colonization of *M. hyopneumoniae* (Thacker *et al.*, 2000), and transmission between vaccinated pigs is not significantly reduced (Meyns *et al.*, 2006; Pieters *et al.*, 2010; Villarreal *et al.*, 2011). Moreover, vaccine efficacy has been shown to vary from herd to herd. One hypothesis raised to explain this variation is the antigenic differences between the strains circulating in pig herds and the vaccine strain (Tamiozzo *et al.*, 2015; Villarreal *et al.*, 2011; Villarreal *et al.*, 2012). In connection with this matter, recent results indicate that vaccination of piglets against *M. hyopneumoniae* does not lead to an important decrease in the diversity of *M. hyopneumoniae* strains in slaughter pigs (Michiels *et al.*, 2017). Altogether, these results suggest that the use of current vaccines alone is not sufficient to eliminate and probably to control *M. hyopneumoniae* infection and there is, thus, "room for improvement".

Actually, investigation on vaccine developments is focused on the use of aerosols for vaccine delivery (Feng *et al.*, 2013) and recombinant subunit or DNA vaccines (Marchioro *et al.*, 2014; Virginio *et al.*, 2014; Woolley *et al.*, 2014).

1.8. *MYCOPLASMA HYOPNEUMONIAE* EXPERIMENTAL MODEL

The host-specificity of *M. hyopneumoniae* makes possible a unique animal model. Hence, artificial administration of *M. hyopneumoniae* can cause MP in pigs (Kobisch and Ross, 1996). Since discovered in 1965 (Goodwin *et al.*, 1965; Maré and Switzer, 1965), *M. hyopneumoniae* experimental models have been widely used to study the pathogenesis, including clinical (Kobisch *et al.*, 1993; Switzer, 1973), microbiological (Etheridge *et al.*, 1979; Goodwin *et al.*, 1968) and immunological (Kristensen *et al.*, 1981; Messier *et al.*, 1990) aspects of the infection. Moreover, this model has been also performed to evaluate *M. hyopneumoniae* interaction with other pathogens (Sibila *et al.*, 2012; Thacker *et al.*, 1999) and to study epidemiological features such as *M. hyopneumoniae* transmission (Meyns *et al.*, 2006; Meyns *et al.*, 2004; Roos *et al.*, 2016) and persistence (Fano *et al.*, 2005; Pieters *et al.*, 2009). Assessment of vaccine (Arsenakis *et al.*, 2016; Woolley *et al.*, 2014) and antibiotics (Del Pozo Sacristán *et al.*, 2012; Hannan *et al.*, 1982) efficacy is another important utility of these models.

Infection animal models are the best strategy to mimic the natural infection scenario (Sande and Zak, 1999). The main determinant of *M.*

hyopneumoniae experimental model success is the percentage of inoculated animals suffering pneumonia as well as its extension. However, this outcome is largely variable and unpredictable between and within *M. hyopneumoniae* experimental models. The latter is especially important in the case of vaccine efficacy compliance, since a significant reduction in the lung lesion score when compared to non-vaccinated animals is required (Ph. Eur., 2013). Henceforth, more severe pneumonia in control pigs increases the chance to observe significant lung lesion reduction in the vaccinated ones. In consequence, a validated *M. hyopneumoniae* experimental model that would enhance severe MP reproduction would be of general interest for both the animal health industry as well as for research institutions.

Chapter 2

Hypothesis and Objectives

Little is still known about the pathogenesis of *M. hyopneumoniae* or the protective mechanisms needed for the host. In addition, the reproduction of MP at experimental level is usually hindered by prevalence, severity and reproducibility variation of pneumonic lesions. This fact impairs the detailed study of aspects related with *M. hyopneumoniae* infection as well as with veterinary medicine product improvements against the infection. While intra-model reproducibility may be more dependent on the host susceptibility, reasons for inter-model outcome variation are not yet known. In addition, the high variability in experimental conditions applied between models makes difficult drawing conclusions in this respect. It was hypothesised, therefore, that success on MP experimental reproduction may partially depend on the specific experimental conditions applied.

Besides, a parameter to estimate the degree of severity of lung lesions in live animals has not been identified yet. Importantly, lung tissue injury in *M. hyopneumoniae* infections seems to be more connected with host inflammatory responses rather than by the direct action of the organism. On top, limited protection has been associated to antibodies raised after infection. Henceforth, it was also hypothesised that humoral immune parameters could be used *ante-mortem* to forecast occurrence and severity of pneumonic lesions associated to *M. hyopneumoniae* infection.

Considering the abovementioned rationales, the general aim of this thesis was to take a step forward on *M. hyopneumoniae* experimental model in terms of exploring triggering and predictor factors for MP. The specific objectives of this PhD thesis were:

- **To identify determinants for the successful reproduction of MP under experimental settings.** In *Chapter 3*, a systematic review approach was used for the comprehensive assessment of *M. hyopneumoniae* experimental model characteristics. Subsequently, the recursive partitioning method was applied to evaluate the influence of experimental conditions on lung lesion outcomes.

- **To optimise the outcome of experimental inoculations with *M. hyopneumoniae*.** This objective was addressed from two different perspectives. On the one hand, in *Chapter 4*, a comparison of the potential to induce MP of three inoculation routes for *M. hyopneumoniae*, namely endotracheal, intranasal and aerosol, was done. On the other hand, in *Chapter 5*, different methodologies were applied to monitor the *in vitro* growth of *M. hyopneumoniae* strains differing in pathogenicity, hereby evaluating them as putative titration techniques for *M. hyopneumoniae* culture.

- **To investigate the potential association between different humoral immune parameters and lung lesions associated with *M. hyopneumoniae***. In *Chapter 6*, the relationship between different specific antibody isotype responses, both at local and systemic levels, with MP compatible lung lesions was addressed in naturally and experimentally infected pigs.

PART II

Studies

"No ens adonem mai del que hem fet, només del que ens queda per fer"

Marie Curie

Chapter 3

Study I

Determinants for swine mycoplasmal pneumonia reproduction under experimental conditions: a systematic review and recursive partitioning analysis

3.1. INTRODUCTION

M. hyopneumoniae experimental swine models have been primarily used to study the pathogenesis of the infection as well as to evaluate the efficacy of antibiotics or vaccines (Kobisch and Ross, 1996). However, variation in the outcome of such experimental models (in terms of number of animals affected by MP and its severity) is a common drawback. In fact, in this scenario, clinical course and pattern of *M. hyopneumoniae* infection have been described to be dependent on several factors such as co-infection with other pathogens (Opriessnig *et al.*, 2004; Thacker *et al.*, 1999; Thacker *et al.*, 2001), study duration (Kobisch *et al.*, 1993; Sørensen *et al.*, 1997), differences in virulence between *M. hyopneumoniae* strains (Meyns *et al.*, 2007; Vicca *et al.*, 2003), inoculum type (Czaja *et al.*, 2002) or dose (Marois *et al.*, 2010). The abovementioned factors together with other conditions highly differ between published experiments, becoming difficult to elucidate specific conclusions regarding their influence on MP development and severity. As a result, up to date, a reference validated model for the experimental MP reproduction in swine is still unavailable. In consequence, the present study sought to identify determinants for the successful reproduction of MP under experimental settings. To reach this goal, a systematic review approach was used for the comprehensive assessment of *M. hyopneumoniae* experimental model characteristics as well as for identification of relevant studies. Subsequently, recursive partitioning was applied to the compiled data to evaluate the influence of experimental conditions on lung lesion outcomes.

3.2. MATERIALS AND METHODS

3.2.1. Data source and literature review

The search term "*Mycoplasma hyopneumoniae*" was used in Medline®/PubMed® database (https://www.ncbi.nlm.nih.gov/pubmed) for peer-reviewed articles describing *M. hyopneumoniae* swine experimental models from January 1990 to May 2016. Those relevant citations contained links to full-text content from PubMed Central, National Center for Biotechnology Information Bookshelf and different publisher web sites. Only articles written in English were considered. In addition, those pertinent studies cited in the reference list of the abovementioned articles were also systematically reviewed. The term "study" was used to define a published *M. hyopneumoniae* experimental inoculation work and "experimental unit" to define a particular set of experimental conditions applied in one or more pigs within a study. While in some studies a unique set of conditions was applied, others included more than one experimental units.

3.2.2. Study inclusion criteria

Eligible studies were those providing: 1) information in regards of MP prevalence and/or macroscopic score (considering also those with no success for MP achievement); and 2) at least one experimental unit including *M. hyopneumoniae*-inoculated pigs (with or without co-infection with other swine pathogens). Therefore, those experimental units in which pigs were mock inoculated, immunostimulated (through vaccination or adjuvantation) or contact-exposed to *M. hyopneumoniae*

were not considered. On the contrary, experimental units within a study meeting the two abovementioned inclusion criteria were systematically introduced into a database (Excel 2013 software, Microsoft Office®).

3.2.3. Data extraction and adequacy

Several variables were annotated from each experimental unit accomplishing the aforesaid inclusion criteria (Table 3-1). For the inoculation route (InRoute) variable, the term intratracheal (IT) referred to those methods placing the inoculum directly into the trachea, including both endotracheal (ET) and transtracheal (TT) systems. In regards to *M. hyopneumoniae* strain (Strain) variable, the term "pool" was applied in those experimental units in which more than one strain were used to inoculate the animals. Lastly, a major setback of the systematic review process was the fact that different lung lesion scoring systems were used along the different compiled studies. In consequence, reported formulae of equivalence (*Annex*) were applied to homogenize mean lung lesion scores between experimental units. Thus, all scores were converted into the European Pharmacopoeia score (Ph. Eur., 2013), which expresses the weight percentage of affected lung tissue by MP.

Table 3-1. Name, description, abbreviation and classification of the variables annotated within experimental units and included in the statistical analyses. Levels within categorical variables are also indicated.

Variable	Description	Abbreviation	Classification	Variable Levels	Abbreviation
Sample size	Number of pigs included in an experimental unit	SamSize	Continuous	---	---
Age at inoculation	Weeks of age of the animals when inoculated	InAge	Continuous	---	---
Inoculation dose	Amount of *M. hyopneumoniae* (expressed in CCU) given to each animal	Dose	Continuous	---	---
Days of inoculation	Number of days in which the inoculum is given	InDays	Continuous	---	---
Necropsy	Post-inoculation week in which animals were necropsied and lung lesions evaluated	Nweek	Continuous	---	---
Lung lesion prevalence	Proportion of affected lungs within an experimental unit	Llprop	Continuous	---	---
Lung lesion score	Mean lung lesion score in each experimental unit expressed as weight percentage of affected tissue (Ph. Eur., 2013)	Llscore	Continuous	---	---
Seronegativity	Seronegative for *M. hyopneumoniae* before inoculation	SerNeg	Categorical	Yes	---
				No	---
Source of animals	Source and health/immunological status of the animals	Asource	Categorical	Conventional	C
				Caesarean-derived, colostrum-deprived	CDCD
				Specific-pathogen free	SPF
Route of inoculation	Route used for the inoculum administration to the animals	InRoute	Categorical	Intratracheal	IT
				Intranasal	IN
				Aerosol	AE
M. hyopneumoniae strain	*M. hyopneumoniae* strain used for challenge	Strain	Categorical	---	---
Type of inoculum	Type of challenge material	InType	Categorical	Lung homogenate	LH
				Culture	CT
				Lung homogenate and pure culture mixed	LH+CT
Co-infection	Additional challenge with other swine pathogens	CoIn	Categorical	Yes	---
				No	---

3.2.4. Data analysis

3.2.4.1. Univariate statistics

Descriptive statistics were used to analyze variables and summarize their main characteristics (Excel 2013 software, Microsoft Office®). Distribution, central tendency and dispersion parameters were calculated for continuous variables whereas frequency distribution bar charts were created for categorical variables.

3.2.4.2. Multivariate statistics

3.2.4.2.1. Decision tree models

The contribution of the previously mentioned variables to the experimental induction of MP was assessed through a recursive partitioning analysis via decision tree models construction. Thus, tree-based models were grown and pruned by using the *rpart* 4.1-10 package (Therneau *et al.*, 2015) in the R software version 3.3.2 (R Core Team, 2016). The pruning procedure reduces the size of the original tree, simplifying and facilitating its interpretation and avoiding overfitting data (De'ath and Fabricius, 2000; Zhang and Singer, 2010). In the present work, decision trees were pruned according to a complexity parameter (CP), defined as the cost (in terms of relative error reduction) of adding another variable to the model. Hence, models were started with a low CP value (CP=0.001) and afterwards, the most parsimonious trees were obtained by using the CP obtained from the model with the lower prediction error after 10 cross validations. Lastly, an overall measure of variable importance (VIP) and the percentage of the observed MP

variability, retained in the final and most parsimonious regression tree models, were calculated.

3.2.4.2.2. Generalized additive model (GAM)

A generalized additive model (Wood, 2006), which describes the non-linear relationships via nonparametric smoothing functions, was applied to further investigate associations between variables. This model was constructed using the R software 3.3.2 version (R Core Team, 2016) and the *mgcv* 1.8-12 package (Wood, 2011).

3.3. RESULTS

3.3.1. Included studies

Study selection flow diagram is shown in Figure 3-1. Electronic search in Medline®/PubMed® database produced an initial list of 643 references until 1990 (last entry in May 2016). An additional article written in Chinese was identified in other sources (Guo-qing *et al.*, 2007). Examination of titles, abstracts and reference lists in the retrieved articles as well as removal of duplicities led to a preliminary list of 136 potentially relevant studies (*M. hyopneumoniae* experimental inoculations reported). After examination of full text, 85 studies, including a total of 261 experimental units, complied with the inclusion criteria and therefore, they were included in the analyses.

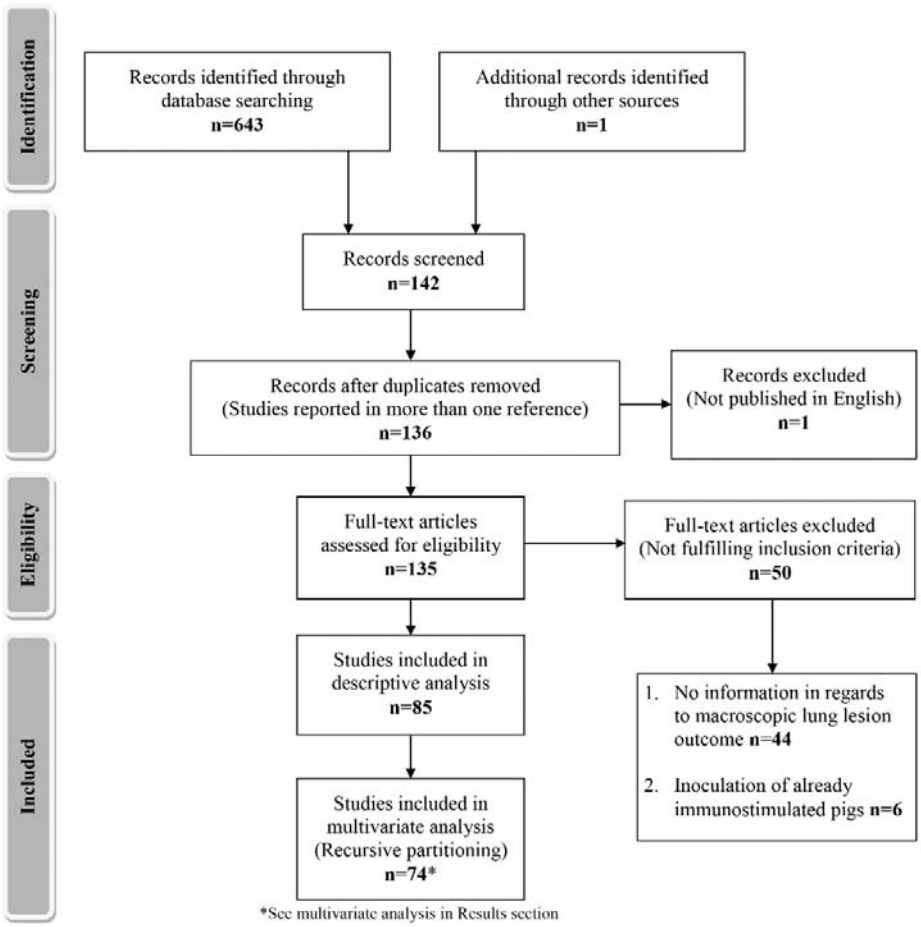


Figure 3-1. Flowchart depicting the flow of information (records identified, included and excluded) through the different phases of the systematic revision. PRISMA guidelines were followed (Moher *et al.*, 2009).

3.3.2. Descriptive analysis

Since different variables were specified in each experimental unit, the number of experimental units considered for the analyses differed between variables.

Main descriptive statistics calculated for continuous variables and the number of experimental units considered for each variable is summarized in Table 3-2, whereas frequency distribution bar charts for categorical variables are shown in Figure 3-2. Only those well identified strains appearing in at least three experimental units not coming from the same study were considered for analyses. Figure 3-3 displays the frequency distribution bar chart of considered *M. hyopneumoniae* strains together with an alphanumerical reference and the countries where they were used.

Table 3-2. Descriptive statistics calculated for continuous variables within each experimental unit and the number of experimental units considered for each variable.

			Distribution	**Central tendency**			**Dispersion**
	No. of experimental units	*Units*	*Range*	*Mean*	*Median*	*Mode*	*Standard deviation*
SamSize	261	No. of pigs	1-116	7.66	5	1	10.10
InAge	258	Week	1-17	6.04	6	6	3.61
Dose	234	CCU/pig	2.19-1.50E+15	3.21E+13	7E+08	1E+07	2.17E+14
InDays	261	Day	1-6	1.56	1	1	0.91
Nweek	252	Week	1-52	6.29	4	4	6.85
Llprop	160	Percentage	0-100	71.96	100	100	39.60
Llscore	227	Percentage	0-51.82	9.83	8.01	0	9.10

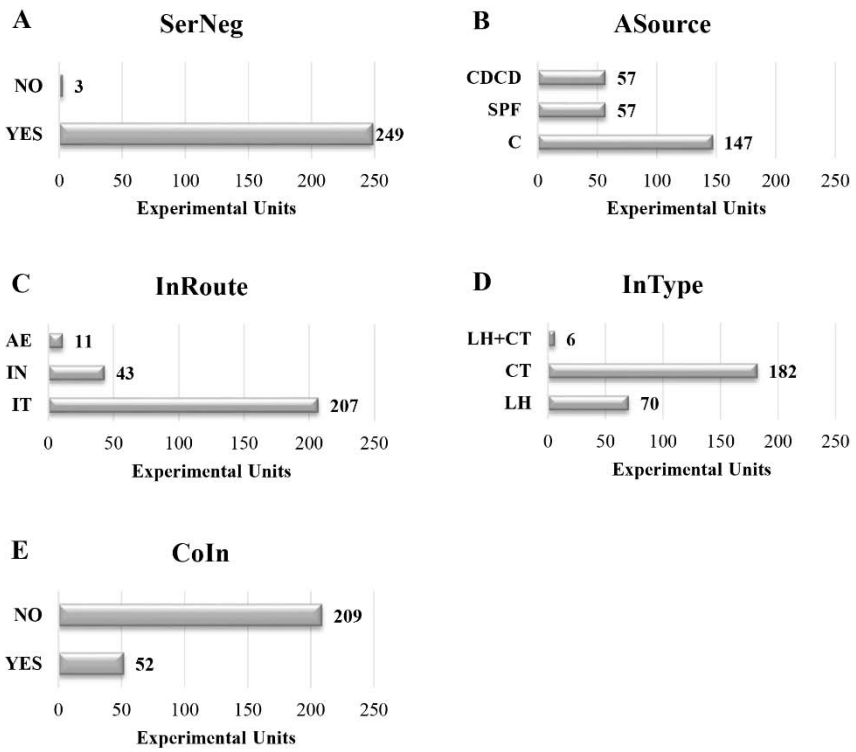


Figure 3-2. Frequency distribution bar charts of SerNeg (A), ASource (B), InRoute (C), InType (D) and CoIn (E) categorical variables.

The mean sample size within experimental units was approximately of 8 inoculated pigs. Nonetheless, experimental units including only one pig were the most prevalent ones. The latter is probably explained because of the impact of a unique study with numerous experimental units of 1 pig each (Kobisch *et al.*, 1993). Conventional (C) pigs were used in more than the half of experimental units and free-*M. hyopneumoniae* antibody animals were principally chosen. In regards of the inoculation procedure, pigs were often challenged at 6 weeks of age through the IT inoculation route in one time.

Strain name	Reference	Experimental units	Countries
98	A	3	The Netherlands, Spain
116	B	18	France
194	C	4	Mexico, USA
232	D	63	Canada, USA
75/2002	E	5	Spain
Beaufort	F	8	Australia
BQ14	G	51	France, Switzerland
E-1	H	21	Japan
F13	I	9	Belgium
J	J	4	USA
F7	K	13	Belgium
Hillcrest	L	3	Australia
JS	M	3	China
P5722-3	N	12	USA
SNU98703	O	7	South Korea
Pool	P	11	Denmark, Belgium, UK
Total		**235**	

Figure 3-3. Frequency distribution bar chart along experimental units of those well-defined *M. hyopneumoniae* strains, the given alphanumerical reference and the countries where their use has been reported.

Concerning the inoculum, *M. hyopneumoniae* culture (CT) was applied in the majority of cases. The dosage given per pig was greatly variable, reaching a maximum of 1.50×10^{15} CCU (Woolley *et al.*, 2012). Such a large dose displaced the mean to a high value, but the median dose per animal was 7×10^{8} CCU, being 10^{7} CCU/pig the most prevalent inoculum dose. Nearly one quarter of the experimental units used co-infection with other viral and bacterial swine respiratory pathogens. In decreasing order of importance, the pathogens co-inoculated were PRRSV, SIV, PCV2 and bacterial pathogens such as *A. pleuropneumoniae* or *P. multocida*.

The mean study duration was found to be about 6 weeks, although in most of the cases the animals were necropsied after 4 wpi. As outcomes, the mean percentage of lungs affected by MP within an experimental unit was 72% and the mean lung lesion score (Ph. Eur., 2013) was around 10% of lung weight damaged. Importantly, from the 261 included experimental units, 30 (11.5%) did not manage to reproduce MP. The use of a given strain was found to be closely related with the laboratory or research group. *M. hyopneumoniae* 232 was the most largely used strain, mainly by the United States research groups. In Europe, a higher variability is seen, but French *M. hyopneumoniae* BQ14 and 116 strains were the most prevalent through experimental units from this continent. Notably, the use of pool of *M. hyopneumoniae* strains, meaning simultaneous or subsequent inoculations of at least two different strains, has been reported in experimental inoculation models only in Europe. In Asia, the Japanese E-1 strain was found to be the most commonly used one. Lastly, two different strains were handled in Australia, named Beaufort and Hillcrest.

3.3.3. Multivariate analysis

Since the Llscore variable was more commonly specified within experimental units (n=227) than the Llprop one (n=160), the former was chosen as response variable in the multivariate analysis (74 studies considered). The explanatory variables were: InAge, Asource, InRoute, Strain, InType, Dose, InDays, CoIn and Nweek. Giving the fact that only 3 experimental units from 2 studies used *M. hyopneumoniae* seropositive

pigs (Sibila *et al.*, 2012; Wilson *et al.*, 2013), SerNeg variable was not considered in recursive partitioning.

In a first obtained tree-based model, in which all explanatory variables were fitted to get the full picture of the model, Nweek was the most important variable by far as shown by an *ad hoc* ANOVA test ($F_{9, 215}$=6.36, p=5.485E-08, R^2=21%). Using GAM, the strong connection between Nweek and Llscore variables was reinforced (F=10.71, edf=5.95, p=3.34E-11, R^2=30%). Indeed, Llscore peaked when necropsies were performed at 4 wpi, dropping later at 8 wpi (Figure 3-4). Thus, the first obtained decision tree established that successful protocols were those assessing MP below 8 wpi; specifically, the top ten Llscore were obtained between 3 and 7 wpi. Consequently, and in order to enable the identification of other putative influencing factors on the Llscore variability, another tree-based model was obtained from the adjustment of data from experimental units with a maximum duration of 8 wpi. As a result, the final decision tree explained 33% of the observed Llscore variability (Figure 3-5) and, in decreasing order of importance, the factors to be accounted to successfully reproduce MP in experimental units of duration below 8 wpi were the following: Strain (VIP=67), InAge (VIP=18), CoIn (VIP=9) and finally, Asource (VIP=5). Overall, *M. hyopneumoniae* strains 116, 75/2002, Beaufort, BQ14, Hillcrest, JS and P5722-3 resulted in higher Llscore values than all the other assessed strains. Importantly, some of the aforesaid strains lead to the highest Llscore in co-infected pigs (Llscore=24%, n=11). Nonetheless, sole inoculation with 75/2002 or JS strains resulted in similar Llscore values

(Llscore=23%, n=8) to those reached in co-infection. All the remaining strains resulted in lower Llscore outcomes, particularly when the age of pigs at inoculation was lower than 3.5 weeks (Llscore=2.6%, n=20). When above 3.5 weeks of age, inoculated specific pathogen-free (SPF) pigs showed higher Llscore values (Llscore=24%, n=11) than their C or cesarean-derived, colostrum-deprived (CDCD) counterparts (Llscore=8.4%, n=102). All the other analyzed variables, namely InRoute, InDays, InType and Dose, were not found relevant to explain Llscore.

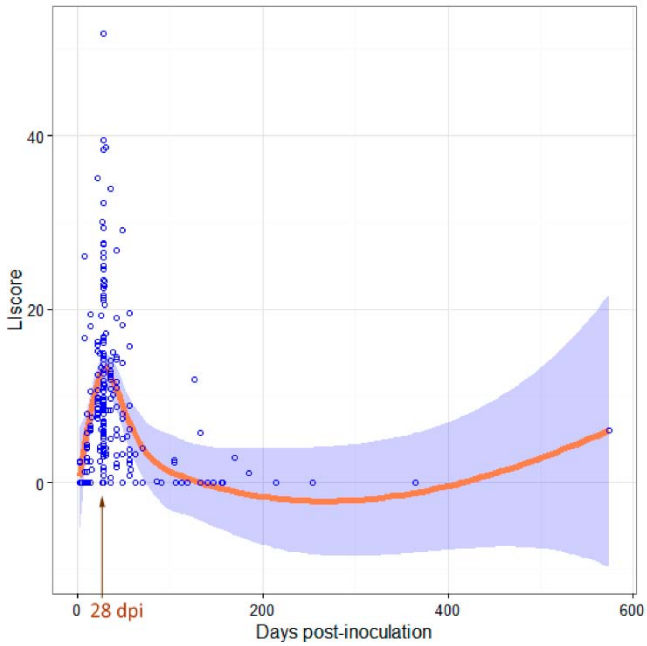


Figure 3-4. Plot representing the distribution of values for the Llscore variable depending on the dpi at which animals were necropsied. Blue dots represent Llscore values (n=227). The arrow indicates that Llscore peaked when necropsies were performed at 28 dpi. The orange line and the blue shadow symbolize the predictions and their range of the GAM at 95% confidence interval, respectively.

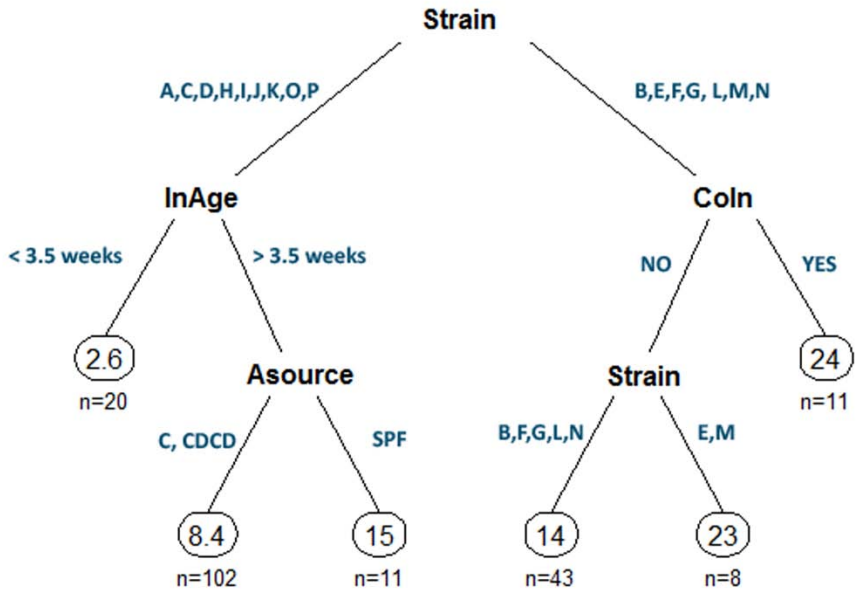


Figure 3-5. Final regression tree resulting from experimental units with a maximum duration of 8 wpi. The numbers into the circles represent the mean value of Llscore variable in "n" experimental units. Blue values aside the lines represent the different categories of the corresponding variables.

3.4. DISCUSSION

Numerous attempts to induce MP at experimental level have been reported and, as evidenced by the present results, variation in both the number of animals affected by MP as well as in severity of such lung lesions do occur. The latter is accompanied by wide differences between *M. hyopneumoniae* experimental models, though highlighting the existing inconstancy between protocols.

Works studying how a unique variable may influence MP under experimental settings have been already made (Czaja *et al.*, 2002; Kobisch *et al.*, 1993; Marois *et al.*, 2010; Sørensen *et al.*, 1997; Vicca *et al.*, 2003). Contrarily, observation and analysis of more than one experimental variables at a time has not been assessed. In consequence, a combination of experimental factors that would increase the chance to successfully achieve MP is not available and the uncertainty around this problem is still far of being mitigated. Therefore, the present work investigated which experimental conditions might be accounted for reproduction of MP, determined by the macroscopic lung lesion score, and proposes the initial bases to build a reproducible and effective *M. hyopneumoniae* challenge model.

In order to accomplish the abovementioned objective, data from a series of studies were summarized through a systematic review and analyzed by means of recursive partitioning. To author's knowledge, this is the first time that such statistical method is applied to evaluate the performance of experimental infections in pigs. Along the same line, Tomás *et al*. (2008) identified factors with a relevant influence on the expression of clinical postweaning multisystemic wasting syndrome (PMWS) under experimental conditions, albeit different statistical techniques were applied (Tomás *et al.*, 2008). Recursive partitioning is particularly well suited for exploring complex relationships, where there is often little knowledge nor predictions regarding which and how variables are related (Zhang and Singer, 2010). In the present work, recursive partitioning was used to create a decision tree that defined an

easy to visualize linear combination of continuous and categorical explanatory variables (InAge, Asource, InRoute, Strain, InType, InDays, Dose, CoIn and Nweek) to predict a continuous response variable (Llscore). Moreover, recursive partitioning provides a better ability to handle missing values in both explanatory and response variables than other regression techniques (De'ath and Fabricius, 2000). Missing data was perhaps one of the most important limitations of the present systematic review, being common within experimental units of different studies. For instance, the pig genetics could not be considered because in most works the breed used was not specified.

In the first fitted regression tree containing all explanatory variables, the most determinant factor for MP achievement at experimental level was found to be the study duration (Nweek). Henceforth, and to further examine such relationship, a complementary analysis was performed by means of GAM. In this way, GAM analysis revealed that lung lesion scores peaked when the Llscore was assessed at 4 wpi, being congruent with those studies indicating that macroscopic lung lesions reach their maximum level around that moment (Kobisch *et al.*, 1993; Sørensen *et al.*, 1997). Hence, the present results support this lapse of time as the standard for MP assessment, tough the time period that should not be exceeded to evaluate MP is 8 wpi. Accordingly, those experiments quantifying lung lesions over 8 wpi aimed principally to assess *M. hyopneumoniae* transmission and persistence instead of MP (Fano *et al.*, 2005; Pieters *et al.*, 2010; Pieters *et al.*, 2009). Taking into account all the above, final tree models were applied to data from experimental units

of less duration than 8 wpi, and the most relevant factors to be accounted for successful MP reproduction appeared to be the *M. hyopneumoniae* strain (Strain) followed by the age of animals at inoculation (InAge), co-infection with other swine pathogens (CoIn) and, finally, the source of the animals (Asource).

Differences in virulence between *M. hyopneumoniae* strains have been evidenced (Meyns *et al*., 2007; Vicca *et al*., 2003). Despite antigenic and genetic heterogeneity between isolates have been demonstrated (Frey *et al*., 1992; Ro and Ross, 1983), it is still not known whether such heterogeneity correlates with dissimilarities in virulence (Charlebois *et al*., 2014; Vicca *et al*., 2003; Vranckx *et al*., 2011). By recursive partitioning, the *M. hyopneumoniae* strains assessed were split into two groups, one leading to higher lung lesion score outcomes than the other. Thus, the obtained results support the already reported high pathogenicity of some *M. hyopneumoniae* strains such as Hillcrest (Woolley *et al*., 2012) or even the lack of pathogenicity of J strain (Bereiter *et al*., 1990; Zielinski and Ross, 1990), from which most of the current *M. hyopneumoniae* commercial vaccines are based (Villarreal *et al*., 2012). On the contrary, the American 232 and the Belgian F7, recognized as pathogenic strains (Meyns *et al*., 2007; Ross *et al*., 1984), were found less appropriate for MP reproduction than other *M. hyopneumoniae* strains. As a distinctive condition, simultaneous or subsequent inoculations with more than one strain did not result in more severe lung lesions. Although some previous studies have suggested an enhanced effect of multiple infections with different *M. hyopneumoniae* strains (Michiels *et al*., 2017;

Villarreal *et al*., 2009; Vranckx *et al*., 2011), the present results upkeep those findings reported by Charlebois *et al*. (2014), in which no link was found between lungs harboring more than one strain and lung lesion severity. Additionally, differences between *M. hyopneumoniae* strains might be explained, among others, by the number of *in vitro* passages, which has been discussed to result in pathogenicity differences (Zielinski and Ross, 1990). Unfortunately, this factor could not be addressed because it was not explicitly mentioned in an extensive number of publications.

Immunopathological events are considered to play an imperative role in the pathogenesis of *M. hyopneumoniae* infection (Damte *et al*., 2011; Thacker and Minion, 2012). Consequently, the higher potential of success obtained for inoculations in pigs older than 3.5 weeks of age could be explained by the fact that some mechanisms from both the innate and acquired immunity are less developed at birth and mature with age (Thacker and Minion, 2012). Indeed, under conditions of a previous study (Piffer and Ross, 1984), no differences were detected in susceptibility of pigs to *M. hyopneumoniae* between 3 and 12 weeks of age. Furthermore, in almost all the analyzed studies, inoculated pigs were tested to be seronegative against *M. hyopneumoniae*, excluding conferred protection of younger pigs by passively acquired maternal antibodies. On the other hand, the use of co-infections have been proven effective to induce MP in several experiments. However, whether co-infection with other swine pathogens influence the severity of *M. hyopneumoniae* infection (macroscopic lung lesions) is still under debate.

Whereas some studies found no potentiation of MP by other microorganisms (Opriessnig *et al.*, 2004; Sibila *et al.*, 2012; Thacker *et al.*, 1999; Zhang *et al.*, 2011), others demonstrated that pneumonia in co-inoculated pigs was more severe than in pigs inoculated only with *M. hyopneumoniae* (Thacker *et al.*, 2001; Thanawongnuwech *et al.*, 2004; Yazawa *et al.*, 2004). Despite such discrepancies, results of the recursive partitioning analysis indicate that co-infection models have a higher potential for reproducing MP than the inoculation of *M. hyopneumoniae* alone. Lastly, the use of SPF pigs resulted to be an important feature to effectively reproduce MP. Unfortunately, criteria applied to define SPF pigs by the authors reporting their use were unknown, becoming difficult to precisely explain the importance of such factor on lung lesion outcome. Nevertheless, and independently of the source, pigs used in *M. hyopneumoniae* experimental models were, in general terms, *M. hyopneumoniae*-free by means of serology and/or PCR techniques. Hence, pigs naive to *M. hyopneumoniae* are probably required to increase the chance to achieve severe MP.

All the other evaluated variables (InRoute, InDays, InType and Dose) were found not relevant to explain MP variability. In agreement, lesion scores were similar between pigs inoculated through the IT and the IN routes in a previous study (Marois *et al.*, 2007). Inoculation with lung homogenate (LH) taken from infected pigs might has the potential to introduce adventitious agents as well as aggravate the inflammatory response due to the administration of foreign antigens (Czaja *et al.*, 2002). However and in accordance to the obtained results, no significant

differences were observed among animals inoculated with LH and CT prepared from the same lung homogenate in terms of lung lesions achieved (Fort *et al.*, 2013). Furthermore, an inoculum dose-dependent response has been reported in *M. hyopneumoniae* challenge studies, in which very high doses are necessary to achieve clinical disease (Marois *et al.*, 2010). In agreement, descriptive statistics revealed an overall high dose (CCU) used per pig.

Importantly, care must be taken in drawing conclusions from the obtained results. The statistical approach performed in the present study was probably hampered by the broad differences in the applied conditions between experimental units, which make difficult to assess the effect of a particular condition in the lung lesion outcome. Moreover, inherent limitations of the included publications and the systematic review process must be also taken into account. In this respect, the trend towards publishing only positive results (e.g. experiments in which MP was successfully achieved) and the exclusion of those studies that did not fulfil all the systematic review inclusion criteria (e.g. experiments in which MP was induced but no information in regards of lung lesion score was provided), probably lead to biases that could have influenced the obtained results. Another important reason for bias is linked to the high prevalence of some particular conditions within a variable, such as the lung lesion assessment at 4 wpi as striking example. Thus, the importance of such widely extended conditions within *M. hyopneumoniae* experimental inoculation protocols could be overestimated in the statistical analysis.

3.5. CONCLUSION

The present study broadens the current understanding in regards to *M. hyopneumoniae* experimental swine models and constitutes the first insight into those conditions supporting MP development under experimental settings. Thus, obtained results might serve as a basis for debate in the search for a universally accepted *M. hyopneumoniae* experimental model. While the *M. hyopneumoniae* strain used may depend basically on the particular research group (strain availability), other easily modifiable conditions might be taken into account in experimental models. Therefore, the highest likelihood to achieve MP would require lung lesion assessment within a period below 8 wpi and include inoculation of SPF (*M. hyopneumoniae* free) pigs older than 3.5 weeks of age and, preferably, in co-infection with another swine respiratory pathogen as a triggering factor.

Chapter 4

Study II

Induction of mycoplasmal pneumonia in experimentally infected pigs by means of different inoculation routes

4.1. INTRODUCTION

One of the most distinguishing features of experimental challenge systems is the *M. hyopneumoniae* inoculation route used. However, its impact on the pathogenesis of the experimentally induced *M. hyopneumoniae* infection has been poorly investigated. There are four inoculation routes reported in the peer-reviewed literature used in swine *M. hyopneumoniae* experimental models: ET, TT, IN and AE. Although both IT methods (ET and TT) are the most widely used, MP has been induced by all models. AE is probably the least extended method, despite it is supposed to mimics better the natural conditions of infection (Czaja *et al*., 2002). Comparisons between *M. hyopneumoniae* challenge models using different inoculation routes are scarce. Marois *et al*. (2007) compared the ET, the TT and the IN routes, but no differences regarding detection and recovery of *M. hyopneumoniae*, clinical signs and lesion scores were evidenced between infection conditions.

This last point should be subjected to further investigation. Therefore, the aim of this chapter was to compare three *M. hyopneumoniae* inoculation routes (ET, IN and AE) for their ability to induce MP. The optimum inoculation route was established by studying colonization, clinical, pathological and immunological parameters.

4.2. MATERIALS AND METHODS

4.2.1. Animals and housing

Animals were obtained from a herd located in North-Eastern Spain that was free from *M. hyopneumoniae* and PRRSV based on serology and clinical history. For animal selection, serology (IDEIA™ *Mycoplasma hyopneumoniae* EIA kit; Oxoid, UK) and a nested PCR for detection of *M. hyopneumoniae* DNA (Calsamiglia *et al.*, 1999) was done on nasal swabs. Thirty 4-week old piglets were selected and transported to the experimental facilities of A.M. ANIMALIA BIANYA S.L. (Girona, Spain). Prior to challenge, animals were randomly distributed (Randbetween function of Excel 2007 software, Microsoft Office®) into four groups equalled according to body weight. Challenged animals were comingled in the same room whereas the control group was placed in a separated room.

4.2.2. Experimental design

At approximately 6 weeks of age, pigs were challenged according to the experimental design detailed in Table 4-1. All animals belonging to the challenged groups (n=24) were inoculated with 5 mL of *M. hyopneumoniae* fresh culture on two consecutive days. Two control animals received 5 mL of sterile phosphate buffered saline (PBS) on two consecutive days by one of the three assessed routes (total of n=6). At 28 dpi, all pigs were euthanized with an intravenous overdose of sodium pentobarbital and subjected to necropsy examination.

Study procedure was approved by the Animal Experimentation Ethics Committee of the *Universitat Autònoma de Barcelona* (n° 5796) and of A.M. ANIMALIA BIANYA S.L. (n° 05/15).

Table 4-1. Experimental design.

Group name	No. of animals	Route of inoculation	Inoculum	
			1st day of challenge	*2nd day of challenge*
Control	2	Endotracheal	5 ml PBS	5 ml PBS
	2	Intranasal		
	2	Aerosol		
ET	8	Endotracheal	5 ml *M. hyopneumoniae* culture	5 ml *M. hyopneumoniae* culture
IN	8	Intranasal		
AE	8	Aerosol		

4.2.3. Inoculum and inoculation procedures

A fresh culture derived from a *M. hyopneumoniae* field strain was used as the inoculum. This strain was isolated in 2010 from a lung of a slaughter-age animal showing CVPC. Inoculum titre was determined by using a limiting dilution method. Briefly, ten-fold dilutions of the inoculum were made and left to grow for 2 weeks at 37 °C. Tubes were tested for *M. hyopneumoniae* by PCR (Mattsson *et al*., 1995) at one and two weeks of incubation in order to indirectly evaluate bacterial growth. From the second week PCR results, the final titre was calculated by means of the Reed and Muench method (Reed and Muench, 1938). The inoculum titre was 8.25 log10 PCR_{50}/mL, in which PCR_{50} represents the limiting dilution of the inoculum that is PCR positive in 50% of its replicates.

For the ET inoculation, a double catheter (an internal with a syringe adapter into an external catheter) (Bastos Medical S.L., Spain) was introduced in the trachea. The inoculum was administered with a syringe through the internal catheter. For the IN inoculation, a mucosal atomization device (MAD Nasal™; Wolfe Tory Medial, Inc., USA) attached to a syringe was used to administrate half of the inoculum volume into each nostril. Animals included in the AE group, were anaesthetised with a combination of 10 mg/kg Ketamine (Imalgene®; Merial, France), 0.4 mg/kg Butorfanol (Torbugesic®-SA; Zoetis, USA) and 6 mg/kg of Azaperone (Stresnil®; Esteve, Spain) and placed in sternal recumbence. The inoculum was administered through an individual mask (Bastos Medical S.L., Spain) connected to an aerosol delivery system (Boy® SX compressor and LC® Sprint nebulizer; Pari GmbH, Germany) with a total output rate of approximately 600 mg/min and a particle mass median diameter of 3.5 μm under a pressure of 1.6 bar.

4.2.4. Clinical evaluation and body weight

After inoculation, pigs were monitored for clinical signs on a weekly basis for four weeks. The focus of clinical observations was on respiratory signs such as dyspnoea and coughing. Body weight was registered prior to the challenge and at necropsy day. ADWG was calculated according to the following formula: body weight at necropsy minus the body weight before challenge divided by the days lapsed between them.

4.2.5. Collection and samples processing

Blood was collected one day before challenge (−1 dpi) and at necropsy day (28 dpi). Laryngeal swabs were obtained as described previously (Roos *et al.*, 2016) at −1 dpi and weekly thereafter (7, 14, 21 and 28 dpi). Once in the laboratory, blood was centrifuged at 1500 *g* for 10 min at 4 °C and sera were stored at −80 °C until used. Laryngeal swabs were resuspended in 1 ml sterile PBS, vortexed and stored at −80 °C.

At necropsy (28 dpi), two lung samples were collected from all each animals: one was fixed in 10% neutral buffered formalin and the second one was frozen. These samples were used for histopathological studies and detection of *M. hyopneumoniae* DNA, respectively. BALF was collected from twelve animals, selecting those three animals showing the most severe lung lesions (when present) within each group. BALF was left for gross mucus sedimentation and supernatant was stored at −80 °C until it was processed. These samples were used for *M. hyopneumoniae* DNA, cytokine and specific IgA antibody detection.

4.2.6. Pathological examination

The extension of gross lung lesions compatible with *M. hyopneumoniae* infection (CVPC) was assessed using the European Pharmacopoeia scoring system (Ph. Eur., 2013). For histopathological studies, formalin-fixed tissues were processed routinely and embedded in paraffin wax. Sections (4 µm) were stained with haematoxylin and eosin and examined under light microscope for BIP. Microscopic scoring was performed as previously described (Sibila *et al.*, 2004). Briefly,

histopathological lung lesions were graded from 0 to 4, where 0 to 2 was classified as non-compatible with MP and 3 to 4 was considered compatible with MP microscopic lung lesions.

4.2.7. Detection of *M. hyopneumoniae*-specific antibodies in serum and BALF

Sera were tested in duplicate for *M. hyopneumoniae* antibodies by means of a commercial competitive inhibition enzyme-linked immunosorbent assay (IDEIA™ *Mycoplasma hyopneumoniae* EIA kit; Oxoid, UK). Samples with mean optical density (OD) <50% of the OD of the buffer control were considered positive. Doubtful (OD from 50 to 64%) and negative (OD≥65%) OD-values were classified as negative in the statistical analysis.

Detection of *M. hyopneumoniae* specific IgA in BALF was performed modifying the *Mycoplasma hyopneumoniae* Antibody Test Kit (BioCheck, UK) with an alkaline phosphatase-labelled goat anti-porcine IgA polyclonal antibody (Bethyl Laboratories, USA) at 1:5000 dilution as secondary antibody. BALF samples were tested undiluted and in duplicate. One hundred μL of each sample were used in the ELISA assay. The cut-off was established at mean OD value of BALF from the control animals plus three folds the standard deviation (SD). Values higher than the cut-off were considered positive. Values below this cut-off were considered negative.

4.2.8. DNA extraction

DNA was extracted from 200 μL of laryngeal swabs suspension or undiluted BALF using BioSprint® 96 DNA Blood kit (Qiagen GmbH, Germany) on the BioSprint 96 workstation (Qiagen GmbH, Germany). Lung tissue was disrupted using TissueLyser (Qiagen GmbH, Germany) for DNA extraction. Approximately 1 g of tissue was homogenized with 600 μL of PBS into plastic tubes containing glass beads. After shaking for 10 min, the lung homogenate was centrifuged at 11 000 *g* for 1 min. DNA was extracted from 200 μL of tissue supernatant (MagMAX™ DNA Multi-Sample Kit, Life Technologies, USA) according to the manufacturer's instructions on the BioSprint 96 workstation (Qiagen GmbH, Germany). To assess potential contamination during the extraction procedure, a negative control was included using PBS as an extraction substrate in each extraction plate.

4.2.9. Quantitative real time PCR

A commercial quantitative *M. hyopneumoniae* real time PCR (qPCR) was performed in laryngeal swabs, BALF and lung tissue samples. The assay was performed using VetMAX™-Plus qPCR Master Mix (Applied Biosystems, USA) and VetMAX™ *M. hyopneumoniae* Reagents (Applied Biosystems, USA), according to the manufacturer's instructions. VetMAX™-Plus qPCR Master Mix kit includes Xeno™ DNA Control, which serves as an internal positive control for DNA purification and qPCR. Runs were carried out in an ABIPRISM® 7500 machine (Applied Biosystems, Singapore). The threshold for the DNA target was set at 10% of the average maximum fluorescence value of the positive control DNA

target. Cycle threshold (Ct) values equal to or lower than 40 were considered positive.

4.2.10. Evaluation of cytokine responses in BALF

Levels of pro-inflammatory cytokines IL-1β, IL-8, IL-6 and TNF-α were determined using commercially available Porcine Quantikine® ELISA kits (R&D Bio-Scientific Pty Ltd, Australia) following manufacturer's recommendations. BALF samples were tested undiluted. Reactions were measured using OD at 450 nm and quantified by the use of a standard curve.

4.2.11. Statistical analyses

Statistical analyses were performed using NCSS software (Hintze, 2004). Normal distribution of continuous variables was evaluated by the Shapiro-Wilk test. An analysis of variance using the Tukey-Kramer test was used for mean comparison of continuous variables (lung score, body weight, ADWG, qPCR Ct, cytokines concentrations, percentage of inhibition and IgA ELISA OD) among groups. The Chi-square or Fischer tests were applied to evaluate the proportion of animals showing MP, seroconversion and qPCR positive results. In order to evaluate the agreement between the qPCR results in laryngeal swabs and lung tissue, the Cohen's kappa coefficient (κ) was calculated. P values ≤0.05 were considered statistically significant, whereas p values >0.05 and ≤0.10 were considered statistical tendencies.

4.3. RESULTS

Control pigs were negative for antibodies, clinical signs, DNA and lesions associated to *M. hyopneumoniae* infection throughout the study.

4.3.1. Clinical signs and body weight

Very sporadic and mild coughing was displayed by challenged pigs from 14 dpi onwards. No other significant clinical signs were observed during the experiment. No significant differences in mean body weight (at challenge and at necropsy) neither in ADWG were observed among groups (data not shown).

4.3.2. Pathological studies

MP was recognised taking into account three criteria concomitantly: 1) Presence of CVPC, based on gross evaluation, 2) presence of BIP determined through microscopic evaluation (score 3 or 4) and 3) detection of *M. hyopneumoniae* DNA by qPCR in lung tissue.

Percentage of animals showing MP and the mean lung score within each group are represented in Table 4-2. MP was observed in all inoculated groups. No significant differences in the lung score were observed among the IN, AE nor Control groups. In contrast, significant differences were obtained regarding the number of animals showing MP as well as in MP severity between the ET and all the other groups ($p<0.05$).

Table 4-2. Proportion (%) of animals showing MP and lung lesion score (mean ± SD) at 28 dpi. Different superscripts within a column indicate significant differences between groups (p<0.05).

Group	No. of animals with MP/Total No. of animals (%)	Ph. Eur. score including all animals per group (mean ± SD)	Ph. Eur. score of animals showing MP per group (mean ± SD)
Control	0/6 (0.0)[a]	0.0 (± 0.0)[a]	0.0 (± 0.0)[a]
ET	7/8 (87.5)[b]	9.5 (± 9.8)[b]	10.8 (± 9.8)[b]
IN	2/8 (25.0)[a]	0.9 (± 1.6)[a]	3.6 (± 0.1)[a]
AE	3/8 (37.5)[a]	1.4 (± 2.7)[a]	3.7 (± 3.3)[a]

4.3.3. Serology and *M. hyopneumoniae*-specific IgA in BALF

None of the animals was seropositive one day before challenge (Figure 4-1). In both IN and AE groups, only one animal out of 8 (12.5%) seroconverted at 4 weeks after challenge (28 dpi). At that time, 5 pigs out of 8 (62.5%) seroconverted within the ET group (p<0.05). The ET mean percentage of inhibition was also statically lower (p<0.01) than all the other groups except for the IN. Interestingly, all animals that seroconverted showed MP regardless the experimental group.

The cut-off value of the indirect IgA ELISA was established at an OD value of 0.21. All BALF samples belonging to the challenged groups (ET, IN and AE) were *M. hyopneumoniae*-IgA positive at 28 dpi at a different levels. The ET group showed a significantly higher (p<0.01) mean OD value than those of the IN, AE and control groups (Figure 4-2). Although mean specific IgA OD values in BALF from AE and IN groups were not statistically different in comparison with the mean value in Controls, a tendency was reported (p≤0.10).

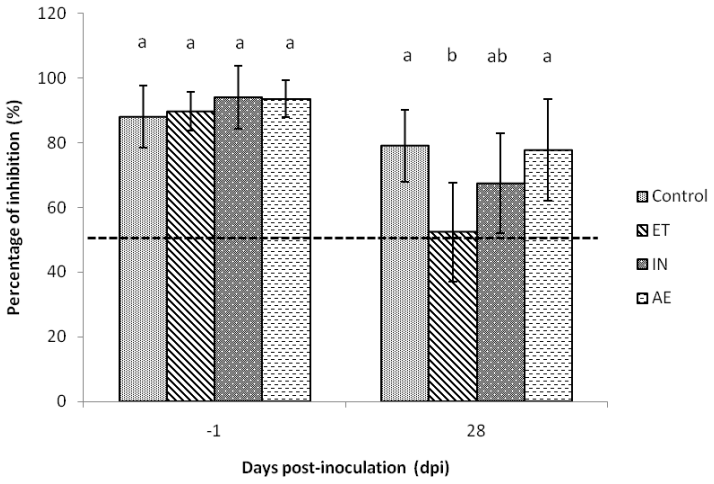


Figure 4-1. Sera percentage of inhibition (mean ± SD) from Control, ET, IN and AE groups one day before challenge (-1 dpi) and at necropsy (28 dpi). Different superscripts indicate significant differences among groups (p<0.05). Discontinuous line represents the positivity threshold.

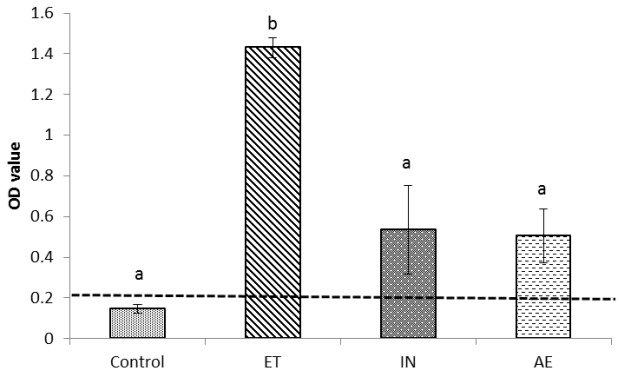


Figure 4-2. *M. hyopneumoniae* IgA antibodies (mean ± SD of OD values at 405 nm) in BALF from Control, ET, IN and AE groups at 28 dpi. Different superscripts indicate significant differences among groups (p<0.05). Discontinuous line represents the positivity threshold.

4.3.4. *M. hyopneumoniae* detection by real time PCR

The percentage of positive animals to *M. hyopneumoniae* qPCR and their Ct values (mean ± SD) in laryngeal swabs along the study, in lung tissue and BALF within each group are represented in Table 4-3.

All the animals included in the study were negative to qPCR in laryngeal swabs before challenge. While in the ET group *M. hyopneumoniae* DNA was detected from 7 dpi onwards, in both IN and AE groups, *M. hyopneumoniae* DNA was not detected until 21 dpi. Independently of the group, all animals that became positive in a certain time point of the study remained positive until the end (28 dpi). The highest percentage of positive animals was detected at 21 dpi in all groups but except for the IN group, in which qPCR positive animals appeared to increase over the last week (28 dpi). In fact, the maximum number of positive animals (75%) within a group was present in the IN group at 28 dpi. All the animals showing MP were qPCR positive in laryngeal swabs at necropsy day, except two animals belonging to the ET group. No significant differences in mean Ct values were observed between groups at any time point. *M. hyopneumoniae* qPCR positive animals in lung tissue were detected in all challenged groups, but the highest percentage of positive animals belonged to the ET one. All animals with MP were qPCR positive in lung tissue at necropsy day regardless of the group. However, three animals without MP from the IN group were also lung qPCR positive.

Table 4-3. Proportion (%) of *M. hyopneumoniae* qPCR positive animals in laryngeal swabs through the study, lung tissue and BALF and Ct values (mean ± SD) from Control, ET, IN and AE groups. Different superscripts within a column indicate significant differences between groups (p<0.05).

Groups	Laryngeal swabs										Lung tissue		BALF	
	-1 dpi		7 dpi		14 dpi		21 dpi		28 dpi					
	Prop. (%)	Ct	Prop. (%)	Ct*	Prop. (%)	Ct*	Prop. (%)	Ct*	Prop. (%)	Ct*	Prop. (%)	Ct*	Prop. (%)	Ct*
Control	0/6 (0.0)^{a}	NA	0/6 (0.0)^{a}	NA	0/6 (0.0)^{a}	NA	0/6 (0.0)^{b}	NA	0/6 (0.0)^{b}	NA	0/6 (0.0)^{b}	NA	0/3 (0.0)^{b}	NA
ET	0/8 (0.0)^{a}	NA	2/8 (25.0)^{a}	38.8 ± 0.4	3/8 (37.5)^{a}	31.8 ± 2.6	5/8 (62.5)^{a}	34.4 ± 1.6^{a}	5/8 (62.5)^{a}	33.6 ± 2.8^{a}	7/8 (87.5)^{a}	26.8 ± 0.9^{a}	3/3 (100)^{a}	24.1 ± 1.0^{a}
IN	0/8 (0.0)^{a}	NA	0/8 (0.0)^{a}	NA	0/8 (0.0)^{a}	NA	2/8 (25.0)^{ab}	33.1 ± 0.8^{a}	6/8 (75.0)^{a}	35.3 ± 3.2^{a}	5/8 (62.5)^{ab}	33.5 ± 6.1^{b}	3/3 (100)^{a}	22.9 ± 2.2^{a}
AE	0/8 (0.0)^{a}	NA	0/8 (0.0)^{a}	NA	0/8 (0.0)^{a}	NA	3/8 (37.5)^{ab}	35.4 ± 4.1^{a}	3/8 (37.5)^{ab}	33.9 ± 5.0^{a}	3/8 (37.5)^{b}	28.5 ± 7.7^{a}	3/3 (100)^{a}	26.2 ± 1.9^{a}

Prop.: Proportion; *Mean Ct value has been calculated considering only those *M. hyopneumoniae* qPCR positive animals

All animals with MP were qPCR positive in lung tissue at necropsy day regardless of the group. However, three animals without MP from the IN group were also lung qPCR positive. From the total of the 15 animals that were *M. hyopneumoniae* positive in lung tissue, 11 were also positive in laryngeal swabs at the same time point of the study (28 dpi), showing both techniques a coincidence of 77% (κ=0.53). Although in the IN group 5 out of 8 animals (62.5%) were qPCR positive, mean lung tissue Ct value was significantly (p<0.05) higher in this group compared to the other two challenged groups.

All the BALF samples from the three challenged groups (ET, IN and AE) were qPCR positive. No significant differences in mean Ct values were observed in BALF between groups.

4.3.5. Cytokine responses in BALF

Four weeks after infection, IL-6 cytokine was not detected in any of the BALF samples tested (data not shown). Mean TNF-α (Figure 4-3A) and IL-8 (Figure 4-3B) cytokine levels were not significantly different between groups. Nevertheless, there was a tendency between mean levels of TNF-α and IL-8 from ET group and those found in Controls (p≤0.10). Remarkably, mean levels of IL-1β (p<0.001) increased significantly in ET-challenged pigs in comparison with mean levels found in all other groups (Figure 4-3C).

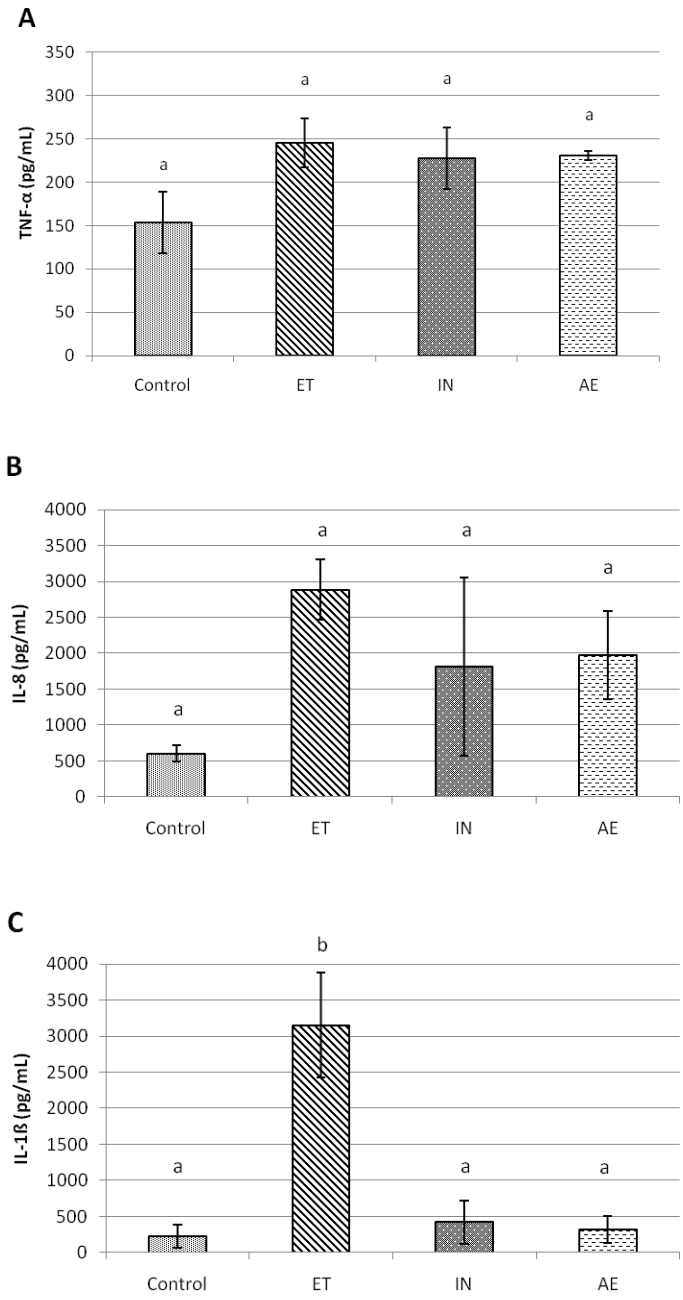


Figure 4-3. Levels (mean ± SD) of TNF-α (A), IL-8 (B) and IL-1β (C) in BALF of pigs from Control, ET, IN and AE at 28 dpi. Different superscripts indicate a significant difference among groups within each graph (p<0.05).

4.4. DISCUSSION

The ability to induce MP in 6-week-old conventional piglets experimentally challenged with *M. hyopneumoniae* using three different inoculation routes was assessed in the present study. Lung lesion scores have been previously reported to be similar between groups of pigs experimentally challenged by means of different inoculation routes (Marois *et al.*, 2007). However, different *M. hyopneumoniae* infectious doses and necropsy timings were used, difficulting comparisons with the present study, as such factors are known to influence the infection pattern and MP severity in pigs (Villarreal *et al.*, 2011). To the best of author's knowledge, this is the first time that the ET, the IN and the AE inoculation routes are compared under the same conditions in pigs challenged with *M. hyopneumoniae*.

The three tested inoculation routes (ET, IN and AE) had been already reported to induce MP (Andrada *et al.*, 2014; Czaja *et al.*, 2002; Marois *et al.*, 2007; Woolley *et al.*, 2012). In agreement with these previous studies and under the settings applied in the present study, all inoculation route models reproduced MP. However, the highest percentage of animals affected and the most severe lung lesions were obtained in the ET inoculated group. These results were accompanied by an earlier respiratory tract colonization and seroconversion, a higher percentage of qPCR positive animals in lung tissue and an enhanced cellular and humoral local immune response. On the other hand, no significant differences in clinical signs neither in mean body weight at necropsy nor in ADWG were observed between groups. The number of animals used,

frequency of clinical signs observation (only weekly) and duration of the study period were probably too limited to observe any effect of the inoculation route on coughing and performance parameters.

M. hyopneumoniae is primarily found on the mucosal surface of the trachea, bronchi and bronchioli (Blanchard *et al.*, 1992), and its adherence to the ciliated epithelium is a prerequisite for initiation of the infection (Maes *et al.*, 2008). Previous studies indicate that tracheo-bronchial swabbing is the most sensitive sampling method for detecting *M. hyopneumoniae* in infected live pigs using nested or real-time PCR assays (Fablet *et al.*, 2010; Kurth *et al.*, 2002; Marois *et al.*, 2007). The use of laryngeal swabs to assess *M. hyopneumoniae* infection has been recently reported (Roos *et al.*, 2016). Under the present conditions and despite being *ante-mortem* and *post-mortem* samples respectively, 77% of concordance (κ=0.53) between laryngeal swabs and lung tissue for *M. hyopneumoniae* detection by qPCR was found at necropsy. In the present study, colonization timing differences were found between groups; *M. hyopneumoniae* DNA was detected in the ET group two weeks earlier (7 dpi) than in the IN and AE groups (21 dpi). Although only two out of eight (25%) IN-challenged animals were showing MP, laryngeal swabs qPCR positive animals appeared to increase over the last week (28 dpi) in this group, accompanied by quite a high proportion (5 out of 8) of qPCR positive animals in lung tissue at that time. In *Chapter 3*, it has been evidenced that *M. hyopneumoniae* gross lung lesions reach their maximum score by 28 dpi. In consequence, in most experimental infection models, pigs are euthanized at this point (Kobisch *et al.*, 1993;

Sørensen *et al.*, 1997; Villarreal *et al.*, 2009; Villarreal *et al.*, 2011; Woolley *et al.*, 2013). Nonetheless, it cannot be ruled out that, in AE and/or IN-challenged animals, longer study periods would have increased the percentage of animals showing MP and its severity.

One limitation of this study was the fact that challenged animals were comingled in the same room and transmission between groups was possible throughout the study. In consequence, whether the infection observed in IN and AE groups is product of the ET *M. hyopneumoniae* shedding cannot be completely excluded. However, this event would be very unlikely since the first evidence of *M. hyopneumoniae* infection was seen at 7 dpi on two animals from the ET group. Considering that necropsies were done 3 weeks after this first detection, the probability that seroconversion and lung lesions observed in AE and IN-challenged animals were due to these two pigs is probably very low.

Among many other factors, development of MP is dependent on the number of organisms that colonize the respiratory tract, which is likely dependent on cumulated infectious doses (Thacker and Minion, 2012). Since all groups were challenged with the same isolate and dose, the present results point out that the inoculation route may have a certain impact on the survival and/or infectivity of *M. hyopneumoniae*, which then is reflected on its colonization and in consequence, on the infection outcome. Information regarding how the inoculation route can affect the survival and infectivity of *M. hyopneumoniae* particles is limited. By means of the ET route, the inoculum penetration into the lower

respiratory tract is expected to be higher, which might enhance colonization. Although IN and, more importantly, the AE inoculation routes mimic better the natural infection conditions, the pig's long and curving respiratory tract may represent a major barrier for such administrations (Feng *et al.*, 2013). Related to this, the size of the aerosol particles is relevant, because it influences the time until they settle as well as the depth of penetration in the respiratory tract upon inhalation (Stärk, 1999). While *M. hyopneumoniae* bacterium mean diameter is 0.20 μm (Kobisch and Friis, 1996), deposition of aerosols in trachea and bronchiole was maximal when aerosol droplet size was 2 to 5 μm (Feng *et al.*, 2013; Hatch, 1961), a range that includes the mean particle size used in the present study (3.5 μm). On the contrary, the mucosal atomization device used in the IN group sprays a fine mist of particles of 30 to 100 μm in size. Although the important variation between particles sizes derived from both devices, in this study no difference in the ability to induce MP was evidenced between the IN and AE inoculation conditions. In fact, a higher colonization was recorded in the IN group at 28 dpi, meaning that other conditions rather than size of particles might influence the *M. hyopneumoniae* deposition in the respiratory tract.

In the current study, a significantly earlier seroconversion and lower percentages of inhibition were found in the ET group. The reason for this finding might be due to the higher cumulated infectious dose achieved via the ET inoculation route, which might imply a faster colonization of *M. hyopneumoniae* and, therefore, a higher exposure to

the mucosal immune system. In fact, all animals that seroconverted at necropsy had also MP. Indeed, it is well known that the concentration of serum antibodies does not correlate with clinical protection against *M. hyopneumoniae* (Djordjevic *et al.*, 1997; Thacker *et al.*, 1998) Nevertheless and similarly to serum results, significantly higher specific IgA levels in BALF were detected in the ET group. Although specific locally secreted IgA after vaccination has been postulated to play a pivotal role in preventing MP development (Marchioro *et al.*, 2013; Martelli *et al.*, 2014; Muneta *et al.*, 2008; Thacker *et al.*, 2000), the results obtained are in accordance to the results reported by Djordjevic *et al*. (1997), where IgA antibody concentrations achieved after *M. hyopneumoniae* challenge in BALF did not prevent MP development. Since the three BALF samples that were taken per group belonged to those animals with the highest lung scores (when MP was present) and comparisons with those animals without lesions within a group could not be performed, BALF results are likely overestimated.

The evaluation of the mucosal immune response was further addressed by the analysis of cytokine levels in BALF. Cytokine responses in BALF from control animals were found to be low and within a clinically normal range by comparing with previously reported data (Muneta *et al.*, 2008; Woolley *et al.*, 2012; Woolley *et al.*, 2013). Increased levels of the pro-inflammatory cytokines in BALF from *M. hyopneumoniae* infected pigs (Asai *et al.*, 1993; Muneta *et al.*, 2008; Okada *et al.*, 2000; Woolley *et al.*, 2012; Woolley *et al.*, 2013) and their relationship with the occurrence of pneumonic lesions (Lorenzo *et al.*, 2006; Rodríguez *et al.*, 2004) have

already been reported. However, the present study aimed to determine whether there was a measurable difference in levels of these cytokines between pigs challenged by different inoculation routes. Although possible overestimation due to the abovementioned reason, IL-1β response was significantly more prominent in the ET group than in the IN and AE challenged groups four weeks after infection. As reported in this study, high BALF levels of IL-1β are associated with tissue damage in the early stages of the infection (Asai *et al.*, 1993; Woolley *et al.*, 2012; Woolley *et al.*, 2013). In contrast, the lower levels of the IL-8 and, more importantly, of IL-1β in BALF of the IN and AE inoculated animals in comparison with the ones from the ET group, also support the pathological data, since inflammation was significantly lower in these two groups (IN and AE).

4.5. CONCLUSION

Under the conditions of this study, ET inoculation route was more effective inducing MP 4 weeks after challenge than the IN or AE ones. ET route is expected to apply a greater inoculum volume in pig's lower respiratory tract, achieving greater infectious doses in shorter times and promoting an earlier *M. hyopneumoniae* colonization and immune response against infection, which at the end is reflected in a higher incidence and more severe MP.

Chapter 5

Study III

Assessment of the *in vitro* growing dynamics and kinetics of the non-pathogenic J and pathogenic 11 and 232 *Mycoplasma hyopneumoniae* strains

5.1. INTRODUCTION

In 1965, Maré and Switzer in the United States and Goodwin in the United Kingdom isolated *M. hyopneumoniae* strains 11 and J, respectively (Rose *et al.*, 1979). Strain J was accepted as the type strain of *M. hyopneumoniae* (Rose *et al.*, 1979). Later on, however, pneumonia was not reproduced when pigs were inoculated with this strain (Bereiter *et al.*, 1990; Zielinski and Ross, 1990), thus, it was recognized as non-pathogenic. Additionally, most of the current commercial bacterins used to control enzootic pneumonia are based on J strain (Villarreal *et al.*, 2012). On the other hand, *M. hyopneumoniae* strain 232, which was isolated from serially passaged porcine lung homogenate containing strain 11 (Zielinski and Ross, 1990), is widely used in experimental studies in the United States and has progressively substituted strain 11, though both are considered pathogenic strains (Zielinski and Ross, 1990). *M. hyopneumoniae* strain 11 is also used as inactivated whole cell concentrate for vaccination (Marchioro, 2013).

Despite comparative genomic (De Castro *et al.*, 2006; Liu *et al.*, 2013; Vasconcelos *et al.*, 2005) and proteomic (Li *et al.*, 2009; Pinto *et al.*, 2009) analyses between *M. hyopneumoniae* pathogenic and non-pathogenic strains have been made, little is still known on the *in vitro* growth of strains differing in pathogenicity. In addition, available information in this respect may result contradictory. On the one hand, a higher pathogenicity of *M. hyopneumoniae* has been associated with a faster *in vitro* growth (Meyns *et al.*, 2007), though this could not be corroborated

afterwards (Calus *et al.*, 2010). On the other hand, low-passaged *M. hyopneumoniae* strains may grow slowly and may yield lower number of cells in medium than do higher-passaged strains, which, in turn, may not be virulent (DeBey and Ross, 1994; Zhang *et al.*, 1995; Zielinski and Ross, 1990). In connection with this matter, non-pathogenic strains protein expression profile has been shown to be more related to metabolism than to infectiousness (Li *et al.*, 2009; Pinto *et al.*, 2009). Therefore, the putative relationship between virulence and *in vitro* growing capacity of *M. hyopneumoniae* deserves further investigation.

Besides, outputs obtained from live animals inoculated with *M. hyopneumoniae* may vary considerably from one experiment to another (Lorenzo *et al.*, 2006; Villarreal *et al.*, 2009; Villarreal *et al.*, 2011; Woolley *et al.*, 2012). Although many other factors might be involved, an inoculum dose-dependent response has been reported in these experiments (Marois *et al.*, 2010). Despite the well-known CCU assay limitations in the assessment of the alive *M. hyopneumoniae* cell titres (Assunção *et al.*, 2005; Calus *et al.*, 2010), this technique continues to be the most frequently used to calculate the inoculum dose given to the animals under experimental settings. Indeed, ATP luminometry (Calus *et al.*, 2010) and flow cytometry (Assunção *et al.*, 2005) assays have been proposed as more reliable, accurate and as real-time titration tools (Assunção *et al.*, 2005; Calus *et al.*, 2010). Notwithstanding, none of these two techniques is specific for *M. hyopneumoniae*. Therefore, a deeper comparison of the CCU with other real-time, accessible and, if possible, *M. hyopneumoniae*-specific techniques remains of general interest.

The aims of the present work were twofold. Firstly, growth dynamics and kinetics of *M. hyopneumoniae* pathogenic (11 and 232) and non-pathogenic (J) strains was described. Secondly, bacterium growth by different methodologies, including ATP luminometry, CCU, CFU and molecular detection methods as qPCR and fluorescent double-stranded DNA (dsDNA) staining, was monitored with the objective to evaluate them as putative techniques for titration of *M. hyopneumoniae* culture.

5.2. MATERIALS AND METHODS

5.2.1. *M. hyopneumoniae* strains and medium for culturing

Type strain J (ATCC®25934™) and strain 11 (ATCC®25095™) were obtained from the American Type Culture Collection (ATCC). Boehringer Ingelheim Veterinary Research Center (BIVRC) GmbH & Co. KG (Hannover, Germany) kindly provided strain 232. All strains were grown in the ATCC recommended conditions: ATCC medium for *M. hyopneumoniae* culture purposes (ATCC® Medium 1699: Revised Mycoplasma medium) at 37°C in an aerobic or 5% CO_2 atmospheres for broth and solid mediums, respectively.

5.2.2. Experimental design

In order to compare the growth curves of these three *M. hyopneumoniae* strains, an initial inoculum of each of them was prepared from a single colony. Thus, a well-shaped and well-separated colony of each strain was localized with the help of an optical microscope and its location marked in the plate. Thereafter, plates were opened under the

laboratory hood and agar was punctured with a sterile 1000 µl size filter pipette tip and immediately resuspended in 1 ml of ATCC broth medium. After approximately 2 weeks of incubation, these cultures turned from red to orange to yellow and were then frozen at -80ºC until used. These frozen cultures were considered the initial inoculums.

For the experiment, *M. hyopneumoniae* strain (J, 11 and 232) initial inoculums were thawed and 100-fold diluted in duplicates (named A and B) in a final volume of 45 ml of ATCC broth medium. A tube containing only 45 ml of broth served as a control. Cultures were grown statically and followed up until reached the senescence phase. Thus, once the initial inoculum dilutions were done, 2 vials of 1 ml each of every culture replicate and control were taken at day 0 post-culture inoculation (D0), at 12 hours after (D0.5) and, afterwards, every 24 hours until the end of the study (D14). In order to avoid cross-contamination between *M. hyopneumoniae* strains, manipulation of each strain culture was done in separate laboratory hoods. One vial of each duplicate was directly frozen at -20°C whereas the other one was freshly and daily used for ATP luminometry, CFU and CCU titre assessments. The frozen vial was used once the experiment was finished for DNA extraction and *M. hyopneumoniae* copies calculation by qPCR and fluorescent double-stranded nucleic acid stain.

5.2.3. CCU assay

Formerly described conditions were followed to assess the number of CCU (Calus *et al.*, 2010) at every single time point. Briefly, ten-fold serial

dilutions (until 10^{-11}) of each *M. hyopneumoniae* strain culture replicate in ATCC broth medium were done. Microtiter plates were then sealed with plastic foil, and incubated at 37°C for 2 weeks. The CCU titre was determined from 2 repeated measurements (A and B) per each strain culture (but for control) and expressed as mean CCU/ml.

5.2.4. CFU assay

At every time point, solid medium plates were inoculated with each strain culture replicate. Plates were divided into eight equal parts. In each segment, a drop of 10 µl of either fresh undiluted culture or a ten-fold serial dilution in ATCC broth medium (from 10^{-1} to 10^{-7}) was spotted onto. In the case of the control, no dilutions were performed and the broth was directly spotted onto the plate. Drops were allowed to become air-dried under the laboratory hood and plates were covered and incubated for 7 days. By means of an optical microscope, *M. hyopneumoniae* colonies were counted until the last dilution in which they were present. The counts of each two strain replicates (A and B) were considered and the mean CFU/ml calculated.

5.2.5. ATP dependent luminometry assay

For luminometry, the BacTiter-Glo™ Microbial Cell Viability assay (Promega, Madison, WI, USA) together with a Fluoroskan Ascent® FL luminometer (Thermo Electron Inc., Milford, MA, USA) were used. For every time point measurement, 100 µl of freshly *M. hyopneumoniae* cultures and control were taken and mixed with 100 µl BacTiter-Glo™ reagent in white opaque 96-well microplates

(PerkinElmer, Waltham, MA, USA). Directly after, the contents were mixed briefly by orbital shaking and incubated at room temperature for five minutes. Luminescence was thereafter measured and recorded as relative light units (RLU). To transform RLUs into ATP concentrations, 10-fold dilutions of r-ATP (Promega, Madison, WI, USA) in ATCC broth medium, from 10 μM to 1 nM, were used as standard. For each plate, sample results were adjusted by subtracting the mean RLUs of the blank (ATCC broth medium). ATP titres of each strain culture were determined as the mean of the replicate measurements (A and B) and expressed as pmol ATP/ml of culture of *M. hyopneumoniae*.

5.2.6. DNA extraction

DNA was extracted for every time point from 1 ml of broth culture of each strain replicate and control using GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, Saint Louis, MO, USA). Once thawed, cultures and control were centrifuged at 10.000 g during 2 minutes and the cellular pellet was then resuspended with 200 μl of the provided resuspension solution. DNA was eluted in 200 μl of elution buffer.

5.2.7. Fluorescent double-stranded DNA stain assay

DsDNA content was determined using the Quan-iT™ PicoGreen® dsDNA kit (Life Technologies, Eugene, OR, USA) together with a Fluoroskan Ascent® FL fluorimeter (Thermo Electron Inc., Milford, MA, USA). Ten μl of the extracted DNA from every time point and strain replicates and control was mixed with 190 μl of Quan-iT™ PicoGreen

reagent in white opaque 96-well microplates (PerkinElmer, Waltham, MA, USA). Afterwards, the contents were mixed briefly by orbital shaking and incubated at room temperature for five minutes. Fluorescence was then measured and recorded as relative fluorescence units (RFU). In order to convert RFUs into dsDNA concentrations, 10-fold dilutions of the provided Lambda DNA standard in the elution buffer used for the DNA extraction (GenElute™ Mammalian Genomic DNA Miniprep Kit) were used for the standard curve (final concentrations from 500 to 0.5 ng/ml). Samples fluorescence value was adjusted by subtracting the mean RFU of the reagent blank (elution buffer). DsDNA concentration of each strain culture was determined as the mean of the replicate measurements (A and B) and expressed as ng/ml. Finally, an online available calculator was used for determining the *M. hyopneumoniae* copy number per ml in each template (http://cels.uri.edu/gsc/cndna.html).

5.2.8. Real time quantitative PCR for *M. hyopneumoniae*

A previously described qPCR specific for *M. hyopneumoniae* was performed (Sibila *et al.*, 2012). DNA extracted from cultures was used as template and plasmid as a standard for the qPCR development. Since sensitivity of the aforesaid qPCR was reported to be between 10^3 and 10^4 *M. hyopneumoniae* copies/ml of plasmid (Sibila *et al.*, 2012), all those samples with a bacteria load below 10^4 *M. hyopneumoniae* copies/ml were considered negative. QPCR results were expressed as the mean of *M. hyopneumoniae* copy numbers per ml of culture of the replicate measurements (A and B).

5.2.9. *M. hyopneumoniae* strains growth modelling

In order to quantitatively describe the *in vitro* growth of *M. hyopneumoniae* strains, a non-linear asymptotic model, namely the Gompertz equation, was fitted to each *M. hyopneumoniae* strain culture titre data obtained from the daily application of the different evaluated methodologies. The Gompertz equation enables to calculate growth kinetic parameters, thereby allowing the comparison of these parameters between the different techniques (Peleg and Corradini, 2011). The aforesaid model was fitted to data with STATISTICA version 8.0 (Stat Soft, Inc., USA) and the equation used can be written in the form (Winsor, 1932): $Y = A*(e(-B*e(-C*t)))$.

The dependent variable Y represents the observed titre value at a time (t); parameter A describes the titre value as it approaches infinity, thus, representing the maximal titre; parameter B is an integration constant defined as the proportion of the asymptotic maximal titre obtained after the initial inoculum dilution (established by the initial titre value and t), and parameter C equals the ratio between the maximum growth rate (μ_{max}) and the maximal titres and determines the slope of the curve. Thus, μ_{max} was obtained by multiplying A and C parameters. Considering *M. hyopneumoniae* as an asynchronic culture (i.e. cells of the total population do not divide simultaneously) mean generation time (G) was estimated by l/μ_{max} as formerly proposed (Baranyi and Roberts, 1995).

Model goodness of fit was evaluated according to the R^2 and the average prediction error (APE). The first determines the percentage of variation

in Y explained by the statistical model whereas the latter quantifies the relative disagreement between observed (Y) and predicted (PY) titre values for each specific t and is calculated as follows: APE% = ([$Y - PY$]/Y) × 100 (Loaiza-Echeverri *et al.*, 2013). In order to evaluate the methodology from which the Gompertz growth model was better fitted to the data, mean APE per technique was calculated by averaging values obtained from each strain culture.

Statistical analyses were performed using STATISTICA version 8.0 (Stat Soft, Inc., USA). Differences between each *M. hyopneumoniae* strain mean value of the parameters obtained from the Gompertz modelling were tested for statistical significance by Kruskal-Wallis test (non-parametric, one-way ANOVA) with post hoc multiple comparisons (Dunn's multiple comparison test). Normal Distribution as well as constant variance were evaluated by the Shapiro–Wilk's test and Levene's test, respectively. P values <0.05 were considered to be significant.

5.2.10. Relationship between assessed techniques

In order to compare the different assessed methodologies, a linear regression analysis between each pair of *M. hyopneumoniae* culture titration techniques was carried out on the log2 transformed data during each strain maximal culture growth, namely logarithmic (log) phase. The period of study days compared was established taking into account the log phase duration from the growth response determined by the ATP assay in each strain culture. From such analysis, regression formulae were acquired to enable conversion of data (culture titre)

obtained by means of different techniques. All statistical analyses were carried out using STATISTICA version 8.0 (Stat Soft, Inc., USA). The significance level (p) was set at 0.05 with statistical tendencies reported when $p<0.10$.

5.3. RESULTS

Negative control (uninoculated ATCC broth medium) had similar ATP values and dsDNA fluorescence reactions than the blank throughout the study (from D0 to D14). Additionally, no colonies were found in any of the agar plates inoculated, no colour change was detected at any time point and qPCR results were also negative. Unfortunately, a fungus-like contamination was detected in replicate A of *M. hyopneumoniae* J strain culture at D7. Therefore, from D7 onwards, different methodology titres were assessed for J strain culture only from the replicate B.

5.3.1. *M. hyopneumoniae* ATP growth response curves

The growth response determined by ATP for each of the three *M. hyopneumoniae* strains is represented in Figure 5-1. It is important to mention that ATP concentration levels between strain initial inocula after thawing were very similar (1222.1 ± 86.2 pmol ATP/ml), which verified that the growth dynamics was started from approximated equal conditions for every *M. hyopneumoniae* strain culture. By means of ATP luminometry, all strains showed growth curves with log, stationary and death phases, and culture colour change (from the original red to orange) firstly detected at the end of the log phase; when yellow, cultures were

established in the stationary phase. *M. hyopneumoniae* J strain reached earlier the stationary phase than 11 and 232 strains. Nonetheless, the latter ones had longer stationary phases followed by less sharp senescence phases in comparison with J strain. Additionally, all three evaluated strains reached similar maximal raw ATP concentrations (1395.5 ± 148.9 pmol ATP/ml) once in the stationary phase.

5.3.2. *M. hyopneumoniae* CCU growth response curves

The growth response determined by the CCU assay for each of the three *M. hyopneumoniae* strains is represented in Figure 5-2. In contrast to ATP luminometry, a lag phase was devised for J and 11 strains. Following such an initial phase, log, stationary and death phases could also be visually differentiated, although not as easily as with ATP concentration along time. Importantly, when all strain cultures colour shift was detected, CCU maximal titres were already reached. Similarly to ATP, all three evaluated strains reached similar maximal raw CCU titres ($4.25 \times 10^9 \pm 1.30 \times 10^9$ CCU/ml) in the stationary phase. Those were also reached earlier by J strain, which again showed a shorter stationary phase than *M. hyopneumoniae* 11 and 232 strains.

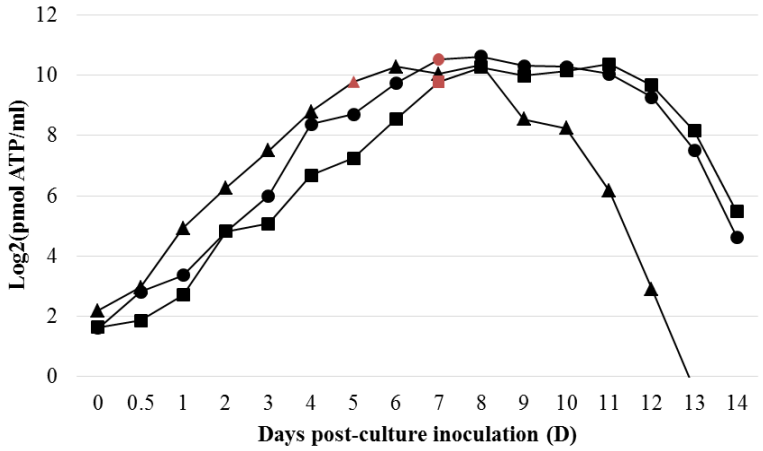


Figure 5-1. Growth curves determined by ATP luminometry of *M. hyopneumoniae* strains J (▲), 11 (■) and 232 (●). Data points represent means of the 2 replicate measurements (A and B) within each strain. The orange data points indicate when the original medium colour change was first visually detected in each strain culture.

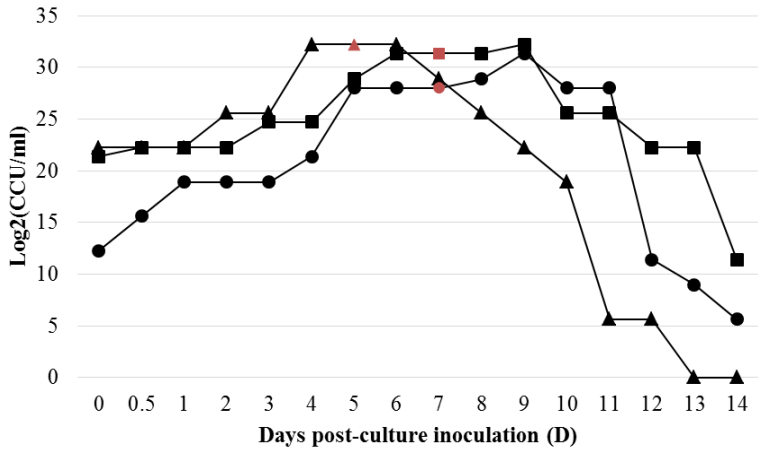


Figure 5-2. Growth curves determined by the CCU assay of *M. hyopneumoniae* strains J (▲), 11 (■) and 232 (●). Data points represent means of the 2 replicate measurements (A and B) within each strain. The orange data points indicate when the original medium colour change was first visually detected in each strain culture.

5.3.3. Growth of *M. hyopneumoniae* on solid media

In this experiment, solid medium enabled the growth of *M. hyopneumoniae* J, 11 and 232 strains. Colonies from such three strains were phenotypically indistinguishable between them through microscopic visualization. Thus, end-point colonies varied in size, from 100 to 240 μm, and were rough with irregular margin, normally lacking a clearly defined nipple (no "fried egg appearance") (Figure 5-3). Colonies were observed as early as D3 for J strain and at D6 and D8 for 11 and 232 strains, respectively. The period along colonies were observed varied between 7 days for J, 3 for 11 and 2 for 232 strain. Hence, the growth curve using viable counts on the solid medium could only be determined by J strain. Since all the growth phases could be well stablished by means of ATP luminometry, counts of CFU per ml of *M. hyopneumoniae* J strain to its ATP growth response along the study period is depicted in Figure 5-4.

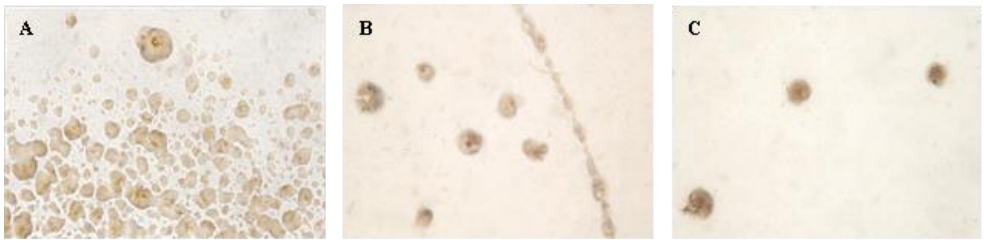


Figure 5-3. Colonies of *M. hyopneumoniae* J strain on solid media at D5 of A) undiluted culture, B) 10^{-1} and C) 10^{-2} dilution of the culture in ATCC medium. In Figure 5-3B, the limit of the culture drop can be differentiated with the colonies observed within such limit. Once inoculated, solid plates were incubated for 7 days at 37 °C and 5% CO_2 and photographed using a microscope at 40x.

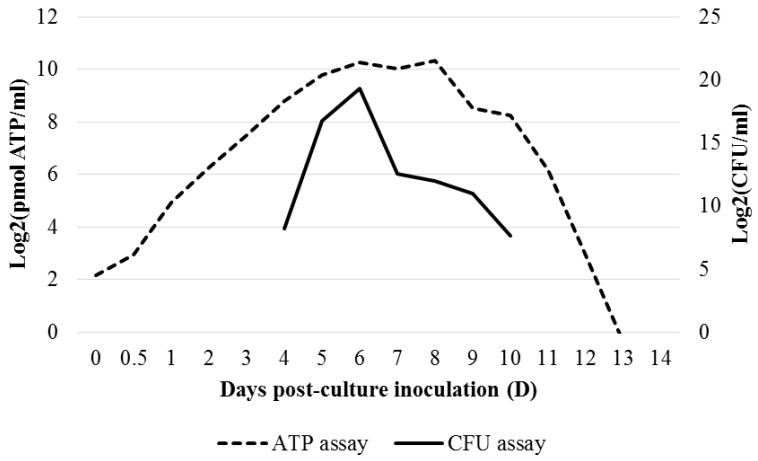


Figure 5-4. Counts of CFU (continuous line) and ATP concentration (discontinuous line) per ml of *M. hyopneumoniae* J strain culture along the study period.

5.3.4. Copies of *M. hyopneumoniae* determination

M. hyopneumoniae copy numbers per ml of the three strain cultures assessed by the fluorescent dsDNA stain assay and qPCR are shown in Figure 5-5 and Figure 5-6, respectively. Both methodologies resulted in similar curves from which a lag and log phases could be recognised. However, the range of copy numbers assessed by qPCR was wider than the one assessed by fluorimetry. Once reached the maximal number of *M. hyopneumoniae* copies/ml, a slightly gradual decrease in the copy numbers was observed as time progressed, though stationary and senescence phases could not be differentiated. Remarkably, the reach of the maximal number of *M. hyopneumoniae* copies/ml of culture concurred approximately in time with the maximal ATP values.

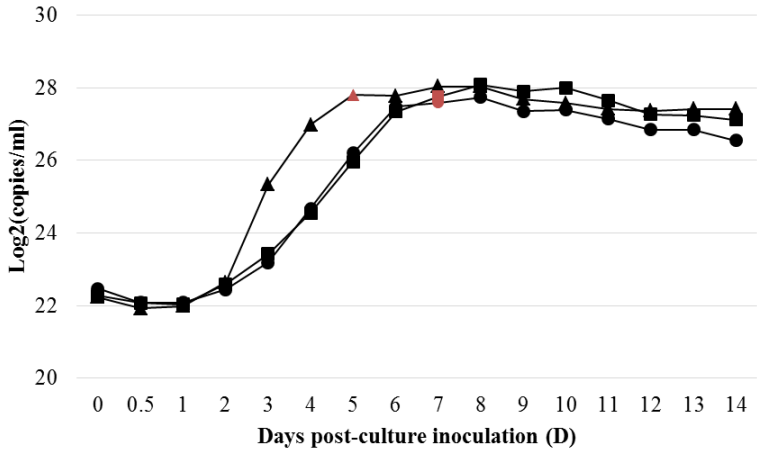


Figure 5-5. ***M. hyopneumoniae*** **strains J (▲), 11 (■) and 232 (●) copies per ml of dsDNA determined by fluorimetry.** Data points represent means of the 2 replicate measurements (A and B) within each strain. The orange data points indicate when the original medium colour change was first visually detected in each strain culture.

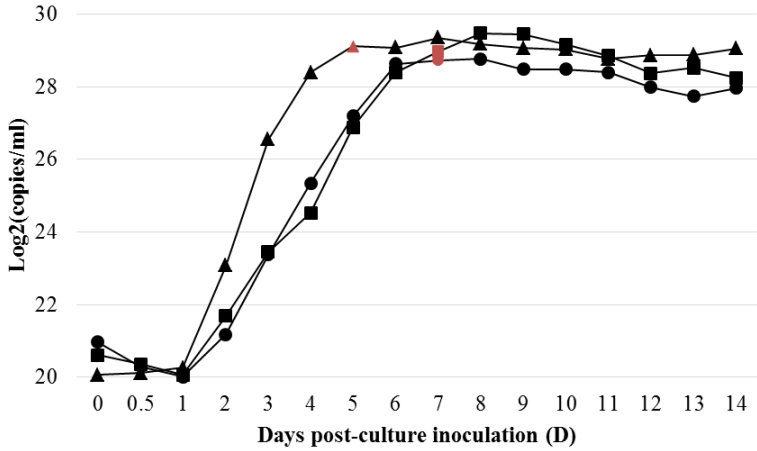


Figure 5-6. ***M. hyopneumoniae*** **strains J (▲), 11 (■) and 232 (●) copies per ml determined by qPCR**. Data points represent means of the 2 replicate measurements (A and B) within each strain. The orange data points indicate when the original medium colour change was first visually detected in each strain culture.

5.3.5. Growth kinetic parameters of *M. hyopneumoniae*

The Gompertz model could not be reliably applied to CFU titres because of the lack of data for 11 and 232 *M. hyopneumoniae* strains and the very short log phase as well as the lack of a stationary phase of the J strain growth curve. For all other techniques, the values of the parameters estimated for the Gompertz model are shown in Table 5-1. All three strains had close values for parameters *A* and *B* in each of the evaluated techniques but for qPCR, in which significant differences between 11 and 232 strains were observed. Remarkably, the kinetic parameters *C*, μ_{max} and G obtained from J culture were significantly lower ($p<0.05$) than the ones corresponding to the 11 strain culture for all the assessed techniques but for CCU. Indeed, none of the parameters obtained from the application of the Gompertz model to the CCU data was significantly different between *M. hyopneumoniae* strains.

All Gompertz regression models had *p* values lower than 0.05 and R^2 values between 0.83 and 1. In general terms, mean APE values of each strain and technique were negative, indicating overall overestimated predictions by the model. Calculated mean APE technique values (average from all strain APE within a method) are shown in Figure 5-7. APE associated with prediction of *M. hyopneumoniae* culture titre at each time showed that differences between predicted and actual titre values alternated in positive and negative sign in all procedures. The highest error, however, was observed in the CCU method. For all the other assessed techniques, better predictions were obtained in the last stage of *M. hyopneumoniae* growing in comparison with the earliest.

Table 5-1. Parameter estimates, coefficient of determination (R^2) and average prediction error (APE) for Gompertz model used to describe *M. hyopneumoniae* strains J, 11 and 232 growths. Different superscripts within a column and technique indicate significant differences between strains (p<0.05).

Technique	Strain	*A**	*B**	*C*◦	μ_{max}†	*G*‡	R^2	APE§
ATP luminometry	J	1493.868	10.626	1.065^{a}	1.171^{a}	14.607^{a}	0.981	-5.118
	11	1677.066	15.485	1.042^{b}	1.121^{b}	20.317^{b}	0.983	5.423
	232	1497.592	15.909	1.056^{ab}	1.159^{ab}	15.756^{ab}	0.985	-3.054
CCU	J	1.25E+10	1.47E+02	1.083	1.310	9.536	0.938	-34.413
	11	9.58E+09	1.48E+03	1.055	1.205	13.788	0.897	-5.401
	232	1.51E+10	5.79E+00	1.026	1.175	14.649	0.829	-70.113
Fluorimetry	J	2.18E+08	1.54E+04	1.143^{a}	1.228^{a}	11.610^{a}	0.997	-4.773
	11	2.54E+08	1.92E+03	1.079^{b}	1.128^{b}	19.256^{b}	0.991	-2.233
	232	1.69E+08	3.23E+04	1.115^{ab}	1.160^{ab}	15.585^{ab}	0.991	-5.161
qPCR	J	7.46E+08^{ab}	2.67E+02^{ab}	1.110^{a}	1.331^{a}	8.162^{a}	0.997	0.074
	11	8.01E+08^{a}	2.27E+02^{a}	1.068^{b}	1.185^{b}	13.597^{b}	0.986	-6.814
	232	4.98E+08^{b}	2.17E+04^{b}	1.103^{ab}	1.252^{ab}	10.535^{ab}	0.983	-12.984

*Pmol ATP, CCU or copies of *M. hyopneumoniae* per ml
◦Pmol ATP, CCU or copies of *M. hyopneumoniae* per ml per hour per unit of maximal titre (parameter *A*)
†Pmol ATP, CCU or copies of *M. hyopneumoniae* per ml per hour
‡Time (hours)
§Percentage (%)

5.3.6. Relation between ATP, CCU, fluorimetry and qPCR assays

Similar to what happened for the Gompertz modelling, the lack of CFU data hampered the comparison of this technique with the others. During the exponential growth phase, there was high correlation (r>0.9) between all the other evaluated methodologies in all three *M. hyopneumoniae* strain cultures and these were statistically associated (p<0.001) (Table 5-2). Equivalences between the assessed methods resulting from the application of the formulae from the linear regression analyses between each pair of techniques in each strain culture are provided in Table 5-3.

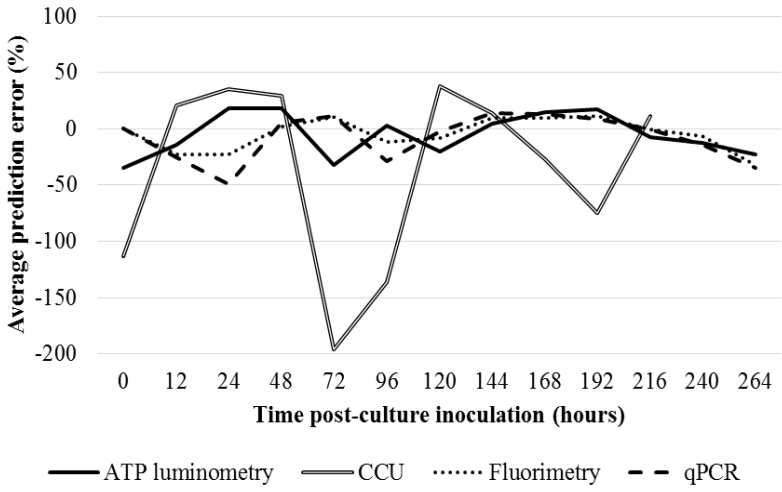


Figure 5-7. Distribution of APE defined by the Gompertz model to *M. hyopneumoniae* titre data from ATP luminometry, CCU, fluorimetry and qPCR techniques.

Table 5-2. Correlation between evaluated techniques considering data from the logarithmic phase of growth (according to ATP luminometry assay) of *M. hyopneumoniae* strain J, 11 and 232 cultures. All systems were statistically associated (p<0.001).

Technique	Strain	Technique			
		ATP	*CCU*	*Fluorimetry*	*qPCR*
ATP	J	---	0.929	0.930	0.961
	11	---	0.924	0.952	0.966
	232	---	0.950	0.945	0.966
CCU	J	---	---	0.958	0.954
	11	---	---	0.985	0.979
	232	---	---	0.933	0.925
Fluorimetry	J	---	---	---	0.982
	11	---	---	---	0.993
	232	---	---	---	0.989

Table 5-3. Values of every evaluated technique corresponding to a unit of a particular method in each *M. hyopneumoniae* strain culture. Equivalences were obtained by fitting the linear regression models of each pair of techniques comparison to data from the maximal culture growth (log phase).

Technique (unit)	**Strain**	**ATP** (pmol ATP/ml)	**CCU** (CCU/ml)	**Fluorimetry** (*M. hyopneumoniae* copies/ml)	**qPCR** (*M. hyopneumoniae* copies/ml)
ATP (pmol ATP/ml)	J	---	1.76E+05	6.09E+05	6.40E+04
	11	---	4.05E+05	1.23E+06	2.09E+05
	232	---	1.66E+03	1.08E+06	1.71E+05
	Mean (±SD)	---	1.94E+05 (±2.02E+05)	9.72E+05 (±3.23E+05)	1.48E+05 (±7.51E+04)
CCU (CCU/ml)	J	2.10E-04	---	9.68E+02	3.79E+00
	11	3.92E-05	---	1.02E+03	4.65E+00
	232	1.23E-02	---	7.79E+04	3.77E+03
	Mean (±SD)	4.17E-03 (±7.00E-03)	---	2.66E+04 (±4.44E+04)	1.26E+03 (±2.17E+03)
Fluorimetry (M. hyopneumoniae copies/ml)	J	7.99E-08	<1	---	<1
	11	6.47E-09	<1	---	<1
	232	1.53E-09	<1	---	<1
	Mean (±SD)	2.93E-08 (±4.39E-08)	NA	---	NA
qPCR (M. hyopneumoniae copies/ml)	J	2.12E-04	<1	3.50E+02	---
	11	2.51E-05	<1	3.69E+02	---
	232	1.18E-05	<1	3.20E+02	---
	Mean (±SD)	8.30E-05 (±1.12E-04)	NA	3.47E+02 (±2.49E+01)	---

5.4. DISCUSSION

In the present study, the course of the *in vitro* growth of *M. hyopneumoniae* J, 11 and 232 strains was evaluated by the application of different techniques with the potential of being used as an alternative to CCU for *M. hyopneumoniae* culture titration. Growth curve kinetics and dynamics of *M. hyopneumoniae* J strain were different from the observed for 11 and 232 strains, which, in turn, showed very similar growth curves regardless the technique applied. Thus, growth curve plots revealed a faster *in vitro* growth of the J strain in comparison with 11 and 232 strains as well as a straighter log phase, and a shorter stationary phase followed by a steeper senescence phase. Nonetheless, raw data analysis showed no differences between strains in terms of maximal titres reached within a particular technique.

Results obtained by those methods that indirectly measure the number of metabolising cells (i.e. CCU and ATP assays) indicated that, once maximal levels were reached, CCU titres dropped earlier than the onset of decrease in ATP titres. The latter supports that *M. hyopneumoniae* lose its capacity to divide (requisite for the CCU titration) before cell lysis and intracellular ATP impairment (Assunção *et al.*, 2006; Calus *et al.*, 2010). Considering ATP growth curves, the medium colour change from red to orange was observed in the last section of the log phase, whereas the shift to yellow was complete in the stationary phase. However, when CCU growth curves are taken into account, the original red shift to orange happened when all strain cultures were already in the

stationary phase. *M. hyopneumoniae* growth phase in which medium turn colour may vary depending on different conditions, such as the medium itself or even the batch of medium used (Calus *et al.*, 2010), as well as on the technique used to assess the growth of the bacterium. Therefore, extrapolation of the growth phase from the colour of the medium should be used with caution. On the other hand, both molecular methods (i.e. fluorimetry and qPCR assays) led to very similar growth curve responses, showing a slightly gradual decrease in the copy numbers once reached the stationary phase. This finding indicates that DNA fragments might be present in culture for longer periods once cells are dead. Henceforth, extrapolation of the growth phase from genomic copy numbers can also be confounding as no clear differentiation could be established between stationary and senesce phases.

In the present work, *M. hyopneumoniae* colonies were seen for all three strains assessed. Nevertheless, a growth curve based on CFU could only be determined by J strain, since growing of 11 and 232 colonies was much more limited. These results indicate that solid medium might support differently the growth of diverse *M. hyopneumoniae* strains. Indeed, *M. hyopneumoniae* growth on solid medium has been considered particularly difficult (Friis, 1975) and solidification of Friis medium with agar may hamper the growth of colonies of *M. hyopneumoniae*, suggesting that agar inhibits the growth or sequesters essential nutrients for growing (Cook *et al.*, 2016). In a recently published work, however, *M. hyopneumoniae* growth curve could be accurately

determined by using viable counts on the solid medium (Cook *et al.*, 2016) and such curve was, actually, similar in shape to the one obtained for strain J in the present study. Strain J culture reached a maximum viability of $6.5x10^5$ CFU/ml at D6, which approximately corresponded to the medium colour shift to orange as well as with the end of the log phase determined by ATP culture concentrations. After this point, there was a decline until D10, from where no more colonies were observed. For 11 and 232 strains, colonies appeared when cultures were in the stationary phase (data not shown). Although depending probably on several conditions such as the *M. hyopneumoniae* strain or the medium used, overall, obtained results suggest that the CFU technique underestimates the number of alive cells, suggesting the inappropriateness of this method for *M. hyopneumoniae* titration.

Microbial growth curve is typically depicted in terms of log numbers and it has a characteristic sigmoid shape. Thus, one way to describe bacteria growth is using nonlinear regression models, such as the Gompertz equation (Mytilinaios *et al.*, 2012; Peleg and Corradini, 2011). This model accommodates measurements in some kinetics parameters and permits appropriate biological interpretation. In the present case and based on the R^2, the Gompertz model was well adjusted to data coming from the application of different titration techniques in all three strain cultures, except for the CFU. As a reference, formerly application of the Gompertz model to *M. hyopneumoniae* ATP growth curves resulted also in high R^2 values (Calus *et al.*, 2010). Importantly, from the comparison between APE values obtained for each technique, the

Gompertz model had poorer fit during the early than at late phases of *M. hyopneumoniae* growth. In the present study, the lack of data at early phases (hours subsequent to the initial inoculum dilution) was perhaps a major limitation of this model. Moreover, CCU was the worst predicted method by the model, probably because progression along time did not always correspond to a further dilution turning colour, not showing a typical bacteria growth curve.

Information related to the association between the *in vitro* growth and the difference in pathogenicity between *M. hyopneumoniae* strains is scarce and can often be difficult to interpret. In fact, the information available on such respect is up to now contradictory (Calus *et al.*, 2010; Li *et al.*, 2009; Meyns *et al.*, 2007; Pinto *et al.*, 2009). In the present study, estimated kinetic parameters by the Gompertz model confirmed the faster *in vitro* performance of the non-pathogenic J strain observed in the growth curve plots. Indeed, all the applied methods mostly agreed that J was the fastest growing strain and the pathogenic 11 the slowest one. In agreement, *M. hyopneumoniae* J strain protein expression profile was found to be related to metabolism (indicative of a non-infective proliferate lifestyle that would fit a better adaptation to the *in vitro* conditions) whereas proteins identified from pathogenic strains (e.g. 232 strain) were predicted to be more associated to the infectious competence (Li *et al.*, 2009; Pinto *et al.*, 2009). This would also agree with the described loss of virulence and adhesion capacity due to successive *in vitro* passages (DeBey and Ross, 1994; Zhang *et al.*, 1995; Zielinski and Ross, 1990). In contradiction to obtained results, the CCU assay revealed

that a highly virulent *M. hyopneumoniae* isolate multiplied faster *in vitro* than a low virulent one (Meyns *et al.*, 2007). Later on, however, the same research group *studied t*he course of growth of those two isolates, together with other isolates proved to be pathogenically (Vicca *et al.*, 2003), genetically (Stakenborg *et al.*, 2006) and phenotypically (Calus *et al.*, 2007) diverse and did not find association between isolates virulence and growth response curves assessed by ATP luminometry (Calus *et al.*, 2010). Overall results suggest a putative relationship between virulence and the *in vitro* growing capacity of *M. hyopneumoniae*, although further studies are needed to confirm results and reinforce this hypothesis.

Although reasons for the former inconsistencies are difficult to elucidate, some aspects should be kept in mind. First, the growing medium used has been reported to be more pronounced on the *M. hyopneumoniae* growth response than the effect of the isolate by itself (Calus *et al.*, 2010). Second, comparisons of the course of growth of different *M. hyopneumoniae* are hindered by the inherent difficulty in obtaining strain initial inoculum with the same amount of *M. hyopneumoniae* alive cells. In the present case, all strain initial inoculum came from single colonies and were used when reached similar maximal ATP values. Moreover, such inoculum were equally diluted at D0 and volumes taken at every time point were the same for all strain cultures. The abovementioned aspects may also partly explain the differences found in the kinetics of the growth curves between this work and a previous one (Calus *et al.*, 2010). While the initial ATP and CCU concentrations were fairly similar between both studies, the former reported growth curves were much

shorter in time than the ones reported in here. Although other many growing conditions might be implicated in such differences, this finding highlights again the effect that the use of different culture media may have on *M. hyopneumoniae in vitro* growth. Besides dissimilarities, it is important to note that previous estimated maximum ATP titres reached values of 1350 pmol ATP/ml (Calus *et al.*, 2010), which were not distant from the maximum values appraised in this work of 1689 pmol ATP/ml.

Comparison of results obtained by the different applied techniques was an important part of the present study. During the log growth phase, titration results of the four assays were highly and significantly correlated and a strong linear relationship was observed. According to the assessed regression models, *M. hyopneumoniae* culture had a mean ATP content of $4.17x10^{-3}$ pmol per colour change of 1 ml culture, whereas another study reported *M. hyopneumoniae* culture to contain $1.77x10^{-6}$ pmol ATP per CCU in 1 ml of culture (Calus *et al.*, 2010). Henceforth, in the present work, higher amount of ATP was needed in comparison to the previous one to achieve the change of colour of 1 ml culture. Neither CCU, ATP nor fluorimetry assays are specific for *M. hyopneumoniae*, which might be problematic when other inoculum type rather than culture is used (e.g. lung homogenate). In this case, *M. hyopneumoniae* copies per ml assessed by qPCR may be the most approximated value to the number of *M. hyopneumoniae* cells. The mean amount of *M. hyopneumoniae* DNA determined by qPCR revealed that 1 CCU/ml correspond to 1260 mycoplasmas. The latter is in agreement to previous data reporting that 1 CCU/ml of *M. hyopneumoniae* strain 116

corresponded to 1000 mycoplasmas (Marois *et al.*, 2010). While qPCR might be a reliable titration technique during the *M. hyopneumoniae* log growing phase, its inability to differentiate between alive and death cells is a major disadvantage during the later stages of the growth. Given that each of the evaluated titration, methodologies have limitations, their use in combination is probably required to optimise *M. hyopneumoniae* culture titration accuracy.

5.5. CONCLUSION

In order to standardise *M. hyopneumoniae* growing protocols in the laboratory as well as inoculum production for experimental applications, it is important to known the *in vitro* growth kinetics and dynamics of a particular strain in a particular medium. Once this information is known, a more reliable approximation of the growing phase status from the culture colour change can be performed. For this purpose, ATP is an economical, rapid, timely and efficient assay that allows the distinction of the different *M. hyopneumoniae* growing phases in culture. Nonetheless, ATP is not a *M. hyopneumoniae* specific technique; thus, its combination with a specific detection method, such the qPCR, might be useful. Lastly, further studies with more strains, both at *in vitro* and *in vivo* levels, are needed to elucidate the putative relationship between *M. hyopneumoniae in vitro* behaviour and virulence.

Chapter 6

Study IV

Potential use of local and systemic humoral immune response parameters to forecast *Mycoplasma hyopneumoniae* associated lung lesions

6.1. INTRODUCTION

CVPC quantification by means of lung lesion scoring is frequently used to estimate the incidence and severity of lung lesions associated to *M. hyopneumoniae* infections, at experimental, herd and abattoir levels (Noyes *et al.*, 1990; Sibila *et al.*, 2009). Nevertheless, *post-mortem* lung evaluation is an end-point parameter that does not provide information on the ongoing respiratory problems in any of the cases (Noyes *et al.*, 1990). Thus, it would be of general interest to find an *ante-mortem* parameter that would provide reliable and real-time information in relation with CVPC prevalence and severity.

Immunopathological events are considered to play an important role in both *M. hyopneumoniae* infection pattern and development of the associated lung lesions (Thacker and Minion, 2012). In connection with this matter, relationship between presence of *M. hyopneumoniae* antibodies and CVPC development has been analyzed in several studies. However, while some studies found a significant positive correlation between both parameters (Fraile *et al.*, 2010; Merialdi *et al.*, 2012; Morris *et al.*, 1995b; Van Til *et al.*, 1991; Yagihashi *et al.*, 1993), others did not find such association (Meyns *et al.*, 2011; Vranckx *et al.*, 2012a). Noteworthy, such relationship has been assessed at a population-based level and scarce information is available at individual level (Van Til *et al.*, 1991). Hence, the relationship between antibody levels against *M. hyopneumoniae* as a tool to assess lung lesions deserves further investigation. On the other hand, most of the commercially available

ELISA kits are designed to detect specific *M. hyopneumoniae* IgG antibodies in serum, but information on other humoral immune parameters and their relationship with CVPC is not available. Therefore, the present study sought to investigate the potential association between different humoral immune parameters, both at local and systemic levels, with the prevalence and severity of CVPC in pigs naturally and experimentally infected with *M. hyopneumoniae*.

6.2. MATERIALS AND METHODS

6.2.1. Study design

In order to accomplish the abovementioned objective, samples from three different published studies were used: 1) samples from *M. hyopneumoniae* experimentally inoculated animals (*Annex*); 2) samples from slaughtered pigs coming from different farms (Fraile *et al.*, 2010) and 3) samples from non-vaccinated pigs belonging to a field study in which chronological necropsies were performed (Sibila *et al.*, 2007b). Samples were tested to evaluate the humoral immune parameters schematized in Figure 6-1 and detailed further below.

Study \ Sample	Lung	Sera		BALF	
	Gross pathology	IgG antibodies	IgG1 and IgG2 antibodies	IgG antibodies	IgA antibodies
Experimental	✓	✓	✓	✓	✓
Slaughterhouse	✓	✓	✓	---	---
Chronological herd	✓	✓	✓	---	---

Figure 6-1. Parameters and samples evaluated within the experimental, slaughterhouse and chronological herd studies. Macroscopic lung lesions were quantified by the European Pharmacopoeia lung scoring system (Ph. Eur., 2013). All humoral immune parameters were assessed by ELISA technique.

6.2.1.1. Experimental study

Ninety seven 6 week-old pigs free from *M. hyopneumoniae* were experimentally inoculated as described (*Annex*). One uninfected group (Control; $n = 6$) received sterile PBS. At 21 (n=37) or 28 (n=60) dpi, all pigs were killed with an intravenous overdose of sodium pentobarbital and subjected to necropsy examination. At that time (21 or 28 dpi), blood and BALF samples were obtained and CVPC evaluation was assessed using the system recommended by the European Pharmacopoeia (Ph. Eur., 2013). Sera were used for specific IgG, IgG1 and IgG2 detection, whereas IgG and IgA antibodies were determined in BALF. Animal care and study procedures were conducted in accordance with the guidelines of Good Experimental Practice, under the approval of the Ethical and Animal Welfare Committee of the *Universitat Autònoma de Barcelona*.

6.2.1.2. Slaughterhouse study

A total of 54 batches of 54 different farms located in North-eastern, Central and South-eastern Spain, were included in the study (Fraile *et al.*, 2010). A batch was defined as a group of pigs (with a mean of 97 animals per batch) belonging to the same farm and sacrificed on the same day at the slaughterhouse. From these 54 farms, 9 farms vaccinated pigs against *M. hyopneumoniae*, 16 did not vaccinate and, from the remaining farms, no data was available (Fraile *et al.*, 2010). The pigs were killed in eight different slaughterhouses according to their own procedures. Percentage of lungs affected by CVPC as well as mean lung lesion score per farm (sum of individual scores/number of scored lungs) were assessed. In addition, blood samples from 20 randomly selected pigs of each batch were taken before being sacrificed and IgG, IgG1 and IgG2 *M. hyopneumoniae* specific antibodies were determined from sera. Farms were then classified as seropositive or seronegative according to the mean S/P ratio obtained from those 20 sampled animals. In addition, mean farm IgG1 and IgG2 OD values were also obtained.

6.2.1.3. Chronological herd study

Fifty-eight pigs from a farrow-to-finish farm located in North-eastern Spain with a history of EP associated respiratory problems were included in the analyses. These piglets belonged to the control group (non-vaccinated) of a previously published work (Sibila *et al.*, 2007b). Blood samples from these animals were taken at 1, 3, 6, 9, 12, 15, 18 and 22 weeks of age. However, the initial number of pigs decreased over time because animals were randomly sacrificed with an intravenous

overdose of sodium pentobarbital, necropsied and CVPC evaluated from 9 weeks of age (n=10) onwards at 12 (n=12), 15 (n=7), 18 (n=13) and 22 (n=16) weeks of age. Specific IgG antibodies were tested from all serum samples in each time point whereas IgG1 and IgG2 specific isotypes were assessed only from sera obtained at the necropsy time, from 9 to 22 weeks of age. Housing, husbandry and slaughtering conditions conformed to the European Union Guidelines and Good Clinical Practices, under the approval of the Ethical and Animal Welfare Committee of the *Universitat Autònoma de Barcelona*.

6.2.2. Pathological examination

In each of these studies, extension of gross lung lesions compatible with *M. hyopneumoniae* infection (CVPC) was assessed using different scoring systems depending on the study. Thus, in order to harmonize all lung lesion scores, those that were obtained through Madec and Kobisch (1982) (slaughterhouse study) and Hannan *et al*. (1982) methodologies were converted to reference European Pharmacopoeia (Ph. Eur., 2013) scores by means of the equivalence formulae provided (*Annex*).

6.2.3. *M. hyopneumoniae* IgG antibody detection

Sera derived from all the studies were tested for *M. hyopneumoniae* IgG antibodies by means of a commercial indirect ELISA (*Mycoplasma hyopneumoniae* Antibody Test Kit; BioChek, UK) and according to the manufacturer's instructions. The relative amounts of specific IgG antibodies in samples were expressed as sample-to-positive (S/P) ratio based on OD as follows: S/P=OD sample-OD mean negative control/ OD

mean positive control-OD mean negative control. Samples with an S/P of ≥0.5 were considered positive whereas samples with an S/P of <0.5 were considered negative. An S/P=0 was given to those samples with OD values below the mean OD of the negative control.

6.2.4. *M. hyopneumoniae* IgG antibody subtype detection

M. hyopneumoniae-specific IgG1 and IgG2 subtypes from sera were measured by using a modification of the above mentioned ELISA (*Mycoplasma hyopneumoniae* Antibody Test Kit; BioChek, UK). Mouse anti-pig IgG1 and IgG2 monoclonal antibodies (Bio-Rad Laboratories, USA) were firstly tested in *M. hyopneumoniae* positive and negative sera diluted 1:30, 1:50, 1:70, 1:90, 1:120 and 1:150 with the sample diluent provided by the kit. As a result, 1:50 dilution (applied for total IgG antibodies determination according to manufacturer's instructions) was appropriate to discriminate between *M. hyopneumoniae* seropositive and seronegative samples. After 30 min incubation, plates were washed and IgG1 and IgG2 antibodies diluted to 1:1000 were added. After incubating for 45 min, plates were washed again and alkaline phosphatase-labeled goat anti-mouse IgG polyclonal antibody (Bio-Rad Laboratories, USA) diluted to 1:1000 was added to each plate well. After incubating for 45 min, plates were washed and the reaction was visualized after 15 min incubation with the kit substrate reagent. Sample absorbance was obtained by reading at 405 nm and results expressed as OD values. For each plate, sample results were adjusted by subtracting the OD of the mean negative control provided by the kit. Furthermore, the cut-off was calculated as mean OD value of negative control plus

three SD. The threshold was established at an OD value of 0.13 for both IgG1 and IgG2 antibodies.Values higher than this cut-off were considered positive whereas values below this cut-off were considered negative.

6.2.5. *M. hyopneumoniae* IgG and IgA antibody detection

Undiluted and previously centrifuged BALF samples from the experimental study were tested for specific IgG antibodies by means of the above mentioned assay (*Mycoplasma hyopneumoniae* Antibody Test Kit; BioChek, UK) and conforming to the manufacturer's instructions but for the initial dilution of the sample. For IgA antibody detection, an alkaline phosphatase-labelled goat anti-porcine IgA polyclonal antibody (Bethyl Laboratories, USA) was used as described previously in *Chapter 4*. Sample absorbance was obtained by reading at 405 nm and results expressed as OD values after subtraction of the mean OD value of BALF from the negative control animals. The cut-off was calculated as mean OD value of the negative control plus three SD for both IgG and IgA BALF antibodies and established at an OD value of 0.34 and 0.29, respectively. Whereas values higher than this cut-off were considered positive, values below this cut-off were considered negative.

6.2.6. Statistical analyses

Statistical analyses were performed using SPSS software, version 15.0 (SPSS Inc., Chicago, Illinois, USA). The significance level was set at $p<0.05$. Variables included in the statistical analyses were classified as categorical (presence or absence of CVPC and positive or negative S/P

ratios and OD values for IgG1 and IgG2 in serum and for IgG and IgA in BALF) or continuous (S/P ratio, and IgG1 and IgG2 OD values in sera and IgG and IgA OD values in BALF, percentage of lungs showing CVPC and Ph. Eur. scores). A non-parametric test (Mann-Whitney) was used to evaluate the effect of any categorical variable over the continuous ones. Besides, in order to study putative associations among continuous variables, a linear regression analysis was carried out. Contingency tables with the corresponding chi-square statistics were performed among categorical variables.

6.3. RESULTS

6.3.1. Experimental study

None of the control pigs showed CVPC. Among inoculated animals, local and systemic *M. hyopneumoniae* humoral immune parameters, percentage of animals showing CVPC as well as the mean lung lesion score of the animals sacrificed at 21 or 28 dpi are represented in Table 6-1.

6.3.1.1. Lung lesion evaluation

CVPC was observed both in pigs sacrificed at 21 and 28 dpi and no significant differences (p>0.05) were found in terms of number of animals affected neither in mean lung lesion scores (Table 6-1).

Table 6-1. Immunopathological data obtained from the experimental study. Number (%) of animals showing CVPC, mean Ph. Eur. score (± SD), mean S/P ratio (± SD) and number (%) of seropositive (IgG) and positive animals to *M. hyopneumoniae*-IgG1 and IgG2 in sera and to IgG and IgA antibodies in BALF at 21 and 28 dpi. Different superscripts within a column indicate significant differences among dpi (p<0.05).

	Lung pathology		**Serology**		**Sera isotypes detection**		**BALF antibodies detection**	
Dpi	*No. of animals with CVPC (%)*	*Mean Ph. Eur. score (± SD)*	*No. of seropositive animals (%)*	*Mean S/P ratio (± SD)*	*No. of IgG1 positive animals (%)*	*No. of IgG2 positive animals (%)*	*No. IgG positive animals (%)*	*No. of IgA positive animals (%)*
21 (n=37)	30 (81.1)[a]	7.7 (± 8.1)[a]	3 (8.1)[a]	0.09 (± 0.22)[a]	0 (0)[a]	0 (0)[a]	34 (91.9)[a]	36 (97.3)[a]
28 (n=60)	48 (80)[a]	9.5 (± 10.3)[a]	12 (20)[a]	0.26 (± 0.34)[b]	18 (30)[b]	32 (53)[b]	37 (61.7)[b]	45 (75)[b]

6.3.1.2. Serology

Number of seropositive animals was higher at 28 than at 21 dpi (p=0.09) (Table 6-1). Although below the positivity threshold, mean S/P ratio at 28 dpi was significantly higher than at 21. While all seropositive pigs (n=15) showed CVPC with different levels of severity (from 0.4 to 32% of lung weight damaged), a total of 63 out of 82 seronegative animals (76.8%) had CVPC. In addition, no significant (p>0.05) correlation was found between individual lung lesion scores and S/P ratios. Nevertheless, the mean S/P ratio of animals with CVPC (0.32 ± 0.36) was significantly higher (p<0.05) than the one from animals without lung lesions (0.03 ± 0.05) at 28 dpi.

6.3.1.3. Serum IgG1 and IgG2 subclasses

Neither IgG1 nor IgG2 against *M. hyopneumoniae* were detected in sera at 21 dpi, even in those three animals that seroconverted for total IgG (Table 6-1). However, all the animals that seroconverted by 28 dpi (n=12) were positive to both isotypes. A significant positive correlation (r=0.77, $p<0.0001$) between OD values of both antibody subclasses was observed, but serum IgG2 amounts were found to be significantly higher ($p<0.05$) than IgG1 ones at 28 dpi. Furthermore, significantly higher ($p<0.05$) IgG1 and IgG2 levels were detected in those animals showing CVPC in comparison with those without lung lesions (Figure 6-2). However, no association between IgG1 or IgG2 OD values and lung lesion scores at individual animal level was observed.

6.3.1.4. Local humoral immune response

Number of positive animals for BALF IgG and IgA antibodies was significantly higher ($p<0.05$) at 21 dpi than at 28 dpi (Table 6-1). A significant increase ($p<0.05$) of both specific IgG and IgA antibodies in BALF was observed in those animals showing CVPC in comparison with those without lung lesions (Figure 6-3A and 6-B, respectively), but for specific IgG BALF antibodies at 21 dpi ($p=0.07$) (Figure 6-3A). Although moderate to low, a significant positive correlation (r=0.49, $p<0.05$) between IgG BALF OD values and lung lesion scores at individual level was observed. Nonetheless, this relationship was not observed with regard to IgA BALF OD values.

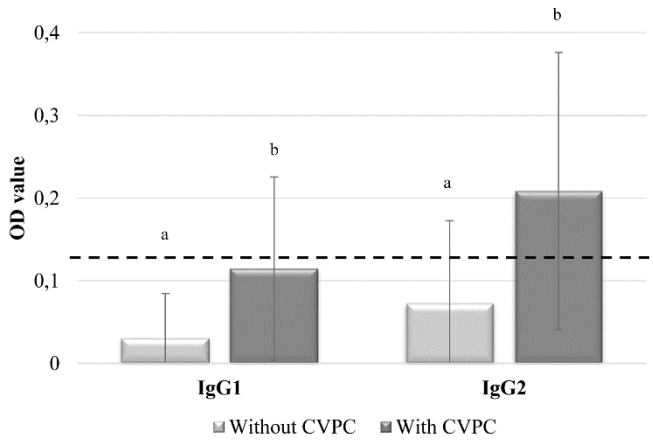


Fig 6-2. Mean IgG1 and IgG2 isotypes OD values (±SD) in animals with and without CVPC at 28 dpi from the experimental study. Different superscripts indicate significant differences of IgG1 and IgG2 antibody levels among animals with and without CVPC (p<0.05). Discontinuous line represents the positivity threshold.

6.3.2. Slaughterhouse study

6.3.2.1. Gross pathology and serology

All the 54 farms included in the present study were found to be affected by CVPC. Globally, mean percentage of lungs affected by CVPC and mean lung lesion score were 68.1 (min 34, max 99) and 5.15 (min 0.19, max 16.38), respectively. Based on the results obtained from the 20 randomly tested serum samples per farm, 40 (74.1%) farms were considered seropositive and the remaining 14 (25.9%) seronegative.

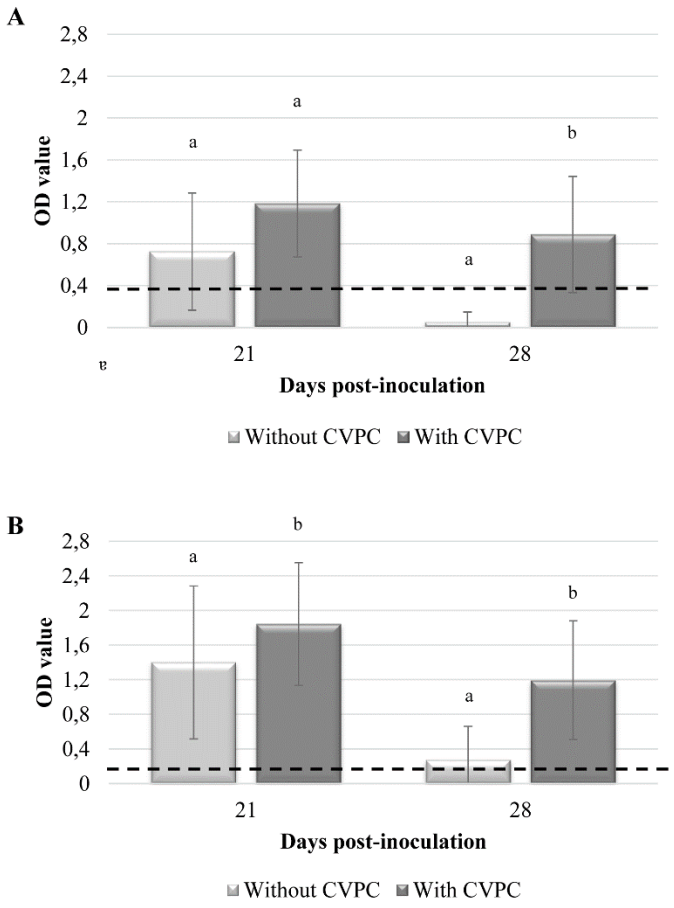


Figure 6-3. Mean *M. hyopneumoniae* IgG (A) and IgA (B) OD values (±SD) in BALF samples from animals of the experimental study with and without CVPC at 21 and 28 dpi. Different superscripts indicate significant differences of antibody levels among animals with and without CVPC at 21 and 28 dpi (p<0.05). Discontinuous line represents the positivity threshold.

Additionally, seropositive farms had a significantly higher (p<0.001) mean lung lesion score as well as percentage of lungs affected by CVPC than seronegative ones (Table 6-2).

However, no significant relationship (p>0.05) was found between the mean S/P value of the 20 randomly collected sera and the mean lung lesion score at individual farm level.

Table 6-2. Mean Ph. Eur. Score (±SD) and percentage of affected lungs by CVPC (±SD) in seropositive and seronegative farms from the slaughterhouse study. Different superscripts within a row indicate significant differences among types of farms (p<0.05).

	Seropositive Farms	**Seronegative Farms**
Mean Ph. Eur. Score (±SD)	6.09 (± 3.89)[a]	2.47 (± 1.91)[b]
Mean percentage of affected lungs (±SD)	71.73 (± 12.78)[a]	57.57 (± 12.07)[b]

6.3.2.2. Serum IgG1 and IgG2 subclasses

All the 40 farms classified as seropositive showed positive IgG2 humoral immune responses and 24 of them (60%) had also IgG1 positive reactions. Similarly to what was observed at experimental level, a significant positive correlation (r=0.59, p<0.0001) between both isotypes was detected and IgG2 response against *M. hyopneumoniae* was found to be significantly (p<0.0001) higher than the IgG1 one. Indeed, a significant positive correlation was found between S/P ratios and IgG2 OD values (r=0.83, p<0.0001) whereas such relationship was not as good when considering IgG1 OD values (r=0.48, p=0.0002). Though, no significant association (p>0.05) between the mean IgG2 OD value and the mean lung lesion score at individual farm level was found.

6.3.3. Chronological herd study

6.3.3.1. Gross pathology and serology

M. hyopneumoniae serological results from all monitored pigs and pathological results obtained along sequential necropsies are summarized in Table 6-3. From 1 to 12 weeks of age, the percentage of seropositive pigs was below 10%. However, from 15 weeks of age onwards, percentage of seropositive pigs increased progressively towards the end of the finishing period. Animals with CVPC began to appear from 12 weeks of age onwards. By the end of the study (22 weeks), the highest percentage of seropositive animals paralleled with the highest mean S/P values. Although not statistically significant (p>0.05), seropositive animals necropsied at 15, 18 and 22 weeks of age had higher lung lesion scores in comparison with the seronegative counterparts. Nonetheless, a statistical tendency between individual S/P ratios and lung lesion scores was observed at 22 weeks of age (r=0.49, p=0.056).

6.3.3.2. Serum IgG1 and IgG2 subclasses

Following the seroconversion pattern, mean *M. hyopneumoniae* IgG2 levels increased progressively from 15 weeks of age until the end of the study (22 weeks), when the mean maximum IgG2 levels in sera were reached (Figure 6-4). On the contrary, IgG1 mean levels maintained below the positivity threshold along the studied period but at 9 weeks of age. Indeed, mean IgG2 serum levels were significantly higher (p<0.05) than IgG1 ones at both 18 and 22 weeks of age. Additionally, a positive

significant correlation was found between S/P ratios and IgG2 OD values in 18 (r=0.83, p=0.0004,) and 22 (r=0.66, p=0.006) week-old pigs. Notwithstanding, individual IgG2 OD values were positively correlated with mean lung lesion scores in 12 (r=0.81, p=0.001), 15 (r=0.83, p=0.04), 18 (r=0.71, p=0.063) and 22 (r=0.62, p=0.01) week-old animals.

Table 6-3. *M. hyopneumoniae* serological results from all pigs at all time points and pathological results obtained along sequential necropsies from the chronological herd study. Different superscripts within a row indicate significant differences between weeks of age (p<0.05).

	Weeks of age							
	1	**3**	**6**	**9**	**12**	**15**	**18**	**22**
No. of animals tested (n)	58	58	58	58	48	36	29	16
No. of seropositive animals (%)	5 (8.6)[a]	3 (5.2)[a]	2 (3.4)[a]	3 (5.2)[a]	2 (4.2)[a]	6 (16.7)[b]	15 (51.7)[c]	11 (68.8)[c]
Mean S/P (±SD)	0.18 ± (0.59)[a]	0.06 ± (0.18)[a]	0.07 ± (0.23)[a]	0.08 ± (0.18)[a]	0.11 ± (0.36)[a]	0.33 ± (0.65)[b]	1.01 ± (1.35)[c]	1.46 ± (1.32)[c]
No. of necropsied animals (n)	NA	NA	NA	10	12	7	13	16
No. of animals with CVPC (%)	NA	NA	NA	0 (0)[a]	4 (33.3)[b]	6 (85.7)[c]	8 (61.5)[b, c]	13 (81.3)[c]
Mean Ph. Eur. Score (±SD)	NA	NA	NA	0,00 ± (0,00)[a]	1.35 ± (2.35)[b]	3.32 ± (2.57)[b]	3.09 ± (7.63)[c]	6.94 ± (8.53)[c]

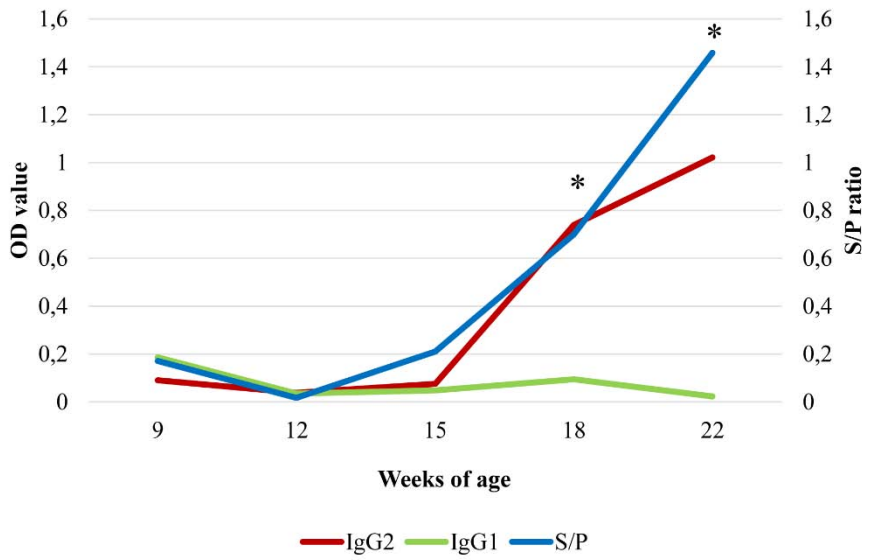


Figure 6-4. Mean S/P ratios and mean IgG1 and IgG2 OD values in sera from pigs sequentially necropsied in the chronological herd study. *Significant differences among mean IgG1 and IgG2 OD values (p<0.05).

6.4. DISCUSSION

This study was motivated by the interest to identify an easy *ante-mortem* parameter that would be used to reliably forecast the prevalence and severity of CVPC during the life of the pig. While serology (particularly useful for economical, rapid and high-throughput sample analysis) is the most common monitoring tool used to determine the *M. hyopneumoniae* status of a pig population (Thacker and Minion, 2012), several studies have reported a positive association between serum antibody levels against *M. hyopneumoniae* and CVPC (Fraile *et al.*, 2010; Merialdi *et al.*, 2012; Morris *et al.*, 1995b; Van Til *et al.*, 1991; Yagihashi *et al.*, 1993). On that basis, this work further studied the value of the humoral immune response to *M. hyopneumoniae* as a CVPC estimator by

describing the association between different specific antibody isotype responses, both at local and systemic levels, and CVPC. Such putative relationship was evaluated in three different scenarios. First, experimental conditions allow modelling the acute phase of *M. hyopneumoniae* infection. Second, the relevance of CVPC is commonly assessed in slaughter-aged pigs (Fraile *et al.*, 2010; Meyns *et al.*, 2011; Noyes *et al.*, 1990), although no information about the *M. hyopneumoniae* infectious status is known. Lastly, pigs chronologically necropsied enabled the assessment of such relationship at different time points along *M. hyopneumoniae* infection dynamics. Despite CVP is frequently attributed to *M. hyopneumoniae* infection (Fraile *et al.*, 2010; Merialdi *et al.*, 2012; Meyns *et al.*, 2011), it might be caused by other microorganisms (e.g. *P. multocida* and SIV) as well as influenced by farm management and housing conditions (Fraile *et al.*, 2010; Merialdi *et al.*, 2012; Meyns *et al.*, 2011; Nathues *et al.*, 2014; Sibila *et al.*, 2007b). Although the present results confirm the presence of *M. hyopneumoniae* circulation in herds from both the slaughterhouse and chronological studies (Fraile *et al.*, 2010; Sibila *et al.*, 2007b), it should be considered that the aforesaid factors may have influenced CVPC and, in turn, the reliability of the association with the humoral immune response to *M. hyopneumoniae*.

At the experimental level, the local humoral immune response was stronger than the systemic one after 21 dpi. Inversely, once the systemic humoral immune response began to increase by 28 dpi, revealed by a higher number of seropositive animals and increased IgG1 and IgG2 responses, a decline of both IgG and IgA antibody levels in BALF was

observed. In the present work, higher levels of specific IgG and IgA mucosal antibodies were detected in those animals with CVPC in comparison with those without lung lesions. In agreement, there is a remarkable increase in the number of cells producing immunoglobulins A and G in the lungs of *M. hyopneumoniae* experimentally infected pigs (Redondo *et al.*, 2009; Sarradell *et al.*, 2003; Suter *et al.*, 1985). Additionally, the number of cells producing specific IgG appeared to be notably higher than the ones producing IgA at lymphoid and lung tissue levels (Suter *et al.*, 1985). Actually, although with low to moderate correlation, specific IgG antibody amounts in BALF were found to be the best parameter to foresee CVPC at the level of the individual pig. While IgA antibodies associate with the mucosal layer in the nasal cavity, trachea and bronchi and would block *M. hyopneumoniae* attachment to epithelial cells, IgG antibodies are mainly found in the alveoli, being more active at lung parenchyma level through participation in opsonization and phagocytosis (Christopher *et al.*, 2012; Walker *et al.*, 1996). Therefore, differential location of IgG and IgA in the respiratory tract might explain why BALF IgG levels were better correlated with CVPC scores. Similarly to earlier results (Feng *et al.*, 2014), the present findings from experimentally inoculated pigs confirm that the humoral respiratory immune response against *M. hyopneumoniae* develops faster than the systemic one, which is commonly delayed in onset but persists longer. Henceforth, in an early stage of *M. hyopneumoniae* infection, a mucosal-based antibody measurement (such in BALF and tracheobronchial lavage samples)

would be probably better as a CVPC predictor than a serological-based one.

While the local humoral immune response could play a major role in the pathogenesis of *M. hyopneumoniae* acute infection, IgG at the systemic level may be more important in later phases. As supporting evidence of this notion, a relationship between pneumonic lesions and IgG serum levels against *Mycoplasma pulmonis* in mice has been indicated (Davis *et al.*, 1985). Noticeable, reduced production of IgG was one of the most prominent characteristics in the immunological function of a mycoplasmal pneumonia resistance Landrace line (Katayama *et al.*, 2011). Results obtained from slaughtered and chronologically necropsied pigs suggest that the amount of *M. hyopneumoniae* specific IgG might be related with the severity of CVPC and that could help to forecast prevalence and severity of EP-like lung lesions using a population based approach. However, the S/P value was not a good measure to predict severity of CVPC at an individual animal or farm level. Unfortunately, the specific relationship between serological responses and lung lesion scores in the 20 sampled slaughtered animals per farm was not possible. Thus, the association between both parameters at an individual slaughter weight animal level could not be assessed.

Lung lesions progress and regress throughout the lives of the pigs (Noyes *et al.*, 1990; Sitjar *et al.*, 1996; Wallgren *et al.*, 1994). While pigs can show recovering lung lesions 8 to 12 weeks after *M. hyopneumoniae*

exposure (Kobisch *et al.*, 1993; Sørensen *et al.*, 1997), serum specific antibodies persist for longer periods (Fano *et al.*, 2005; Pieters *et al.*, 2009). In fact, CVPC severity may depend on the time elapsed from *M. hyopneumoniae* seroconversion to the slaughter (Andreasen *et al.*, 2001; Sørensen *et al.*, 1997; Wallgren *et al.*, 1994). Thus, the main limitations to describe CVPC prevalence and severity in slaughtered pigs are due to the fact that CVPC consequence of early *M. hyopneumoniae* infections would probably escape detection, whereas animals infected close to the slaughter date might show CVPC but no seroconversion. For a better understanding of the humoral immunity value to forecast lifetime CVPC, samples from pigs that were followed up from birth to 22 weeks of age with sequential necropsies were evaluated. In such scenario, CVPC appeared earlier (at 12 weeks of age) than evident seroconversion (15 weeks of age) and both the percentage of seropositive pigs and animals with lung lesions increased progressively towards the end of the finishing period. Henceforth and similar to what was seen in slaughtered pigs, seropositivity was linked with more severe CVPC, but no significant correlation was observed between individual S/P ratios and lung lesion scores at any time point. While the chronological study was performed in non-vaccinated pigs, slaughtered animals belonged to batches from vaccinated and non-vaccinated herds. Although CVPC in slaughtered pigs was not importantly affected by *M. hyopneumoniae* vaccination (Fraile *et al.*, 2010), we cannot completely rule out that vaccination could affect the association pattern between serology and CVPC.

A novel approach aimed to determine whether there was a measurable difference in IgG1 and/or IgG2 subclasses in pig sera with and without CVPC was raised. Predominance of one IgG subclass over another in an immune response suggests Th1/Th2 bias (Crawley and Wilkie, 2003). Dominant secretion of IgG1 antibodies conveys differentiation of naïve Th cells into Th2, which are involved mainly in the activation of the humoral part of the immune response. In contrast, major detection of IgG2 is related to Th1 cells activation, which preferentially promotes the cellular immune response (Crawley and Wilkie, 2003; Raymond and Wilkie, 2004). The relevant immune response type and IgG subclass distribution could be critical for protection against a particular disease (Furesz *et al.*, 1998). However, the involvement of IgGl and IgG2 subclass responses to *M. hyopneumoniae* infection and the ratio of these subclasses with respect to protection are not yet known. In the murine respiratory mycoplasma infection model, specific Th2 cell responses were found to be involved in the development of inflammatory lung lesions. In contrast, the ability to generate a Th1 cell-mediated lung immune response was related with more protection (Woolard *et al.*, 2004). Remarkably, while this functional polarization of the Th cells has been extensively studied in mice and humans, in pigs it is less defined (Raymond and Wilkie, 2004).

In the present study, the systemic humoral immune response in both experimental and natural *M. hyopneumoniae* infections was predominate the IgG2 subclass. In fact, IgG2 OD values and S/P values (total IgG) were found to be significantly and positively correlated. Moreover, the

chronological study revealed that this antibody subclass level developed in parallel with the specific IgG response, indicating that IgG2 might probably be the main component of the systemic humoral immune response against *M. hyopneumoniae*. Contrarily, previous studies have reported a higher IgG1 than IgG2 antibody response in pigs challenged with *M. hyopneumoniae* at local (Muneta *et al.*, 2008; Okamba *et al.*, 2010) and systemic (Okamba *et al.*, 2010) levels. Although reasons for such inconsistencies are unknown, while in such previous studies IgG1 and IgG2 subclasses against P46 (Muneta *et al.*, 2008) or P97 (Okamba *et al.*, 2010) proteins were detected, in the present case, detected antibodies were directed to a recombinant *M. hyopneumoniae* antigen. The latter might have had a certain influence on the observed IgG isotype bias, as it has been demonstrated that the antigen may determine the type of immune response that develops (Raymond and Wilkie, 2004). Notwithstanding, IgG2 OD values were found to be better estimators of lifetime pneumonia than S/P values. Indeed, the IgG2 OD value was the humoral immune parameter that better correlated with the lung lesion score. Despite the fact that a Th1 shifted immune response has been described in a MP resistant Landrace genetic line (Katayama *et al.*, 2011), present results suggest the absence of a protective role of IgG2 subclass against *M. hyopneumoniae*. Interestingly, Th1 immune response is required for host defense against intracellular pathogens (Christopher *et al.*, 2012; Raymond and Wilkie, 2004). Although *M. hyopneumoniae* is regarded as an extracellular bacteria (Park *et al.*, 2002), it has been recently speculated as an intracellular pathogen (Raymond *et al.*, 2016). Altogether, the present finding of IgG

isotype bias as a correlate of *M. hyopneumoniae* associated lung lesions may provide an incentive for further evaluation of the Th immune response in *M. hyopneumoniae* infection scenarios.

6.5. CONCLUSION

The present study provides insight into those potential mechanisms of immunity that might be implicated in the pathogenesis of *M. hyopneumoniae* infection in pigs. To our knowledge, this is the first study examining the involvement of different *M. hyopneumoniae* specific antibody isotype responses in lung lesion development. The results indicated an increased antibody-specific production that most likely is associated to lung lesion severity during infection. Since *M. hyopneumoniae* systemic humoral immune response is delayed in onset, a mucosal-based antibody measurement would be preferably chosen to predict lung lesion severity at early stages of infection. Being *M. hyopneumoniae* monitoring by ELISA one of the most common practices on swine farms, the obtained results may give an indication of the lung lesion severity in a certain batch. In addition, there was a clear trend towards the IgG2 response, which may reflect a dominant Th1-controlled antibody-mediated immune response. However, the measurement of other parameters related with the cellular immune response would be required to further study the role that the Th1/Th2 bias could have in *M. hyopneumoniae* infections.

PART III

General Discussion and Conclusions

"Escriure no és més que buscar a les palpentes el món real"

Maria Aurèlia Capmany i Farnés

Chapter 7

General Discussion

While *M. hyopneumoniae* remains as an important and wide-spread pathogen in today's swine industry, the various mechanisms of pathogenesis involved with its infection are still unclear. However, long-term colonization of airway epithelium, stimulation of a prolonged inflammatory reaction, suppression and modulation of the innate and adaptive immune responses, as well as interaction with other infectious agents, are considered key components in the pathogenesis of this bacterium (Thacker and Minion, 2012). Altogether reveals a particular and complex relationship between the pathogen and the host, highlighting, at the same time, the multifactorial nature of the lung pathology outcome resulting from the infection with *M. hyopneumoniae*.

One of the many consequences of this complex interaction is the variability observed in prevalence as well as in severity of MP within and between *M. hyopneumoniae* experimental models. Since experimental models are specifically designed to be as much repeatable as possible, the variable MP outcome is considered a major concern, especially when testing veterinary medicine products against the infection. Therefore, an experimental model designed to ensure the successful reproduction of MP in challenged animals is needed, becoming the corner-stone justification for this thesis. As a result, the present dissertation is in part focussed on identifying tangible measures to improve the *M. hyopneumoniae* model outcome in terms of increased prevalence, severity and reproducibility of MP.

In order to reach this goal, a systematic review to compile reported *M. hyopneumoniae* experimental models as well as to identify those putative MP triggering factors was carried out (*Chapter 3*). In a systematic review, firstly, a clear set of rules to search for studies is defined and secondly, the studies accomplishing specific criteria are selected (Borenstein *et al.*, 2009). Since an element of subjectivity can appear when stablishing such criteria, selection decisions should be transparently explained (Borenstein *et al.*, 2009). A key element in most systematic reviews is the statistical synthesis of the data, which basically consist of a meta-analysis (Borenstein *et al.*, 2009; Harrison, 2011). For a meta-analysis, each of the considered variables should have had an "effect size" over the outcome. Such an effect size is represented by a value (e.g. Pearson's correlation coefficient, *r*, Cohen's *d*, or the odds ratio) that reflects the magnitude of relationship between both the variable and the outcome (Borenstein *et al.*, 2009; Harrison, 2011). In the absence of such value in the vast majority of the evaluated *M. hyopneumoniae* experimental models, another statistical approach, namely recursive partitioning, was applied. This approach is a statistical method for multivariable analysis based on decision trees development. This kind of analysis has been widely utilized to develop prediction models in various medical fields to enable better clinical decision-making (Marshall, 2001). To date, however, this method has not been used to evaluate the performance of experimental infections in pigs.

As the outcome for recursive partitioning was MP severity measured as macroscopic lung lesion score, one of the major inconveniences faced

was derived from the variability observed in the scoring systems used. In order to overcome such problem, MP resultant from a *M. hyopneumoniae* experimental model was assessed by the most frequently used lung scoring systems and afterwards, equivalence formulae between them were obtained (*Annex*). These formulae, in fact, made possible the statistical assessment of the scores obtained by different experimental models included in the systematic review by unifying them to a unique reference value. Besides the utility in this specific study, provided equivalence formulae would allow comparisons (prospective or retrospective) of lung lesions evaluated by different scoring systems, not only at experimental, but also at abattoir and herd levels (*Chapter 6*).

Decision trees derived from the recursive partitioning analysis revealed that the time elapsed from *M. hyopneumoniae* artificial inoculation to MP assessment at necropsy is an important factor to be taken into account. Understanding that certain time is needed for lung lesions to develop, pneumonia should be evaluated no later than 8 wpi. Indeed, results obtained from a complementary statistical analysis, namely GAM, upkept the 4 wpi as the best moment for MP assessment. Notwithstanding, severe MP has been also obtained after 1 wpi (Kobisch *et al.*, 1993; Lorenzo *et al.*, 2006), suggesting that other experimental conditions may influence this outcome. In agreement, further statistical analyses considering only experimental models of less duration than 8 wpi revealed that other conditions may partly explain the observed lung lesion score variability. In order of importance, these

are: the *M. hyopneumoniae* strain used for the inoculation, the age of the animals at inoculation, the animals' co-infection status with other swine pathogens, and the animal source.

M. hyopneumoniae strains used in experimental studies are normally field strains coming from pigs with CVPC, isolated by research groups, and whose pathogenicity has been already proved. Therefore, the *M. hyopneumoniae* strain inoculated is often a fixed factor not easily interchangeable in experiments performed by a particular research group. With regard to the other conditions, while inoculation of *M. hyopneumoniae*-free pigs older than 3.5 weeks of age is fairly feasible, the use of another swine respiratory pathogen to co-inoculate the animals may not fit the objective of various experiments. As a striking example, the use of co-infections is not considered to accomplish with the efficacy test for the EP inactivated vaccine (Ph. Eur., 2013), probably because the presence of another infectious agent could confound the results obtained. Importantly, other experimental conditions more directly related with the aetiological agent, such as the inoculum type, dose or the inoculation route used, were not found relevant to explain MP variability.

MP outcome may be also probably explained by individual host susceptibility. Indeed, a recently selected MP resistant Landrace line (Miyagino L2) has been established based on reduced incidence of pulmonary lesions (Kadowaki *et al.*, 2012). This genetic line has been shown to have a different immunophenotype to combat *M.*

hyopneumoniae infection (Katayama *et al.*, 2011), which highlights the potential relevance of the host immune response on MP development. Unfortunately, the impact of animal genetics on MP could not be addressed due to the lack of information in the literature regarding this aspect, although it is probably an important aspect to consider in upcoming *M. hyopneumoniae* experimental models.

So far, no statistical model is free of limitations, and it is important to be aware about these as well as of the merits of the model. Results obtained in *Chapter 3* provide a descriptive picture of which *M. hyopneumoniae* experimental model conditions are mostly reported in the literature as well as a first approach on the triggering factors for MP experimental development. However, care must be taken in drawing general conclusions. Since the effect of some particular conditions in MP could escape this first multivariable statistical approach, the next step was to individually evaluate those experimental factors with potential to have a certain impact on the MP outcome but found no significant by the recursive partitioning analysis. Thus, subsequently, the effect of the inoculation route into MP (*Chapter 4*) and the *M. hyopneumoniae* inoculum titration assessment (*Chapter 5*) were investigated.

In *Chapter 4*, the ability to induce MP in pigs experimentally inoculated with *M. hyopneumoniae* by three different inoculation routes (i.e. ET, IN and AE) was assessed. Importantly, the three inoculation routes were compared under the same experimental conditions, thus, avoiding interferences of any other experimental condition in the outcome. As a

result, MP could be reproduced by means of the three inoculation routes, although animals belonging to the ET group displayed a significantly higher prevalence of pneumonia as well as a higher mean lung lesion score. Nevertheless, these results should not ignore the fact that IN and AE routes are supposed to mimic better the natural *M. hyopneumoniae* route of infection. Notably, AE route can be applied by anaesthetising the animals and using an individual mask (Steenhard *et al.*, 2009) or by placing them into specially designed chambers (Czaja *et al.*, 2002). The first option (chosen in this study) may provide more accuracy in dosing pigs individually. Nevertheless, the second option allows the use of non-anesthetised animals, which might be an advantage. Taking into account that anaesthesia is known to reduce the respiratory rate, it is not really known how it may affect the result of the inoculation. In fact, the best reported MP outcome using the AE route has been achieved by using an aerosolisation chamber (Czaja *et al.*, 2002).

In support to the notion that lung tissue injury in *M. hyopneumoniae* infections is caused more importantly by the host inflammatory response rather than by the direct action of the organism (Thacker and Minion, 2012), animals inoculated by means of the ET route showed an increased immune response to *M. hyopneumoniae* in comparison with those inoculated by the IN or AE routes. Thus, higher levels of pro-inflammatory cytokines (i.e. TNF-α, IL-8 and IL-1β) as well as specific IgA antibodies at local level were found in ET inoculated animals, which was paralleled with an earlier immune response at systemic level.

Altogether emphasise the key role that the host immune response against *M. hyopneumoniae* plays in pathogenesis.

Outstandingly, *M. hyopneumoniae* detection in laryngeal swabs revealed an earlier colonization of those pigs inoculated through the ET route. Since colonization of the epithelial linings of the porcine respiratory tract is a prerequisite for infection (Thacker and Minion, 2012), such an earlier colonization may explain the earlier and more intense immune response against *M. hyopneumoniae* and, in turns, the most severe lung lesions observed at 4 wpi in the ET group. *M. hyopneumoniae* colonization also took place in IN and AE inoculated pigs, but later on time, suggesting that MP assessment beyond 4 wpi could have led to more severe pneumonic lesions in those animals. While the time that elapses until MP assessment at necropsy is a critical factor in *M. hyopneumoniae* experimental models (*Chapter 3*), 4 wpi as the time period to achieve the most severe lung lesions may not be suitable for all experimental conditions (e.g. when the IN or AE routes are used). Similarly, Villarreal *et al*. (2011) proposed a period longer than 4 wpi to study clinical signs caused by low virulent *M. hyopneumoniae* strains. Furthermore, results obtained in this chapter confirm that experimental conditions with a certain impact on the MP outcome could escape the multivariable statistical assessment done in *Chapter 3*, thus, any of those not relevant conditions should be underestimated.

From *Chapter 4* it can be also subtracted that the inoculum penetration into the lower respiratory tract is better if applied by the ET route, which

probably allowed greater cumulated infectious doses and, consequently, enhance colonization of the pathogen. Following this rationale, it can be hypothesised that higher inoculum doses would increase the chance to achieve MP in an experiment. Indeed, an inoculum dose-dependent response has been already reported in the literature and a minimal dose of 10^5 CCU per pig of *M. hyopneumoniae* 116 strain culture was settled (Marois *et al*., 2010). Anyway, it is very likely that the minimal dose is strain dependent. Unfortunately, there are still many limitations that hamper an accurate and reliable *M. hyopneumoniae* culture titration and, thus, dosing of individuals.

Since Friis (1975) described the medium for growth and isolation of *M. hyopneumoniae*, hardly any improvement has been made to it despite this pathogen grows slowly and low cell yields are obtained (Calus *et al*., 2010). Indeed, Friis medium composition serves as basis for *M. hyopneumoniae* cultivation but, at the very end, each research group uses its own receipt, adding another source of variability. Moreover, *M. hyopneumoniae* culture titration is based on the colour change of the medium used for cultivation, becoming then the medium of special importance in regards of inoculum dosing. Aside, at least two weeks of incubation are needed for *M. hyopneumoniae* inoculum titration by means of the CCU assay. Therefore, the use of this technique implies either to inoculate the animals without knowing the titre (since it is calculated retrospectively) or to use a previously frozen, tittered inoculum. However, whether freezing can affect or not *M. hyopneumoniae* viability is unknown. In addition, previous results

indicate that the link between colour and growth phase should be used with caution since it may depend on the medium and medium batch, as well as on the container used for cultivation (Calus *et al*., 2010). As a result, other assays, such a as ATP luminometry (Calus *et al*., 2010) or flow cytometry (Assunção *et al*., 2006), have been proposed for *M. hyopneumoniae* titration, but they did not gain wide acceptance and the CCU technique continues to be extensively used through experimental models. Henceforth, in *Chapter 5*, different techniques with potential to be utilized to titrate a *M. hyopneumoniae* culture were evaluated at the same time that the *in vitro* growth dynamics and kinetics of pathogenic and non-pathogenic *M. hyopneumoniae* strains was described.

In respect *M. hyopneumoniae* culture titration, obtained results are in agreement with those formerly provided by Calus *et al*. (2010), sustaining the ATP assay as a valuable and robust alternative for reproducible real-time titre assessment of freshly grown *M. hyopneumoniae* cultures. It is likely faster, more accurate and cost-efficient, and less time-consuming compared to the CCU assay. For a particular research group working with specific *M. hyopneumoniae* strains and medium, the establishment of the strains' *in vitro* growth dynamics and kinetics through the application of the ATP luminometry assay is highly recommended. This may allow the establishment of a culture backup from which the *in vitro* growth behaviour is known, becoming especially useful to better standardise and describe *M. hyopneumoniae* inoculum productions for experimental applications. Nevertheless, whether the growth stage of the *M. hyopneumoniae*

inoculum can affect the MP outcome is still unknown. Since the growing stages of *M. hyopneumoniae* culture are easily established by ATP measurement, further studies evaluating the MP outcome in pigs inoculated with *M. hyopneumoniae* culture at different points along the log and the stationary phases of growing would be of interest. It should be kept in mind that this study was performed with standard *M. hyopneumoniae* strain pure cultures grown in a specific medium. Therefore, different results may probably be obtained if other inoculum types (i.e. lung homogenate), other strains less adapted to *in vitro* environments or other cultivation medium compositions are used. Remarkably, Marois *et al*. (2010) was first in using qPCR to quantify *M. hyopneumoniae* in culture. However, this technique has not been further evaluated for titration purposes. Despite its inability to differentiate between death and live cells, the additional use of qPCR may be advantageous in the case of using lung homogenate, as lung tissue is not sterile and it may interfere with results provided by other non-specific titration techniques. Its application, together with another technique indicating viability, is also recommended in culture, since this is easily contaminated.

Notably, results provided in *Chapter 5* also suggest a relationship between the ability to grow under *in vitro* conditions and pathogenicity of *M. hyopneumoniae*, evidenced by the presumed better growing capacity of the non-pathogenic J strain in comparison with the pathogenic 11 and 232 strains. However, this growing kinetics difference between high and low pathogenic *M. hyopneumoniae* strains

was not observed by Calus *et al*. (2010). Perhaps, a very high number of *in vitro* passages, such as to J strain, are needed to observe such growing differences. Regrettably, limited information regarding the *in vitro* passages needed for *M. hyopneumoniae* strains to loss virulence is available. Further works comparing in parallel the number of passages, the *in vitro* growth ability and the pathogenicity of *M. hyopneumoniae* strains would probably provide useful knowledge to better upkeep *M. hyopneumoniae* strains under artificial conditions and procuring, to the extent possible, ensure their ability to induce disease.

Besides identification and optimization of experimental factors that may have an influence on MP reproduction, nowadays there is not a reliable parameter to predict such outcome along the study period. Hence, the success of the experimental model is unknown until necropsies are performed. The same can be also transferred to field conditions, where retrospective *post-mortem* lung lesion inspections do not provide information on the ongoing respiratory problems. Thus, the present dissertation also sought to identify an *ante-mortem* parameter that would provide reliable and real-time information in relation with prevalence and severity of lung lesions associated with *M. hyopneumoniae* infection.

The evidence provided implies that the host immune response developed against *M. hyopneumoniae* is likely involved in pathogenesis. Moreover, previous data pointed out a possible relationship between antibody values against the bacterium and CVPC (Fraile *et al*., 2010;

Merialdi *et al.*, 2012; Morris *et al.*, 1995b; Van Til *et al.*, 1991; Yagihashi *et al.*, 1993). Given the convenience and the ease of antibody detection tests, the value of the humoral immune response to *M. hyopneumoniae* as a CVPC estimator was assessed in *Chapter 6*. Henceforth, the relationship between different specific antibody isotype responses, both at local and systemic levels, and CVPC was evaluated. In this chapter, the focus was not exclusively at experimental level, but also considering *M. hyopneumoniae* naturally infected pigs.

At experimental level and taking into consideration the standardised study period of 4 wpi (*Chapter 3*), a mucosal-based antibody measurement would be the best choice to estimate CVPC severity. In this case, both IgG and IgA antibody levels in BALF were higher in those animals showing pneumonia. This is in agreement to what was observed in *Chapter 4*, where those pigs showing the most severe MP had also significantly higher IgA antibody levels in BALF. Due to the intrinsic limitations in collecting BALF *in vivo*, other more practical respiratory mucosal samples, such as nasal or tracheobronchial lavage, should be evaluated for this purpose. Since the local humoral immune response against *M. hyopneumoniae* develops faster but persists shorter than the systemic one, blood specific IgG antibodies may be a better CVPC predictor in later phases of the infection. Nonetheless, the S/P value was not found to be a good measure to foresee severity of CVPC at an individual animal or herd levels, as higher S/P values did not translate into higher lung affection. Even so, seropositive pigs chronologically necropsied as well as seropositive herds at the time of

slaughter showed more severe MP than their seronegative counterparts. Interestingly, individual IgG2 OD values were positively and significantly correlated with lung lesion scores in chronologically necropsied pigs, which may offer prospects to serology for being used as EP-like lung lesions estimator.

Although both Th1 and Th2 cytokine profiles have been described in *M. hyopneumoniae* infection (Lorenzo *et al.*, 2006; Muneta *et al.*, 2008; Redondo *et al.*, 2009; Shen *et al.*, 2017; Woolley *et al.*, 2013), a new insight provided by this last chapter was the systemic IgG2 subclass predominance, which suggests a dominant Th1-mediated immune response against *M. hyopneumoniae*. In agreement, in *Chapter 4*, both IL-8 and IL-1β (pro-inflammatory Th1-like cytokines) levels in BALF were found significantly higher in pigs experimentally inoculated with *M. hyopneumoniae*. Indeed, pigs inoculated via the ET route showing the most severe lung lesions had significantly higher IL-1β levels in BALF in comparison with their counterparts inoculated via the IN or AE routes. This putative predominance of a Th1-mediated immune response to *M. hyopneumoniae* encounters discrepancies in the literature (Katayama *et al.*, 2011; Muneta *et al.*, 2008; Okamba *et al.*, 2010), although support recent findings proposing *M. hyopneumoniae* as an intracellular bacterium (Raymond *et al.*, 2016). Consequently, further studies would be required to confirm the role that the Th1/Th2 bias could have in *M. hyopneumoniae* infections.

So far, the multifactorial nature of the lung pathology outcome consequence of the *M. hyopneumoniae* infection has been evidenced, thus, many different factors play probably a role in its development. Under experimental settings, conditions applied may be optimized to increase the chance to successfully reproduce MP. In *Chapter 3*, the application of a statistical model to data systematically collected enabled the identification of determinant experimental factors for the successful reproduction of MP. However, intrinsic limitations both of the statistical model as well as of the analysed data hampered the assessment of the effect of particular conditions in MP. Hence, *Chapter 4* was raised to individually address the effect that the inoculation route could have in MP. Due to the limitations that the *M. hyopneumoniae* culture titration is still facing, *Chapter 5* sought to provide progress in this aspect; regrettably, the achieved insights could not be addressed by means of their application in a *M. hyopneumoniae* experimental model. Lastly, *Chapter 6* describes the potential of humoral immune response parameters to forecast lung lesions associated to *M. hyopneumoniae* infection and open a window towards further evaluation of the Th immune response in *M. hyopneumoniae* infection scenarios. Overall, the present dissertation broadens the current understanding in regards to *M. hyopneumoniae* experimental models and constitutes the first insight into conditions that may be optimized and standardised to successfully reproduce MP under experimental settings. In addition, the exposed results may serve as a basis for debate in the search for a validated and accepted *M. hyopneumoniae* experimental model.

Chapter 8

General Conclusions

1. The MP outcome resulting from the artificial inoculation with *M. hyopneumoniae* is partly explained by the experimental conditions applied. In order of importance, the most influencing factors are the time elapsed from the inoculation to lung lesion assessment, the inoculated *M. hyopneumoniae* strain, the age of the animals at inoculation, co-infection with other swine pathogens and source of pigs.

2. *M. hyopneumoniae* experimental models seeking the reproduction of severe MP should assess lung lesions within a period below 8 wpi and include inoculation of *M. hyopneumoniae*-free pigs older than 3.5 weeks of age and co-infected with another swine respiratory pathogen.

3. MP can be reproduced by inoculating pigs through different routes, named ET, IN and AE routes, although the ET route is the most effective inducing severe MP in a period of 4 wpi.

4. The establishment of the *in vitro* growth kinetics and dynamics of a particular *M. hyopneumoniae* strain in a specific medium is useful to standardise the process to prepare *M. hyopneumoniae* seed material. The use of a technique providing an on-time evaluation of the *M. hyopneumoniae* growth together with a specific technique for *M. hyopneumoniae* can be reliably used for this purpose.

5. The non-pathogenic *M. hyopneumoniae* J strain is better adapted to the *in vitro* growing conditions than the 11 and the 232 pathogenic *M. hyopneumoniae* strains.

6. In a population based approach, mucosal and serological-based antibody measurements help forecasting prevalence and severity of *M. hyopneumoniae* associated lung lesions at early and later phases of the infection, respectively. The best *M. hyopneumoniae*-specific humoral immune parameter to potentially predict the CVPC outcome is the IgG2 OD value.

7. The *M. hyopneumoniae* specific systemic humoral immune response is predominated by the IgG2 subclass, which suggests a dominant Th1-mediated immune response.

Annex

Assessment of *Mycoplasma hyopneumoniae*-induced pneumonia using different lung lesion scoring systems

Introduction

Several lung scoring methods are currently in place for evaluating CVPC at the farm, abattoir and experimental inoculation levels. At the farm level, necropsy examination followed by lung lesion scoring is highly recommended when severe coughing is observed in the animals. In such a scenario, the presence of CVPC may be indicative of *M. hyopneumoniae* involvement. However, since other microorganisms can produce similar lesions, laboratory testing is needed to confirm involvement (Sibila *et al.*, 2009; Thacker, 2004). At the abattoir level, evaluating lung lesions is commonly used to estimate the prevalence and severity of respiratory diseases and their impact on carcass market price, risk factor assessment and vaccine efficacy (Merialdi *et al.*, 2012; Sibila *et al.*, 2009). Abattoir surveillance may also be useful for detecting subclinical disease (i.e. no apparent clinical signs, but presence of lung lesions) or incipient cases, but it does not necessarily provide information about on-going respiratory problems at the farm level (i.e. during the rearing period as lesions may have healed until animals are slaughtered) (Noyes *et al.*, 1990; Sibila *et al.*, 2009). In experimental conditions, lung lesion scoring is used to study the pathogenesis of the infection and/or to assess antibiotic or vaccine efficacy (Kobisch and Friis, 1996).

Different CVPC scoring methods have been described in the literature, but there have been few comparisons made between them (Davies *et al.*, 1995; Morrison *et al.*, 1985; Mousing and Christensen, 1993). Such limited information might imply that differences in lung lesions found between studies may just reflect differences in scoring techniques (Morrison *et al.*, 1985). Seven CVPC scoring systems reported in the scientific literature were compared in the present study. Additionally, the system recommended by the European Pharmacopoeia (Ph. Eur., 2013) for the evaluation of porcine EP inactivated vaccine efficacy was included as the reference scoring system. Interestingly, this method has never been compared with other lung scoring systems for evaluation of CVPC. For a detailed description of the lung scoring systems compared refer to *Chapter 1*, section "*1.6.3. Cranio-ventral pulmonary consolidation scoring systems*".

CVPC not only has negative effects on the health of pigs, but also on their ADWG and, therefore, on the profit margin for each animal (Maes *et al.*, 1996; Thacker and Minion, 2012). However, information related to the precise economic losses derived from the presence of such lesions can be difficult to interpret. Until now, the information available has been contradictory (Maes *et al.*, 1996; Thacker and Minion, 2012).

The aim of the present study was therefore to evaluate eight lung lesion scoring systems used to assess pneumonia associated with *M. hyopneumoniae* by applying them to the lungs of 76 pigs with experimentally induced MP.

Comparison of lung lesion scoring systems

Ninety-five animals free of *M. hyopneumoniae* were inoculated via ET route on two consecutive days with 5 ml of fresh Friis medium culture of *M. hyopneumoniae* with a dose of 10^7 to 10^8 CCU/ml. At 21 or 28 dpi, animals were killed with an overdose of intravenous pentobarbital. Body weight was recorded at the time of infection and at necropsy examination and ADWG was calculated according to the following formula: weight at the time of necropsy examination minus the weight at the time of infection divided by the days between these time points. At necropsy examination, lungs were removed, weighed and evaluated for CVPC. Lung lesions were scored in a blinded fashion by the same veterinary pathologist according to seven scoring systems. Digital images were taken of all lungs for CVPC quantification by image analysis (Sibila *et al.*, 2014). An example of a lung with lesions derived from the *M. hyopneumoniae* inoculation and the corresponding value of each scoring system used is given in Figure A-1.

A linear regression analysis between each pair of lung lesion scoring systems was carried out to compare the different systems. For this purpose, only data from 76 out of 95 pigs that showed CVPC after experimental infection with *M. hyopneumoniae* were used. It was considered that the different day of necropsy examination did not interfere with the results, since each comparison was established with each lung. Moreover, a formula of equivalence between lung scoring methods and the reference method (Ph. Eur., 2013) was calculated from the regression analysis.

In addition, regression analysis was also used to assess the relationship between lung lesion severity scored by the reference method with: (1) the ratio of lung weight/body weight ratio (LW/BW) (Sibila *et al.*, 2014), (2) individual lung lobe score, and (3) ADWG. All statistical analyses were carried out using the SAS system V.9.1.3 (SAS institute Inc., Cary, North Carolina, USA) and the individual pig as the experimental unit. *P* was set at 0.05 with statistical tendencies reported when $p<0.10$.

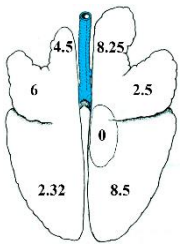
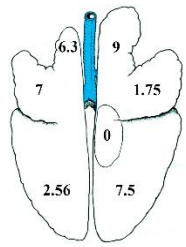
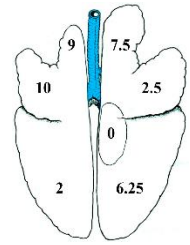
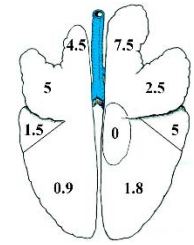
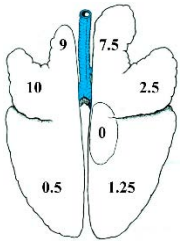
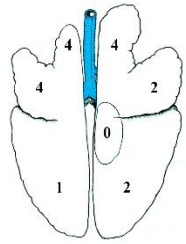
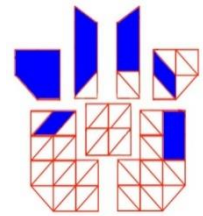
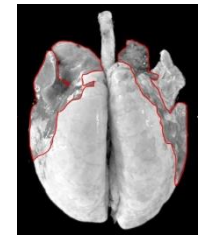
Reference method (Ph. Eur., 2013)	**Morrison *et al.* (1985)**	**Straw *et al.* (1986)**	**Christensen *et al.* (1999)**
			
Total scoring= 32.07%	Total scoring= 34.11%	Total scoring= 37.25%	Total scoring= 28.7%
Goodwin *et al.* (1969)	**Madec and Kobisch (1982)**	**Hannan *et al.* (1982)**	**Sibila *et al.* (2014)**
			
Total scoring= 30.75/55 points	Total scoring= 17/28 points	Total scoring= 15.86/35 points	Total scoring= 30.21%

Figure A-1. Example of a lung with lesions derived from an experimental *M. hyopneumoniae* inoculation and the corresponding value of each scoring system used. Lung drawing modified from König and Liebich (2005).

Correlation between the scoring systems

There was high correlation (r>0.9) between the lung lesion scoring systems, with the exception of image analysis (Table A-1). Very high correlations were observed between the scoring systems based on measuring the surface percentage of affected lung (Christensen *et al.*, 1999; Morrison *et al.*, 1985; Straw *et al.*, 1986), including the reference method (Ph. Eur., 2013). In addition, the reference method showed the highest correlations with the other two three-dimensional methods (Christensen *et al.*, 1999; Morrison *et al.*, 1985). The score devised by Christensen *et al.* (1999), where the cranial area of the diaphragmatic lobes is scored separately, showed no significant difference when compared with other similarly calculated methods. In relation to the methods where points are given per lobe instead of a percentage, the scoring systems proposed by Goodwin *et al.* (1969) and Hannan *et al.* (1982) showed higher correlations with the other seven methods than that of Madec and Kobisch (1982). Considering all studied lung scoring methods, the image analysis method described by Sibila *et al.* (2014) showed the poorest correlation with the other scoring techniques. This finding may be explained by the fact that the extent of lesions was quantified only from images of the dorsal surface of the lung and, in consequence, lesions located in the accessory lobe were not considered. If the image analysis had been applied to both surfaces of the lungs, the correlations may have improved. Formulae of equivalence and their determination coefficient (R^2) between the reference score and the other seven methods were calculated (Table A-2), so the results obtained with different lung scoring methods could be converted to a unique reference

value (Ph. Eur., 2013), making them comparable. Additionally, the relationship between CVPC scored by the reference method (Ph. Eur., 2013) and the LW/BW ratio was studied. Davies *et al.* (1995) reported a close association between a volumetric method and a subjective method (visual determination of CVPC as a percentage). However, in the present work, a significant association (p<0.001), but moderate to low correlation (r=0.710) was observed. In comparison with the correlation found between the scoring systems evaluated, the LW/BW ratio was the least informative method. However, as this was the first time that the LW/BW ratio has been evaluated to assess experimentally induced *M. hyopneumoniae* lung lesions, further studies would be of interest to validate this method of assessing CVPC.

Table A-1. Correlation between lung scoring systems to evaluate the presence and extension of CVPC in peer-reviewed literature. All lung lesion scoring systems were statistically associated (p<0.001).

Lung Scoring	*Ph. Eur. (2013)*	*Goodwin et al. (1969)*	*Hannan et al. (1982)*	*Madec and Kobisch (1982)*	*Morrison et al. (1985)*	*Straw et al. (1986)*	*Christensen et al. (1999)*
Ph. Eur. (2013)	---	---	---	---	---	---	---
Goodwin et al. (1969)	0.966	---	---	---	---	---	---
Hannan et al. (1982)	0.948	0.962	---	---	---	---	---
Madec and Kobisch (1982)	0.909	0.922	0.925	---	---	---	---
Morrison et al. (1985)	0.994	0.975	0.956	0.919	---	---	---
Straw et al. (1986)	0.984	0.982	0.974	0.926	0.993	---	---
Christensen et al. (1999)	0.992	0.961	0.954	0.907	0.987	0.983	---
Sibila et al.(2014)	0.824	0.825	0.835	0.725	0.825	0.825	0.822

Table A-2. Formulae of equivalence and determination coefficients (R^2) between the reference method (Ph. Eur., 2013) and the other seven methods (x variable) included in the comparison.

	Reference method (Ph. Eur., 2013)	R^2 (%)
Goodwin *et al*. (1969)	0.11 + 1.18*x	93.5
Hannan *et al*. (1982)	-1.39 +1.84*x	89.9
Madec and Kobisch (1982)	-2.42 + 1.83*x	82.7
Morrison *et al*. (1985)	0.3 + 0.98*x	98.9
Straw *et al*. (1986)	-0.29 + 0.87*x	97.9
Christensen *et al*. (1999)	-0.01 + 1.03*x	98.6
Sibila *et al*. (2014)	2.35 + 0.84*x	67.9

CVPC in Each Lung Lobe Related to Total Lung Score

CVPC was observed more frequently in the right side of the lung (68.75%) than in the left (57%). Additionally, right lobes showed more extensive CVPC than their corresponding left lobes (Table A-3). These results reflect the anatomical differences between the left and right porcine lung. Regression analysis was performed to study how the individual score of each lung lobe contributes to the entire score of the lung using the reference method (Ph. Eur., 2013). A significant correlation (p<0.05) between each lung lobe score and the total lung score was observed with coefficients of determination ranging from 0.359 to 0.696. Right lobe scores showed higher correlations with the corresponding total lung score than the left lobe scores, except for the intermediate left lung lobe, which showed a slightly higher correlation in comparison with the intermediate right lobe (Table A-3).

These results are in accordance with those of Mousing and Christensen (1993), who suggested scoring the right lung if just one side of the lung was to be evaluated.

Table A-4. Proportion (%) of each lung lobe with CVPC from the lungs showing CVPC derived from an experimental *M. hyopneumoniae* inoculation and the correlation among each lung lobe score and the corresponding total lung score using the reference method (Ph. Eur., 2013).

Lung lobe	Presence of CVPC/Total of lungs affected by CVPC	Mean lung score (Ph. Eur., 2013)	Correlation with the total lung score
Apical right	47/76 (63.2)	2.5%	0.779
Apical left	38/76 (51.3)	1.1%	0.672
Intermediate right	62/76 (81.6)	3.5%	0.733
Intermediate left	54/76 (71.1)	1.6%	0.730
Diaphragmatic right	45/76 (59.2)	3.5%	0.834
Diaphragmatic left	38/76 (50)	2.8%	0.709
Accessory	55/76 (72.4)	2.2%	0.599

Association of the Reference Scoring Method with ADWG

There is general acceptance that *M. hyopneumoniae* affects growth performance, but studies of this fact are controversial (Maes *et al.*, 1996; Thacker and Minion, 2012). In the present study, a significant association, but a poor correlation (r=0.353) was found between CVPC severity and ADWG. This result is in agreement with previous studies where no detrimental effects of *M. hyopneumoniae* on overall growth performance under experimental (Escobar *et al.*, 2002) or natural (Scheidt *et al.*, 1990; Straw *et al.*, 1990) settings was reported. In contrast,

in other studies a negative correlation between these two parameters was observed (Hill *et al.*, 1992; Paisley *et al.*, 1993; Pointon *et al.*, 1985; Straw *et al.*, 1989). The ability of *M. hyopneumoniae* to depress growth may be dependent on a number of factors, including co-infection with other organisms (Escobar *et al.*, 2002), as well as the duration of the infection (Davies *et al.*, 1995) and/or individual susceptibilities. Additionally, it must be considered that in the present study, ADWG was recorded at 3 or 4 weeks after *M. hyopneumoniae* infection. Further studies are needed to assess if the strength of such relationship is greater for longer periods post infection.

Conclusions

The present work reports good correlation between different scoring systems to quantify *M. hyopneumoniae* induced lung lesions. The strongest correlations were seen between scoring methods assessing the affected area or weight percentage within each lung lobe. If higher levels of objectivity, repeatability and precision are desired, a method using a schematic map of the lung or image analysis would be preferable. Since no universally accepted method for assessing the extent of CVPC exists, formulae of equivalence between the system recommended by the European Pharmacopoeia and the other seven lung scoring methods were calculated. These formulae would allow comparisons (prospective or retrospective) of lung lesions evaluated by the different scoring systems.

References

Adams, C., Pitzer, J., Minion, F.C., 2005. *In vivo* expression analysis of the P97 and P102 paralog families of *Mycoplasma hyopneumoniae*. Infect Immun 73, 7784-7787.

Ameri-Mahabadi, M., Zhou, E.M., Hsu, W.H., 2005. Comparison of two swine *Mycoplasma hyopneumoniae* enzyme-linked immunosorbent assays for detection of antibodies from vaccinated pigs and field serum samples. J Vet Diagn Invest 17, 61-64.

Andrada, M., Quesada-Canales, O., Suárez-Bonnet, A., Paz-Sánchez, Y., Espinosa de los Monteros, A., Rodríguez, F., 2014. Cyclooxygenase-2 expression in pigs infected experimentally with *Mycoplasma hyopneumoniae*. J Comp Pathol 151, 271-276.

Andreasen, M., Mousing, J., Krogsgaard Thomsen, L., 2001. No simple association between time elapsed from seroconversion until slaughter and the extent of lung lesions in Danish swine. Prev Vet Med 52, 147-161.

Arsenakis, I., Panzavolta, L., Michiels, A., Del Pozo Sacristán, R., Boyen, F., Haesebrouck, F., Maes, D., 2016. Efficacy of *Mycoplasma hyopneumoniae* vaccination before and at weaning against experimental challenge infection in pigs. BMC Vet Res 12, 63.

Asai, T., Okada, M., Ono, M., Irisawa, T., Mori, Y., Yokomizo, Y., Sato, S., 1993. Increased levels of tumor necrosis factor and interleukin 1 in bronchoalveolar lavage fluids from pigs infected with *Mycoplasma hyopneumoniae*. Vet Immunol Immunopathol 38, 253-260.

Asai, T., Okada, M., Ono, M., Mori, Y., Yokomizo, Y., Sato, S., 1994. Detection of interleukin-6 and prostaglandin E2 in bronchoalveolar lavage fluids of pigs experimentally infected with *Mycoplasma hyponeumoniae*. Vet Immunol Immunopathol 44, 97-102.

Assunção, P., Diaz, R., Comas, J., de Galarreta, C.M., González-Llamazares, O.R., Poveda, J.B., 2005. Evaluation of *Mycoplasma hyopneumoniae* growth by flow cytometry. J Appl Microbiol 98, 1047-1054.

Assunção, P., Rosales, R.S., Rifatbegovic, M., Antunes, N.T., de la Fe, C., Ruiz de Galarreta, C.M., Poveda, J.B., 2006. Quantification of mycoplasmas in broth medium with sybr green-I and flow cytometry. Front Biosci 11, 492-497.

Baccaro, M.R., Hirose, F., Umehara, O., Goncalves, L.C., Doto, D.S., Paixao, R., Shinya, L.T., Moreno, A.M., 2006. Comparative efficacy of two single-dose bacterins in the control of *Mycoplasma hyopneumoniae* in swine raised under commercial conditions in Brazil. Vet J 172, 526-531.

Baranyi, J., Roberts, T.A., 1995. Mathematics of predictive food microbiology. Int J Food Microbiol 26, 199-218.

Batista, L., Pijoan, C., Ruiz, A., Utrera, V., Dee, S., 2004. Assessment of transmission of *Mycoplasma hyopneumoniae* by personnel. J Swine Health Prod 12, 75-77.

Bayliss, C.D., Palmer, M.E., 2012. Evolution of simple sequence repeat-mediated phase variation in bacterial genomes. Ann N Y Acad Sci 1267, 39-44.

Bereiter, M., Young, T.F., Joo, H.S., Ross, R.F., 1990. Evaluation of the ELISA and comparison to the complement fixation test and radial immunodiffusion enzyme assay for detection of antibodies against *Mycoplasma hyopneumoniae* in swine serum. Vet Microbiol 25, 177-192.

Bin, L., Luping, D., Bing, S., Zhengyu, Y., Maojun, L., Zhixin, F., Yanna, W., Haiyan, W., Guoqing, S., Kongwang, H., 2014. Transcription analysis of the porcine alveolar macrophage response to *Mycoplasma hyopneumoniae*. PLoS One 9, e101968.

Bischof, D.F., Vilei, E.M., Frey, J., 2009. Functional and antigenic properties of GlpO from *Mycoplasma mycoides* subsp. *mycoides* SC: characterization of a flavin adenine dinucleotide-binding site deletion mutant. Vet Res 40, 35.

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

Borenstein, M., Hedges, L.V., Higgins, J.P.T., Rothstein, H.R., 2009. Introduction to meta-analysis, 1st ed. Wiley.

Browne, C., Loeffler, A., Holt, H.R., Chang, Y.M., Lloyd, D.H., Nevel, A., 2017. Low temperature and dust favour *in vitro* survival of *Mycoplasma hyopneumoniae*: time to revisit indirect transmission in pig housing. Lett Appl Microbiol 64, 2-7.

Burnett, T.A., Dinkla, K., Rohde, M., Chhatwal, G.S., Uphoff, C., Srivastava, M., Cordwell, S.J., Geary, S., Liao, X., Minion, F.C., Walker, M.J., Djordjevic, S.P., 2006. P159 is a proteolytically processed, surface adhesin of *Mycoplasma hyopneumoniae*: defined domains of P159 bind heparin and promote adherence to eukaryote cells. Mol Microbiol 60, 669-686.

Buttenschon, J., Friis, N.F., Aalbaek, B., Jensen, T.K., Iburg, T., Mousing, J., 1997. Microbiology and pathology of fibrinous pericarditis in Danish slaughter pigs. Zentralbl Veterinarmed A 44, 271-280.

Calsamiglia, M., Collins, J.E., Pijoan, C., 2000. Correlation between the presence of enzootic pneumonia lesions and detection of *Mycoplasma hyopneumoniae* in bronchial swabs by PCR. Vet Microbiol 76, 299-303.

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

Calsamiglia, M., Pijoan, C., Trigo, A., 1999. Application of a nested polymerase chain reaction assay to detect *Mycoplasma hyopneumoniae* from nasal swabs. J Vet Diagn Invest 11, 246-251.

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

Calus, D., Maes, D., Meyns, T., Pasmans, F., Haesebrouck, F., 2009. *In vivo* virulence of *Mycoplasma hyopneumoniae* isolates does not correlate with *in vitro* adhesion assessed by a microtitre plate adherence assay. J Appl Microbiol 106, 1951-1956.

Calus, D., Maes, D., Vranckx, K., Villareal, 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. J Microbiol Methods 83, 335-340.

Caruso, J.P., Ross, R.F., 1990. Effects of *Mycoplasma hyopneumoniae* and *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* infections on alveolar macrophage functions in swine. Am J Vet Res 51, 227-231.

Cattani, A.M., Siqueira, F.M., Guedes, R.L., Schrank, I.S., 2016. Repetitive elements in *Mycoplasma hyopneumoniae* transcriptional regulation. PLoS One 11, e0168626.

Cook, B.S., Beddow, J.G., Manso-Silvan, L., Maglennon, G.A., Rycroft, A.N., 2016. Selective medium for culture of *Mycoplasma hyopneumoniae*. Vet Microbiol 195, 158-164.

Crawley, A., Wilkie, B.N., 2003. Porcine Ig isotypes: function and molecular characteristics. Vaccine 21, 2911-2922.

Czaja, T., Kanci, A., Lloyd, L.C., Markham, P.F., Whithear, K.G., Browning, G.F., 2002. Induction of enzootic pneumonia in pigs by the administration of an aerosol of *in vitro*-cultured *Mycoplasma hyopneumoniae*. Vet Rec 150, 9-11.

Charlebois, A., Marois-Crehan, C., Helie, P., Gagnon, C.A., Gottschalk, M., Archambault, M., 2014. Genetic diversity of *Mycoplasma hyopneumoniae* isolates of abattoir pigs. Vet Microbiol 168, 348-356.

Choi, Y.K., Goyal, S.M., Joo, H.S., 2003. Retrospective analysis of etiologic agents associated with respiratory diseases in pigs. Can Vet J 44, 735-737.

Christensen, G., Sørensen, V., Mousing, J., 1999. Diseases of the respiratory system, In: Diseases of swine, 8th ed. Iowa State University Press, pp. 913-940.

Christopher, C.L., Chase, Lunney, J.K., 2012. Immune system, In: Diseases of swine, 10th ed. Wiley-Blackwell, pp. 227-347.

Damte, D., Lee, S.J., Hwang, M.H., Gebru, E., Choi, M.J., Lee, J.S., Cheng, H., Park, S.C., 2011. Inflammatory responses to *Mycoplasma hyopneumoniae* in murine alveolar macrophage cell lines. N Z Vet J 59, 185-190.

Davies, P.R., Bahnson, P.B., Grass, J.J., Marsh, W.E., Dial, G.D., 1995. Comparison of methods for measurement of enzootic pneumonia lesions in pigs. Am J Vet Res 56, 09-14.

Davis, J.K., Parker, R.F., White, H., Dziedzic, D., Taylor, G., Davidson, M.K., Cox, N.R., Cassell, G.H., 1985. Strain differences in susceptibility to murine respiratory mycoplasmosis in C57BL/6 and C3H/HeN mice. Infect Immun 50, 647-654.

De'ath, G., Fabricius, K.E., 2000. Classification and regression trees: a powerful yet simple technique for ecological data analysis. Ecology 81, 3178-3192.

De Castro, L.A., Rodrigues Pedroso, T., Kuchiishi, S.S., Ramenzoni, M., Kich, J.D., Zaha, A., Henning Vainstein, M., Bunselmeyer Ferreira, H., 2006. Variable number of tandem aminoacid repeats in adhesion-related CDS products in *Mycoplasma hyopneumoniae* strains. Vet Microbiol 116, 258-269.

DeBey, M.C., Ross, R.F., 1994. Ciliostasis and loss of cilia induced by *Mycoplasma hyopneumoniae* in porcine tracheal organ cultures. Infect Immun 62, 5312-5318.

Del Pozo Sacristán, 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. Vet J 194, 420-422.

Deutscher, A.T., Jenkins, C., Minion, F.C., Seymour, L.M., Padula, M.P., Dixon, N.E., Walker, M.J., Djordjevic, S.P., 2010. Repeat regions R1 and R2 in the P97 paralogue Mhp271 of *Mycoplasma hyopneumoniae* bind heparin, fibronectin and porcine cilia. Mol Microbiol 78, 444-458.

Diedershagen, M., Overbeck, S., Arlt, S., Plumakers, B., Lintges, M., Rink, L., 2007. *Mycoplasma arthritidis*-derived superantigen (MAM) displays DNase activity. FEMS Immunol Med Microbiol 49, 266-271.

Djordjevic, S.P., Eamens, G.J., Romalis, L.F., Nicholls, P.J., 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. Aust Vet J 75, 504-511.

Dos Santos, L.F., Sreevatsan, S., Torremorell, M., Moreira, M.A., Sibila, M., Pieters, M., 2015. Genotype distribution of *Mycoplasma hyopneumoniae* in swine herds from different geographical regions. Vet Microbiol 175, 374-381.

Escobar, J., Van Alstine, W.G., Baker, D.H., Johnson, R.W., 2002. Growth performance and whole-body composition of pigs experimentally infected with *Mycoplasma hyopneumoniae*. J Anim Sci 80, 384-391.

Etheridge, J.R., Cottew, G.S., Lloyd, L.C., 1979. Isolation of *Mycoplasma hyopneumoniae* from lesions in experimentally infected pigs. Aust Vet J 55, 356-359.

Fablet, C., Marois-Crehan, C., Simon, G., Grasland, B., Jestin, A., Kobisch, M., Madec, F., Rose, N., 2012. Infectious agents associated with respiratory diseases in 125 farrow-to-finish pig herds: a cross-sectional study. Vet Microbiol 157, 152-163.

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. Vet Microbiol 143, 238-245.

Fano, E., Pijoan, C., Dee, S., 2005. Dynamics and persistence of *Mycoplasma hyopneumoniae* infection in pigs. Can J Vet Res 69, 223-228.

Fano, E., Pijoan, C., Dee, S., Deen, J., 2007. Effect of *Mycoplasma hyopneumoniae* colonization at weaning on disease severity in growing pigs. Can J Vet Res 71, 195-200.

Fano, E., Pijoan, C., Dee, S., Deen, J., 2012. Longitudinal assessment of two *Mycoplasma hyopneumoniae* enzyme-linked immunosorbent assays in challenged and contact-exposed pigs. J Vet Diagn Invest 24, 383-387.

Feng, Z.X., Bai, Y., Yao, J.T., Pharr, G.T., Wan, X.F., Xiao, S.B., Chi, L.Z., 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. Vet J 202, 128-133.

Feng, Z.X., Wei, Y.N., Li, G.L., Lu, X.M., Wan, X.F., Pharr, G.T., Wang, Z.W., Kong, M., Gan, Y., Bai, F.F., Liu, M.J., Xiong, Q.Y., Wu, X.S., Shao, G.Q., 2013. Development and validation of an attenuated *Mycoplasma hyopneumoniae* aerosol vaccine. Vet Microbiol 167, 417-424.

Ferrarini, M.G., Siqueira, F.M., Mucha, S.G., Palama, T.L., Jobard, E., Elena-Herrmann, B., AT, R.V., Tardy, F., Schrank, I.S., Zaha, A., Sagot, M.F., 2016. Insights on the virulence of swine respiratory tract mycoplasmas through genome-scale metabolic modeling. BMC Genomics 17, 353.

Ferreira, H.B., Castro, L.A.d., 2007. A preliminary survey of *M. hyopneumoniae* virulence factors based on comparative genomic analysis. Genet Mol Biol 30, 245-255.

Fort, M., Mombarg, M., Balasch, M., Taylor, L., Urniza, A., 2013. Comparison of two different challenge procedures (transtracheal injection Vs. endotracheal inoculation) and two different inoculum preparations (lung homogenate Vs. fresh M. hyo. culture)

for the induction of *Mycoplasma hyopneumoniae* infection in young pigs, In: Proceedings of the 5th European Symposium of Porcine Health Management, Edinburgh, United Kingdom.

Fraile, L., Alegre, A., López-Jiménez, R., Nofrarias, M., Segalés, J., 2010. Risk factors associated with pleuritis and cranio-ventral pulmonary consolidation in slaughter-aged pigs. Vet J 184, 326-333.

Frey, J., Haldimann, A., Nicolet, J., 1992. Chromosomal heterogeneity of various *Mycoplasma hyopneumoniae* field strains. Int J Syst Bacteriol 42, 275-280.

Friis, N.F., 1974. *Mycoplasm suipneumoniae* and *Mycoplasma flocculare* in comparative pathogenicity studies. Acta Vet Scand 15, 507-518.

Friis, N.F., 1975. Some recommendations concerning primary isolation of *Mycoplasma suipneumoniae* and *Mycoplasma flocculare* a survey. Nord Vet Med 27, 337-339.

Furesz, S.E., Wilkie, B.N., Mallard, B.A., Rosendal, S., MacInnes, J.I., 1998. Anti-haemolysin IgG1 to IgG2 ratios correlate with haemolysin neutralization titres and lung lesion scores in *Actinobacillus pleuropneumoniae* infected pigs. Vaccine 16, 1971-1975.

Gomes Neto, J.C., Strait, E.L., Raymond, M., Ramirez, A., Minion, F.C., 2014. Antibody responses of swine following infection with *Mycoplasma hyopneumoniae*, *M. hyorhinis*, *M. hyosynoviae* and *M. flocculare*. Vet Microbiol 174, 163-171.

Goodwin, R.F., 1985. Apparent reinfection of enzootic-pneumonia-free pig herds: search for possible causes. Vet Rec 116, 690-694.

Goodwin, R.F., Pomeroy, A.P., Whittlestone, P., 1968. Attempts to recover *Mycoplasma suipneumoniae* from experimental and natural cases of enzootic pneumonia in pigs. J Hyg (Lond) 66, 595-603.

Goodwin, R.F.W., Pomeroy, A.P., Whittlestone, P., 1965. Production of enzootic pneumonia in pigs with a mycoplasma. Vet Rec 77, 1247.

Grosse 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*. Prev Vet Med 88, 255-263.

Guo-qing, S., Mao-jun, L., Pei-yuan, S., Ji-chun, W., Gai-mei, D., Yong-qi, Z., Dong-xia, L., 2007. The establishment of an experimental swine model of swine mycoplasma pneumonia. J Microb Infect 4, 011.

Hames, C., Halbedel, S., Hoppert, M., Frey, J., Stulke, J., 2009. Glycerol metabolism is important for cytotoxicity of *Mycoplasma pneumoniae*. J Bacteriol 191, 747-753.

Hannan, P.C., Bhogal, B.S., Fish, J.P., 1982. Tylosin tartrate and tiamutilin effects on experimental piglet pneumonia induced with pneumonic pig lung homogenate containing mycoplasmas, bacteria and viruses. Res Vet Sci 33, 76-88.

Hansen, M.S., Pors, S.E., Jensen, H.E., Bille-Hansen, V., Bisgaard, M., Flachs, E.M., Nielsen, O.L., 2010. An investigation of the pathology and pathogens associated with porcine respiratory disease complex in Denmark. J Comp Pathol 143, 120-131.

Harrison, F., 2011. Getting started with meta-analysis. Methods Ecol Evol 2, 1-10.

Hatch, T.F., 1961. Distribution and deposition of inhaled particles in respiratory tract. Bacteriol Rev 25, 237-240.

Hill, M.A., Scheidt, A.B., Teclaw, R.F., Clark, L.K., Knox, K.E., Jordan, M., 1992. Association between growth indicators and volume of lesions in lungs from pigs at slaughter. Am J Vet Res 53, 2221-2223.

Hintze, J., 2004. NCSS and PASS. Number cruncher statistical systems. http://www.NCSS.com.

Hirahara, K., Nakayama, T., 2016. CD4+ T-cell subsets in inflammatory diseases: beyond the Th1/Th2 paradigm. Int Immunol 28, 163-171.

Hsu, T., Minion, F.C., 1998. Molecular analysis of the P97 cilium adhesin operon of *Mycoplasma hyopneumoniae*. Gene 214, 13-23.

Hwang, M.H., Damte, D., Cho, M.H., Kim, Y.H., Park, S.C., 2010. Optimization of culture media of pathogenic *Mycoplasma hyopneumoniae* by a response surface methodology. J Vet Sci 11, 327-332.

Hwang, M.H., Damte, D., Lee, J.S., Gebru, E., Chang, Z.Q., Cheng, H., Jung, B.Y., Rhee, M.H., Park, S.C., 2011. *Mycoplasma hyopneumoniae* induces pro-inflammatory cytokine and nitric oxide production through NFkappaB and MAPK pathways in RAW264.7 cells. Vet Res Commun 35, 21-34.

Hwang, M.H., Lim, J.H., Yun, H.I., Kim, J.C., Jung, B.Y., Hsu, W.H., Park, S.C., 2006. The effect of polyclonal antibody on intracellular calcium increase induced by *Mycoplasma hyopneumoniae* in porcine tracheal cells. Vet J 172, 556-560.

Jenkins, C., Wilton, J.L., Minion, F.C., Falconer, L., Walker, M.J., Djordjevic, S.P., 2006. Two domains within the *Mycoplasma hyopneumoniae* cilium adhesin bind heparin. Infect Immun 74, 481-487.

Kadowaki, H., Suzuki, E., Kojima-Shibata, C., Suzuki, K., Okamura, T., Onodera, W., Shibata, T., Kano, H., 2012. Selection for resistance to swine mycoplasmal pneumonia over 5 generations in Landrace pigs. Livestock Sci 147, 20-26.

Katayama, M., Fukuda, T., Okamuara, T., Suda, Y., Suzuki, E., Uenishi, H., Suzuki, K., 2011. Immunophenotype characterization for swine selected line, which is resistant for the mycoplasma pneumonia. Asian Australas J Anim Sci 24, 889-897.

Kim, J., Chung, H.K., Chae, C., 2003. Association of *porcine circovirus 2* with porcine respiratory disease complex. Vet J 166, 251-256.

King, K.W., Faulds, D.H., Rosey, E.L., Yancey, R.J., Jr., 1997. Characterization of the gene encoding Mhp1 from *Mycoplasma hyopneumoniae* and examination of Mhp1's vaccine potential. Vaccine 15, 25-35.

Kobisch, M., Blanchard, B., Le Potier, M.F., 1993. *Mycoplasma hyopneumoniae* infection in pigs: duration of the disease and resistance to reinfection. Vet Res 24, 67-77.

Kobisch, M., Friis, N.F., 1996. Swine mycoplasmoses. Rev Sci Tech 15, 1569-1605.

Kobisch, M., Ross, R.F., 1996. Experimental infections of swine, In: Molecular and diagnostic procedures in mycoplasmology, 2nd ed. Academic Press, pp. 371-376.

König, H.E., Liebich, H.G., 2005. Aparato respiratorio, In: Anatomía de los animales domésticos: texto y altlas en color (volumen 2). Médica Panamericana, pp. 96-102.

Kristensen, B., Paroz, P., Nicolet, J., Wanner, M., de Weck, A.L., 1981. Cell-mediated and humoral immune response in swine after vaccination and natural infection with *Mycoplasma hyopneumoniae*. Am J Vet Res 42, 784-788.

Kurth, K.T., Hsu, T., Snook, E.R., Thacker, E.L., Thacker, B.J., Minion, F.C., 2002. Use of a *Mycoplasma hyopneumoniae* nested polymerase chain reaction test to determine the optimal sampling sites in swine. J Vet Diagn Invest 14, 463-469.

Lannek, N., Wesslen, T., Kikas, E., 1955. Histological examinations of tissue cultures inoculated with cytopathogenic agent from swine enzootic pneumonia. Acta Pathol Microbiol Scand 36, 343-351.

Le Carrou, J., Laurentie, M., Kobisch, M., Gautier-Bouchardon, A.V., 2006. Persistence of *Mycoplasma hyopneumoniae* in experimentally infected pigs after marbofloxacin treatment and detection of mutations in the parC gene. Antimicrob Agents Chemother 50, 1959-1966.

Leon, E.A., Madec, F., Taylor, N.M., Kobisch, M., 2001. Seroepidemiology of *Mycoplasma hyopneumoniae* in pigs from farrow-to-finish farms. Vet Microbiol 78, 331-341.

Li, Y.Z., Ho, Y.P., Chen, S.T., Chiou, T.W., Li, Z.S., Shiuan, D., 2009. Proteomic comparative analysis of pathogenic strain 232 and avirulent strain J of *Mycoplasma hyopneumoniae*. Biochemistry (Mosc) 74, 215-220.

Lin, J.H., Chen, S.P., Yeh, K.S., Weng, C.N., 2006. *Mycoplasma hyorhinis* in Taiwan: diagnosis and isolation of swine pneumonia pathogen. Vet Microbiol 115, 111-116.

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.

Loaiza-Echeverri, A.M., Bergmann, J.A., Toral, F.L., Osorio, J.P., Carmo, A.S., Mendonca, L.F., Moustacas, V.S., Henry, M., 2013. Use of nonlinear models for describing scrotal circumference growth in Guzerat bulls raised under grazing conditions. Theriogenology 79, 751-759.

Lorenzo, H., Quesada, O., Assunção, P., Castro, A., Rodríguez, F., 2006. Cytokine expression in porcine lungs experimentally infected with *Mycoplasma hyopneumoniae*. Vet Immunol Immunopathol 109, 199-207.

Madec, F., Kobisch, M., 1982. Bilan lesionnel des poumons de porcs charcutiers a l'abattoir. Journ Rech Porc Fr 14, 405-412.

Madsen, M.L., Puttamreddy, S., Thacker, E.L., Carruthers, M.D., Minion, F.C., 2008. Transcriptome changes in *Mycoplasma hyopneumoniae* during infection. Infect Immun 76, 658-663.

Maes, D., Segalés, J., Meyns, T., Sibila, M., Pieters, M., Haesebrouck, F., 2008. Control of *Mycoplasma hyopneumoniae* infections in pigs. Vet Microbiol 126, 297-309.

Maes, D., Verdonck, M., Deluyker, H., de Kruif, A., 1996. Enzootic pneumonia in pigs. Vet Q 18, 104-109.

Makhanon, M., Tummaruk, P., Thongkamkoon, P., Thanawongnuwech, R., Prapasarakul, N., 2012. Comparison of detection procedures of *Mycoplasma hyopneumoniae*, *Mycoplasma hyosynoviae*, and *Mycoplasma hyorhinis* in lungs, tonsils, and synovial fluid of slaughtered pigs and their distributions in Thailand. Trop Anim Health Prod 44, 313-318.

Marchioro, S.B. 2013. Immune responses following vaccination of pigs and mice against *Mycoplasma hyopneumoniae*, Ghent University.

Marchioro, S.B., Fisch, A., Gomes, C.K., Jorge, S., Galli, V., Haesebrouck, F., Maes, D., Dellagostin, O., Conceicao, F.R., 2014. 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. Vet Microbiol 173, 166-171.

Marchioro, S.B., Maes, D., Flahou, B., Pasmans, F., Del Pozo Sacristán, R., Vranckx, K., Melkebeek, V., Cox, E., Wuyts, N., Haesebrouck, F., 2013. Local and systemic immune

responses in pigs intramuscularly injected with an inactivated *Mycoplasma hyopneumoniae* vaccine. Vaccine 31, 1305-1311.

Maré, C.J., Switzer, W.P., 1965. New species: *Mycoplasma hyopneumoniae*; a causative agent of virus pig pneumonia. Vet Med Small Anim Clin 60, 841-846.

Marois, C., Dory, D., Fablet, C., Madec, F., Kobisch, 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. J Appl Microbiol 108, 1523-1533.

Marois, C., Le Carrou, J., Kobisch, M., Gautier-Bouchardon, A.V., 2007. Isolation of *Mycoplasma hyopneumoniae* from different sampling sites in experimentally infected and contact SPF piglets. Vet Microbiol 120, 96-104.

Marshall, R.J., 2001. The use of classification and regression trees in clinical epidemiology. J Clin Epidemiol 54, 603-609.

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. Vet Microbiol 168, 357-364.

Mateusen, B., Maes, D., Van Goubergen, M., Verdonck, M., de Kruif, A., 2002. Effectiveness of treatment with lincomycin hydrochloride and/or vaccination against *Mycoplasma hyopneumoniae* for controlling chronic respiratory disease in a herd of pigs. Vet Rec 151, 135-140.

Mattsson, J.G., Bergstrom, K., Wallgren, P., Johansson, K.E., 1995. Detection of *Mycoplasma hyopneumoniae* in nose swabs from pigs by *in vitro* amplification of the 16S rRNA gene. J Clin Microbiol 33, 893-897.

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

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

Mebus, C.A., Underdahl, N.R., 1977. Scanning electron microscopy of trachea and bronchi from gnotobiotic pigs inoculated with *Mycoplasma hyopneumoniae*. Am J Vet Res 38, 1249-1254.

Merialdi, G., Dottori, M., Bonilauri, P., Luppi, A., Gozio, S., Pozzi, P., Spaggiari, B., Martelli, P., 2012. Survey of pleuritis and pulmonary lesions in pigs at abattoir with a focus on the extent of the condition and herd risk factors. Vet J 193, 234-239.

Messier, S., Ross, R.F., Paul, P.S., 1990. Humoral and cellular immune responses of pigs inoculated with *Mycoplasma hyopneumoniae*. Am J Vet Res 51, 52-58.

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. Vet Microbiol 120, 87-95.

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. Prev Vet Med 66, 265-275.

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. Vet J 187, 388-392.

Michiels, A., Vranckx, K., Piepers, S., Del Pozo Sacristán, R., Arsenakis, I., Boyen, F., Haesebrouck, F., Maes, D., 2017. Impact of diversity of *Mycoplasma hyopneumoniae* strains on lung lesions in slaughter pigs. Vet Res 48, 2.

Minion, F.C., Jarvill-Taylor, K.J., Billings, D.E., Tigges, E., 1993. Membrane-associated nuclease activities in mycoplasmas. J Bacteriol 175, 7842-7847.

Minion, F.C., Lefkowitz, E.J., Madsen, M.L., Cleary, B.J., Swartzell, S.M., Mahairas, G.G., 2004. The genome sequence of *Mycoplasma hyopneumoniae* strain 232, the agent of swine mycoplasmosis. J Bacteriol 186, 7123-7133.

Moher, D., Liberati, A., Tetzlaff, J., Altman, D.G., 2009. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. Bmj 339, b2535.

Moorkamp, L., Hewicker-Trautwein, M., Grosse 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. Transbound Emerg Dis 56, 54-56.

Morris, C.R., Gardner, I.A., Hietala, S.K., Carpenter, T.E., 1995a. Enzootic pneumonia: comparison of cough and lung lesions as predictors of weight gain in swine. Can J Vet Res 59, 197-204.

Morris, C.R., Gardner, I.A., Hietala, S.K., Carpenter, T.E., Anderson, R.J., Parker, K.M., 1995b. Seroepidemiologic study of natural transmission of *Mycoplasma hyopneumoniae* in a swine herd. Prev Vet Med 21, 323-337.

Morrison, R.B., Hilley, H.D., Leman, A.D., 1985. Comparison of methods for assessing the prevalence and extent of pneumonia in market weight swine. Can Vet J 26, 381-384.

Mousing, J., Christensen, G., 1993. Pathological lesions in the right and left porcine lung: evaluation of an alternative method for scoring pneumonic lesions based on right lung examination. Acta Vet Scand 34, 151-158.

Muneta, Y., Minagawa, Y., Shimoji, Y., Nagata, R., Markham, P.F., Browning, G.F., Mori, Y., 2006. IL-18 expression in pigs following infection with *Mycoplasma hyopneumoniae*. J Interferon Cytokine Res 26, 637-644.

Muneta, Y., Minagawa, Y., Shimoji, Y., Ogawa, Y., Hikono, H., Mori, Y., 2008. Immune response of gnotobiotic piglets against *Mycoplasma hyopneumoniae*. J Vet Med Sci 70, 1065-1070.

Muneta, Y., Uenishi, H., Kikuma, R., Yoshihara, K., Shimoji, Y., Yamamoto, R., Hamashima, N., Yokomizo, Y., Mori, Y., 2003. Porcine TLR2 and TLR6: identification and their involvement in *Mycoplasma hyopneumoniae* infection. J Interferon Cytokine Res 23, 583-590.

Munoz, R., Yarza, P., Ludwig, W., Euzéby, J., Amann, R., Schleifer, K.H., Glöckner, F.O., Rosselló-Móra, R., 2011. Release LTPs104 of the all-species living tree. Syst Appl Microbiol 34, 169-170.

Muto, A., Ushida, C., 2002. Transcription and translation, In: Molecular biology and pathogenicity of mycoplasmas. Springer, pp. 323-345.

Mytilinaios, I., Salih, M., Schofield, H.K., Lambert, R.J., 2012. Growth curve prediction from optical density data. Int J Food Microbiol 154, 169-176.

Nathues, H., Chang, Y.M., Wieland, B., Rechter, G., Spergser, J., Rosengarten, R., Kreienbrock, L., Grosse 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. Transbound Emerg Dis 61, 316-328.

Nathues, H., Grosse 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. Vet Microbiol 152, 338-345.

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

Nathues, H., Woeste, H., Doehring, S., Fahrion, A.S., Doherr, M.G., Beilage, E., 2013. Herd specific risk factors for *Mycoplasma hyopneumoniae* infections in suckling pigs at the age of weaning. Acta Vet Scand 55, 30.

Noyes, E.P., Feeney, D.A., Pijoan, C., 1990. Comparison of the effect of pneumonia detected during lifetime with pneumonia detected at slaughter on growth in swine. J Am Vet Med Assoc 197, 1025-1029.

Nuntaprasert, A., Mori, Y., Fujita, K., Yoneda, M., Miura, R., Tsukiyama-Kohara, K., Kai, C., 2004. Characterization of specific antibodies and the establishment of sandwich ELISA and ELISPOT systems for swine IL-4. Comp Immunol Microbiol Infect Dis 27, 457-470.

Okada, M., Asai, T., Ono, M., Sakano, T., Sato, S., 2000. Cytological and immunological changes in bronchoalveolar lavage fluid and histological observation of lung lesions in pigs immunized with *Mycoplasma hyopneumoniae* inactivated vaccine prepared from broth culture supernate. Vaccine 18, 2825-2831.

Okamba, F.R., Arella, M., Music, N., Jia, J.J., Gottschalk, M., Gagnon, C.A., 2010. Potential use of a recombinant replication-defective adenovirus vector carrying the C-terminal portion of the P97 adhesin protein as a vaccine against *Mycoplasma hyopneumoniae* in swine. Vaccine 28, 4802-4809.

Opriessnig, T., Gimenez-Lirola, L.G., Halbur, P.G., 2011. Polymicrobial respiratory disease in pigs. Anim Health Res Rev 12, 133-148.

Opriessnig, T., Thacker, E.L., Yu, S., Fenaux, M., Meng, X.J., Halbur, P.G., 2004. Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by dual infection with *Mycoplasma hyopneumoniae* and *porcine circovirus type 2*. Vet Pathol 41, 624-640.

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. Vet Microbiol 145, 198-208.

Pabst, R., Binns, R.M., 1994. The immune system of the respiratory tract in pigs. Vet Immunol Immunopathol 43, 151-156.

Paisley, L.G., Vraa-Andersen, L., Dybkjaer, L., Moller, K., Christensen, G., Mousing, J., Agger, J.F., 1993. An epidemiologic and economic study of respiratory diseases in two conventional Danish swine herds. II: Associations between lesions present at slaughter

and mean daily gains during specific intervals of the growth period. Acta Vet Scand 34, 331-344.

Palzer, A., Ritzmann, M., Wolf, G., Heinritzi, K., 2008. Associations between pathogens in healthy pigs and pigs with pneumonia. Vet Rec 162, 267-271.

Pantoja, L.G., Pettit, K., Dos Santos, L.F., Tubbs, R., Pieters, M., 2016. *Mycoplasma hyopneumoniae* genetic variability within a swine operation. J Vet Diagn Invest 28, 175-179.

Park, S.C., Yibchok-Anun, S., Cheng, H., Young, T.F., Thacker, E.L., Minion, F.C., Ross, R.F., Hsu, W.H., 2002. *Mycoplasma hyopneumoniae* increases intracellular calcium release in porcine ciliated tracheal cells. Infect Immun 70, 2502-2506.

Peleg, M., Corradini, M.G., 2011. Microbial growth curves: what the models tell us and what they cannot. Crit Rev Food Sci Nutr 51, 917-945.

Peters, I.R., Helps, C.R., McAuliffe, L., Neimark, H., Lappin, M.R., Gruffydd-Jones, T.J., Day, M.J., Hoelzle, L.E., Willi, B., Meli, M., Hofmann-Lehmann, R., Tasker, S., 2008. RNase P RNA gene (rnpB) phylogeny of *Hemoplasmas* and other *Mycoplasma* species. J Clin Microbiol 46, 1873-1877.

Ph. Eur., 2013. Porcine enzootic pneumonia (inactivated) vaccine (monograph no. 04/2013:2448), In: European Pharmacopoeia 8.0, 8th ed, pp. 1001-1003.

Pieters, M., Fano, E., 2016. *Mycoplasma hyopneumoniae* management in gilts. Vet Rec 178, 122-123.

Pieters, M., Fano, E., Pijoan, C., Dee, S., 2010. An experimental model to evaluate *Mycoplasma hyopneumoniae* transmission from asymptomatic carriers to unvaccinated and vaccinated sentinel pigs. Can J Vet Res 74, 157-160.

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. Vet Microbiol 134, 261-266.

Pieters, M., Rovira, A., 2013. Comparison of various samples types for detection of *Mycoplasma hyopneumoniae* in recently infected pigs, In: Proceedings Allen D. Leman Swine Conference, St. Paul, MN, US.

Piffer, I.A., Ross, R.F., 1984. Effect of age on susceptibility of pigs to *Mycoplasma hyopneumoniae* pneumonia. Am J Vet Res 45, 478-481.

Pilo, P., Vilei, E.M., Peterhans, E., Bonvin-Klotz, L., Stoffel, M.H., Dobbelaere, D., Frey, J., 2005. A metabolic enzyme as a primary virulence factor of *Mycoplasma mycoides* subsp. *mycoides* small colony. J Bacteriol 187, 6824-6831.

Pinto, P.M., Chemale, G., de Castro, L.A., Costa, A.P., Kich, J.D., Vainstein, M.H., Zaha, A., Ferreira, H.B., 2007. Proteomic survey of the pathogenic *Mycoplasma hyopneumoniae* strain 7448 and identification of novel post-translationally modified and antigenic proteins. Vet Microbiol 121, 83-93.

Pinto, P.M., Klein, C.S., Zaha, A., Ferreira, H.B., 2009. Comparative proteomic analysis of pathogenic and non-pathogenic strains from the swine pathogen *Mycoplasma hyopneumoniae*. Proteome Sci 7, 45.

Pointon, A.M., Byrt, D., Heap, P., 1985. Effect of enzootic pneumonia of pigs on growth performance. Aust Vet J 62, 13-18.

Pollack, J.D., Williams, M.V., McElhaney, R.N., 1997. The comparative metabolism of mollicutes (mycoplasmas): the utility for taxonomic classification and the relationship of putative gene annotation and phylogeny to enzymatic function in the smallest free-living cells. Crit Rev Microbiol 23, 269-354.

R Core Team, 2016. R: A language and environment for statistical computing. https://www.R-project.org/.

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

Raymond, B.B.A., Turnbull , L., Whitchurch, C.B., Padula, M., Djordjevic, S.P., 2016. *Mycoplasma hyopneumoniae*: a recently discovered intracellular pathogen, In: Proceedings of the 4th VETPATH Conference on the Pathogenesis of Bacterial Diseases of Animals, Prato, Italy.

Raymond, C.R., Wilkie, B.N., 2004. Th-1/Th-2 type cytokine profiles of pig T-cells cultured with antigen-treated monocyte-derived dendritic cells. Vaccine 22, 1016-1023.

Razin, S., Glaser, G., Amikam, D., 1984. Molecular and biological features of mollicutes (mycoplasmas). Ann Microbiol (Paris) 135a, 9-15.

Razin, S., Yogev, D., Naot, Y., 1998. Molecular biology and pathogenicity of mycoplasmas. Microbiol Mol Biol Rev 62, 1094-1156.

Redondo, E., Masot, A.J., Fernández, A., Gázquez, A., 2009. Histopathological and immunohistochemical findings in the lungs of pigs infected experimentally with *Mycoplasma hyopneumoniae*. J Comp Pathol 140, 260-270.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. Am J Epidemiol 27, 493-497.

Ro, L.H., Ross, R.F., 1983. Comparison of *Mycoplasma hyopneumoniae* strains by serologic methods. Am J Vet Res 44, 2087-2094.

Rodríguez, F., Batista, M., Hernández, J.N., Afonso, A.M., Poveda, J.B., 2016. Relationship between expression of interleukin-5 and interleukin-13 by epithelial cells and bronchiolar changes in pigs infected with *Mycoplasma hyopneumoniae*. J Comp Pathol 154, 165-168.

Rodríguez, F., Quesada, O., Poveda, J.B., Fernández, A., Lorenzo, H., 2007. Immunohistochemical detection of interleukin-12 and interferon-gamma in pigs experimentally infected with *Mycoplasma hyopneumoniae*. J Comp Pathol 136, 79-82.

Rodríguez, F., Ramírez, G.A., Sarradell, J., Andrada, M., Lorenzo, H., 2004. Immunohistochemical labelling of cytokines in lung lesions of pigs naturally infected with *Mycoplasma hyopneumoniae*. J Comp Pathol 130, 306-312.

Roos, L.R., Fano, E., Homwong, N., Payne, B., Pieters, M., 2016. A model to investigate the optimal seeder-to-naïve ratio for successful natural *Mycoplasma hyopneumoniae* gilt exposure prior to entering the breeding herd. Vet Microbiol 184, 51-58.

Rose, D.L., Tully, J.G., Wittler, R.G., 1979. Taxonomy of some swine mycoplasmas: *Mycoplasma suipneumoniae* Goodwin *et al*. 1965, a later, objective synonym of *Mycoplasma hyopneumoniae* Maré and Switzer 1965, and the status of *Mycoplasma flocculare* Meyling and Friis, 1972. Int J Syst Evol Microbiol 29, 83-91.

Ross, R.F., Zimmermann-Erickson, B.J., Young, T.F., 1984. Characteristics of protective activity of *Mycoplasma hyopneumoniae* vaccine. Am J Vet Res 45, 1899-1905.

Rutz, S., Ouyang, W., 2016. Regulation of interleukin-10 expression. Adv Exp Med Biol 941, 89-116.

Sande, M.A., Zak, O., 1999. Handbook of animal models of infection: experimental models in antimicrobial chemotherapy. Elsevier

Sarradell, J., Andrada, M., Ramírez, A.S., Fernández, A., Gómez-Villamandos, J.C., Jover, A., Lorenzo, H., Herraez, P., Rodríguez, F., 2003. A morphologic and immunohistochemical study of the bronchus-associated lymphoid tissue of pigs naturally infected with *Mycoplasma hyopneumoniae*. Vet Pathol 40, 395-404.

Savic, B., Ivetic, V., Milicevic, V., Pavlovic, I., Zutic, M., Gagrcin, M., 2010. Genetic diversity of *Mycoplasma hyopneumoniae* isolates from conventional farrow-to-finish pig farms in Serbia. Acta Vet Hung 58, 297-308.

Scheidt, A.B., Mayrose, V.B., Hill, M.A., Clark, L.K., Cline, T.R., Knox, K.E., Runnels, L.J., Frantz, S., Einstein, M.E., 1990. Relationship of growth performance to pneumonia and atrophic rhinitis detected in pigs at slaughter. J Am Vet Med Assoc 196, 881-884.

Schmidt, J.A., Browning, G.F., Markham, P.F., 2007. *Mycoplasma hyopneumoniae* mhp379 is a $Ca^{(2+)}$-dependent, sugar-nonspecific exonuclease exposed on the cell surface. J Bacteriol 189, 3414-3424.

Seymour, L.M., Deutscher, A.T., Jenkins, C., Kuit, T.A., Falconer, L., Minion, F.C., Crossett, B., Padula, M., Dixon, N.E., Djordjevic, S.P., Walker, M.J., 2010. A processed multidomain *Mycoplasma hyopneumoniae* adhesin binds fibronectin, plasminogen, and swine respiratory cilia. J Biol Chem 285, 33971-33978.

Seymour, L.M., Falconer, L., Deutscher, A.T., Minion, F.C., Padula, M.P., Dixon, N.E., Djordjevic, S.P., Walker, M.J., 2011. Mhp107 is a member of the multifunctional adhesin family of *Mycoplasma hyopneumoniae*. J Biol Chem 286, 10097-10104.

Seymour, L.M., Jenkins, C., Deutscher, A.T., Raymond, B.B., Padula, M.P., Tacchi, J.L., Bogema, D.R., Eamens, G.J., Woolley, L.K., Dixon, N.E., Walker, M.J., Djordjevic, S.P., 2012. Mhp182 (P102) binds fibronectin and contributes to the recruitment of plasmin(ogen) to the *Mycoplasma hyopneumoniae* cell surface. Cell Microbiol 14, 81-94.

Sheldrake, R.F., Gardner, I.A., Saunders, M.M., Romalis, L.F., 1990. Serum antibody response to *Mycoplasma hyopneumoniae* measured by enzyme-linked immunosorbent assay after experimental and natural infection of pigs. Aust Vet J 67, 39-42.

Shen, Y., Hu, W., Wei, Y., Feng, Z., Yang, Q., 2017. Effects of *Mycoplasma hyopneumoniae* on porcine nasal cavity dendritic cells. Vet Microbiol 198, 1-8.

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 Vet Res 10, 165.

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. Vet Microbiol 127, 165-170.

Sibila, M., Calsamiglia, M., Segalés, J., Rosell, C., 2004. Association between *Mycoplasma hyopneumoniae* at different respiratory sites and presence of histopathological lung lesions. Vet Rec 155, 57-58.

Sibila, M., Fort, M., Nofrarias, 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. J Comp Pathol 147, 285-295.

Sibila, M., Nofrarias, M., López-Soria, S., Segalés, J., Riera, P., Llopart, D., Calsamiglia, M., 2007a. Exploratory field study on *Mycoplasma hyopneumoniae* infection in suckling pigs. Vet Microbiol 121, 352-356.

Sibila, M., Nofrarias, M., 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. Vet Microbiol 122, 97-107.

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

Simionatto, S., Marchioro, S.B., Maes, D., Dellagostin, O.A., 2013. *Mycoplasma hyopneumoniae*: from disease to vaccine development. Vet Microbiol 165, 234-242.

Siqueira, F.M., Thompson, C.E., Virginio, V.G., Gonchoroski, T., Reolon, L., Almeida, L.G., da Fonseca, M.M., de Souza, R., Prosdocimi, F., Schrank, I.S., Ferreira, H.B., de Vasconcelos, A.T., Zaha, A., 2013. New insights on the biology of swine respiratory tract mycoplasmas from a comparative genome analysis. BMC Genomics 14, 175.

Sitjar, M., Noyes, E.P., Simon, X., Pijoan, C., 1996. Relationship among seroconversion to *Mycoplasma hyopneumoniae*, lung lesions, and production parameters in pigs. Swine Health Prod 4, 273-277.

Sørensen, V., Ahrens, P., Barfod, K., Feenstra, A.A., Feld, N.C., Friis, N.F., Bille-Hansen, V., Jensen, N.E., Pedersen, M.W., 1997. *Mycoplasma hyopneumoniae* infection in pigs: duration of the disease and evaluation of four diagnostic assays. Vet Microbiol 54, 23-34.

Spellberg, B., Edwards, J.E., Jr., 2001. Type 1/Type 2 immunity in infectious diseases. Clin Infect Dis 32, 76-102.

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. Vet Microbiol 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. J Microbiol Methods 66, 263-275.

Stärk, K.D., 1999. The role of infectious aerosols in disease transmission in pigs. Vet J 158, 164-181.

Stärk, K.D., Miserez, R., Siegmann, S., Ochs, H., Infanger, P., Schmidt, J., 2007. A successful national control programme for enzootic respiratory diseases in pigs in Switzerland. Rev Sci Tech 26, 595-606.

Steenhard, N.R., Jungersen, G., Kokotovic, B., Beshah, E., Dawson, H.D., Urban, J.F., Jr., Roepstorff, A., Thamsborg, S.M., 2009. *Ascaris suum* infection negatively affects the

response to a *Mycoplasma hyopneumoniae* vaccination and subsequent challenge infection in pigs. Vaccine 27, 5161-5169.

Stemke, G.W., Laigret, F., Grau, O., Bove, J.M., 1992. Phylogenetic relationships of three porcine mycoplasmas, *Mycoplasma hyopneumoniae*, *Mycoplasma flocculare*, and *Mycoplasma hyorhinis*, and complete 16S rRNA sequence of *M. flocculare*. Int J Syst Bacteriol 42, 220-225.

Strait, E.L., Madsen, M.L., Minion, F.C., Christopher-Hennings, J., Dammen, M., Jones, K.R., Thacker, E.L., 2008. Real-time PCR assays to address genetic diversity among strains of *Mycoplasma hyopneumoniae*. J Clin Microbiol 46, 2491-2498.

Straw, B.E., Bäckström, L., Leman, A.D., 1986. Examination of swine at slaughter. Part II. Findings at slaughter and their significance. Compend Contin Educ Pract Vet 8, 106-112.

Straw, B.E., Shin, S.J., Yeager, A.E., 1990. Effect of pneumonia on growth rate and feed efficiency of minimal disease pigs exposed to *Actinobacillus pleuropneumoniae* and *Mycoplasma hyopneumoniae*. Prev Vet Med 9, 287-294.

Straw, B.E., Tuovinen, V.K., Bigras-Poulin, M., 1989. Estimation of the cost of pneumonia in swine herds. J Am Vet Med Assoc 195, 1702-1706.

Suter, M., Kobisch, M., Nicolet, J., 1985. Stimulation of immunoglobulin-containing cells and isotype-specific antibody response in experimental *Mycoplasma hyopneumoniae* infection in specific-pathogen-free pigs. Infect Immun 49, 615-620.

Switzer, W.P., 1973. Response of swine to *Mycoplasma hyopneumoniae* infection. J Infect Dis 127, Suppl:S59-60.

Tajima, M., Yagihashi, T., 1982. Interaction of *Mycoplasma hyopneumoniae* with the porcine respiratory epithelium as observed by electron microscopy. Infect Immun 37, 1162-1169.

Tajima, M., Yagihashi, T., Nunoya, T., Takeuchi, A., Ohashi, F., 1984. *Mycoplasma hyopneumoniae* infection in pigs immunosuppressed by thymectomy and treatment with antithymocyte serum. Am J Vet Res 45, 1928-1932.

Tamiozzo, P., Zamora, R., Lucchesi, P.M., Estanguet, A., Parada, J., Carranza, A., Camacho, P., Ambrogi, A., 2015. MLVA typing of *Mycoplasma hyopneumoniae* bacterins and field strains. Vet Rec Open 2, e000117.

Tassis, P.D., Papatsiros, V.G., Nell, T., Maes, D., Alexopoulos, C., Kyriakis, S.C., Tzika, E.D., 2012. Clinical evaluation of intradermal vaccination against porcine enzootic pneumonia (*Mycoplasma hyopneumoniae*). Vet Rec 170, 261.

Thacker, E.L., 2001. Immunology of the porcine respiratory disease complex. Vet Clin North Am Food Anim Pract 17, 551-565.

Thacker, E.L., 2004. Diagnosis of *Mycoplasma hyopneumoniae*. Anim Health Res Rev 5, 317-320.

Thacker, E.L., Halbur, P.G., Ross, R.F., Thanawongnuwech, R., Thacker, B.J., 1999. *Mycoplasma hyopneumoniae* potentiation of *porcine reproductive and respiratory syndrome virus*-induced pneumonia. J Clin Microbiol 37, 620-627.

Thacker, E.L., Minion, F.C., 2012. Mycoplasmosis, In: Diseases of swine, 10th ed. Wiley-Blackwell, pp. 779-797.

Thacker, E.L., Thacker, B.J., Boettcher, T.B., Jayappa, H., 1998. Comparison of antibody production, lymphocyte stimulation, and protection induced by four commercial *Mycoplasma hyopneumoniae* bacterins. J Swine Health Prod 6, 107-112.

Thacker, E.L., Thacker, B.J., Janke, B.H., 2001. Interaction between *Mycoplasma hyopneumoniae* and *swine influenza virus*. J Clin Microbiol 39, 2525-2530.

Thacker, E.L., Thacker, B.J., Kuhn, M., Hawkins, P.A., Waters, W.R., 2000. Evaluation of local and systemic immune responses induced by intramuscular injection of a *Mycoplasma hyopneumoniae* bacterin to pigs. Am J Vet Res 61, 1384-1389.

Thanawongnuwech, R., Thacker, B., Halbur, P., Thacker, E.L., 2004. Increased production of proinflammatory cytokines following infection with *porcine reproductive and respiratory syndrome virus* and *Mycoplasma hyopneumoniae*. Clin Diagn Lab Immunol 11, 901-908.

Thanawongnuwech, R., Thacker, E.L., 2003. Interleukin-10, interleukin-12, and interferon-gamma levels in the respiratory tract following *Mycoplasma hyopneumoniae* and PRRSV infection in pigs. Viral Immunol 16, 357-367.

Therneau, T., Atkinson, B., Ripley, B., 2015. rpart: recursive partitioning and regression trees. R package version 4.1-10. https://cran.r-project.org/web/packages/rpart/rpart.pdf.

Tomás, A., Fernándes, L.T., Valero, O., Segalés, J., 2008. A meta-analysis on experimental infections with *porcine circovirus type 2* (PCV2). Vet Microbiol 132, 260-273.

Underdahl, N.R., Kennedy, G.A., Ramos, A.S., Jr., 1980. Duration of *Mycoplasma hyopneumoniae* infection in gnotobiotic pigs. Can Vet J 21, 258-261.

Van Alstine, W.G., 2012. Respiratory System, In: Diseases of swine, 10th ed. Wiley-Blackwell, pp. 348-362.

Van Til, L.D., Dohoo, I.R., Morley, R.S., 1991. Epidemiological associations between *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* antibody titers and lung lesions in Prince Edward Island swine herds. Can J Vet Res 55, 347-351.

Vasconcelos, A.T., Ferreira, H.B., Bizarro, C.V., Bonatto, S.L., Carvalho, M.O., Pinto, P.M., Almeida, D.F., Almeida, L.G., Almeida, R., Alves-Filho, L., Assuncao, E.N., Azevedo, V.A., Bogo, M.R., Brigido, M.M., Brocchi, M., Burity, H.A., Camargo, A.A., Camargo, S.S., Carepo, M.S., Carraro, D.M., de Mattos Cascardo, J.C., Castro, L.A., Cavalcanti, G., Chemale, G., Collevatti, R.G., Cunha, C.W., Dallagiovanna, B., Dambros, B.P., Dellagostin, O.A., Falcao, C., Fantinatti-Garboggini, F., Felipe, M.S., Fiorentin, L., Franco, G.R., Freitas, N.S., Frias, D., Grangeiro, T.B., Grisard, E.C., Guimaraes, C.T., Hungria, M., Jardim, S.N., Krieger, M.A., Laurino, J.P., Lima, L.F., Lopes, M.I., Loreto, E.L., Madeira, H.M., Manfio, G.P., Maranhao, A.Q., Martinkovics, C.T., Medeiros, S.R., Moreira, M.A., Neiva, M., Ramalho-Neto, C.E., Nicolas, M.F., Oliveira, S.C., Paixao, R.F., Pedrosa, F.O., Pena, S.D., Pereira, M., Pereira-Ferrari, L., Piffer, I., Pinto, L.S., Potrich, D.P., Salim, A.C., Santos, F.R., Schmitt, R., Schneider, M.P., Schrank, A., Schrank, I.S., Schuck, A.F., Seuanez, H.N., Silva, D.W., Silva, R., Silva, S.C., Soares, C.M., Souza, K.R., Souza, R.C., Staats, C.C., Steffens, M.B., Teixeira, S.M., Urmenyi, T.P., Vainstein, M.H., Zuccherato, L.W., Simpson, A.J., Zaha, A., 2005. Swine and poultry pathogens: the complete genome sequences of two strains of *Mycoplasma hyopneumoniae* and a strain of *Mycoplasma synoviae*. J Bacteriol 187, 5568-5577.

Vicca, J., Stakenborg, T., Maes, D., Butaye, P., Peeters, J., de Kruif, A., Haesebrouck, F., 2003. Evaluation of virulence of *Mycoplasma hyopneumoniae* field isolates. Vet Microbiol 97, 177-190.

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 highly virulent *M. hyopneumoniae* isolate. Vaccine 27, 1875-1879.

Villarreal, I., Meyns, T., Dewulf, J., Vranckx, K., Calus, D., Pasmans, F., Haesebrouck, F., Maes, D., 2011. The effect of vaccination on the transmission of *Mycoplasma hyopneumoniae* in pigs under field conditions. Vet J 188, 48-52.

Villarreal, I., Vranckx, K., Calus, D., Pasmans, F., Haesebrouck, F., Maes, D., 2012. Effect of challenge of pigs previously immunised with inactivated vaccines containing homologous and heterologous *Mycoplasma hyopneumoniae* strains. BMC Vet Res 8, 2.

Virginio, V.G., Gonchoroski, T., Paes, J.A., Schuck, D.C., Zaha, A., Ferreira, H.B., 2014. Immune responses elicited by *Mycoplasma hyopneumoniae* recombinant antigens and DNA constructs with potential for use in vaccination against porcine enzootic pneumonia. Vaccine 32, 5832-5838.

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. J Clin Microbiol 49, 2020-2023.

Vranckx, K., Maes, D., Del Pozo Sacristán, R., Pasmans, F., Haesebrouck, F., 2012a. A longitudinal study of the diversity and dynamics of *Mycoplasma hyopneumoniae* infections in pig herds. Vet Microbiol 156, 315-321.

Vranckx, K., Maes, D., Marchioro, S.B., 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 Vet Res 8, 24.

Walker, J., Lee, R., Mathy, N., Doughty, S., Conlon, J., 1996. Restricted B-cell responses to microbial challenge of the respiratory tract. Vet Immunol Immunopathol 54, 197-204.

Wallgren, P., Beskow, P., Fellstrom, C., Renstrom, L.H., 1994. Porcine lung lesions at slaughter and their correlation to the incidence of infections by *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* during the rearing period. Zentralbl Veterinarmed B 41, 441-452.

Wilson, S., Van Brussel, L., Saunders, G., Runnels, P., Taylor, L., Fredrickson, D., Salt, J., 2013. Vaccination of piglets up to 1 week of age with a single-dose *Mycoplasma hyopneumoniae* vaccine induces protective immunity within 2 weeks against virulent challenge in the presence of maternally derived antibodies. Clin Vaccine Immunol 20, 720-724.

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 week of age with an inactivated *Mycoplasma hyopneumoniae* vaccine reduces lung lesions and improves average daily gain in body weight. Vaccine 30, 7625-7629.

Wilton, J., Jenkins, C., Cordwell, S.J., Falconer, L., Minion, F.C., Oneal, D.C., Djordjevic, M.A., Connolly, A., Barchia, I., Walker, M.J., Djordjevic, S.P., 2009. Mhp493 (P216) is a proteolytically processed, cilium and heparin binding protein of *Mycoplasma hyopneumoniae*. Mol Microbiol 71, 566-582.

Winsor, C.P., 1932. The Gompertz curve as a growth curve. Proc Natl Acad Sci USA 18, 1-8.

Wood, S., 2006. Generalized additive models: an introduction with R, 1st ed. Taylor & Francis.

Wood, S., 2011. Fast stable restricted maximum likelihood and marginal likelihood estimation of semiparametric generalized linear models. J R Stat Soc Series B Stat Methodol 73, 3-36.

Woolard, M.D., Hodge, L.M., Jones, H.P., Schoeb, T.R., Simecka, J.W., 2004. The upper and lower respiratory tracts differ in their requirement of IFN-gamma and IL-4 in controlling respiratory mycoplasma infection and disease. J Immunol 172, 6875-6883.

Woolley, L.K., Fell, S., Gonsalves, J.R., Walker, M.J., Djordjevic, S.P., Jenkins, C., Eamens, G.J., 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 isolates. Vet Microbiol 161, 186-195.

Woolley, L.K., Fell, S.A., Djordjevic, S.P., Eamens, G.J., Jenkins, C., 2013. Plasmin activity in the porcine airways is enhanced during experimental infection with *Mycoplasma hyopneumoniae*, is positively correlated with proinflammatory cytokine levels and is ameliorated by vaccination. Vet Microbiol 164, 60-66.

Woolley, L.K., Fell, S.A., Gonsalves, J.R., Raymond, B.B., Collins, D., Kuit, T.A., Walker, M.J., Djordjevic, S.P., Eamens, G.J., 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.

Yagihashi, T., Kazama, S., Tajima, M., 1993. Seroepidemiology of mycoplasmal pneumonia of swine in Japan as surveyed by an enzyme-linked immunosorbent assay. Vet Microbiol 34, 155-166.

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*. Vet Microbiol 98, 221-228.

Young, T.F., Thacker, E.L., Erickson, B.Z., Ross, R.F., 2000. A tissue culture system to study respiratory ciliary epithelial adherence of selected swine mycoplasmas. Vet Microbiol 71, 269-279.

Zhang, H., Lunney, J.K., Baker, R.B., Opriessnig, T., 2011. Cytokine and chemokine mRNA expression profiles in tracheobronchial lymph nodes from pigs singularly infected or coinfected with *porcine circovirus type 2* (PCV2) and *Mycoplasma hyopneumoniae* (MHYO). Vet Immunol Immunopathol 140, 152-158.

Zhang, H., Singer, B., 2010. Recursive partitioning and applications, 2nd ed. Springer.

Zhang, Q., Young, T.F., Ross, R.F., 1994. Glycolipid receptors for attachment of *Mycoplasma hyopneumoniae* to porcine respiratory ciliated cells. Infect Immun 62, 4367-4373.

Zhang, Q., Young, T.F., Ross, R.F., 1995. Identification and characterization of a *Mycoplasma hyopneumoniae* adhesin. Infect Immun 63, 1013-1019.

Zielinski, G.C., Ross, R.F., 1990. Effect of growth in cell cultures and strain on virulence of *Mycoplasma hyopneumoniae* for swine. Am J Vet Res 51, 344-348.

Zielinski, G.C., Ross, R.F., 1993. Adherence of *Mycoplasma hyopneumoniae* to porcine ciliated respiratory tract cells. Am J Vet Res 54, 1262-1269.