Universitat Autònoma de Barcelona

Expanding knowledge on Mycoplasma hyopneumoniae gilt acclimation, vaccination and genetic variability

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Tesi doctoral presentada per **Laura Garza Moreno** per accedir al grau de Doctor 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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Que la memòria titulada "**Expanding knowledge on *Mycoplasma hyopneumoniae* gilt acclimation, vaccination and genetic variability**" presentada per **Laura Garza Moreno** per a l' obtenció del grau de Doctor en Medicina i Sanitat Animals, s'ha realitzat sota la seva supervisió i tutoria, i autoritzen la seva presentació per tal de que sigui valorada per la comissió establerta

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LIST OF ABBREVIATIONS

A. pleuropneumoniae: Actinobacillus pleuropneumoniae

A: Adenine

ADWG: Average daily weight gain

AFLP: Amplified fragment length polymorphism

AIAO: All-in-all-out

AP-PCR: Arbitrary primed PCR

ATCC: American Type Culture Collection

BALF: Broncho-alveolar lavage fluid

C: Cytosine

CF: Continuous flow

CLP: Ceva Lung Program

Ct: Cycle threshold

CVPC: Cranio-ventral pulmonary consolidation

DEAE-dextran: Diethylaminoethyl-dextran

DNA: Deoxyribonucleic acid

Dpi: Days post-infection

DSU: detection and synchronization unit

ELISA: Enzyme-linked immunosorbent assay

EP: Enzootic pneumonia

EP-index: Enzootic pneumonia index

FS: Previous batches of own replacement

G: Guanine

GDU: Gilt development unit

H. parasuis: Haemophilus parasuis

Hk: Housekeeping genes

IFN: Interferon

IL: Interleukin

M. flocculare: Mycoplasma flocculare

M. hyopneumoniae: Mycoplasma hyopneumoniae

M. hyorhinis: Mycoplasma hyorhinis

M. hyosynoviae: Mycoplasma hyosynoviae

MLST: Multilocus sequence typing

MLVA: Multilocus variable number tandem repeat analysis

MP: Mycoplasmal pneumonia

NA: Non-applicable

NR: No reported

NV: Non-vaccinated

OD: Optical density

PAM: Porcine alveolar macrophages

PBS: Phosphate buffer saline

PCR: polymerase chain reaction

PCR-RFLP: PCR combined with restricted fragments length polymorphism

PCV-2: *Porcine circovirus type 2*

PFGE: Pulsed-field gel electrophoresis

PI: Percentage of inhibition

PK-15: Porcine kidney epithelial cells

PRDC: Porcine respiratory disease complex

PRRS: *Porcine reproductive and respiratory syndrome virus*

RAPD: Random amplified polymorphic DNA

R_n: Reproduction ratio

RR: Repeat region

Rt-PCR: real time-PCR

SD: Standard deviation

SIV: *Swine influenza virus*

T: Thymine

TAE: Tris-Acetate-EDTA

TNF: Tumor necrosis factor

TP: Typing profile

V: Vaccinated

VNTR: Variable number of tandem repeats

WG: Whole genome

wpe: Weeks post entry

SUMMARY

Despite several measures have been used for controlling *Mycoplasma hyopneumoniae (M. hyopneumoniae)*, this respiratory bacterium is still causing important economic losses to the swine industry worldwide, mainly at growing and finishing periods. The severity of respiratory problems at those mentioned production stages has been proposed to be linked with the *M. hyopneumoniae* prevalence at weaning. Currently, replacements are considered the main source of *M. hyopneumoniae* infection for their offspring. The control of *M. hyopneumoniae* within herds by means of an adequate gilt acclimation against this bacterium has been suggested as key to control the infection. Therefore, the aim of the present Thesis was to expand the knowledge about the current gilt acclimation strategies carried out in Europe and the effect of the most common one (vaccination) on replacements and their piglets at weaning. Furthermore, the presence of several *M. hyopneumoniae* strains has been suggested to be associated with more severe respiratory problems in pigs. Therefore, *M. hyopneumoniae* genetic variability in non-vaccinated and vaccinated pigs showing *M. hyopneumoniae*-like lung lesions, as well as in the vaccines used in each farm, were also explored.

The purpose of the first study was to identify the current gilt acclimation strategies against *M. hyopneumoniae* performed in European pig farms. To reach that goal, a questionnaire focused on different features of the recipient herd, incoming replacements and acclimation process, was designed. This survey was distributed among the European swine veterinarians and it was voluntarily responded. Results from this survey indicated *M. hyopneumoniae* is still a concern for the European swine industry. Interestingly, most respondents introduced seropositive replacements into the herds. However, gilt shedding status was unknown by a majority of them. Gilt acclimation against *M.*

hyopneumoniae were performed by most respondents. Further, obtained results would indicate that the most common strategy used for *M. hyopneumoniae* gilt acclimation was vaccination based on multiple doses programs. However, a minority of the participants verified the effect of the acclimation process used.

In order to assess the effect of vaccination against *M. hyopneumoniae* during the acclimation period, different gilt vaccination schedules were evaluated in the second study of this Thesis. One hundred eighty *M. hyopneumoniae* naïve gilts were selected at the acclimation unit in a *M. hyopneumoniae* positive farm. Gilts were distributed according to the vaccination program into three groups: (A) four doses of a commercial vaccine against *M. hyopneumoniae* at 2, 4, 6 and 8 weeks post entry (wpe) into acclimation unit, (B) two vaccine doses at 2 and 6, and two doses of phosphate-buffered saline (PBS) at 4 and 8 wpe, and (C) four doses of PBS at the same time points. Laryngeal swabs to assess the *M. hyopneumoniae* shedding and bacterium variability, and blood samples for the evaluation of humoral immunity, were taken from gilts at 1 (at entry), 14 (after acclimation), 27 (pregnancy confirmation) and 34 wpe (weeks prior farrowing). Also, laryngeal swabs and blood from six of their piglets at weaning were obtained. Vaccinated groups (A and B) showed significantly lower proportion of shedding gilts compared to the non-vaccinated group after acclimation (14 to 34 wpe). In addition, lower *M. hyopneumoniae* genetic variability was detected in the monitored gilt batch compared with two previous studied batches. Significantly higher levels of antibodies (lower values of percentage of inhibition [PI] by a competitive ELISA) were detected in vaccinated gilts compared to non-vaccinated ones throughout the study. However, no significant differences were found between vaccination programs with four or two doses in terms of ELISA PI values. Regarding piglets at weaning, they were *M. hyopneumoniae* negative by real time PCR (rt-PCR) independently of gilt vaccination program

administered. In contrast, the proportion of seropositive piglets and levels of antibodies were higher in those coming from vaccinated groups. The present work represents the first attempt to demonstrate that gilt vaccination against *M. hyopneumoniae* can be an effective tool to reduce the infectious pressure and bacterium variability into replacement batches, as well as provide a long-term humoral immunity to gilts and their offspring.

The third study aimed to evaluate the *M. hyopneumoniae* genetic variability in non-vaccinated and vaccinated pigs showing *M. hyopneumoniae*-like lung lesions at slaughterhouses, as well as in the vaccines used in each vaccinated farm. To achieve this objective, ten vaccinated and ten non-vaccinated fattening farms with clinical respiratory problems associated to *M. hyopneumoniae* were selected. At slaughterhouse, *M. hyopneumoniae*-like lung lesions from one batch per farm were scored and the three lungs showing the most extensive lesion from each batch were collected for *M. hyopneumoniae* detection. In addition, the genetic variability of commercial vaccines against *M. hyopneumoniae* (used in vaccinated farms) was also assessed. Positive samples were genotyped through sequencing by counting the variable number of tandem repeats (VNTR) of two (P97, P146) or four loci (P97, P146, H1 and H5). A *M. hyopneumoniae* typing profile (TP) was assigned to a unique combination of the VNTR of these two or four loci. High number of TPs were found in slaughtered pigs coming from non-vaccinated and vaccinated farms using two and four loci. Interestingly, the higher the number of tested loci, the higher the variability. Despite this, only one TP was detected per lung with *M. hyopneumoniae*-like lesions and the TPs detected in pigs from vaccinated farms were different from the strain of the corresponding vaccine used. Based on these results, this study describes for the first time the *intra* and *inter*-farm *M. hyopneumoniae* genetic variability among non-vaccinated and vaccinated fattening farms in Spain, and their comparison with commercial vaccines and

vaccinated farm. Furthermore, this study provides novel and interesting data for future discussion regarding the influence of the number of loci tested on the *M. hyopneumoniae* genetic variability.

In summary, from the studies included in this Thesis, it was concluded that gilt acclimation against *M. hyopneumoniae* was a common strategy carried out in Europe, and the vaccination is the main method used. In fact, gilt vaccination against *M. hyopneumoniae* reduced the bacterial gilt shedding and provided humoral immunity to gilts that was subsequently transferred to their piglets. Moreover, *M. hyopneumoniae* genetic variability was detected within replacement batches of the same farm, slaughtered pigs with different vaccination status and bacterins used in vaccinated tested farms.

RESUMEN

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) sigue siendo un patógeno respiratorio causante de importantes pérdidas económicas en la industria porcina de todo el mundo, principalmente durante los períodos de crecimiento y cebo. La gravedad de estos problemas respiratorios se ha asociado con la prevalencia de *M. hyopneumoniae* al destete. Hoy en día, las primíparas se consideran la principal fuente de infección por *M. hyopneumoniae* para sus lechones. Por tanto, se considera que el control de *M. hyopneumoniae* mediante una adecuada adaptación de la reposición frente a esta bacteria es clave. De este modo, el objetivo de esta Tesis fue ampliar el conocimiento sobre las estrategias de adaptación de la reposición utilizadas en Europa en la actualidad, así como evaluar el efecto de la estrategia más común (la vacunación) en la reposición y sus lechones al destete. Además, la presencia de diferentes cepas de *M. hyopneumoniae* también se ha relacionado con problemas respiratorios más graves en cerdos de engorde. Por tanto, también se investigó la variabilidad genética de *M. hyopneumoniae* en cerdos no vacunados y vacunados que presentaban lesiones pulmonares asociadas a *M. hyopneumoniae* en matadero, así como en las vacunas usadas en cada granja.

El objetivo del primer estudio consistió en identificar las estrategias actuales de adaptación de la reposición frente *M. hyopneumoniae* utilizadas en las granjas porcinas de Europa. Para alcanzar dicho objetivo, se diseñó un cuestionario centrado en las diferentes características de la explotación, la reposición y el proceso de adaptación. Esta encuesta fue distribuida entre veterinarios porcinos europeos, colectándose finalmente información sobre 321 granjas. Los resultados de esta encuesta indicaron que *M. hyopneumoniae* sigue siendo una preocupación para la industria porcina europea representada en esta encuesta. Curiosamente, la mayoría de los encuestados introducían reposición

seropositiva en las explotaciones, aunque la mayoría de ellos desconocía el estado de infección de la misma. No obstante, la mayoría de los encuestados respondió que realizaba la adaptación de la reposición frente a *M. hyopneumoniae*. En particular, los resultados obtenidos indicaron que la vacunación frente *M. hyopneumoniae* con múltiples dosis, fue la estrategia más utilizada, sola o en combinación con otros métodos, para la adaptación de la reposición frente a este patógeno en Europa. Solo una minoría de los participantes verificó el efecto del proceso de adaptación utilizado.

Con el fin de conocer el efecto de la vacunación de la reposición frente a *M. hyopneumoniae* en la adaptación, se evaluaron diferentes programas vacunales en el segundo estudio de esta Tesis. Para ello, se seleccionaron 180 cerdas negativas frente a *M. hyopneumoniae* en la unidad de adaptación de una granja positiva a *M. hyopneumoniae*. Las nulíparas se distribuyeron según el programa vacunal en tres grupos: (A) cuatro dosis de una vacuna comercial contra *M. hyopneumoniae* a las 2, 4, 6 y 8 semanas tras la entrada (spe) en la unidad de adaptación, (B) dos dosis de vacuna a las 2 y 6, y dos inyecciones de solución salina tamponada con fosfato (PBS) a las 4 y 8 spe, y (C) cuatro dosis de PBS a los mismos tiempos. Se tomaron hisopos laríngeos para evaluar la excreción de *M. hyopneumoniae* y su variabilidad genética, y muestras de sangre para la evaluación de la inmunidad humoral de las nulíparas a las spe 1 (entrada), 14 (después de la adaptación), 27 (confirmación de gestación) y 34 (semana previa al parto). Además, se tomaron hisopos laríngeos y sangre de seis de sus lechones al destete. Los grupos vacunados (A y B) mostraron una proporción significativamente más baja de nulíparas excretoras en comparación con el grupo no vacunado después de la adaptación (de las spe 14 a 34). Además, se detectó una menor variabilidad genética de *M. hyopneumoniae* en el lote de nulíparas monitorizadas en comparación con dos lotes estudiados previamente. Los niveles de anticuerpos fueron significativamente más altos (medido a través

de porcentaje de inhibición [PI] de un ELISA competitivo) en nulíparas vacunadas que en las no vacunadas durante todo el estudio. Sin embargo, no se encontraron diferencias significativas entre los protocolos de vacunación con cuatro o dos dosis en términos de valores de ELISA (PI). Respecto a los lechones al destete, todos fueron negativos a *M. hyopneumoniae* por PCR en tiempo real, independientemente del programa de vacunación administrado a sus madres. Por otro lado, la proporción de lechones seropositivos y los niveles de anticuerpos fueron mayores en los lechones provenientes de los grupos vacunados. Este estudio demostró por primera vez que la vacunación de la reposición frente a *M. hyopneumoniae* puede ser una herramienta eficaz para reducir la presión de infección y la variabilidad de *M. hyopneumoniae* en lotes de reposición, así como proporcionar una inmunidad humoral a largo plazo para la reposición y sus lechones.

El tercer estudio de esta Tesis tuvo como objetivo evaluar la variabilidad genética de *M. hyopneumoniae* en cerdos no vacunados y vacunados que presentaban lesiones pulmonares asociadas a *M. hyopneumoniae* en los mataderos. Asimismo, se evaluó la variabilidad genética de las vacunas comerciales frente a *M. hyopneumoniae* utilizadas en las granjas vacunadas. Para lograr este objetivo, se seleccionaron diez granjas de cerdos de engorde vacunadas y diez no vacunadas, todas ellas con problemas respiratorios clínicos asociados a *M. hyopneumoniae*. Una vez en el matadero, se evaluaron las lesiones pulmonares compatibles con *M. hyopneumoniae* de un lote por granja y se recogieron muestras de los tres pulmones con la lesión más extensa. Los pulmones positivos a *M. hyopneumoniae* por PCR se genotiparon mediante secuenciación contando el número variable de repeticiones en tándem (del inglés, *variable number of tandem repeats*, VNTR) en dos (P97, P146) o cuatro genes (P97, P146, H1 y H5). Posteriormente se asignó un perfil de tipificación (TP) de *M. hyopneumoniae* a la combinación única de VNTR de estos dos o

cuatro genes. Se detectaron varios TPs en las granjas no vacunadas y vacunadas, tanto cuando se utilizaron cuatro o dos genes. Cuanto mayor fue el número de genes estudiados, mayor variabilidad. A pesar de esto, solo se detectó un TP por pulmón y los TPs detectados en cerdos de granjas vacunadas fueron diferentes de la cepa vacunal utilizada en la granja. Este estudio describe por primera vez la variabilidad genética de *M. hyopneumoniae* dentro y entre- granjas de engorde no vacunadas y vacunadas en España. Además, los resultados confirmarían que la cepa presente en las granjas vacunas es diferente a la de la vacuna comercial utilizada. Además, este estudio proporciona datos novedosos e interesantes sobre el efecto del número de genes analizados sobre la variabilidad genética de *M. hyopneumoniae*, dando pie a futuras discusiones.

En resumen, a partir de los estudios incluidos en esta Tesis, se puede concluir que la adaptación de la reposición frente a *M. hyopneumoniae* es una estrategia comúnmente realizada en Europa, y que la vacunación es el método más utilizado para dicha adaptación. De hecho, los resultados de esta Tesis mostraron que la vacunación de la reposición frente a *M. hyopneumoniae* puede ser una herramienta eficaz para reducir la excreción bacteriana y proporcionar inmunidad humoral a las nulíparas y los lechones. Por último, la variabilidad genética de *M. hyopneumoniae* se detectó en lotes de reposición, así como en cerdos sacrificados con lesiones asociadas a *M. hyopneumoniae* provenientes de granjas de engorde con diferentes estados de vacunación.

PUBLICATIONS

Scientific outcomes derived from this Thesis have been published or submitted for publication in international scientific peer-reviewed journals:

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

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

- Garza-Moreno, L., Pieters, M., López-Soria, S., Carmona, M., Krejci, R., Segalés, J., Sibila, M. Comparison of vaccination protocols against *Mycoplasma hyopneumoniae* during the gilt acclimation period. Veterinary Microbiology, 2019, 229, 7–13.

- Garza-Moreno, L., Segalés, J., Aragón, V., Correa-Fiz, F., Pieters, M., Carmona, M., Krejci, R., Sibila, M. Characterization of *Mycoplasma hyopneumoniae* strains in vaccinated and non-vaccinated pigs from Spanish slaughterhouses. Under revision.

CHAPTER 1

General Introduction

Mycoplasmas belong to the class Mollicutes, a group of bacteria characterized by a complete lack of cell wall and, thus, by their resistance to antimicrobials targeting it, such as β-lactams (Razin *et al.,* 1998; Thacker and Minion, 2012). Nowadays, mycoplasmas are the smallest prokaryote known. Their cytoplasm is surrounded by a plasma membrane with a variety of morphologies predominantly spherical (Razin *et al.,* 1998). Although important differences between mycoplasmas and other bacteria have been described in the literature, it is generally accepted that mycoplasmas and gram-positive bacteria have a common ancestor, as demonstrated by the similarities between transcription and translation mechanisms (Muto and Ushida, 2002).

The mycoplasma genome is also the smallest known one (580-2,220 kb) for self-replicating organisms. The low guanine (G) and cytosine (C) contents (23-40%) of the genome and the high levels of adenine (A) and thymine (T) suggest biased mutation pressure from G+C to A+T pairs, which would be related to DNA replication and repairing system (Muto and Ushida, 2002). The fact that only a part of that genome is used for biosynthetic pathways means that mycoplasmas require a direct association with the mammalian host for the synthesis of mycoplasmal structures during bacterial multiplication. However, mycoplasmas are also able to grow under *in vitro* conditions using supplemented media with proteins, cholesterol and certain fatty acids, among others nutrients. These nutritional elements are usually provided by the addition of serum to the culture media (Razin *et al.,* 1998). Nevertheless, the *in vitro* growth of the majority of mycoplasmas is still considered complex and further research into culture and isolation methods is needed (Razin *et al.,* 1998; Thacker and Minion, 2012).

These microorganisms are highly host specific and, although they can be present in different tissues, mycoplasmas have affinity for mucosal surfaces

(Muto and Ushida, 2002). Different mycoplasmal species have been detected in swine, being *Mycoplasma hyopneumoniae (M. hyopneumoniae*) the most relevant one for the swine industry. This bacterium causes an important chronic respiratory disease in pigs leading to significant economic losses (Opriessnig *et al.,* 2011; Thacker and Minion, 2012). Another porcine mycoplasma frequently detected in the upper respiratory tract of young pigs is *Mycoplasma hyorhinis* (*M. hyorhinis;* Ross and Young, 1993; Thacker and Minion, 2012). However, it is not considered as a primary pathogen able to cause pneumonia (Opriessnig *et al.,* 2011; Luehrs *et al.,* 2017). Besides being an upper tract colonizer, *M. hyorhinis* can produce polyserositis and arthritis, generally in nursery pigs (Thacker and Minion, 2012). Similarly, *Mycoplasma hyosynoviae* (*M. hyosynoviae)* can be detected in the oral and nasal cavity of healthy animals, persisting in the tonsils of carrier pigs, as well as in synovial fluids in cases of arthritis in growing-fattening pigs (Whittlestone *et al.,* 1979; Nielsen *et al.,* 2005). Other mycoplasmas, such as *Mycoplasma flocculare (M. flocculare)*, are considered commensal microorganisms due to their assumed non-pathogenic capability (Thacker and Minion, 2012). The importance of *M. flocculare* is related to its similarity with *M. hyopneumoniae* at genomic, transcriptomic, proteomic and antigenic level (Gómez-Neto *et al.,* 2014; Siqueira *et al.,* 2013,2014). The fact that common antigenic proteins have been detected in both *M. hyopneumoniae* and *M. flocculare* implies a significant cross-reactivity among both mycoplasmas and it could interfere in the reliability of results in diagnostic antibody-based assays (Freeman *et al.,* 1984; Bereiter and Young, 1990; Gómez-Neto *et al.,* 2014). Finally, infections with *Mycoplasma suis* have been associated with anaemia that affects mainly weaned and feeder pigs as well as sows before or immediately after farrowing (Hoelzle *et al.,* 2014).

1.1. *Mycoplasma hyopneumoniae*

1.1.1. Characteristics of *M. hyopneumoniae*

Mycoplasma hyopneumoniae is one of the smallest bacterial species included under genus *Mycoplasma* (Tajima and Yagihashi, 1982). This bacterium shows an oval morphology both under *in vitro* and *in vivo* scenarios (Figure 1-1; Blanchard *et al.,* 1992; Tajima and Yagihashi, 1982). Moreover, under *in* vivo conditions, *M. hyopneumoniae* seems to possess radial fibrils on its membrane that facilitate the adhesion to epithelial cells and/or the interconnection between other mycoplasmal cells (Tajima and Yagihashi, 1982).

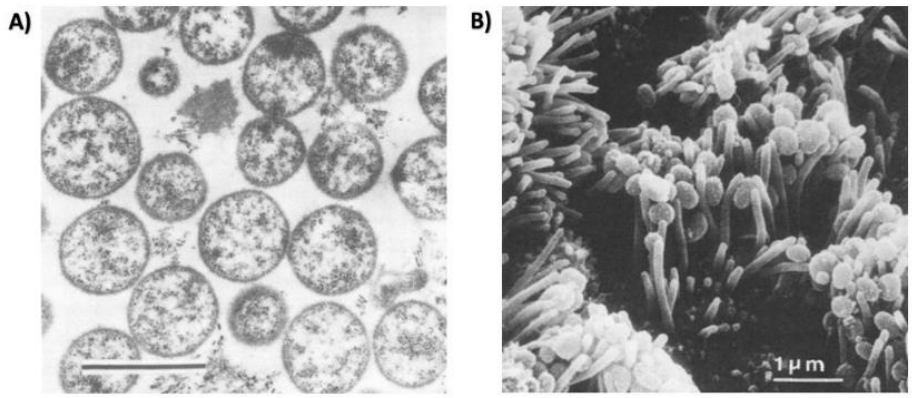

Figure 1-1. Scanning electron microscopy images of *M. hyopneumoniae.* **A)** Culture of VPP 11 strain (Tajima and Yagihashi, 1982). **B)** Trachea surface at 2 weeks post-inoculation where *M. hyopneumoniae* appears closely associated with the upper part of cilia, bar=1µm (Blanchard *et al.,* 1992).

The first reports of *M. hyopneumoniae* isolation were performed by Mare and Switzer (1965) in the United States (USA) and by Goodwin (1965) in the United Kingdom. In these reports, the two strains known as strain 11 (Mare and Switzer, 1965) and strain J (Goodwin, 1965) were isolated from pneumonic lungs. While strain 11 is still considered as pathogenic, strain J was recognized

as non-pathogenic after several *in vitro* passages, due to the fact that lung lesions were not observed after its inoculation (Bereiter *et al.,* 1990). Anyway, these two strains are still considered as reference strains and several current commercial bacterins to control *M. hyopneumoniae* infections are based on them (Garza-Moreno *et al.,* 2018; Maes *et al.,* 2018).

As most mycoplasmas, the isolation and culture of *M. hyopneumoniae* under *in vitro* conditions are complicated due to its slow growth and the potential contaminations with other bacteria or mycoplasmas such as *M. hyorhinis*, frequently present in the pig respiratory tract (Kobisch and Friis, 1996; Thacker and Minion, 2012). Moreover, *M. hyopneumoniae* requires a highly enriched media known as the Friis medium (Friis, 1975), which is supplemented with serum (Goodwin *et al.,* 1965) and kanamycin to prevent the overgrowth of *M. hyorhinis* (Friis, 1971; Williams, 1978). Generally, growing of *M. hyopneumoniae* from clinical samples is firstly attempted in Friis liquid media (Figure 1-2), requiring a period of 3-10 days for color media shift from pink (phenol red) to yellow as well as producing certain turbidity (McAuliffe *et al.,* 2006; Thacker and Minion, 2012) or not (Assunção *et al.,* 2005; Cook *et al.,* 2016). Afterwards, *M. hyopneumoniae* in liquid media can be transferred to agar for growth and colony visualization (Figure 1-2). The solid media used is Friis medium with agar, but previous studies suggested that such agar may inhibit the *M. hyopneumoniae* growth by sequestering essential nutrients (Maglennon *et al.,* 2013). This fact implies that a high and purified bacterial concentration is required for the culture of *M. hyopneumoniae* in solid media. Recently, an improvement of the solid media using purified agar treated with diethylaminoethyl (DEAE)-dextran and kanamycin (2 µg/mL) has been proposed, and colonies have been detected after 8 days of inoculation (Figure 1-2, Cook *et al.,* 2016).

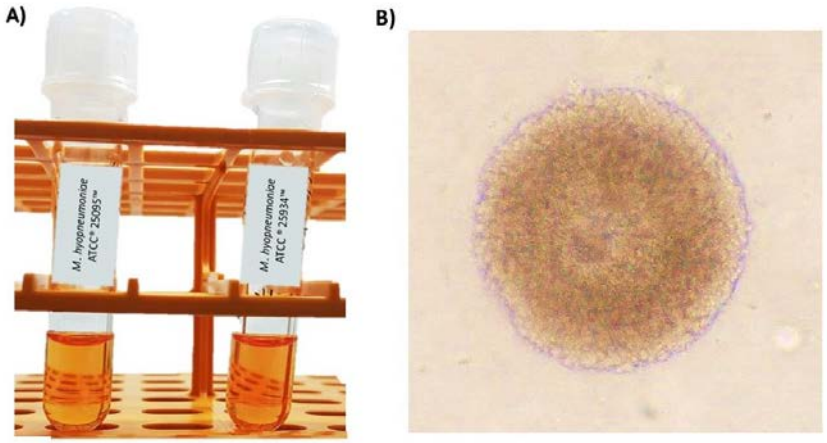

Figure 1-2. *Mycoplasma hyopneumoniae* in liquid and solid media (source: IRTA-CReSA). **A)** *M. hyopneumoniae* reference strains (ATCC® 25095™ and 25934™). **B)** Colony of *M. hyopneumoniae* on solid media.

1.1.2. Variability

The occurrence and severity of *M. hyopneumoniae* associated disease is influenced by different factors, including the specific *M. hyopneumoniae* strain/s involved in the process. Several approaches have been used to characterize *M. hyopneumoniae* strains in order to gain insight on the virulence of the strain and the epidemiology of the infection (Sibila *et al.,* 2009). These studies have described the variability of *M. hyopneumoniae* circulating strains at genomic, antigenic, proteomic and pathogenic levels (Vicca *et al.,* 2003; Minion *et al.,* 2004; Assunção *et al.,* 2005; Calus *et al.,* 2007; Pinto *et al.,* 2009; Charlebois *et al.,* 2014).

1.1.2.1. Genomic

Several genotyping techniques have demonstrated *M. hyopneumoniae* heterogeneity of different isolates from clinical samples obtained from naturally and experimentally infected animals (Maes *et al.,* 2018). These

techniques are based on the detection and characterization of complete or partial genome using different targets. The sequencing of complete genome has been accomplished so far in six strains: one reference strain (strain J), and other five field strains (232, 7448, 168, 7422 and KM014) (Minion *et al.,* 2004; Vasconcelos *et al.,* 2005; Liu *et al.,* 2011; Siqueira *et al.,* 2013; Han *et al.,* 2017). While the strain 232 was isolated from a pig inoculated with the reference strain 11, the other strains were isolated from field cases of disease (Mare and Switzer, 1965).

The techniques most utilized to describe the genetic diversity of *M. hyopneumoniae* are summarized in Table 1-1. Among them, Multi-Locus Variable Analysis (MLVA) is the most commonly technique employed in the most recent studies (Vranckx *et al.,* 2011; Nathues *et al.,* 2011a; Vranckx *et al.,* 2012b; Charlebois *et al.,* 2014; Dos Santos *et al.,* 2015; Tamiozzo *et al.,* 2015; Galina-Pantoja *et al.,* 2016; Takeuti *et al.,* 2017a; Michiels *et al.,* 2017a; Felde *et al.,* 2018).The MLVA allows the genetic characterization of *M. hyopneumoniae* without prior cultivation or isolation (Vranckx *et al.,* 2011) by estimation of the number of repetitions of different variable number of tandem repeats (VNTR). This estimated number is used to classify the detected *M. hyopneumoniae* DNA for each analyzed locus and each particular combination of those analyzed loci is considered a distinct variant (Michiels *et al.,* 2017a).

Table 1-1. Overview of the published results obtained with the different techniques used to describe *M. hyopneumoniae* genetic variability.

Reference	Method	Target	*M. hyopenumoniae* variability	
			Interfarm	Intrafarm
Artiushin and Minion, 1996	AP-PCR	WG[a]	High	NE
Stakenborg et al. 2005	PFGE	WG[b]	High	Low
Stakenborg et al. 2006	AFLP	WG[b]	High	High
	PFGE	WG[b]	High	Low
	RAPD	WG[b]	High	High
	VNTR	P97-RR1,RR2	High	High
Mayor et al. 2007	RAPD	WG[b]	High	Low
Mayor et al. 2008	MLST	*9 Hk* genes	High	Low
Nathues et al. 2011a	RAPD	WG[b]	High	High
	MLVA	P97-RR1, P146-RR3,H4	High	High
Vranckx et al. 2011	MLVA	P97-RR1; P146-RR3; H1; H5-RR2	High	High
Vranckx et al. 2012b	MLVA	P97-RR1; P146-RR3; H1, H5-RR2	High	Low
Charlebois et al. 2014	PCR-RFLP	P146	High	Low
	MLVA	Locus 1, 2; P97-RR1,RR2	High	Low
Dos Santos et al. 2015	MLVA	P97-RR1; P146-RR3	High	High
Tamiozzo et al. 2015	MLVA	P95, P146-RR1; H4, H5	High	High

Table 1-1. Overview of the published results obtained with the different techniques used to describe *M. hyopneumoniae* genetic variability (continued).

Reference	Method	Target	*M. hyopenumoniae* variability	
			Interfarm	Intrafarm
Galina Pantoja et al*.,* 2016	MLVA	P97-RR1; P146-RR3	Low	Low
Takeuti et al. 2017a	MLVA	P97-RR1; P146-RR3	High	High
Michiels et al. 2017a	MLVA	P97-RR1; P146-RR3; H1; H5-RR2	High	High
Felde et al. 2018	MLST MLVA VNTR	10 *Hk* P97-RR1, locus1 P146	High	High

WG: whole genome; AP-PCR: arbitrary primed PCR; PFGE: pulsed-field gel electrophoresis; AFLP: Amplified fragment length polymorphism; RAPD: random amplified polymorphic DNA; VNTR: variable number of tandem repeats; MLST: multilocus sequence typing; MLVA: multilocus variable number tandem repeat analysis; PCR-RFLP: PCR combined with restricted fragments length polymorphism; [a]Using arbitrary primers (OPA); [b] using restriction enzymes; *Hk*: housekeeping genes; High= more and low =less than one strain, variant or clonal variants were detected.

Results obtained using MLVA are fairly variable (Table 1-1). On the one hand, in some studies, a high genetic variability between isolates and field samples from different countries has been detected (Stakenborg *et al.,* 2005, 2006; Mayor *et al.,* 2007, 2008; Dos Santos *et al.,* 2015). In fact, no common strains characterized by MLVA were detected in countries such as Brazil, Mexico, Spain and the USA (Dos Santos *et al.,* 2015). Regarding to variability at inter-farm level, contradictory results have been obtained. Several studies carried out in regions with high pig density detected a high genetic *M. hyopneumoniae* heterogeneity between farms (Nathues *et al.,* 2011a; Charlebois *et al.,* 2014; Michiels *et al.,* 2017a) and within these farms (Michiels *et al.,* 2017a; Nathues

et al., 2011a; Tamiozzo *et al.,* 2015) as well as these variants were different from the ones within bacterins (Charlebois *et al.,* 2014; Tamiozzo *et al.,* 2015). On the contrary, other studies concluded that the strain detected was similar or indistinguishable among or within farms (Galina-Pantoja *et al.,* 2016) and a low diversity of *M. hyopneumoniae* was noted into the studied farms (Stakenborg *et al.,* 2006; Charlebois *et al.,* 2014; Galina-Pantoja *et al.,* 2016 also, clonal variants were described within herds (Vranckx *et al.,* 2011, 2012b). Additionally, multiple strains have been detected at batch and pig level (Nathues *et al.,* 2011a; Michiels *et al.,* 2017a) as well as at the same sample (Nathues *et al.,* 2011a).

Notwithstanding, different type and number of loci as well as terminology (isolate, strains, variants or clonal variants) have been utilized to describe the *M. hyopneumoniae* genetic variability. Overall, most techniques target genes encoding proteins involved in the adhesion of *M. hyopneumoniae* to the cilia (Table 1-1), mainly P97 and P146. These issues together with the facts that management and vaccination practices, presence of co-infections and/or environmental conditions were different in each study, could influence the obtained results and complicate the interpretation and comparison of results from different published studies up to now. Indeed, the fact that some discrepancies have been found among studies with respect of potential virulence markers leads to definitive association between these genomic characteristics and the virulence has not been established.

1.1.2.2. Antigenic

Antigenic diversity is the ability of a microorganism to alter its surface components, causing different host immune response (Razin *et al.,* 1998). Antigenic variation among *M. hyopneumoniae* strains has been evaluated using different serologic and proteomic assays and different proteins with

antigenic properties have been identified (Ro and Roos, 1983; Stipkovits *et al.,* 1991; Scarman *et al.,* 1997; Assunçao *et al.,* 2005; Pinto *et al.,* 2007; Petersen *et al.,* 2016).

Differences on the immunoreactivity of these antigenic proteins, including cytosolic, membrane and adhesion proteins, were identified. Indeed, the cytosolic protein P36, named lactate dehydrogenase (LDH; Hadimann *et al.,* 1993), was detected in *M. hyopneumoniae* field strains from Switzerland, Hungary, France and Canada (Stipkovits *et al.,* 1991), whereas this protein was not detected in the *M. hyopneumoniae* field strains from Spain (Assunção *et al.,* 2005). Regarding to membrane proteins, the protein P46 and P74 are considered as potential antigen for ELISA kits to detect the presence of antibodies against the pathogen, due to the fact these proteins seem to be conserved in most isolates (Bereiter and Young, 1990; Assunçao *et al.,* 2005; Okada *et al.,* 2005). Noteworthy, P74 is currently used in a commercial ELISA kit against *M. hyopneumoniae* (Oxoid, Thermo Fisher Scientific). Similarly, adhesins proteins such as P97 have been detected in all tested isolates (Zhang *et al.,* 1995; Assunção *et al.,* 2005) and differences on antigenic profiles such as the variation in its size (Wise and Kim, 1987; Zhang *et al.,* 1995; Assunção *et al.,* 2005) and/or its expression (Rosengarten and Yogev, 1996; Razin *et al.,* 1998), have been associated to adaptation mechanisms of *M. hyopneumoniae* against environmental changes such as culture passages (Razin *et al.,* 1998; Assunção *et al.,* 2005).

The existence of cross-reactive antigens between *M. hyopneumoniae* and other swine mycoplasmas like *M. hyorhinis*, *M. hyosynoviae* and *M. flocculare* has been documented (Ro and Ross, 1983; Wise and Kim, 1987; Stipkovits *et al.,* 1991; Scarman *et al.,* 1997; Gómez-Neto *et al.,* 2014; Petersen *et al.,* 2016). Although, the closest antigenic relationship was described between *M.*

hyopneumoniae and *M. flocculare* isolates (Ro and Ross, 1983; Petersen *et al.,* 2016), one recent study indicated that only 3 out of 39 tested *M. hyopneumoniae* proteins were exclusively present in *M. hyopneumoniae* and did not cross-react with *M. hyorhinis*, *M. hyosynoviae* and/or *M. flocculare* (Petersen *et al.,* 2016).

The *M. hyopneumoniae* antigenic diversity described in the abovementioned studies may have several implications. Although the mechanisms of *M. hyopneumoniae* pathogenesis have not been fully understood, the heterogeneity on surface proteins might play a crucial role in the *M. hyopneumoniae* adhesion to the ciliated epithelium (Wise and Kim, 1987) and to evade the host immune system (Maes *et al.,* 2018). Indeed, the ability of *M. hyopneumoniae* to induce immune responses seems to be different according to the variation of antigenic profile as it has been observed under experimental (Vicca *et al.,* 2002; Strait *et al.,* 2003) and natural (Ameri *et al.,* 2006) conditions. Likewise, it is still unclear whether putative antigenic variations may imply differences in antibody profiles generated by field isolates when compared to strains used in bacterins (Villarreal *et al.,* 2012). Furthermore, the cross-reactivity detected between *M. hyopneumoniae* and other swine mycoplasmas may interfere with the results of serological diagnostic methods (Rosengarten and Yogev, 1996; Assunçao *et al.,* 2005; Petersen *et al.,* 2016). Therefore, these findings indicate the need to identify conserved *M. hyopneumoniae* antigens as targets to develop highly accurate serological diagnostic tests as well as new vaccines against this bacterium.

1.1.2.3. Proteomic

Proteomics studies have demonstrated high variability among the very limited number of *M. hyopneumoniae* isolates so far tested (Calus *et al.,* 2007). This approach has allowed characterizing the total proteins of *M. hyopneumoniae*, and identifying its intracellular and surface proteins and post-translational modifications (PTMs) as well as characterizing potential antigenic proteins (Calus *et al.,* 2007; Pinto *et al.,* 2007, 2009; Tacchi *et al.,* 2016).

The variability found on the total protein profile among the *M. hyopneumoniae* isolates was not associated with virulence (Calus *et al.,* 2007; Pinto *et al.,* 2007, 2009). However, differences on PTMs seem to be relevant for *M. hyopneumoniae* since they regulate the bacterium surface variability and might play an important role in the adhesion to the epithelium as well as in the recognition by host immune response (Maes *et al.,* 2018). Such PTMs have been observed in cytosolic proteins ("moonlighting" proteins), lipoproteins at the cell surface and in adhesin proteins from the P97/P102 paralogs families (Seymour *et al.,* 2010; Bogema *et al.,* 2011, 2012; Simionatto *et al.,* 2013; Tacchi *et al.,* 2016; Maes *et al.,* 2018).

Recently, proteomic studies have been also used to described the host-pathogen interaction using the secretome (Paes *et al.,* 2017; Leal-Zimmer *et al.,* 2018), a collection of transmembrane and secreted proteins into the extracellular space (Mukherjee and Mani, 2013). A comparative analysis between *M. hyopneumoniae* (pathogenic) and *M. flocculare* (commensal) showed differences in their secretome profile, suggesting different putative virulence factors in each species (Paes *et al.,* 2017). In line with this hypothesis, differences in the number of adhesins (e.g. P97 and P102) and hypothetical proteins related with pathogenicity between one non-pathogenic (strain J)

and another pathogenic (strain 7448) *M. hyopneumoniae* strains infecting a swine tracheal cell line were detected (Leal-Zimmer *et al.,* 2018).

1.1.2.4. Pathogenicity and virulence

Mycoplasma hyopneumoniae is considered a major pathogen because it can cause respiratory disease by itself (Opriessnig *et al.,* 2011). Nevertheless, different pathogenicity among *M. hyopneumoniae* strains based on their potential to produce respiratory disease in specific pathogen free pigs by means of experimental inoculation have been observed (Goodwin *et al.,* 1965; Mare and Switzer, 1965). In fact, as abovementioned, the reference strain J was initially isolated from a pneumonic lung (Goodwin, 1965), but after several *in vitro* passages was recognized as non-pathogenic (Bereiter *et al.,* 1990). In contrast, strain 11 is still considered as pathogenic strain (Zielinski and Ross, 1990).

Differences on virulence or the severity of clinical signs and lesions caused among *M. hyopneumoniae* strains has been demonstrated (Vicca *et al.,* 2003; Meyns *et al.,* 2004; Villareal *et al.,* 2009, 2011a; Woolley *et al.,* 2012). Indeed, Vicca et al. (2003) evaluated the virulence of six field isolates of *M. hyopneumoniae* from Belgium by experimental pig inoculation. These isolates were classified as low, moderate and highly virulent according to the observed respiratory signs, lung lesion score, histopathology, immunofluorescence and serology (Vicca *et al.,* 2003). Subsequent experimental studies also described differences on the virulence of two Australian field isolates: Hillcrest strain was recognized as highly virulent compared to the Beaufort strain (Scarman *et al.,* 1997; Seymour *et al.,* 2011; Woolley *et al.,* 2012).

Since differences on virulence of *M. hyopneumoniae* strains have been detected, several genomic, antigenic and proteomic approaches have been performed to determine the potential existence of virulence markers. In fact, previous studies suggested that the presence of a 5000 bp RAPD fragment (Vicca *et al.,* 2003) or the existence of Locus 1 (Charlebois *et al.,* 2014), among others, were associated with more virulent strains. Additionally, the analysis of the metabolic routes of *M. hyopneumoniae, M. hyorhinis* and *M. flocculare* revealed differences on the metabolism between these three species considered as pathogenic (*M. hyopneumoniae* and *M. hyorhinis)* and commensal (*M. flocculare*). Such difference was mainly related to the ability of *M. hyopneumoniae* to produce cytotoxic hydrogen peroxide (H_2O_2), compared to *M. hyorhinis* and *M. flocculare* (Ferrarini *et al.,* 2016, 2018). Therefore, the production of this metabolite might be linked to the pathogenicity and might be considered as a virulence marker of *M. hyopneumoniae* (Ferrarini *et al.,* 2018). Nevertheless, all these hypothetical virulence markers have not been fully confirmed by subsequent studies (Maes *et al.,* 2018).

Therefore, all these findings would reinforce the idea that an integrated approach, including genomic, antigenic, transcriptomic, proteomic and metabolomic analyses, is needed to provide new insights into *M. hyopneumoniae* variability and its association with the pathogenicity and virulence of this respiratory bacterium.

1.2. Epidemiology

Respiratory disease associated to *M. hyopneumoniae* is considered endemic in most European countries with an intensive swine production (Rautiainen *et al.,* 2001; Fraile *et al.,* 2010; Meyns *et al.,* 2011; Fablet *et al.,* 2012a, 2012b;

Nathues *et al.,* 2014). Different studies have estimated the prevalence of *M. hyopneumoniae* infection by monitoring lung lesions associated to this bacterium at slaughterhouses (Fraile *et al.,* 2010; Meyns *et al.,* 2011; Fablet *et al.,* 2012a). Results from those studies revealed the presence of *M. hyopneumoniae*-compatible lung lesions in 55.7% of the lungs evaluated in Spain (Fraile *et al.,* 2010), 23.85% in France (Meyns *et al.,* 2011) and 69.3% in Belgium (Fablet *et al.,* 2012a). Such high prevalence of *M. hyopneumoniae*-like lesions at abattoirs was also described in other studies by the measurement of antibodies against this pathogen. These reports showed that 82% of the fattening tested farms were seropositive in Spain (Fraile *et al.,* 2010), 71% in France (Fablet *et al.,* 2012a), 79% in Belgium (Meyns *et al.,* 2011) and 63% in Germany (Nathues *et al.,* 2014). In contrast, Scandinavian countries such as Finland and Norway as well as other European countries as Switzerland, are considered free of EP due to the strict control and eradication programs against *M. hyopneumoniae* implemented (Rautiainen *et al.,* 2001; Stärk *et al.,* 2007; Luehrs *et al*., 2017; Maes *et al.,* 2018). Nevertheless, the prevalence of respiratory problems associated with *M. hyopneumoniae* seems to be slightly increasing lately in Switzerland (Overesch and Kuhnert, 2017).

The prevalence of *M. hyopneumoniae* in terms of antibody presence reaches the highest level at growing and finishing period (Sibila *et al.,* 2004). In previous stages as lactation and nursery, the presence of maternally derived antibodies makes impossible to assess infection through the measurement of antibodies. Therefore, the prevalence of *M. hyopneumoniae* should be assessed by the detection of the pathogen. The detection of *M. hyopneumoniae* in sucking piglets is variable, ranging from 0.5-3.8% in nasal swabs (Sibila *et al.,* 2007a,b) to 12.3% in broncho-alveolar lavage fluid (BALF; Moorkamp *et al.,* 2009). Likewise, *M. hyopneumoniae* can be detected in the nasal cavity of recently weaned pigs to a very different percentage, ranging

from >1% up to 51% (Sibila *et al.,* 2004, 2007a; Fano *et al.,* 2007). Similarly, detection by tracheo-bronchial mucus samples offered also diverging results from 1.1% to 16.7% (Vangroenweghe *et al.,* 2015a, b).

Considering that piglets are *M. hyopneumoniae*-free at birth, the main transmission route during lactation period would be the direct contact with a positive dam (Calsamiglia and Pijoan, 2000; Rautiainen and Wallgren, 2001). Some epidemiological studies have suggested that gilts and younger parity sows are the main *M. hyopneumoniae* shedders (Fano *et al.,* 2007; Sibila *et al.,* 2007b; Boonsoongnern *et al.,* 2012). In fact, it has described that gilts play an important role in maintaining the circulation of *M. hyopneumoniae* as well as destabilizing the health status and immunity of the recipient herd by infected gilt purchases (Nathues *et al.,* 2013, 2014). In consequence, gilt acclimation focused on homogenizing the immune status and reducing the bacterium shedding at farrowing seems to be necessary (Pieters and Fano, 2016). Although the prevalence of seropositive sows was proposed as a possible indicator of infection pressure within herds (Grosse-Beilage *et al.,* 2009), the sow serological status does not seem to be associated with the infection of suckling piglets (Sibila *et al.,* 2007b). In contrast, the prevalence of nasal colonization at weaning has been correlated to the severity of the disease associated to *M. hyopneumoniae* in growing and fattening pigs (Fano *et al.,* 2007; Sibila *et al.,* 2007b).

Once the suckling piglets are infected, a subsequent and progressive horizontal transmission between infected and susceptible piglets can occur among or within pen-mates (Maes *et al.,* 1996; Maes *et al.,* 2018). The spread of *M. hyopneumoniae* is characterized by a slow transmission and a long persistent infection (Meyns *et al.,* 2004; Pieters *et al.,* 2009; Pieters and Fano, 2016). The *M. hyopneumoniae* transmission ratio has been calculated by an

adjusted reproduction ratio (R_n) varying among 1.16-1.28 under experimental conditions (Meyns *et al.,* 2004; Roos *et al.,* 2016) and between 0.56 and 0.71 under field conditions (Villarreal *et al.,* 2011b). The differences between values may be explained by the higher infection dose of *M. hyopneumoniae* used in the experimental inoculation, compared to the natural infection occurring under field conditions (Villarreal *et al.,* 2011). Moreover, a single experimental study has shown that pigs infected with *M. hyopneumoniae* can shed the bacterium for up to 214 days post-infection (dpi), reaching total pathogen clearance by 254 dpi (Pieters *et al.,* 2009).

The long duration and slow transmission facilitates *M. hyopneumoniae* to circulate among the newly introduced gilts, the recipient herd and the offspring, maintaining the infection within herds (Nathues *et al.,* 2016). Additionally, others factors such as the type of *M. hyopneumoniae* strain involved (Vicca *et al.,* 2003; Meyns *et al.,* 2004; Nathues *et al.,* 2016) and/or management practices such as cross-fostering (Alexander *et al.,* 1980; Maes *et al.,* 2018), and the piglet age at weaning (Nathues *et al.,* 2013; Pieters *et al.,* 2014) could influence this infection dynamics.

In addition to direct contact, airborne transmission has been described in the literature. This way of transmission was suggested as the main route of *M. hyopneumoniae* introduction in naïve herds when an infected farm was located within a distance of 3.2 km or shorter (Goodwin, 1985). Subsequent studies demonstrated that *M. hyopneumoniae* can be detected over larger distances such as 4.7 km (Dee *et al.,* 2009) and 9.2 km (Otake *et al.,* 2010) after intra-tracheal inoculation of *M. hyopneumoniae* in pigs. Moreover, the potential pathogen viability was confirmed by bioassay in inoculated pigs using the 9.2 km sample (Otake *et al.,* 2010).

The role of farm personnel and fomites in *M. hyopneumoniae* transmission is not clearly described in the literature. Although the pathogen has been detected in nasal swabs from farm workers (Nathues *et al.,* 2011b), a previous study reported that a *M. hyopneumoniae* naïve herd using standard biosecurity procedures (i.e. change of clothing and showering previous entry) were not become infected by personnel exposed to infected herds (Batista *et al.,* 2004; Pitkin *et al.,* 2011). In addition, a recent study demonstrated that *M. hyopneumoniae* can survive for up to 8 days at 4ºC on various surface materials commonly encountered in pig farms such as glass, general purpose polystyrene, dust, stainless steel and polypropylene copolymer (Browne *et al.,* 2016). However, basic biosecurity practices as cleaning and disinfection protocols seem to be sufficiently effective against *M. hyopneumoniae* (Browne *et al.,* 2016; Maes *et al.,* 2018).

1.3. Pathogenesis

The adherence of *M. hyopneumoniae* to the ciliated epithelium is required to initiate the respiratory disease (Tajima and Yagihashi, 1982; Blanchard *et al.,* 1992; DeBey and Ross, 1994; Razin *et al.,* 1998; Thacker and Minion, 2012). Although the exact mechanism of this adhesion is poorly known, different components of the *M. hyopneumoniae* cell membrane seem to be involved in the process, such as the presence of a polysaccharide capsule with fine fibrils and multiple binding proteins (Tajima and Yagihashi, 1982; Blanchard *et al.,* 1992; Zhang *et al.,* 1995; Kobisch and Friis, 1996; Hsu and Minion, 1998; Sarradell *et al.,* 2003; Bogema *et al.,* 2012).

One of the most relevant binding proteins is the cilium adhesin protein P97, a 97 kDa surface protein located among the fibrils (Zhang *et al.,* 1995). This protein is encoded by the *mhp183* gene and has two repeat regions (RR, RR1

and RR2) with significant implications in the binding process (Hsu *et al.,* 1997; Hsu and Minion, 1998; Deutscher *et al.,* 2010). The RR1 domain has been proposed as the responsible for cilium binding (Hsu *et al.,* 1997; Hsu and Minion, 1998) and a minimum of eight pentapeptide (AAKP[EV]) repeats in this region are supposedly required for a significant *M. hyopneumoniae* binding to porcine cilia (Minion *et al.,* 2000). Despite the apparent importance of P97, *M. hyopneumoniae* adherence to cilia is still possible when the P97 binding site is blocked by monoclonal antibodies, indicating that other adhesins are also implicated in this process (Zhang et al., 1995; King et al., 1997).

The adhesin P102 is a protein which is also involved in cilia binding. This 102 kDa protein is encoded by *mhp182*, a gene located in the same operon as the mentioned *mhp183* gene. Interestingly, the two genes encoding P97 and P102 have six paralogs within the *M. hyopneumoniae* genome (Adams *et al.,* 2005). The P97/P102 paralog family facilitates the modification of the *M. hyopneumoniae* surface architecture and, presumably, the evasion of host immune responses (Djordjevic *et al.,* 2004; Deutscher *et al.,* 2010; Seymour *et al.,* 2010, 2011, 2012; Woolley *et al.,* 2014). Such variations in the surface topography have been related with the fact that most proteins from the P97/P102 paralog family are post-translationally processed and cleaved (Seymour *et al.,* 2010; Bogema *et al.,* 2012). Besides the repertoire of cleaved fragments generated, the presence of multifunctional "moonlighting" proteins increase the diversity on *M. hyopneumoniae* surface, evading the immune response (Bogema *et al.,* 2012; Simionatto *et al.,* 2013). Among these multifunctional proteins, the P146 lipoprotein encoded by *mhp684* gene has been recognized as a significant regulator of surface topography (Bogema *et al.,* 2012). The variability observed among strains in a repeat region of this protein (RR3) would support the interest of this locus as target for *M. hyopneumoniae* genotyping (Stakenborg *et al.,* 2006; Mayor *et al.,* 2008;

Kuhnert *et al.,* 2011; Bogema *et al.,* 2012; Dos Santos *et al.,* 2015; Galina-Pantoja *et al.,* 2016; Michiels *et al.,* 2017a; Overesch and Kuhnert, 2017; Felde *et al.,* 2018).

In addition to the abilities of *M. hyopneumoniae* surface to vary, these proteins can also recruit mucin, heparin, plasminogen and fibronectin to the surface of *M. hyopneumoniae*, facilitating the respiratory epithelium colonization (Deutscher *et al.,* 2010; Bogema *et al.,* 2012). Recently, a study concluded that the adhesive proteoforms generated from these P97/P102 paralogs seem to be associated with the formation of biofilms in the swine respiratory tract (Raymond *et al.,* 2018). Such biofilm formation might explain the different susceptibility to some antimicrobials, as well as the evasion to the host immune response and the survival of *M. hyopneumoniae* on surfaces under *in vitro* conditions (McAuliffe *et al.,* 2006; Simmons and Dybvig, 2007; Tassew *et al.,* 2017). However, the potential effect of *M. hyopneumoniae* biofilms under *in vivo* conditions has not been explored yet.

Once *M. hyopneumoniae* is attached to the ciliated epithelium, several degenerative changes are observed (Figure 1-3). Firstly, the bacterium attachment to the cilia is associated with a disruption of potassium (K^+) channels, which results in ciliostasis (DeBey and Ross, 1994). This reduction of the ciliary activity causes a decrease in mucosal clearance of respiratory tract, facilitating *M. hyopneumoniae* colonization and multiplication (DeBey and Ross, 1994). Afterwards, different hypotheses regarding to the damage mechanism of *M. hyopneumoniae* have been proposed. The rise of intracellular calcium (Ca^{2+}) after adhesion was identified as an intracellular marker to determine the destruction of cilia (Zhang *et al.,* 1994; Park *et al.,* 2002). Moreover, and according to a metabolic model, H_2O_2 derived from the use of glycerol as carbon source can also act as a damaging mechanism

(Ferrarini *et al.,* 2016). However, such hypothesis has not been confirmed by other authors (Maes *et al.,* 2018).

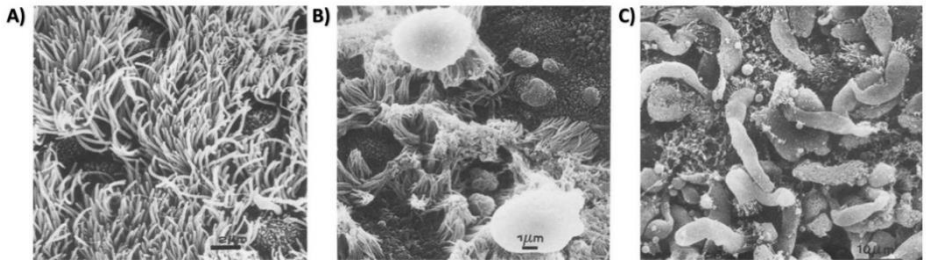

Figure 1-3. Scanning electron microscopy images of the respiratory tract of piglets. **A)** Trachea from a control piglet (not inoculated with *M. hyopneumoniae*) showing healthy epithelial cells with microvilli, bar=2 µm. **B)** Trachea from an inoculated piglet with *M. hyopneumoniae* at 2 weeks post-inoculation. Epithelial cells have lost cilia and *M. hyopneumoniae* is attached to remaining cilia bar=1 µm. **C)** Bronchi epithelium surface from an inoculated piglet with *M. hyopneumoniae* at 8 weeks post-inoculation. Epithelium surface is cilia and mucus free, bar=10 µm (Blanchard *et al.,* 1992).

Despite the fact that *M. hyopneumoniae* is considered an extracellular respiratory pathogen (Blanchard *et al.,* 1992; Kwon and Chae, 1999), recent studies suggested that *M. hyopneumoniae* is able to penetrate into porcine kidney epithelial cells (PK-15) and reside intracellularly (Raymond *et al.,* 2016, 2018). This finding could be related to the structural changes on actin filaments of cytoskeleton that allows the organism being phagocytosed and located intracellularly (Tacchi *et al.,* 2016; Raymond et al. 2018). Moreover, this fact might explain the *M. hyopneumoniae* detection and eventual isolation in other internal organs apart from lung, such as brain, liver, spleen, and kidneys of pigs infected experimentally (Friis, 1974; Le Carrou *et al.,* 2006;

Marois *et al.,* 2007; Woolley *et al.,* 2012). Nevertheless, curiously, those tissues did not show any lesion potentially associated to this bacterial infection. Recently, Pieters *et al.* (2018) also detected from testicles and tails of commercial piglets at processing age (1-5 days of age). These results could be in agreement with previous studies that suggested the spread of *M. hyopneumoniae* via lymphatic or blood may occur (Le Carrou *et al.,* 2006). Another possibility would be that this bacterium might be present in the environment contaminating those tissues samples (Pieters *et al.,* 2018).

1.4. Respiratory diseases associated to *M. hyopneumoniae*

Respiratory diseases are one of the most important concerns for the swine industry worldwide due to the generated economic losses. Indeed, a previous study carried out in the USA reported *Porcine reproductive and respiratory syndrome virus* (PRRSV), *Swine influenza virus* (SIV) and *M. hyopneumoniae* as the most important respiratory pathogens in the different pig production systems (Holtkamp *et al.,* 2007). Overall, respiratory disease is the result of combined infection with viruses and bacteria, which can be divided into primary and secondary (or opportunistic) pathogens according to their capability to induce clinical signs and lesions in the respiratory tract (Brockmeier *et al.,* 2002; Opriessnig *et al.,* 2011). *Mycoplasma hyopneumoniae* is considered primary and a major respiratory pathogen due to its ability to cause disease in pigs by itself such as mycoplasmal (MP) and enzootic pneumonia (EP) as well as contributing as one of the main players to develop other, more severe, respiratory diseases such as the porcine respiratory disease complex (PRDC; Opriessnig *et al.,* 2011).

1.4.1. Mycoplasmal and enzootic pneumoniae

Mycoplasma hyopneumoniae is the etiological agent of MP, a respiratory process subclinically or clinically characterized by a relatively mild dry and non-productive cough (Sibila *et al.,* 2009; Garza-Moreno *et al.,* 2018; Maes *et al.,* 2018) with little or no impact on performance parameters (Maes *et al.,* 1996). Nevertheless, when this respiratory process is complicated by other respiratory bacteria such as *Pasteurella multocida, Actinobacillus pleuropneumoniae (A. pleuropneumoniae), Streptococcus suis, M. hyorhinis, Bordetella bronchiseptica,* and/or *Haemophilus parasuis (H. parasuis)*, the disease is named as EP (Sibila *et al.,* 2009; Thacker and Minion, 2012; Maes *et al.,* 2018) and the impact on performance parameters is increased.

Mycoplasmal pneumonia is generally observed under experimental conditions, where the disease is reproduced as infection model to assess vaccine effectiveness, among other purposes (Garcia-Morante *et al.,* 2016b). In contrast, EP is commonly observed under field conditions where the co-infections of *M. hyopneumoniae* with respiratory bacterial pathogens mentioned above frequently occur. Although EP can also take a subclinical or clinical course depending on the concomitant pathogens involved (Straw *et al.,* 1989; Maes *et al.,* 1996). Enzootic pneumonia affects mainly growing and fattening pigs and it is characterized by a chronic, dry and non-productive cough, mild fever as well as a reduction of feed intake and growth performance parameters such as average daily weight gain (ADWG) and feed efficiency (Maes *et al.,* 2018). Indeed, more severe respiratory disease with high fever, labored breathing, and prostration has been also related to EP (Maes *et al.,* 1996; Thacker and Minion, 2012).

Although observed coughing is not exclusive of *M. hyopneumoniae* (Nathues *et al.,* 2012; Thacker and Minion, 2012), the severity of both MP and EP is

dependent on the health status of the animals, management practices, housing conditions and *M. hyopneumoniae* strain/s involved (Vicca et al., 2003; Thacker and Minion, 2012; Woolley et al., 2012; Michiels *et al.,* 2015; Michiels *et al.,* 2017a; Maes *et al.,* 2018). In endemic farm scenarios, *M. hyopneumoniae* infections with high morbidity and low mortality are usually observed (Sibila *et al.,* 2009; Thacker and Minion, 2012). Nevertheless, when *M. hyopneumoniae* infects a naïve herd, pigs suffering this outbreak can display coughing, dyspnea, pyrexia, anorexia and even death. In this last scenario, pigs of different ages could be affected (Maes *et al.,* 1996).

Coughing is the consequence of the lung lesions generated by *M. hyopneumoniae* (Maes *et al.,* 2018). Macroscopically, *M. hyopneumoniae*-like lung lesions consist of purple to grey areas of consolidation, known as cranio-ventral pulmonary consolidation (CVPC; Fraile *et al.,* 2010; Meyns *et al.,* 2011). These are usually found bilaterally in the apical, intermediate, accessory and the anterior parts of diaphragmatic lobes (Figure 1-4; Maes *et al.,* 2008). Notwithstanding, these lung lesions associated to *M. hyopneumoniae* are not exclusively of this bacteria, and others pathogens as SIV can cause similar lung lesions (Sibila *et al.,* 2009; Thacker and Minion, 2012; Maes *et al.,* 2018;). In EP cases, a complication of such lesions with a catarrhal or mucopurulent exudate associated to the presence of other bacterial respiratory pathogens is often observed at advanced stages (Thacker and Minion, 2012).

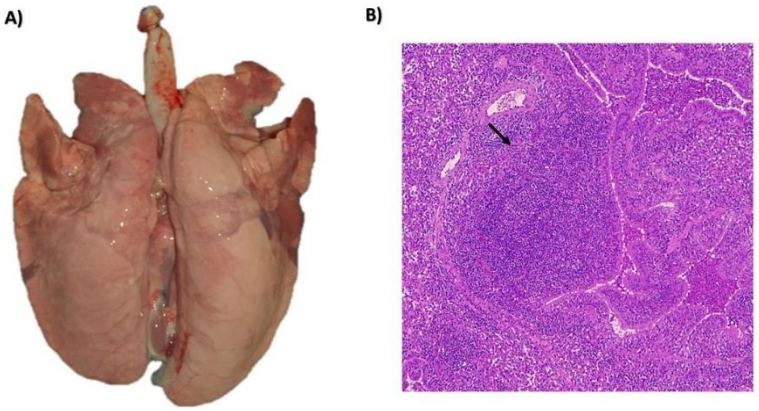

Figure 1-4. Gross and microscopic lung lesions compatibles with *M. hyopneumoniae* infection (source: IRTA-CReSA). **A)** Lung from *M. hyopneumoniae* experimentally inoculated pig. **B)** Experimentally infected pig showing broncho-interstitial pneumonia with presence of lymphoid follicles peribronchiales (indicated by the arrow).

At microscopic level, a broncho-interstitial pneumonia is observed in MP cases where *M. hyopneumoniae* is the only respiratory pathogen involved (Figure 1-4). Such lesions consist of lymphoplasmacytic hyperplasia in perivascular, peribronchiolar and alveolar septa. The accumulation of neutrophils, macrophages and plasma cells with oedema fluid in the alveolar spaces is also observed (Blanchard *et al.,* 1992; Sarradell *et al.,* 2003). More advanced lesions consist of an evident peribronchial and perivascular lymphocytic hyperplasia, the presence of lymphoid follicles, an increase of Goblet cells and hyperplasia of submucosal glands (Sibila *et al.,* 2007a; Thacker and Minion, 2012). Regarding EP-like lesions, suppurative bronchopneumonia with mucous and neutrophilic exudate in alveoli and airways, varying according the type of co-infection and disease stage, is frequently observed (Thacker and Minion, 2012). Further, collapsed and/or emphysematous alveoli, hyperplastic

lymphoid nodules as well as fibrosis can be observed in chronic or recovering lesions displaying EP (Whittlestone *et al.,* 1972).

1.4.2. Porcine respiratory disease complex

The term PRDC is used to describe a clinical entity characterized by respiratory clinical signs in the postweaning stages (mainly in the fatteners), which is etiologically associated to mixed respiratory infections of viral and bacterial origin (Opriessnig *et al.,* 2011; Thacker and Minion, 2012). *Mycoplasma hyopneumoniae* is one of the most frequently found bacterial pathogens in PRDC, and can be detected together with other respiratory bacterial and viral pathogens such as PRRSV, SIV and/or *Porcine circovirus 2* (PCV-2) (Brockmeier *et al.,* 2002; Opriessnig *et al.,* 2011).

Although clinical signs are similar to MP and EP, its severity and the mortality rate in PRDC is higher than in the abovementioned infectious respiratory processes in section 1.4.1 (Harms *et al.,* 2002). In relation to PRDC-like lung lesions, these are also frequently located in cranio-ventral parts of the lung (Fraile *et al.,* 2010). In addition, a bronchopneumonia in combination with interstitial pneumonia is often observed in PRDC cases due to the combination of bacterial and viral infections (Opriessnig *et al.,* 2011).

Similar to MP and EP, severity of PRDC can also vary depending on the age of pig, potential environmental stressors, farm management, and the type of pathogens involved in the process (Opriessnig *et al.,* 2011). Indeed, previous studies demonstrated that co-infections with *M. hyopneumoniae* and PRRSV (Thacker *et al.,* 1999) or PCV-2 (Opriessnig *et al.,* 2004) seemed to potentiate respiratory clinical signs and lesions, whereas other experimental study did not confirm such link to PCV-2 (Sibila *et al.,* 2012). Finally, interaction between *M. hyopneumoniae* and SIV seems to be minimal (Thacker *et al.,* 2001) and

may depend on the viral subtype involved (e.g. *M. hyopneumoniae* and SIV H1N1 might act synergistically, but not *M. hyopneumoniae* and SIV H1N2; Fablet *et al*., 2012; Deblanc *et al*., 2012)

1.5. Immune response

Despite the exact mechanism is not fully understood, *M. hyopneumoniae* is able to modulate the porcine innate and adaptive immune responses (Maes *et al.,* 1996; Thacker *et al.,* 2001). Such modulation allows *M. hyopneumoniae* evading host defenses, persisting and proliferating into the respiratory tract of infected pigs (Thacker and Minion, 2012; Woolley *et al.,* 2013). Moreover, the immune reaction against this pathogen plays an important role in the development of associated pneumonia (Livingston *et al.,* 1972; Sarradell *et al.,* 2003).

1.5.1. Innate immunity

The host innate immune response represents the first line of recognition and defense against pathogens. Once *M. hyopneumoniae* colonizes the respiratory tract, this bacterium is recognized by the host, causing both immunostimulatory and inhibitory effects on the immune system (Caruso and Ross, 1990; Hwang *et al.,* 2011; Bin *et al.,* 2014). On one hand, *M. hyopneumoniae* infection stimulates pulmonary alveolar macrophages (PAM) and monocytes to secrete different pro-inflammatory cytokines (Th-1) and other metabolites such as nitric oxide (Muneta *et al.,* 2003; Hwang *et al.,* 2011; Bin *et al.,* 2014). These increased levels of cytokines from Th-1 cells, including interleukin (IL)-1 (α and β), IL-2, IL-4, IL-6, IL-8, IL-10, IL-18, IL-12, interferon (IFN)- γ, tumor necrosis factor (TNF)-α, have been detected in BALF and lung tissue (Asai *et al.,* 1993, 1994; Muneta *et al.,* 2006, 2008; Thanawongnuwech

et al., 2004; Rodríguez *et al.,* 2004, 2007; Lorenzo *et al.,* 2009; Woolley *et al.,* 2013). While these cytokines contribute to the elimination of pathogens by regulation of numerous cell types, its overproduction can lead to excessive inflammatory responses and lymphoid hyperplasia (Okada *et al.,* 2000; Sarradell *et al.,* 2003; Choi *et al.,* 2006).

On the other hand, *M. hyopneumoniae* is also able to suppress the immune response by inhibiting the capacity of PAM to phagocytose (Caruso and Ross, 1990). These macrophages process and present antigen for the induction of specific immune response, regulating the innate immune response (Murtaugh *et al.,* 2002). Such inhibition of PAM functionality caused by *M. hyopneumoniae* infection implies a reduction of bacterial clearance, and consequently, pigs are more susceptible to *M. hyopneumoniae* and secondary pathogen infections (Caruso and Ross, 1990; Asai *et al.,* 1993).

1.5.2. Adaptive immunity

1.5.2.1. Humoral immune response

Since newborn piglets are naïve to *M. hyopneumoniae*, maternally derived antibodies after colostrum intake would be one of the earliest defense in the piglets (Bandrick *et al.,* 2008, 2011; 2014; Martelli *et al.,* 2016). This passive immunity confers partial protection to the offspring, but it does not prevent the infection. The median half-life of these maternal antibodies was estimated approximately 16 in days (Morris *et al.,* 1994). However, such antibody persistence in piglets apparently depends on the titers of maternal antibodies, being more persistent when the level of antibodies of sow is high (Wallgren *et al.,* 1998; Martelli *et al.,* 2006; Bandrick *et al.,* 2014).

Once piglets are exposed to *M. hyopneumoniae*, they also develop a local and systemic humoral immune responses. Mucosal immunity seems to play an important role in the protection against *M. hyopneumoniae* (Sarradell *et al.,* 2003), although data available in this respect is contradictory. Previous experimental studies revealed that the humoral mucosal immune response, in terms of IgG and IgA antibodies, appeared at 1-3 weeks post-*M. hyopneumoniae* inoculation (wpi) and declined after 4 wpi (Redondo *et al.,* 2009; Sarradell *et al.,* 2009; Garcia-Morante *et al.,* 2016b; Shen *et al.,* 2017). The presence of IgG presumably enhances the opsonization and phagocytosis of *M. hyopneumoniae* by PAM (Walker *et al.*, 1996), whereas IgA secretion is associated with the prevention of *M. hyopneumoniae* adhesion to respiratory cilia (Sheldrake *et al.*, 1990, 1993; Sarradell *et al.,* 2003). In fact, some studies suggested that IgA elicited by vaccination might be key in protection (Marchioro *et al.,* 2013; Martelli *et al.,* 2014). In contrast, other studies concluded IgA in *M. hyopneumoniae* challenged pigs did not prevent lung lesions (Djordjevic *et al.,* 1997). Recently, a study concluded that the measurement of mucosal immunity may have an association with the prevalence of lung lesions at early stages of infections (Garcia-Morante *et al.,* 2017). In this study, the mucosal IgG generated in later infection stages was the parameter with the highest correlation with the lung lesion score.

The systemic humoral immune response onset is delayed but lasts for longer than the local one. While seroconversion appears between 2 and 4 wpi experimentally (Sibila *et al.,* 2009), seroconversion would start at 1 to 6, even 9 weeks post infection under natural conditions (Leon *et al.,* 2001; Fano *et al.,* 2005). This delay on seroconversion might be explained by the extracellular condition of this pathogen and/or the potential delay in presenting the antigen to lymphocytes and activating the immune response (Janeway *et al.,* 2001). The IgG2 is the antibody subclass predominant in the humoral immune

response against *M. hyopneumoniae* infections, as described García-Morante et al. (2017). Since this antibody class is related to the activation of Th-1 cells, this finding might indicate cellular immune response could be important for protection against *M. hyopneumoniae* (García-Morante *et al.,* 2017).

1.5.2.2. Cellular immune response

The passive and active cell-mediated immune response also plays an important role in the protection against *M. hyopneumoniae* (Bandrick *et al.,* 2008). In fact, maternal derived cell-mediated immunity transferred to newborn piglets by colostrum of their biological dam seems to stimulate the specific cell responses in those piglets subsequent exposed to the bacterium (Bandrick *et al.,* 2011, 2014). Interestingly, since piglets can receive the *M. hyopneumoniae*-specific cell mediated immunity only from their biological dam, cross-fostering practices should be considered after 12 to 20h to ensure the reception of maternal derived cell-immunity (Bandrick *et al.,* 2011).

The active cell-mediated response developed upon *M. hyopneumoniae* infections, in particular the T-cell dependent immune response, seems to be a key element in the pathogenesis of this bacterium (Sarradell *et al.,* 2003). *Mycoplasma hyopneumoniae* stimulates Th-1 cells to secrete cytokines (see section 1.5.1), and afterwards, those stimulate T lymphocytes and activate B cells to produce IgG2 antibodies (Spellberg and Edwards, 2001; Hirahara and Nakayama, 2016; García-Morante *et al.,* 2017). Moreover, cytokines such as IL-1 (α and β) and TNF-α have been associated with the activation of phagocytosis (Lorenzo *et al.,* 2006) and IL-12 with the proliferation of macrophages (Murtaugh and Foss, 2002). Besides, IL-12 stimulates IFN-γ in T lymphocytes (Stern *et al.,* 1996) and the feedback effect between these two cytokines implies a constant Th-1 based response.

On the other hand, anti-inflammatory cytokines (Th-2) regulate the expression of Th-1 cytokines and induce IgG1 secretion (Crawley and Wilkie, 2003). One of the most important anti-inflammatory cytokines is IL-10, which is associated with clinical protection and reduction in the severity of lung lesions (Morrison *et al.*, 2000).

1.6. Diagnosis

Diagnosis of *M. hyopneumoniae* infection can be technically challenging as the disease associated with this respiratory pathogen can take subclinical and clinical courses (Sibila *et al.,* 2009; Garza-Moreno *et al.,* 2018). Moreover, the presence of other pathogens may complicate the diagnostic scenario. This means that the diagnostic approach for *M. hyopneumoniae* infection should be based on a combination of observational and laboratory analyses (Table 1-2; Garza-Moreno *et al.,* 2018). Additionally, the fact that clinical signs and lung lesions mentioned are indicative, but not exclusively, of *M. hyopneumoniae* infection reinforces the importance of confirming the presumptive observational diagnosis with a definitive laboratory diagnosis (Maes *et al.,* 2008).

Several laboratory diagnostic methodologies have been described to assess *M. hyopneumoniae* infection (Table 1-2). Bacteriological culture from lung tissue is considered the "gold standard" (Thacker and Minion, 2012). Nevertheless, it is not used for routine diagnosis due to the media growth and time requirements (4-8 weeks), the low isolation rate and the frequent contamination by the overgrowth of other Mycoplasma spp. (Friis, 1975). Nowadays, the most commonly strategy to diagnose and monitor *M. hyopneumoniae* infection in herds is based on a combination of abattoir

surveillance (i.e. macroscopic lung lesions scoring), serologic testing and pathogen detection (Sibila *et al.,* 2009).

Mycoplasma hyopneumoniae infection is usually monitored by scoring of lung lesions associated to this bacterium. Different lung lesion scoring methods are available, being most of them based on the extension and/or weight of the affected lung tissue; their scores are expressed in points or percentages (Garcia-Morante *et al.,* 2016a). Despite discrepancies between different scoring methods have been observed (Morrison *et al.,* 1985), a recent comparison between different lung scoring systems for *M. hyopneumoniae* demonstrated high correlation among them (Garcia-Morante *et al.,* 2016a).

Table 1-2. Summary of the monitoring strategies for *M hyopneumoniae* diagnosis (adapted from Garza-Moreno *et al.,* 2018).

	Monitoring strategy	Parameters	Samples
Observational diagnosis / Monitoring	Clinical examination	Presence of dry and non-productive cough	None
	Lung examination	Observation of CVPC	Entire lungs
		Broncho-interstitial pneumonia with bronchus-associated lymphoid tissue hyperplasia	Lung tissue
Laboratory diagnosis / Conclusive diagnosis	Bacterial isolation	*M. hyopneumoniae* growth	Lung tissue
	Immunofluorescence	Detection of *M. hyopneumoniae* antigen	Lung tissue
	Immunohistochemistry		
	In situ hybridization	Detection of *M. hyopneumoniae* nucleic acid	
	PCR	Detection of *M. hyopneumoniae* nucleic acid	Respiratory tract[a]
	ELISA	Detection of antibody response against *M. hyopneumoniae*	Serum

CVPC: cranio-ventral pulmonary consolidation; [a]Different samples from respiratory tract can be used.

Antibody detection through enzyme linked immunosorbent assay (ELISA) is the most commonly used method for *M. hyopneumoniae* antibody detection. This technique provides evidence of exposure to *M. hyopneumoniae*, but without distinguishing maternally derived antibodies from antibodies elicited by infection and/or vaccination (Bandrick *et al.,* 2011; Thacker and Minion, 2012). A limitation of the ELISA is the potential occurrence of false negatives (Thacker, 2004; Pieters *et al.,* 2017) and positives due to the extensive cross-reactivity with *M. flocculare* (Bereiter and Young, 1990; Gómez-Neto *et al.,* 2014; Petersen *et al.,* 2016). Further, current ELISA tests are not able to detect antibodies at the early-stage infection (Sitjar *et al.,* 1996; Kurth *et al.,* 2002; Pieters *et al.,* 2017). This fact, together to the high variability in seroconversion timing (Thacker, 2004; Sibila *et al.,* 2009), suggests that the absence of antibodies is not necessarily indicative of a *M. hyopneumoniae* free status. This limitation is especially critical during elimination processes and/or replacement entries in which a *M. hyopneumoniae* well-known health status should be warranted (Pieters *et al.,* 2017; Garza-Moreno *et al.,* 2018). Thus, the *M. hyopneumoniae* detection is required for a conclusive diagnosis.

To confirm the presence of *M. hyopneumoniae* in lung tissue, different laboratory techniques have been described such as immunohistochemistry, immunofluorescent assay and *in situ* hybridization (Boye *et al.,* 2001; Opriessnig *et al.,* 2004). Some limitations of these methods include the use of *post mortem* samples as histologic sections of lung tissue are required (Sibila *et al.,* 2009). Moreover, since only small lung sample portions can be evaluated, the risk of false-negative increases (Cai *et al.,* 2007).

The detection of *M. hyopneumoniae* by polymerase chain reaction (PCR) and specifically the real-time PCR (rt-PCR; Dubosson *et al.,* 2004; Strait *et al.,* 2008) has been proposed as the most sensitive and useful strategy to confirm the

pathogen presence (Sibila *et al.,* 2009), even during the early-stages infection prior to seroconversion and the observation of clinical signs (Pieters *et al.,* 2017). One important advantage of PCR testing is the possibility to detect *M. hyopneumoniae* on different respiratory tract samples (Sibila *et al.,* 2009; Pieters *et al.,* 2017). This means that PCR can be used for both *post mortem* and *in vivo* diagnosis (Sibila *et al.,* 2009). Nevertheless, there is a lack of consensus on which sample shows the highest sensitivity to detect *M. hyopneumoniae*. Samples from lower respiratory tract, where *M. hyopneumoniae* attachment to the cilia occurs, seem to offer a higher sensitivity (Calsamiglia *et al.,* 1999; Fablet *et al.,* 2010). Indeed, a recent study showed a high correlation between lung tissue and bronchial swabs for *M. hyopneumoniae* detection (Burrough *et al.,* 2018). Thus, in dead animals, lung tissue, bronchial swab and BALF have been proposed as the best samples to detect *M. hyopneumoniae* (Kurth *et al.,* 2002; Moorkamp *et al.,* 2008). In alive animals, tracheo-bronchial (Fablet *et al.,* 2010; Vangroenweghe *et al.,* 2015a,b) and laryngeal swabs (Pieters *et al.,* 2017) have demonstrated the highest sensitivity for *M. hyopneumoniae* detection, whereas oral fluids showed the lowest (Pieters *et al.,* 2017). On the contrary, other studies determined the usefulness of oral fluids as pen-based surveillance tool for *M. hyopneumoniae* detection within farms affected by PRDC (Cheong *et al.,* 2017; Hernández-García *et al.,* 2017; Rawal *et al*., 2018).

Notably, some authors have suggested the quantification of coughing (known as coughing index) together with serologic testing as an EP diagnostic approach (Nathues *et al.,* 2012). However, given that subclinical infections can occur, the most adequate strategy for monitoring *M. hyopneumoniae* status of a herd would be the combination of attentive clinical-pathological observations (clinical signs and lung lesions) coupled with laboratory analyses (ELISA and PCR).

1.7. Treatment and prevention strategies

The occurrence of *M. hyopneumoniae* infections as well as the development and severity of disease associated with this bacterium is influenced by several risk factors (Nathues *et al.,* 2014; Pieters *et al.,* 2014) such as the stocking density (Tuovinen *et al.,* 1990), the distance to other farms (Maes *et al.,* 2000), co-infections with other bacteria and/or viruses, as well as housing (Nathues *et al.,* 2013) and climatic conditions (Segalés *et al.,* 2012; Rawal *et al.,* 2018), among others. In consequence, different approaches such as the optimization of management practices and farm housing conditions, antimicrobial use and vaccination strategies have been proposed to control *M. hyopneumoniae* infections within herds (Maes *et al.,* 2008; Maes *et al.,* 2018).

1.7.1. Management practices and housing conditions

The improvement of management practices and housing conditions, as well as the general herd characteristics, are essential for preventing and controlling *M. hyopneumoniae* infections. Different studies evaluated the influence of these risk factors on *M. hyopneumoniae* infection at weaning (Grosse Beilage *et al.,* 2009; Nathues *et al.,* 2013; Pieters *et al.,* 2014) as well as the occurrence and severity of associated respiratory disease such as EP in posterior production stages (Nathues *et al.,* 2014, 2016). A summary of the most important risk factors is shown in Table 1-3.

Table 1-3. Summary of the most important management practices and housing conditions influencing the risk of *M. hyopneumoniae* infection and the occurrence of associated respiratory diseases.

		Factors increasing the risk of *M.hyopneumoniae* infection and associated diseases	**Factors decreasing the risk of *M.hyopneumoniae* infection and associated diseases**
Herd features	Production system	Farrow-to-finish	Multiple sites
	Pig density area	High	Low
	Replacement purchase	External	Own/Known
		Health status unknown	Health status verification
	Replacement rate	High (>120 gilts/year)	<120 gilts/year
Management practices	Pig flow	Continuous flow	AIAO
	Restocking	Yes, with different ages	No restocking
	Cross-fostering	Highly practiced	No practiced
	Age of weaning	> 4 weeks of age	Early weaning
	Quarantine	No quarantine	Time > 30 days
	Gilt acclimation	No acclimation	Yes (method used, time)
	Co-infections	PRRSV, SIV, PCV-2, App	No co-infections
	Treatment and/or vaccination	No treatment and/or vaccination	Reduction the presence of *M. hyopneumoniae*
Farm housing conditions	Biosecurity and hygiene measures	No basic biosecurity measure	Strict biosecurity and hygiene measures
	Temperature	Fluctuations	30-32ºC for piglets 20-25ºC for fattening pigs
	Ammonia concentration	>10 ppm	5-10 ppm
	Density	Farrowing pens per compartment >16	>0.7m^2/pig at fattening period

AIAO: all-in/ all-out policy; PRRSV: *Porcine reproductive and respiratory syndrome virus*; SIV: *Swine influenza virus*; PCV-2: *Porcine circovirus 2*; App: *Actinobacillus pleuropneumoniae.*

One of the most critical management practices for preventing and controlling diseases in general, and *M. hyopneumoniae* infection in particular, is the introduction of replacement (Nathues *et al.,* 2013, 2014, 2016; Pieters *et al.,* 2014). In fact, the risk of herd destabilization for *M. hyopneumoniae* increases when replacement rates are high or gilts are coming from different origin

sources (Maes *et al.,* 2008; Nathues et al, 2013, 2016). To decrease such risk, an isolation period or quarantine of replacement at arrival is recommended to avoid the entrance of new pathogens, and/or different strains, into the recipient herd. Afterwards, gilt acclimation period is required to develop an active immunity against the pathogens that are present in the recipient herd to maintain the breeding stability. Such acclimation period allows for an effective exposure to the pathogens and the development of immune responses, minimizing clinical disease and facilitating the recovery from infectious period (Pieters and Fano, 2016).

Given the epidemiologic importance of this process, special attention should be paid to the health status of the replacement animals and of recipient herds. In a recent review, Garza-Moreno et al. (2018) proposed a *M. hyopneumoniae* status classification based on observational and laboratory diagnosis in farms and its replacement (Table 1-4).

Table 1-4. Proposed classification according to *M. hyopneumoniae* health status (Garza-Moreno *et al.,* 2018).

Classification		Clinical signs	Lung lesions	ELISA result [a]	PCR result
Negative		Not observed	Not observed	Negative	Negative
Provisionally negative		Not observed	Not observed	Positive	Negative
Positive	**Subclinically infected I**	Not observed	Not observed	Positive/Negative	Positive
	Subclinically infected II	Not observed	Observed	Positive/Negative	Positive
	Clinically affected	Observed	Observed	Positive/Negative	Positive

[a]ELISA results (negative/positive) could depend on infection pattern in the farm and sampling time point.

According to this classification, different situations may be in place depending on the health status of recipient herd and incoming replacement. Importantly, a variety of management strategies have been proposed to deal with these types of farms (Table 1-5; Garza-Moreno *et al.,* 2018). Nevertheless, the efficacy of gilt acclimation controlling *M. hyopneumoniae* infection as well as the most adequate strategy to acclimate gilts are fairly unknown.

Table 1-5 Scenarios for replacement introduction within breeding herd farms according to *M. hyopneumoniae* health status (Garza-Moreno *et al.,* 2018).

<table>
<tr><th colspan="2" rowspan="2"></th><th colspan="2">Recipient herd</th></tr>
<tr><th>Negative/Provisional negative</th><th>Subclinically infected and clinically affected</th></tr>
<tr><td rowspan="2">Incoming gilts</td><td>Negative / Provisional negative</td><td>Isolation period to warrant gilts are M. hyopneumoniae negative and any antibodies against the pathogen is detected</td><td rowspan="2">Gilt acclimation is required:
- Entry into acclimation unit as early as possible
- Exposure at least 210-240 before farrowing
- Vaccination against M. hyopneumoniae to stimulate and homogenize the immune response
- Identification of M. hyopneumoniae source (“shedders”)
- Process verification</td></tr>
<tr><td>Subclinically infected and clinical affected</td><td>Gilt entrance should be avoided. If it is not possible, gilt entrance will be postponed until the infection is cleared:
- No clinical signs
- Shedding is ceased
- Lack of antibodies against the bacterium</td></tr>
</table>

1.7.2. Antibiotics

Due to the lack of cell wall in *M. hyopneumoniae*, antimicrobials targeting cell wall precursors, such as β-lactam, must not be used. The common classes of antimicrobials used against *M. hyopneumoniae* in the swine industry include macrolides, tetracyclines, lincosamides, pleuromutilins, fluoroquinolones,

amphenicols, aminoglycosides and aminocyclitols (Holst *et al.,* 2015; Maes *et al.,* 2018). Characteristics and mechanism of action for each antimicrobial are summarized in Table 1-6.

Table 1-6. Characteristics summary of the most common used antimicrobials against *M. hyopneumoniae* (adapted from Holst *et al.,* 2015).

	Antimicrobial class	Mechanism of action	Common antimicrobial used in the swine industry	Route of administration
Bacteriostatic	Macrolides	Inhibition of protein synthesis	Erytromycin Tulathromycin Tylosin Tylvalosin	P, MF or MW P P, MF or MW MW
	Tetracyclines	Inhibition of protein synthesis	Chlortetracycline Doxycycline Oxytetracycline	MF or MW MF or MW P, MF or MW
	Lincosamides	Inhibition of protein synthesis	Lincomycin	P, MF or MW
	Pleuromutilins	Inhibition of protein synthesis	Tiamulin	MF or MW
	Fluoroquinolones	DNA synthesis inhibition	Enrofloxacin Marbofloxacin	P P
	Amphenicoles	Inhibition of protein synthesis	Florfenicol	P or MW
Bactericidal	Aminoglycosides	Inhibition of protein synthesis	Gentamicine Neomycine Streptomycine	P MW P or MW
	Aminocyclitols	Inhibition of protein synthesis	Spectinomycin	P or MW

P: Parenteral; MF: Mixed in feed; MW: Mixed in water.

Antimicrobials against *M. hyopneumoniae* can be applied individually and/or to the whole herd group, and the route of administration can be parenteral (frequently used for individual treatments) or mixed in feed/water (commonly

used for group treatments) depending on the choice of antibiotic given (Table 1-6). The efficacy of the described antimicrobials for the treatment of *M. hyopneumoniae* has been evaluated in several experimental and field studies. The main described effects are improvement of ADWG and feed conversion efficiency as well as reduction of bacterial load, clinical signs and lung lesions (McKelvie *et al.,* 2005; Nanjani *et al.,* 2005; Nutsch *et al.,* 2005; Thacker 2006; Ciprián *et al.,* 2012; Pallarés *et al.,* 2015). This efficacy justifies the use of antimicrobials for treating pigs suffering clinical signs, normally associated with EP and PRDC. Some studies showed that the use of antimicrobials do not completely eliminate *M. hyopneumoniae* from the pig (Overesch and Kuhnert, 2017), and the bacterium could still be isolated after cessation of the treatment (Le Carrou *et al.,* 2006; Ciprián *et al.,* 2012). Therefore, the promotion of more effective alternative strategies to antimicrobials for controlling *M. hyopneumoniae* is required.

Another common approach for controlling *M. hyopneumoniae* infections under field conditions is the use of strategic medication. It is based on medication of herds with the aim of decreasing the *M. hyopneumoniae* shedding, and minimize the pathogen transmission (Thacker and Minion, 2012). This metaphylactic treatment is commonly utilized in gilts and sows prior to farrow to reduce the *M. hyopneumoniae* shedding to the offspring (Holst *et al.,* 2015). In fact, some authors have also considered this strategy as prophylactic treatment (Karriker *et al.,* 2012). Nevertheless, the inappropriate usage of antimicrobials leads to the development of antimicrobial resistance, and thus, its use should be limited to a therapeutic objective. Indeed, *M. hyopneumoniae* resistance to lincosamides, macrolides, tetracyclines and fluoroquinolones has been already reported (Stakenborg *et al.,* 2005; Le Carrou *et al.,* 2006; Vicca *et al.,* 2007; Del Pozo Sacristán *et al.,* 2012; Thongkamoon *et al.,* 2013; Qiu *et al.,* 2018). Since antimicrobial resistance is

one of the most important concerns for the public health (World Health Organization, 2017), public authorities are proposing initiatives focused on the responsible and reduced antimicrobial use. Some of these proposed initiatives are the prohibition of certain uses of antimicrobials such as the prophylactic and metaphylactic use of antimicrobials (European Commission, 2018).

1.7.3. Vaccination

Vaccination against *M. hyopneumoniae* is one of the most commonly used strategy to control its infection in swine production worldwide (Maes *et al.,* 2018). Different studies have demonstrated the efficacy of vaccination showing reduction of clinical sign severity, lung lesion extension and *M. hyopneumoniae* bacterial load in the respiratory tract (Vranckx *et al.,* 2012a, Woolley et al., 2013; Michiels et al., 2017b). Such reduction of respiratory signs also implies an improvement of productive parameters such as ADWG, feed conversion ratio and mortality rate (Maes *et al.,* 1999; Pallarés *et al.,* 2015; Maes *et al.,* 2018). Moreover, vaccination against *M. hyopneumoniae* represents the most feasible alternative to antibiotic reduction in the future.

Most commercial vaccines against *M. hyopneumoniae* are inactivated whole-cell preparations or bacterins (Maes *et al.,* 2018). The main differences among these vaccines are the *M. hyopneumoniae* strain, adjuvant used and the vaccination schedule proposed by the manufacturer (Table 1-7). Although the administration route of most of these vaccine products is intramuscular, there is one vaccine licensed to be applied intradermically. Interestingly, some of these vaccines are combined with other pathogens (i.e. PCV-2, *H. parasuis* or PRRSV*)* that can be ready-to-use or ready-to-mix.

Table 1-7. Summary of most common used commercial vaccines (bacterins) against *M. hyopneumoniae* available in Europe (adapted from Garza-Moreno *et al.,* 2018).

Manufacturing company	Vaccine name	Antigen	Licensed for	Dosage and route of application	Dose and Schedule proposed by the manufacturer	
Boehringer Ingelheim	Ingelvac Mycoflex® [a]	Strain J	Pigs	1 ml, IM	Single	3w
Ceva	Hyogen®	Strain 2940	Pigs	2 ml, IM	Single	>3w
Elanco	Stellamune® One	Strain NL1042	Pigs	2 ml, IM	Single	3d/3w
	Stellamune®Mycoplasma		Pigs	2 ml, IM	Double	1w + 3w
HIPRA	Mypravac® Suis	Strain J	Pigs	2 ml, IM	Double	1w + 3w
MSD Animal Health	M + PAC®	Strain J	Pigs	2 ml, IM 1 ml, IM	Single Double	3w 1w + 3w
	Porcilis® Mhyo	Strain 11	Pigs	2 ml, IM	Double	>1w + 3w
	Porcilis® Mhyo ID ONCE		Pigs	0.2 ml, ID	Single	3w
	Porcilis® PCV-Mhyo[b]	Strain J	Pigs	2 ml, IM	Single	3w
zoetis	Suvaxyn® MH-One/Mono	Strain P-5722-3	Pigs	2 ml, IM	Single	>1w
	Suvaxyn® Mhyo		Pigs	2 ml, IM	Double	1w + 3w
	Suvaxyn® Circo+MH[bc]		Pigs	2 ml, IM	Single	3w
	Suvaxyn® MHYO-PARASUIS[d]		Pigs	2 ml, IM	Double	>7d + 2w

[a] This vaccine can be used as ready-to-mix product with PCV-2 and PRRSV vaccines; [b] Combined with PCV-2; [c] Vaccine based on soluble antigens of *M. hyopneumoniae*; [d] Combined with *H. parasuis*; IM: Intramuscular; ID: Intradermal; w: weeks; d: days; m: months.

A potential alternative to commercial vaccines could be autogenous vaccines. In this case, these vaccines are prepared from lung tissue homogenate obtained from pigs showing CVPC associated to *M. hyopneumoniae* infections. However, given the difficulty for *M. hyopneumoniae* isolation and growth, the usage of autogenous vaccine is limited (and not allowed in certain parts of the world). Furthermore, in a previous study where the immunization efficacy with homologous and heterologous strains against *M. hyopneumoniae* infection challenge was compared, no differences in terms of clinical signs, lung lesions, pig colonization and serological response were detected (Villarreal *et al.,* 2012).

Despite the well-known advantages associated with vaccination, this strategy does not prevent *M. hyopneumoniae* colonization (Thacker *et al.,* 1998). Anyway, commercial vaccines against *M. hyopneumoniae* induce humoral and cellular immune responses (Thacker *et al.,* 1998). Several studies have demonstrated that these vaccines induce humoral immune response in terms of IgG, IgM and IgA antibodies (Thacker, 2000; Vranckx *et al.,* 2012a; Marchioro *et al.,* 2013; Michiels *et al.,* 2017b). However, vaccine induced immune response has been suggested to target different cell-surface proteins of *M. hyopneumoniae* (Assunçao *et al.,* 2005; Martelli *et al.,* 2006; Calus *et al.,* 2007; Nathues *et al.,* 2011a; Vranckx *et al.,* 2011, 2012a). Some authors suggested such immune response may vary according to the strain used in the vaccine as well as the strain that is present in the farm (Assunção *et al.,* 2005). Therefore, the diversity in such immunogenic surface proteins among *M. hyopneumoniae* strains might explain the inconsistent efficacy of antibodies elicited by vaccination between herds (Minion *et al.,* 2002; Assunçao *et al.,* 2005; Vranckx *et al.,* 2012a).

Likewise, cell-mediated immune responses induced by vaccines are also considered important for protection since are claimed to reduce the production of pro-inflammatory cytokines, especially TNF-α (Thacker *et al.,* 2000; Vranckx *et al.,* 2012a; Machioro *et al.,* 2013). Moreover, higher number of IL-10 and IL-12 secreting cells were detected in vaccinated pigs from one study (Machioro *et al.,* 2013). Despite both humoral and cellular responses are involved in induction of protection after vaccination, the exact mechanism of immune response is not fully understood (Thacker, 2000; Machioro et al. 2013; Maes *et al.,* 2018). Therefore, *M. hyopneumoniae* vaccination is recommended to be applied coupled with abovementioned management control measures for a global disease control.

Although different vaccination strategies have been used to control *M. hyopneumoniae* (Maes *et al.,* 2018), ideally the first step before implementing a vaccination program would be the determination of *M. hyopneumoniae* infection dynamics within farms. Vaccination should be applied before *M. hyopneumoniae* exposure to induce immunity against the pathogen (Maes *et al.,* 2008). Moreover, the use of vaccination during an extended period is recommended as benefits of vaccination are observed several months after administration (Haesebrouck *et al.,* 2004). Further, an analysis of cost-benefit of vaccination should be considered to choose the adequate vaccination program (Maes *et al.,* 2003).

1.7.3.1. Piglet

Piglet vaccination against *M. hyopneumoniae* is practiced worldwide (Maes *et al.,* 2018). Piglets can be vaccinated during the first days of life or at weaning (3-4 weeks of age). In particular, only one dose during the first days of life or at weaning is applied in single vaccination programs. However, piglets receive

the first shot at first days or week of age and the second dose two weeks later in two-shot vaccination programs (Table 1-7). Although the number of peer-reviewed studies comparing both strategies is rather limited, both vaccination programs showed comparable results under similar infectious pressure (Roof *et al.,* 2001, 2002; Alexopaulos *et al.,* 2004; Grenier *et al.,* 2011). Nevertheless, single vaccination is applied more frequently as implies less labor and is more welfare friendly since the animals are handled once (Alarcon *et al.,* 2014).

The efficacy of piglet vaccination prior weaning (Reynolds *et al.,* 2009; Kim *et al.,* 2011; Wilson *et al.,* 2012; Del Pozo Sacristan *et al.,* 2014) and at weaning (Maes *et al.,* 1999; Pallarés *et al.,* 2000; Strait *et al.,* 2008; Michiels *et al.,* 2017; Jeong *et al.,* 2018) have been demonstrated efficacious under experimental and field conditions. Importantly, recent studies concluded that the stress caused by the weaning process may influence the vaccine efficacy in terms of performance parameters (Arsenakis *et al.,* 2016, 2017). In this way, piglet vaccination applied shortly before weaning should be considered (Arsenakis *et al.,* 2017). In contrast, some authors suggested that the early vaccination of piglets in presence of high maternally derived antibodies levels (e.g. first weeks of age) could interfere with the immune response elicited by the vaccine (Wallgren *et al.,* 1998; Hodgins *et al.,* 2004; Grosse-Beilage and Schereiber, 2005). However, the maternally derived immunity seems to have an apparent little or no effect on piglet immune response to the vaccination (Martelli *et al.,* 2006; Bandrick *et al.,* 2008, 2014).

Another strategy is pig vaccination at post-weaning or early growing-fattening period. This late vaccination (4-10 weeks of age) is mainly recommended in farms where late *M. hyopneumoniae* infections occur, i.e. multi-site production system when segregated weaning is practice (Haesebrouck *et al.,*

2004; Maes *et al.,* 2008). However, this strategy is not recommended in herds where piglets are infected early (i.e. one-site system).

1.7.3.2. Breeding herd

Vaccination of breeding herds against *M. hyopneumoniae* is becoming commonly used for a global disease control. Nevertheless, there is limited information in this respect in the peer-reviewed literature. Particularly, there is not available information on the effect of vaccination in gilts during the acclimation period as well as in boars and/or barrows used for heat detection in breeding herds. In contrast, sow vaccination has been evaluated in different studies by the application of different sow vaccination programs against *M. hyopneumoniae* prior to farrowing.

The objective of sow vaccination against *M. hyopneumoniae* is to reduce vertical transmission (nose-to-nose) from the dam to the offspring (Ruiz *et al.,* 2003; Sibila *et al.,* 2008) as well as passively immunize their offspring by the transference of maternally derived immunity through the colostrum (Martelli *et al.,* 2006; Bandrick *et al.,* 2008). In these investigations, vaccination of diverse parity sows with different commercial vaccines was evaluated using different number of doses and application timings. Some authors evaluated one dose sow vaccination protocol at 2 weeks (Martelli *et al.,* 2006) prior farrowing. In contrast, in other studies, multiple vaccine doses were applied at 8 and 4 (Grosse Beilage and Schereiber, 2005), 6 and 3 (Kristensen *et al.,* 2004), 5 and 3 (Ruiz *et al.,* 2003; Sibila *et al.,* 2008) or 4 and 2 (Hodgins *et al.,* 2004) weeks prior to farrowing. All these studies concluded that sow vaccination elicited higher levels of antibodies in sows and their piglets compared to non-vaccinated groups. Moreover, a numerically reduction of *M.*

hyopneumoniae colonization in piglets coming from vaccinated sows has been reported (Ruiz *et al.,* 2003; Sibila *et al.,* 2008).

Regarding gilt vaccination, immunization may be applied during the acclimation period as well as prior to the first farrowing. The aim of gilt vaccination during acclimation is to induce immunity against *M. hyopneumoniae* and reduce the shedding at first farrowing (Pieters and Fano, 2016). Moreover, this strategy homogenizes the immune status of the replacement batch prior entry into herd, avoiding the presence of a seronegative population and, therefore, to decrease the risk of herd immunity destabilization after gilt entry (Bargen, 2004).

Sows with different parity can shed *M. hyopneumoniae* (Calsamiglia and Pijoan, 2000), but gilts are considered the main *M. hyopneumoniae* shedders (Boonsoongnern *et al.,* 2012). In consequence, an adequate acclimation is key for controlling *M. hyopneumoniae* within herds. Different gilt vaccination protocols during acclimation have been proposed in terms of number of doses and timing of application (Alfonso *et al.,* 2004; Yeske, 2007). Despite there is little information regarding the adequate gilt vaccination protocol and its effect on vertical transmission, two surveys carried out in the USA and Mexico determined vaccination as the main strategy used to acclimate gilts (Centeno *et al.,* 2016; Fano and Payne, 2015). Further research is needed to describe the effect of gilt vaccination and establishing and adequate gilt vaccination protocol.

1.7.4. Exposure to *M. hyopneumoniae*

Another strategy to control *M. hyopneumoniae* within farms is to acclimate gilts by a natural and/or controlled exposure to the bacterium (Dalquist, 2014;

Fano and Payne 2015, Centeno *et al.,* 2016; Sponheim, 2017). The natural exposure attempts to exposure the incoming replacement to the *M. hyopneumoniae* strain/s present within farm by the contact with infected and shedding cull sows and/or piglets. Due to the slow and low transmission ratio of *M. hyopneumoniae* (Meyns *et al.,* 2004; Roos *et al.,* 2016), the shedding status of the considered shedders and the time of exposure are crucial to achieve an effective exposure (Roos *et al.,* 2016). An inconsistent exposure to the bacteria might not guarantee that all gilts get infected and may lead to the presence of negative subpopulation, and consequently, a potential farm destabilization. In order to avoid such scenario, another approach based on a controlled exposure by administering (intratracheally) lung tissue homogenate containing *M. hyopneumoniae (*Fano and Payne, 2015; Centeno *et al.,* 2016) or by inoculating fresh *M. hyopneumoniae* culture derived from a *M. hyopneumoniae* field strain (Schleper *et al.*, 2018) has been proposed. Recently, the exposure of groups of gilts to *M. hyopneumoniae* via aerosol has been described (Sponheim, 2017). This controlled exposure would favour the *M. hyopneumoniae* infection, but further research is needed to determine the efficacy and safety of each of these deliberate exposure methods.

CHAPTER 2

Hypothesis and objectives

Mycoplasma hyopneumoniae associated disease is one of the most important health and economic concerns for the pig industry worldwide. Although different measures for preventing and controlling the disease have been implemented, *M. hyopneumoniae* is still causing several respiratory problems. One of the most critical point for controlling *M. hyopneumoniae* within farms is the first farrowing because gilts are considered as main shedders (Boonsoongnern *et al.,* 2012; Pieters and Fano, 2016). An appropriate gilt acclimation focused on reducing the *M. hyopneumoniae* shedding has been proposed as attempt to control this disease (Pieters and Fano, 2016). However, little is known about gilt acclimation methods for *M. hyopneumoniae* used in practice.

In addition, the severity of respiratory diseases associated with *M. hyopneumoniae* is presumably linked to *M. hyopneumoniae* strain/s circulating within herds. Currently, *M. hyopneumoniae* genetic diversity has been detected at country, farm, batch, pig and even at sample level (Nathues *et al.,* 2011a; Charlebois *et al.,* 2014; Dos Santos *et al.,* 2015; Michiels *et al.,* 2017a). Nevertheless, minimal information is available regarding *M. hyopneumoniae* genetic variability considering the pig vaccination status as well as the strains used in *M. hyopneumoniae* vaccines.

Considering the abovementioned rationales, the general aim of this PhD Thesis was to get novel insights regarding gilt acclimation practices to control *M. hyopneumoniae* infections and on the impact of the genetic variability on lung lesions under different scenarios (vaccination versus non-vaccination). The specific objectives of this PhD thesis were:

- To describe the gilt acclimation strategies for *M. hyopneumoniae* performed in Europe (*Chapter 3*).

- To compare the effect of different gilt vaccination protocols against *M. hyopneumoniae* on gilt and piglet infection and seroconversion as well as on the genetic variability within a positive clinically affected farm (*Chapter 4).*
- To determine the *M. hyopneumoniae* genetic variability in vaccinated and non-vaccinated slaughtered pigs showing CVPC (*Chapter 5*).

CHAPTER 3

Study I

Survey on *Mycoplasma hyopneumoniae* gilt acclimation practices in Europe

3.1. Introduction

The prevalence and severity of *M. hyopneumoniae* lung lesions at growing and finishing stages have been correlated with the *M. hyopneumoniae* colonization of piglets at weaning (Fano *et al.,* 2007; Sibila *et al.,* 2007b). The exposure of piglets to *M. hyopneumoniae* during the lactation period is mainly due to contact with the dam (Nathues *et al.,* 2016). In fact, gilts are considered the main source of *M. hyopneumoniae* introduction a farm (Calsamiglia and Pijoan, 2000; Boonsoongnern *et al.,* 2012) and the first farrowing has been proposed as a key point to control *M. hyopneumoniae* transmission within herds (Pieters and Fano, 2016). Additionally, the absence of acclimation period has been rated as an important risk factor for *M. hyopneumoniae* infections at weaning (Nathues *et al.,* 2013, 2016; Pieters *et al.,* 2014). Hence, an adequate gilt acclimation focused on minimizing gilt shedding at first farrowing could aid in decreasing the colonization of piglets at weaning and consequently, the severity of the disease (Pieters and Fano *et al.,* 2006).

Information on the procedures used for *M. hyopneumoniae* gilt acclimation is scarce worldwide. Up to now, there are only two non-peer reviewed reports describing the strategies most frequently used for *M. hyopneumoniae* gilt acclimation in the USA and Mexico (Fano and Payne, 2015; Centeno *et al.,* 2016). In contrast, there is no published information about gilt acclimation procedures used at European level. Therefore, the objective of the present work was to expand the knowledge about replacement stock status in regards of *M. hyopneumoniae* and to identify the gilt acclimation strategies performed in the European continent.

3.2. Materials and methods

The survey on *M. hyopneumoniae* European gilt acclimation practices was based on a questionnaire. Such questionnaire was submitted by e-mail and/or by web link (https://es.surveymonkey.com/r/3QxMQ8Z) to swine veterinarians across Europe. E-mail contacts were obtained from databases from *Centre de Recerca en Sanitat Animal* (CReSA), European College of Porcine Health Management (ECPHM) and European Association of Porcine Health Management (EAPHM). Collected data was the product of the voluntary participation of veterinarians and is reported in a descriptive fashion. Each questionnaire represented data from one single farm and was counted as such.

The questionnaire included 15 questions, 10 of them closed (e.g. yes/no or multiple choice questions) and 5 semi-closed (e.g. days of exposure to the acclimation strategy). In the first part of the questionnaire, information related with production system and herd size was requested. In the second part of the document, questions were focused to four main topics:

- Farm status in regards *M. hyopneumoniae* infection. The objective of this question was to ascertain the knowledge of practitioners about the status of their farms and how this status was assessed (by clinical signs, lung lesions, PCR, ELISA and/or others).
- Gilt replacement origin and status. In this section, information concerning to type of replacement (own, purchased or mixed), age of replacement on arrival (days), frequency of replacement entrance into the farm (per year) and number of replacement animals (per entrance) was asked. Additionally, *M. hyopneumoniae* health status of gilts on arrival and the method used to check this status was requested.

- Acclimation strategies and timing. This section asked for the availability of isolation sites for gilt acclimation in the farms (yes or no), management practices used in these facilities (all-in-all-out [AIAO] sites or continuous flow [CF]), protocol applied (vaccines, live animals or others) and the time of exposure of animals to these strategies (if used).

- Methods used to assess the effect of such strategies. The potential verification of *M. hyopneumoniae* acclimation in the farms, as well as the assessment method (PCR or ELISA), was also demanded.

3.3. Results

A total of 321 questionnaires were voluntarily completed by 108 veterinarians from 18 countries from the European continent (Figure 3-1), representing globally 482,391 sows and 140,839 gilts. The median number of questionnaires per veterinarian (Min – Max) was 3.3 (1 – 15).

Country	No. vets
Austria	4
Belarus	1
Belgium	6
Czech Republic	1
Denmark	6
France	1
Germany	2
Greece	3
Hungary	2
Italy	6
Netherlands	2
Poland	3
Portugal	1
Romania	4
Russia	1
Spain	59
Sweden	1
UK	5
Total	**108**

Figure 3-1. Number of questionnaires collected (*n* = 321) and number of responding veterinarians per European country.

General data of farms represented in the survey is shown in Table 3-1. From these 321 farms, 225 were from Southern European countries (Portugal, Italy, Spain and Greece; 70.1%) and 96 were from the rest of participant countries (29.9%).

Table 3-1. Number of farms included in the survey based on production system type and sow-farm size.

Type of production system		n (%)	Size of sow farm		Number of gilts per sow farm	
			Median values	Range[a]	Median values	Range[a]
One site	Farrow to Finish	135 (42.1)	525	75 – 7000	130	7 – 2450
	Farrow to Wean	109 (34.0)	1000	160 – 12000	352	10 –4400
	Wean to Finish[b]	19 (5.9)	1200	390 – 3500	285	50 – 1925
	Finish	3 (0.9)	NR	NR	NR	NR
Multiple sites	Farrow to Wean + Wean to Finish	4 (1.2)	2000	600 – 8000	700	100 –4000
	Farrow + Wean + Finish	51 (15.9)	1040	400 - 6500	400	110 – 3380
Total		321	NA	NA	NA	NA

[a] Range indicates minimum and maximum values [b] Number of sows and gilts in this production system indicates the number of sows and gilts from those breeding farms where piglets came from; NA: Non-applicable; NR: Non-reported.

3.3.1. Assessment of *M. hyopneumoniae* herd status

The assessment of *M. hyopneumoniae* farm status was reported by all but one of the farms (320 out of 321, 99.7%). Among these 320 farms, *M. hyopneumoniae* farm status was evaluated using one (80 [25.0%]), two (148 [46.3%]), three (51 [15.9%]), four (39 [12.2%]) and even five (2 [0.6%]) methods, respectively (Table 3-2).

Table 3-2. Number of farms (%) according to number of methods used by responders to assess *M. hyopneumoniae* status of farms.

No. of methods	Methods					Total	%
	Clinical signs	Lung lesions	PCR	ELISA	Others		
0						1	0.3
1	✓					39	12.1
		✓				14	4.4
				✓		25	7.8
					✓	2	0.6
2	✓	✓				95	29.6
	✓			✓		22	6.9
	✓		✓			25	7.8
		✓		✓		4	1.2
	✓				✓	1	0.3
3		✓	✓			1	0.3
	✓		✓	✓		17	5.3
	✓	✓		✓		15	4.7
	✓	✓	✓			8	2.5
		✓	✓	✓		7	2.2
	✓	✓			✓	2	0.6
	✓			✓	✓	2	0.6
4	✓	✓	✓	✓		36	11.2
	✓	✓		✓	✓	2	0.6
		✓	✓	✓	✓	1	0.3
5	✓	✓	✓	✓	✓	2	0.6
Total	266	187	97	118	12	321	100.0

3.3.2. Replacement origin and status

Approximately half of the surveyed farms introduced external replacement gilts (Table 3-3), whereas one-third used own replacement. The rest of the farms had a mixed (purchased + own) replacement practice. *M. hyopneumoniae* health status of replacement on arrival was known by 280 out of 321 (87.2%) farms, from which 161 (57.5%) were seropositive. Importantly, only 79 out these 280 (28.2%) farms confirmed the theoretical gilt status upon arrival. The most

frequently used method to verify this status was ELISA (69 out of 79, 87.3%). Results of such verification were not requested.

Age of replacement on arrival at sow farm (in case of farms with purchased replacement) or at internal selection site (in case of farms with own replacement) differed among studied farms, varying from 0 to 210 days (Figure 3-2), being animals older than 100 days the most frequent one. Frequency of replacement batch entry into the herd also varied, from annual to weekly or as needed without further specification (Figure 3-3). Gilt introduction every three or four months was the most frequent practice (228 out of 321, 71.0%).

Table 3-3. Number of farms (%) based on replacement origin, *M. hyopneumoniae* health status and the verification of replacement status.

No. of farms according to the replacement source (%)	No. of farms according to *M. hyopneumoniae* health status (%)			No. of farms which verify the health status of the replacement (%)	Method used for replacement verification (%)			
					ELISA	PCR	ELISA+PCR	Others
Purchased 145 (45.2)	Known 126 (86.9)	Positive	61/126 (48.4)	4/61 (6.6)	2/4 (50.0)	0/4 (0.0)	2/4 (50.0)	0/4 (0.0)
		Negative	36/126 (28.6)	10/36 (27.8)	8/10 (80.0)	1/10 (10.0)	1/10 (10.0)	0/10 (0.0)
		Non-specified	29/126 (23.0)	24/29 (82.8)	24/24 (100.0)	0/24 (0.0)	0/24 (0.0)	0/24 (0.0)
	Unknown 19 (13.1)	NA	NA	NA	NA	NA	NA	NA
Own 103 (32.1)	Known 86 (83.5)	Positive	60/86 (69.8)	12/60 (20.0)	10/12 (83.3)	2/12 (16.7)	0/12 (0.0)	0/12 (0.0)
		Negative	25/86 (29.1)	22/25 (88.0)	21/22 (95.5)	0/22 (0.0)	0/22 (0.0)	1/22 (4.5)
		Non-specified	1/86 (1.1)	0/86 (0.0)	NA	NA	NA	NA
	Unknown 17 (16.5)	NA	NA	NA	NA	NA	NA	NA
Mixed 73 (22.7)	Known 68 (93.2)	Positive	40/68 (58.8)	2/40 (5.0)	1/2 (50.0)	0/2 (0.0)	0/2 (0.0)	1/2 (50.0)
		Negative	27/68 (39.7)	4/27 (14.8)	3/4 (75.0)	1/4 (25.0)	0/4 (0.0)	0/4 (0.0)
		Non-specified	1/68 (1.5)	1/1 (100.0)	0/1 (0.0)	0/1 (0.0)	0/1 (0.0)	1/1 (100.0)
	Unknown 5 (6.8)	NA	NA	NA	NA	NA	NA	NA

NA: non-applicable

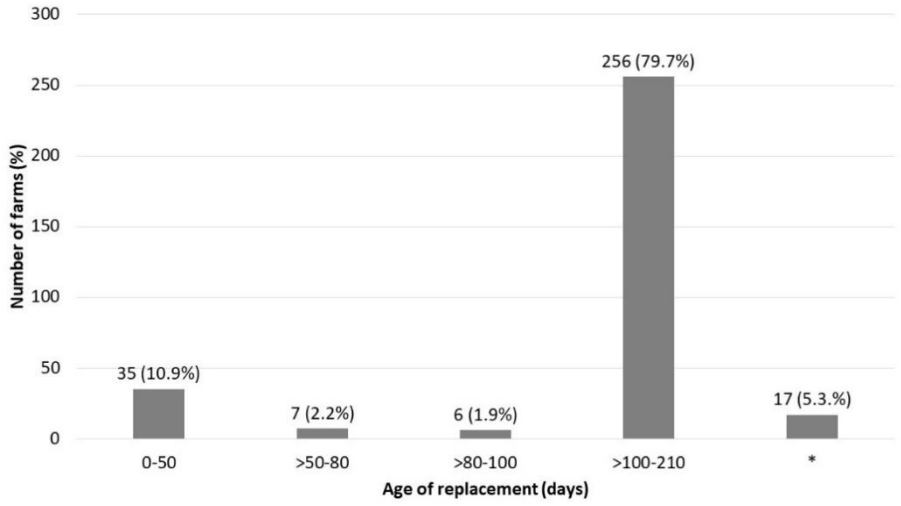

Figure 3-2. Number of farms (%) included in the survey according to the age of replacement gilts on arrival/internal selection site (days). Asterisk (*) indicates that responders did not specify the age of replacement on arrival/internal selection site.

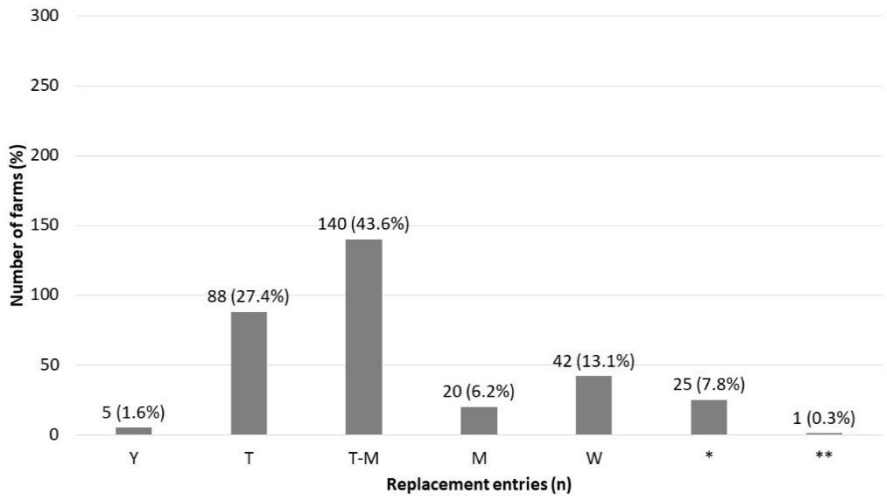

Figure 3-3. Number of farms (%) based on the frequency of replacement entry into farms included in the survey. Y: year; T: trimester; T-M: trimester to monthly; M: monthly; W: weekly; *: non-specified; ** when needed.

3.3.3. Acclimation strategies

Information of *M. hyopneumoniae* acclimation strategies performed is shown in Table 3-4. From the 321 farms for which information was obtained in the questionnaire, 278 (86.6%) had gilt isolation sites available for acclimation, which were managed mainly by AIAO practices. From these 278 farms, 225 (80.9%) farms, performed a specific acclimation process for *M. hyopneumoniae*. From these 225 farms, 159 (70.7%) were located in Southern European countries and 66 (29.3%) in the rest of European participating countries. However, there were 24 farms that, although not having isolation units or not answering this question, performed acclimation process for *M. hyopneumoniae* (no more information was available regarding the specific site used to acclimate gilts). Additionally, from these 249 farms performing acclimation strategies, *M. hyopneumoniae* status of gilts on arrival was investigated in 224 farms (224/249, 90.0%).

Vaccination against *M. hyopneumoniae* was the most used acclimation procedure (145/249, 58.2%), followed by vaccination and animal exposure, with either culled sows (53/249, 21.3%) or pigs (13/249, 5.2%), and a combination of vaccination and both types of live animal exposure (31/249, 12.4%) (Table 3-5). The number of vaccine doses used during gilt acclimation varied among one (71 out of 243, 29.2%), two (77 out of 243, 31.7%) or three (38 out of 243, 15.6%). There were 57 (23.4%) farms for which no data were available on how many doses were administered to gilts.

Table 3-4. Information of *M. hyopneumoniae* acclimation strategies performed by the respondents.

	Availability of isolation units (%)	Management practices in isolation units				No. of farms performing *M. hyopneumoniae* acclimation strategies (%)	Mean (range) of duration of acclimation period (days)	No. of farms verifying acclimation (%)	Methods used for acclimation verification (%)		
		AIAO	CF	AIAO/CF	NR				ELISA	PCR	ELISA+PCR
Collected data	Yes 278 (86.6)	122/278 (43.9)	82/278 (29.5)	1/278 (0.4)	73/278 (26.2)	224/278 (80.6)	28.3 (7 – 180)	53/224 (23.7)	22/53 (41.5)	4/53 (7.5)	27/53 (50.9)
	No 32 (10.0)	NA	NA	NA	NA	19/32 (59.4)	37 (21 – 60)	0/19 (0.0)	NA	NA	NA
	Unknown 11 (3.4)	NA	NA	NA	NA	5/11 (45.5)	NR	0/5 (0.0)	NA	NA	NA
Total	321	122	82	1	73	249	NA	54	22	4	27

AIAO: all in-all out; CF: Continuous flow; NA: non-applicable; NR: non-reported

Table 3-5. Number of farms (%) according the methods used for replacement gilt acclimation in terms of *M. hyopneumoniae*.

No. methods	Vaccination	Selected sows for slaughter	Pigs	Others	Total	%
0					72	22.4
1	✓				145	45.2
		✓			2	0.6
			✓		1	0.3
				✓	1	0.3
2	✓	✓			53	16.5
	✓		✓		13	4.1
		✓	✓		2	0.6
	✓			✓	1	0.3
3	✓	✓	✓		31	9.7
Total	243	88	47	2	321	100.0

Mycoplasma hyopneumoniae specific acclimation was performed by 76.4% (172/225) and 80.2% (77/96) of farms from Southern and rest of Europe, respectively. In both geographic regions vaccination was the most frequently used method, reaching values of around of 95%. Nevertheless, whereas in Southern regions combined vaccination with animal exposure (cull sows and/or pigs) was practiced, farms located in the rest of Europe mainly used vaccination alone.

Finally, considering the farms where the initial status was known and the acclimation process was carried out, the verification of the effect of such strategies was performed in only 23.7% of the farms (53/224), being the combination of ELISA and PCR the most frequently used method. Importantly, the confirmation of theoretical gilt status upon arrival and after gilt acclimation to verify the effect was checked in 49.4% farms (39/79). Results of such verification were not requested.

3.4. Discussion

The aim of the present study was to gain knowledge on *M. hyopneumoniae* gilt status and acclimation practices conducted in European farms by means of a descriptive study. To date, no information about this issue is available in Europe and, in fact, minimal data do exist from around the world, with surveys performed in the USA (Fano and Payne, 2015) and Mexico (Centeno *et al.,* 2016).

Questionnaires were voluntarily responded, thus, they may have some inherent biases in the responses (Eisele *et al.,* 2013). Indeed, in this investigation, most of the veterinarians (70.7%) were from Southern European countries. Representation of Central and Northern European countries was more limited. This finding was already expected for some Scandinavian countries due to the low or no prevalence of *M. hyopneumoniae* in pig farms (O. Peltoniemi, Finland, and Carl-Andreas Grøntvedt, Norway, personal communications). Although the European situation might not be fully represented (especially regarding small pig farms with non-specialized swine veterinarians), no significant differences in terms of gilt acclimation practices were detected between the South of Europe (Portugal, Spain, Italy and Greece) and the rest of participating countries (data not shown). Nevertheless, information obtained about *M. hyopneumoniae* current status on gilt acclimation should help depicting measures that can be potentially applied elsewhere. Additionally, the facts that all but one of the participants evaluated *M. hyopneumoniae* status of their farm and more than 80% were aware of their replacement status regarding this pathogen, suggest that EP is still a concern to swine industry. Notwithstanding, this assumption could be influenced by the fact that only concerned veterinarians on *M. hyopneumoniae* completed the questionnaire.

Most of the questionnaire respondents reported that the assessment of farms was based on presence of clinical signs accompanied with lung lesion scoring at

slaughterhouse. Noteworthy, non-productive dry coughing and cranio-ventral pulmonary consolidation (CVPC), the usual clinical signs and lung lesions attributed to *M. hyopneumoniae* infection, can also be produced by other respiratory pathogens (Garza-Moreno *et al.,* 2018), and these parameters do not allow detecting a potential subclinical infection. In consequence, clinical disease assessment should be supplemented with the laboratory confirmation of *M. hyopneumoniae* involvement in clinical signs and lesions (Sibila *et al.,* 2009). A total of 151 out of 320 (47.1%) farms in which *M. hyopneumoniae* status was evaluated based their assessment only on non-specific methods (clinical signs or lung lesions scoring at abattoir). This finding suggested that most European farms represented in this study performed an incomplete assessment of *M. hyopneumoniae* health status. This percentage is lower than Mexican survey (no data is available for US), since in that study, 71% of the respondents evaluated *M. hyopneumoniae* farms situation only according to clinical signs (Centeno *et al.,* 2016).

The introduction of external replacement into a swine herd is considered a potential risk of new pathogen introduction and farm health destabilization (Maes *et al.,* 2008), as well as for becoming infected or re-infected with different strains (Nathues *et al.,* 2013, 2014). However, more than 40% of the evaluated farms purchased external replacement, being in most of the cases seropositive against *M. hyopneumoniae*. Comparatively, percentage of positive replacement in the assessed European farms was similar (161/280, 57.5%) to that in the USA (55%), but lower than in Mexico (90%) (Fano and Payne 2015; Centeno *et al.,* 2016).

Interestingly, most farms (80.9%) had isolation facilities to acclimate, being the most utilized the AIAO system. In terms of type of management systems used to acclimate gilts, a clear difference between participating European farms, the

USA and Mexico was observed. Whereas 75% and 72% of Mexican (Centeno *et al.,* 2016) and US (Fano and Payne, 2015) farms used CF to acclimatize gilts, respectively, only 29.5% of European farms of the present survey performed such strategy. These differences probably reflect the different production systems used in each country.

Replacement gilt acclimation methods used in Southern European farms were mainly based on vaccination alone or in combination with live animal exposure (culled sows or pigs). However, farms from rest of participant countries utilized vaccination exclusively. These results were in line with the ones reported by US and Mexican studies (Fano and Payne 2015; Centeno *et al.,* 2016), in the sense that vaccination was the most used approach. The rationale behind this strategy would be linked to the reduction of the number of animals showing CVPC, reduction of clinical signs (coughing) and decrease of number of microorganisms and bacterial shedding (Meyns *et al.,* 2004). However, current vaccines against *M. hyopneumoniae* are not able to prevent bacterial colonization and the transmission between vaccinated pigs seems not to be significantly altered (Thacker *et al.,* 1998; Maes *et al.,* 2008; Villarreal *et al.,* 2011a,b; Pieters *et al.,* 2009). Presumably infected culled sows or pigs were utilized as potential *M. hyopneumoniae* shedders in 27.4% and 14.6% of studied farms, respectively; however, their shedding status was not required in the questionnaire. These results are in agreement with previous studies in the USA (34% of respondents utilized culled sows to acclimatize) and Mexico (27% of responders used culled sows and 10% piglets) (Fano and Payne 2015; Centeno *et al.,* 2016). Acclimation strategies based on others (unspecified methods) were very scarce (0.3%).

Finally, the relatively low percentage of farms verifying the acclimation process (23.7%) indicated that most of the surveyed farms did not evaluate gilt infection and shedding status at first farrowing. This situation coincided with the

information reported by Mexican and US studies, where only 20% and 14% of responders, respectively, validated the acclimation process. An inadequate acclimation process could imply that gilts would be a potential source of infection for their offspring. Therefore, such scenario may lead to an outbreak of *M. hyopneumoniae* in seronegative farms or *M. hyopneumoniae* re-circulation/re-infection in seropositive ones.

In conclusion, the present study shows that most of the European farms introduced *M. hyopneumoniae* positive replacement stock, but only a minority assessed its health status on arrival. Likewise, most of participating farms performed a specific gilt acclimation procedure against *M. hyopneumoniae*. Moreover, the verification of this process was not a common practice.

CHAPTER 4

Study II

Comparison of vaccination protocols against *Mycoplasma hyopneumoniae* during gilt acclimation period

4.1. Introduction

Gilt vaccination against *M. hyopneumoniae* has been identified as the most common strategy used for acclimation in America and Europe (Garza-Moreno *et al.,* 2018). Despite vaccination against *M. hyopneumoniae* does not fully prevent the infection, this strategy facilitates the development of immune responses and the homogenization of the immunity of the vaccinated population (Maes *et al.,* 2003). Moreover, vaccination minimizes clinical signs caused by *M. hyopneumoniae* and decreases the impact of this bacterium within herds (Maes *et al.,* 2018).

Different gilt vaccination programs against *M. hyopneumoniae* during the acclimation period have been described among farms in terms of number of administered doses and application timings (*Chapter 3*). In fact, gilt vaccination protocols based on multiple doses are becoming commonly used, particularly, in the USA (Alfonso *et al.,* 2004; Yeske, 2007; *Chapter 3*). However, the efficacy of these gilt vaccination programs has not been evaluated and, thus, is not available in the literature. Therefore, the objectives of the present study were: a) to compare different gilt vaccination schedules on the *M. hyopneumoniae* gilt seroconversion and shedding at different times post-vaccination, as well as piglet antibody detection and colonization at weaning, and b) to investigate the potential effect of vaccination on the genetic diversity of *M. hyopneumoniae* within the studied farm.

4.2. Materials and methods

4.2.1. Farm management and housing conditions

A conventional *M. hyopneumoniae* positive, clinically affected (Garza-Moreno *et al.,* 2018) farrow-to-finish farm introducing external negative and own positive replacement gilts was selected. The selected farm had a gilt development unit (GDU) for acclimation with a duration approximately of 10 weeks, which followed AIAO management practices. After acclimation, external and own replacement gilt batches were moved to oestrus detection and synchronization unit (DSU) and were allocated sharing pens (60 gilts per pen). At the DSU, gilt oestrus was synchronized and gilts were divided in weekly batches of 90 gilts according to oestrus detection time. Gilts were artificially inseminated at the second oestrus and pregnancy diagnosis was performed 4 weeks after insemination. Once pregnancy was confirmed, pregnant gilts were moved to the gestation unit and housed in pens (60 gilts/pen). Finally, one week previous to delivery, gilts were moved to the farrowing units. In these facilities, with continuous flow and weekly batch management, a total of 50 gilts and sows of different parities were housed.

4.2.2. *Mycoplasma hyopneumoniae* infectious status previous to the start of the study

To confirm *M. hyopneumoniae* gilt infection during acclimation, laryngeal swabs from a total of 20 gilts (10 gilts/batch) showing clinical signs of dry coughing from two previous batches of own replacement (FS1 and FS2) were collected at 14 weeks post-entry (wpe) in the GDU. In these samplings, 19 out of 20 (95%) gilts were *M. hyopneumoniae* positive by rt-PCR, being 10 out of 10 (100%) positive gilts in FS1 and 9 out of 10 (90%) in FS2. The Ct values varied from 30.7 to 36.9 in FS1 and from 27.5 to 36.9 in FS2.

4.2.3. Animal selection and study design

Blood samples and laryngeal swabs from a total of 180 six month-old gilts coming from an external *M. hyopneumoniae* negative farm were collected at 1 wpe at the GDU (Figure 4-1). Laryngeal swabs and blood samples were tested by rt-PCR and ELISA, for detection of the pathogen and the antibodies against it, respectively. Afterwards, gilts were randomly divided into three groups (A: four vaccine doses; B: two vaccine doses and C: no vaccinated) balanced according to *M. hyopneumoniae* antibodies measured by ELISA percentage of inhibition (PI). Animals received intramuscularly 2 mL per dose of a *M. hyopneumoniae* commercial vaccine (Hyogen®, CEVA Santé Animale, Libourne, Cedex, France) and/or 2 mL of phosphate-buffered saline (PBS) according to their experimental group and time points (Table 4-1).

Table 4-1. Vaccination schedules against *M. hyopneumoniae* performed during the gilt acclimation period.

Group name	No. of gilts	Vaccine doses	Vaccination schedule			
			2 wpe	4 wpe	6 wpe	8 wpe
A	60	4	Hyogen®	Hyogen®	Hyogen®	Hyogen®
B	60	2	Hyogen®	PBS	Hyogen®	PBS
C	60	0	PBS	PBS	PBS	PBS

wpe: weeks post-entry; PBS: phosphate-buffered saline.

Gilts included in this study entered at the DSU splitted into two batches (n=90 each batch) separated by one week. Piglets were ear tagged at birth according to their maternal treatment and cross-fostering was allowed only between sows within the same treatment. One day prior to weaning, six randomly selected piglets per sow were monitored and sampled. No antimicrobials against *M. hyopneumoniae* were administrated to gilts and piglets under study conditions. Study procedures were approved by the Animal Experimentation Ethics Committee of the Generalitat de Catalunya (*Departament de Territori i*

Sostenibilitat, Direcció General de Polítiques Ambientals i Medi Natural; Reference 9336).

4.2.4. Sample collection and processing

Blood samples and laryngeal swabs were collected from gilts at 1, 14, 27 and 34 wpe, and from 6 piglets of each of them at weaning (38 wpe, Figure 4-1). Once in the laboratory, blood was centrifuged at 1500 *g* for 10 min at 4ºC and sera was aliquoted and stored at -20ºC until used. Laryngeal swabs were re-suspended in 1 mL of PBS, vortexed, and stored at -20ºC until DNA extraction was performed.

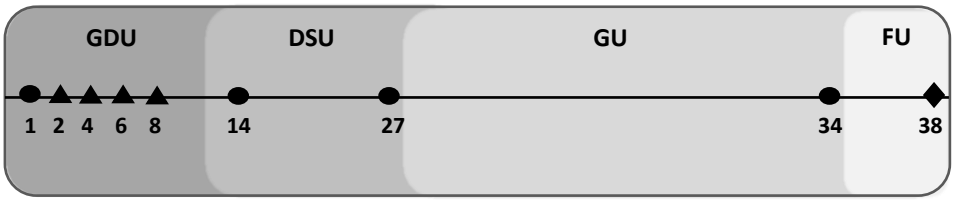

Figure 4-1. Study design, housing location and sampling points of gilts and piglets included in the study. Housing sites are gilt development unit (GDU), detection and synchronization unit (DSU), gestation unit (GU) and farrowing unit (FU). Shapes represent gilt vaccination (▲) and sampling points of gilts (●) at different weeks post-entry (wpe) into GDU are indicated by numbers. Additionally, piglets (◆) were sampled at weaning (equivalent to 38 wpe of gilts).

4.2.5. Detection of *M. hyopneumoniae*-specific antibodies in serum

Sera were tested in duplicate for the presence of antibodies against *M. hyopneumoniae* by means of a commercial competitive ELISA (IDEIA™ *M. hyopneumoniae*, EIA kit, Oxoid, UK). ELISA results were expressed as percentage of inhibition (PI). The PI was calculated considering the mean optical density (OD) of each sample and the buffer control following the formula: % PI = 100*(mean sample OD/ mean buffer control OD). Samples with PI < 50% were considered to be positive, whereas doubtful (PI from 50 to 64%) and negative samples (PI≥65%) were classified as negative. PI median and ranges of tested samples were calculated.

4.2.6. DNA extraction and *M. hyopneumoniae* detection by real time PCR

DNA was extracted from 200 µL of laryngeal swab suspension using MagMax™ DNA Multi-Sample Kit (Life Technologies, USA) according to the manufacturer's instructions, on the BioSprint 96 workstation (Qiagen GmbH, Germany). Two different positive extraction controls were used in each extraction: a laryngeal swab spiked with *M. hyopneumoniae* strain 11 (ATCC®25095™) and a commercial internal positive control (Xeno™, included in VetMax™-Plus qPCR Master Mix kit). Negative controls (PBS) were also included to assess potential contamination during extraction.

Extracted DNA was tested by a commercial rt-PCR for *M. hyopneumoniae* detection: VetMax™-Plus qPCR Master Mix (Life Technologies, USA) and VetMax™ *M. hyopneumoniae* Reagents (Life Technologies, USA), according to the manufacturer's instructions. Rt-PCR runs were carried out in ABI PRISM® 7500 machine (Applied Biosystems, Singapore). The rt-PCR threshold was set at 10% of the maximum fluorescence value of the commercial DNA positive control. Samples with cycle threshold (Ct) values equal or lower than 40 were

considered positive. Ct ranges were calculated considering only rt-PCR positive samples.

4.2.7. *Mycoplasma hyopneumoniae* genetic variability

Positive rt-PCR laryngeal swabs were genotyped by Sanger sequencing method and the VNTR of three loci (P97, P146 and H1) were counted. Moreover, the reference strain 11 (ATCC® 25934™) was also included as technique positive control. Primers used were previously described by Vranckx *et al.,* (2011). These three loci were individually amplified in a final volume of 50 µL. Reaction mixtures contained 1X PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate, 0.4 µM of each primer, 1.5 U of GoTaq® G2 Flexi DNA Polymerase (Promega, Madison, USA) and, finally, 6µL of extracted DNA dilution (1:10). Cycling conditions were 4 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 53°C and 30 s at 72°C, then a final extension step of 7 min at 72°C. The typing PCR products were analyzed by electrophoresis on 2% agarose gel in Tris-Acetate-EDTA (TAE)-buffer and stained with ethidium bromide. These PCR products were purified by ExoSAP-IT® (Isogen Life Science, The Netherlands) according to manufacturer's instructions and sequenced using a ABI PRISM 3130xl (Applied Biosystems, Singapore) genetic analyzer.

Nucleotide sequences were aligned and translated to aminoacid sequences using FingerPrinting II Informatix software (Applied Maths, Saint-Martens-Latem, Belgium). VNTR per each locus were counted and a typing variant profile (TP) was assigned according to the combination of the three loci. The TP was considered different when the combination of VNTR per each locus was unique. A minimum spanning tree (MST) was also constructed to visualize the similarity among TP using PHYLOViZ 2.0 (Ribeiro-Gonçalves *et al.,* 2016).

4.2.8. Statistical analyses

Bivariate analysis using the Kruskal-Wallis test was applied for median comparison of PI among gilt groups (A, B, and C) at different sampling points. The homogeneity of PI values in each group through the study was evaluated by *F* values. The Chi square test was used to evaluate the proportion of positive rt-PCR samples between treatments at different sampling points. When significant results were obtained, *a posteriori* contrast analysis 2 to 2 was performed. *Post hoc* pairwise comparisons were computed using Tukey's Honestly Significant Difference. Additionally, a linear mixed model was used to assess the effect of different gilt vaccination programs on piglet colonization and humoral immunity at weaning, considering sow as a random effect. Statistical analyses were performed with SAS v9.4 (SAS Institute Inc., Cary, NC, USA). The significance level was set to $p<0.05$.

4.3. Results

4.3.1. Detection of antibodies against *M. hyopneumoniae* in gilts and piglets

Studied gilts were seronegative at 1 wpe (Table 4-2, Figure 4-2). At 14 wpe all gilts with the exception of one in group C had seroconverted (179/180, 99.4%). By 27 wpe, the number of gilts were reduced from 180 to 152 (52, 49, and 51 gilts in groups A, B and C, respectively) since 28 gilts were culled (due to lack of pregnancy and lameness, mainly). From 146 gilts (49, 47, and 50 gilts in groups A, B and C, respectively) that reached one week prior to farrowing sampling (34 wpe), all vaccinated gilts (groups A and B) remained seropositive, whereas the percentage of non-vaccinated seropositive gilts was slightly lower (44/50, 88.0%). Statistical differences ($p<0.05$) in terms of proportion of seropositive gilts among vaccinated (A and B) and non-vaccinated (C) groups were detected at 27 and 34 wpe.

Table 4-2. Proportion (%) of *M. hyopneumoniae* seropositive gilts and piglets and median (range) of percentage of inhibition (PI) at different sampling points.

Groups	Sampling points									
	1 wpe		14 wpe		27 wpe		Pre-farrowing (34 wpe)		Piglets (at weaning)	
	Prop (%)	PI (Range)	Prop (%)	PI (Range)	Prop (%)	PI (Range)	Prop (%)	PI (Range)	Prop (%)	PI (Range)
A	0/60 0%[a]	67.1 (51.2-89.4)[a]	60/60 100%[a]	4.8 (3.1-19.8) [a]	52/52 100%[a]	6.8 (3.4-39.3)[a]	49/49 100%[a]	5.7 (3.6-17.8) [a]	230/252 91%[a]	15.5 (2.1-98.7)[a]
B	0/60 0%[a]	67.1 (50.6-85.5)[a]	60/60 100%[a]	5.5 (3.7-13.5)[b]	49/49 100%[a]	8.07 (2.8-22.4)[a]	47/47 100%[a]	6.2 (4.0-36.6) [a]	200/252 79%[a]	23.9 (2.0-94.3)[a]
C	0/60 0%[a]	66.9 (50.4-85.2)[a]	59/60 98%[a]	14.3 (3.3-53.2)[c]	45/51 88%[b]	31.7 (6.6-82.1)[b]	44/50 88% [b]	28.7 (5.0-61.8)[b]	85/240 35% [b]	27.3 (1.0-99.0)[b]
Total	0/180	-	179/180 (99.4)	-	146/152 (96.1)	-	140/146 (95.9)	-	515/744 (69.2)	-

wpe: week post-entry; *Prop*: Proportion; Range: minimum-maximum; Different superscripts within each column indicate significant differences among groups at different time points (p<0.05).

Mean PI values (±SD) for each group at different sampling time points are detailed in Table 4-2. The PI values were statistically different (p<0.05) between vaccinated (A and B) and non-vaccinated (C) groups at 14, 27 and 34 wpe (Table 4-2; Figure 4-2). Statistical differences (p<0.05) among all three groups (A, B and C) were detected in terms of PI at 14 wpe, showing differences between four and two vaccine doses. The F values showed statistical higher homogeneity of IP values (Figure 4-2) through this study in vaccinated group A (F=1.27) compared to group B (F=3.94) and C (F=5.49).

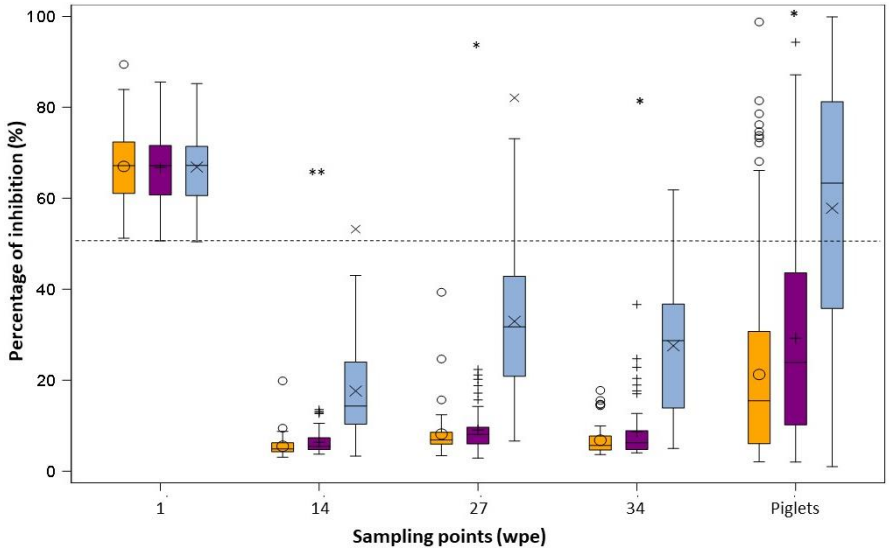

Figure 4-2. Percentages of inhibition (%) of gilts from groups A (■), B (■) and C (■) at 1, 14, 27 and 34 wpe; and from their piglets at weaning. Statistically significant differences (p<0.05) were observed between vaccinated (A and B) and non-vaccinated groups (C) (*) as well as among all three groups (**). The discontinuous line represents ELISA seropositivity threshold.

A total of 744 piglets (252 from gilts of group A [n=42], 252 from gilts of group B [n=42] and 240 from gilts of group C [n=40]) born to the 124 gilts that reached farrowing were sampled one day prior to weaning. The differences in the proportion of seropositive and PI of piglets from vaccinated and non-vaccinated gilts were statistically significant. Additionally, a higher number of seropositive piglets coming from gilts from group A was detected compared to the ones from group B (Table 4-2). A total of 229 piglets were seronegative (30.8%), where 23 (10%) of them came from seronegative non-vaccinated sows (group C) and the remaining 206 (22 [9.6%], 52 [22.7%] and 132 [57.7 %] from groups A, B and C, respectively) came from seropositive sows (Table 4-2).

4.3.2. *Mycoplasma hyopneumoniae* detection in laryngeal swabs in gilts and piglets

Mycoplasma hyopneumoniae positive *s*hedding gilts were detected at 14 and 27 wpe (Table 4-3). The proportions of rt-PCR positive gilts at 14 wpe in vaccinated groups A (1/60, 1.7%) and B (2/60, 3.3%) were significantly lower ($p<0.05$) compared to the non-vaccinated group C (27/60, 45%). However, no statistical differences were found between vaccinated groups (A and B) with different number of doses. The proportion of rt-PCR positive gilts was reduced at 27 wpe and only one positive gilt (1/51, 2.2%) was detected in the group C. Finally, none of the gilts was positive at 34 wpe for rt-PCR.

All laryngeal swab samples from piglets (n=744) were negative for *M. hyopneumoniae* by rt-PCR.

Table 4-3. Proportion (%) of *M. hyopneumoniae* rt-PCR positive gilts and piglets and Ct values at different sampling points of the study.

Groups	Sampling points									
	1 wpe		14 wpe		27 wpe		Pre-farrowing (34 wpe)		Piglets (at weaning)	
	Prop (%)	Ct range (Max – Min)	Prop (%)	Ct range (Min– Max)	Prop (%)	Ct range (Max – Min)	Prop (%)	Ct range (Max – Min)	Prop (%)	Ct range (Max – Min)
A	0/60 0%[a]	NA	1/60 2%[a]	38.8-38.8 [a]	0/52 0%[a]	NA	0/49 0%[a]	NA	0/252 0%[a]	NA
B	0/60 0%[a]	NA	2/60 3%[a]	32.2-37.8[a]	0/49 0%[a]	NA	0/47 0%[a]	NA	0/252 0%[a]	NA
C	0/60 0% [a]	NA	27/60 45%[b]	29-1-38.7 [a]	1/51 2%[a]	37.5-37.5	0/50 0%[a]	NA	0/240 0%[a]	NA
Total	0/180	-	30/180 (16.7)	-	1/152 (0.6)	-	0/146	-	0/744	-

wpe: week post-entry; *Prop*: Proportion; NA*:* Non-applicable; Different superscript within each column indicate significant differences among groups (p<0.05). Ct range has been calculated considering only rt-PCR positive animals.

4.3.3. Characterization of *M. hyopneumoniae* genotypes through the study

All rt-PCR positive samples detected into previous batches (FS1=10 and FS2=9), 14 wpe (n=1 in group A, n=2 in group B and n=27 in group C) and 27 wpe (n=1 in group C) were used for *M. hyopneumoniae* genetic characterization. From these 50 samples, 43 (86.0%) were successfully sequenced by the three selected loci (Table 4-4).

Table 4-4. Variable number of tandem repeat (VNTR) and typing profile (TP) in *M. hyopneumoniae* positive gilts using three loci.

Sampling point	Number of gilts carrying the variant (%)	VNTR			TP	Group	
		P97	P146	H1		Treatment	n
FS1	1 (2.3)	11	38	6	1	NA	10
	1 (2.3)	11	42	6	2		
	2 (4.5)	11	45	6	3		
	5 (11.3)	11	46	6	4		
	1 (2.3)	11	47	6	5		
FS2	1 (2.3)	11	22	4	6	NA	9
	1 (2.3)	11	43	6	7		
	5 (11.3)	11	46	6	4		
	1 (2.3)	11	47	6	5		
	1 (2.3)	9	22	6	8		
14 wpe	1 (2.3)	9	20	4	9	C	1
	19 (43.2)	9	20	6	10	B	2
						C	17
	4 (9.0)	9	20	7	11	C	4
RF	1 (2.3)	14	21	10	RF	NA	1
Total	44 (100.0)	NA	NA	NA	12	NA	44

TP: Typing profile assigned*; wpe*: week post-entry; RF: Reference strain; *NA:* Non-applicable.

Eleven different genotypes were detected among samples. Five TP were detected in each batch FS1 (n=10) and FS2 (n=9), although two variants (TP4 and TP5) were identified in both batches. Three variants were detected at 14 wpe, being different from those detected in previous batches (Table 4-4). No TP was

able to be characterized from the positive gilt from group A at 14 wpe. A minimum spanning tree was generated to show the genetic variation over time (Figure 4-3).

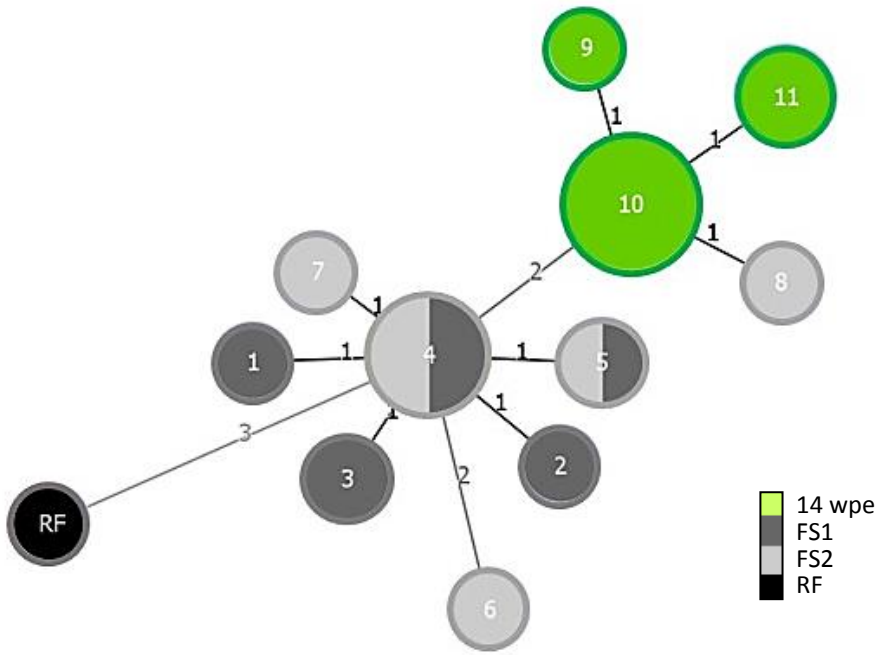

Figure 4-3. Minimum spanning tree showing different *M. hyopneumoniae* variant profiles detected. RF: Reference strain 11 used as technique control. Each circle represents a variant profile. The size of the circle is proportional with the number of samples belonging to each variant profile. Absolute distances among variant profiles are represented by link label.

Regarding VNTR per each locus, the P97 locus showed limited heterogeneity due to the fact that only two VNTR (9 and 11 repeats) were identified (Table 4-4). The P146 locus showed higher variability at FS1 and FS2, ranging from 38 to 47 and from 22 to 47 repeats, respectively. The VNTR of P146 at 14 wpe was homologous (20 repeats) in all identified variants. For locus H1, the VNTR varied from 4 to 7 repeats.

4.4. Discussion

One of the key points to control EP within farms is the *M. hyopneumoniae* transmission between the dam and her piglets. Since gilts are considered the main *M. hyopneumoniae* shedders, acclimation strategies focused on reducing the bacterial shedding at first farrowing have been proposed (Pieters and Fano, 2016). Vaccination has been determined as the most used strategy for acclimation as well as elimination, although different number of doses and application timings are being used (Garza-Moreno *et al., 2018*). However, its effect on gilt shedding, humoral immune response, as well as the maternal derived immunity transfer to piglets was not assessed in any of those studies. The number of vaccine doses and the application timing proposed in the past were 2 doses at 1 and 3 wpe to GDU (Yeske, 2007), or 3 doses at 55 and 220 days of age of gilts, and another last dose 2 weeks prior to farrowing (Alfonso *et al.,* 2004). On the other hand, the effect of sow vaccination against *M. hyopneumoniae* infection at 8 and 4 (Grosse Beilage and Schereiber, 2005), or 5 and 3 weeks prior to farrowing (Ruiz *et al.,* 2003; Sibila *et al.,* 2008) has been evaluated. All the three studies agreed that sow vaccination against *M. hyopneumoniae* enhanced levels of antibodies in vaccinated sows as well as in their piglets. In addition, Ruiz *et al.* (2003) and Sibila *et al.* (2008) concluded that sow vaccination could reduce, numerically, the prevalence of piglet colonization. However, Grosse-Beilage and Schereiber (2005) did not evaluate this parameter. Therefore, the objective of the present study was to evaluate the effect of gilt vaccination against *M. hyopneumoniae* using different vaccination programs during the acclimation period on gilt shedding, and consequently, on piglet's colonization at weaning.

The current study used vaccination protocols based on multiple doses (four and two) since a number of field studies have hypothesized that a high number of vaccine doses could induce a strong immune response, reduce colonization, and

better control the disease (Alfonso *et al.,* 2004; Yeske, 2007). The interval between the first and booster vaccination in the present study was 2 weeks to complete the vaccination protocol within a rather usual timing of acclimation at GDU (Garza-Moreno *et al.,* 2018). In order to minimize the effect of the previous humoral immune status, gilts were distributed into three groups according to ELISA PI values at entry. At 14 wpe, all vaccinated gilts had seroconverted, as expected with commercial vaccines. From the non-vaccinated group, all gilts but one also seroconverted, indicating that *M. hyopneumoniae* natural exposure occurred soon after entry. Significant differences of humoral responses in terms of PI were detected between vaccinated and non-vaccinated groups at all sampling points, as previously described by Kristensen et al. (2004) in sows. Interestingly, all vaccinated gilts remained seropositive during all study duration. On the contrary, the proportion of seropositive gilts in the non-vaccinated group decreased slightly over time (from 98% at 14 wpe to 88% in both 27 and 34 wpe). These findings suggest that vaccination may provide a longer duration of humoral immunity compared to that of natural infection. Furthermore, the statistically significant differences found between animals vaccinated with four or two vaccine doses at 14 wpe suggests that repeated vaccination elicited stronger immune response (lower PI). Indeed, PI values from vaccinated gilts with four doses remained statistically more homogeneous (lower *F* values) than those vaccinated with two doses followed by the non-vaccinated group, suggesting that vaccination helped homogenizing the immune status of the studied population.

A significantly higher percentage of seropositive piglets at weaning from gilts vaccinated two or four times was detected compared to those from non-vaccinated gilts. This finding is in agreement with other studies in sows (Kristensen *et al.,* 2004; Grosse Beilage and Schereiber, 2005), fitting with the hypothesis that vaccinated gilts show higher antibody levels against *M.*

hyopneumoniae (lower PI) than non-vaccinated gilts in colostrum. Nevertheless, no statistical differences were identified between piglets coming from gilts from groups A and B, suggesting the passive humoral transfer might be fairly independent of 2 or 4 doses applied. Additionally, 206 seronegative piglets were from seropositive sows at 34 wpe. This fact could be explained by a poor colostrum intake (Quesnel, 2011) and/or the decay of *M. hyopneumoniae* maternal antibodies since previous studies reported a median half-live of maternally derived antibodies of 15 days and, therefore, the amount of transferred antibodies was dependent on the dam's serological status (Morris *et al.,* 1995).

Mycoplasma hyopneumoniae shedding was first detected at 14 wpe, with the proportion of shedding gilts significantly lower in the vaccinated than in non-vaccinated groups. This finding could be associated with the reduction of *M. hyopneumoniae* bacterial load by vaccination, as previously reported Woolley et al. (2014). Moreover, an absolute reduction of shedding gilts was detected at 27 wpe in vaccinated groups, and only one non-vaccinated gilt was a shedder. *Mycoplasma hyopneumoniae* was not detected in any studied group at 34 wpe. The fact that all gilts from the non-vaccinated group seroconverted indicates that *M. hyopneumoniae* infection occurred, and this probably took place in a relatively short period of time, since by 27 wpe only one gilt was detected as PCR positive. Taking this into account, gilts from this trial could have been naturally exposed to *M. hyopneumoniae* around 10-12 wpe, shortly after mixing them with the positive own replacement. The peak of shedding might have been reached at 14 wpe (4 weeks after this exposure) and the complete cease of shedding would have occurred between 27 and 34 wpe (17-24 weeks after exposure). The duration of gilt shedding in this study seemed to be shorter compared to a previous study that detected shedders up to 200 days post-infection under experimental conditions (Pieters *et al.,* 2009). The difference in

the duration of shedding may be explained by the lower *M. hyopneumoniae* load in naturally infected gilts under field conditions compared to experimental conditions, where the bacterial load administered endo-tracheally might be much higher. Moreover, given the limited number of samplings over time of this study, the proposed timings about *M. hyopneumoniae* infection dynamics within this breeding herd should be considered as an estimation. Further research is needed to gain insight into infection dynamics of gilts under field conditions.

Previous studies have suggested that *M. hyopneumoniae* piglet colonization at weaning is correlated with respiratory disease and lung lesions at fattening stages (Fano *et al.,* 2007; Sibila *et al.,* 2007b). However, in the present field study, *M. hyopneumoniae* gilt shedding one week prior to farrowing (34 wpe) was not detected by rt-PCR and all piglets from vaccinated and non-vaccinated gilts were rt-PCR negative. Thus, association between gilt shedding at farrowing and piglet colonization at weaning could not be assessed. Obtained results are in agreement with previous studies with similar *M. hyopneumoniae* infection dynamics in which piglets were also negative at weaning (Takeuti *et al.,* 2017a,b).

Taking all these results together, the usage of two or four vaccination dose protocols in gilts seemed to be effective strategies for decreasing *M. hyopneumoniae* shedding and infectious pressure within a farm. Furthermore, results using four doses were slightly better (from a numeric point of view) than using two doses regarding ELISA results. Notwithstanding, these differences were only statistically significant in terms of PI at 14 wpe, suggesting that vaccination with these two extra doses is not apparently justified from an infection and seroconversion points of view.

The intra-farm genetic diversity of *M. hyopneumoniae* has been previously described using different methods and bacterium loci and thus, results are diverse. Several studies described a high intra-farm genetic diversity (Stakenborg *et al*., 2006; Nathues *et al.,* 2011a; Dos Santos *et al.,* 2015; Tamiozzo *et al.,* 2015; Michiels *et al.,* 2017a; Takeuti *et al.,* 2017a) whereas others concluded that the variability was limited within the same farm (Stakenborg *et al.,* 2005; Mayor *et al.,* 2007, 2008; Vranckx *et al.,* 2012b; Charlebois *et al.,* 2014; Galina-Pantoja *et al.,* 2016). Results from the current study showed high *M. hyopneumoniae* variability within the farm. In the previous gilts batches (FS1 and FS2), higher variability was detected compared to monitored gilts at 14 wpe. Despite variants being different, similarity between TP 8 from FS2 and the more prevalent variant at 14 wpe (TP 10) might indicate that TP 10 was the result of mutations for each locus along this study. These findings are in agreement with previous reports that concluded that Mollicutes can exhibit high mutation and recombination rates by modification of environmental conditions (Razin *et al.,* 1998). Furthermore, this similarity also suggests that the source of infection of gilts included in the study at entry was the own replacement (infected already based on data from FS1 and FS2) located in the same DSU. Unfortunately, information about *M. hyopneumoniae* variants harbored by own replacement over time was not assessed in the present study. Finally, analyzing the VNTR obtained per locus, results of P97 and P146 loci are in accordance with previously published reports in which Spanish *M. hyopneumoniae* strains showed approximately 20 repeats in P97 and more than 30 repeats in P146 (Dos Santos *et al.,* 2015). No previous data regarding VNTR for H1 are available.

In conclusion, the present study showed that *M. hyopneumoniae* gilt vaccination at acclimation period significantly reduced the *M. hyopneumoniae* shedding of gilts at 14 wpe and increased antibody levels (low PI) of dams and their piglets. Since *M. hyopneumoniae* shedding in gilts was not detected at 34 wpe, the lack

of bacterial piglet colonization was expectable. Despite the fact that *M. hyopneumoniae* vaccination does not provide full protection, the infectious pressure within the gilt population of the studied herd was significantly reduced. Gilt vaccination protocol with four doses showed slightly better numerical results than the protocol with two doses during all study. Therefore, these results suggested that the vaccination with two doses seems to be sufficient to reduce the infectious pressure and to induce strong and humoral immune response in gilts. Finally, the characterization of *M. hyopneumoniae* strains confirmed high genetic variability of this bacterium within the studied farm.

CHAPTER 5

Study III

Characterization of *Mycoplasma hyopneumoniae* strains in vaccinated and non-vaccinated pigs from Spanish slaughterhouses

5.1. Introduction

The severity of EP depends on the presence of co-infections, management and housing conditions, and probably on the virulence of the *M. hyopneumoniae* strain involved in the process (Vicca *et al.,* 2002, 2003; Maes *et al.,* 2008). Strain genetic diversity has been studied using different genotyping techniques (see section 1.1.2.1). The use of many different techniques covering different targets of detection, different power of discrimination and reproducibility have complicated the comparison and interpretation of results published to date (Stakenborg *et al.,* 2006). However, in the latest peer-reviewed studies, the more frequently used techniques are based on MLVA of loci related with adhesion to the host cells (Minion *et al.,* 2000; Bogema *et al.,* 2012).

Variability of *M. hyopneumoniae* has been detected at farm, batch, pig and even at sample level, with variable inter and intra farm genetic variability (Nathues *et al.,* 2011a; Vranckx *et al.,* 2012b; Charlebois *et al.,* 2014; Dos Santos *et al.,* 2015; Michiels *et al.,* 2017a). Such genetic diversity has been studied in different scenarios including non-vaccinated (Vranckx *et al.,* 2012b; Overesch and Kuhnert, 2017) and vaccinated pigs (Charlebois *et al.,* 2014; Tamiozzo *et al.,* 2015; Michiels *et al.,* 2017a), as well as in pigs with unknown vaccination status (Nathues *et al.,* 2011a). Additionally, some of the *M. hyopneumoniae* strain used as bacterins have also been genotyped (Charlebois *et al.,* 2014; Tamiozzo *et al.,* 2015). However, a contemporaneous comparison of *M. hyopneumoniae* variability between non-vaccinated and vaccinated animals, and the strain of the vaccine used in the vaccinated farms has not been assessed. Therefore, the aim of the present study was to compare, using conventional sequencing of different loci, the *M. hyopneumoniae* TP detected in vaccinated and non-vaccinated slaughtered pigs showing CVPC lesions, as well as in the vaccines used in such farms.

5.2. Materials and methods

5.2.1. Farm selection

Ten vaccinated and ten non-vaccinated fattening farms experiencing clinical respiratory signs compatible with *M. hyopneumoniae* infection (dry cough and presence of animals with CVPC in lungs at slaughterhouse) were selected. A fattening farm was included as vaccinated when pigs were vaccinated against *M. hyopneumoniae* at weaning. Pigs from non-vaccinated fattening farms did not receive *M. hyopneumoniae* vaccination at any point in the production cycle. Information on farm batch, vaccine products used as well as authorization for the slaughter checks were obtained from the practitioner and/or the producer. Farms included in the study were located in north-eastern Spain.

5.2.2. Lung lesion scoring and sample collection at slaughterhouse

Twenty lung batches (from 10 non-vaccinated and 10 vaccinated farms) of finishing pigs with *M. hyopneumoniae* compatible lung lesions (purple to grey pulmonary consolidation areas, generally located bilaterally in the cranio-ventral areas; Maes *et al.,* 2008) were individually scored at slaughterhouse. The scoring system used to quantify the *M. hyopneumoniae*-like lung lesions was the Ceva Lung Program (CLP), a lung scoring software based on two methods previously described (Madec and Kobisch, 1982; Christensen *et al.,* 1999). In the CLP system, each lobe was scored from 0 to 4 points according to the following classification: 0) no lesion, 1) lesion affecting <25% of the lobe surface, 2) lesion affecting ≥25% to <50% of the lobe surface, 3) lesion affecting ≥50% to <75% of the lobe surface and 4) lesion affecting ≥75% of the lobe surface (Madec and Kobisch, 1982). Each lobe score was finally normalized by its relative volume (Christensen *et al.,* 1999). Moreover, an additional point to the total lung score was considered when a scar was present in any lung lobe. The EP-index of each farm was calculated as the mean score of all evaluated lungs.

Three lungs showing the most extensive CVPC lesions within each batch were selected for *M. hyopneumoniae* strain variability detection and characterization. From those lungs, a portion of each lobe (including affected and healthy tissue) were collected to increase the rate of bacterium detection. Samples were transported in refrigeration to the laboratory where they were stored at -80ºC until used.

5.2.3. Vaccines and reference strains

Five commercial vaccines (bacterins) against *M. hyopneumoniae* (A, B, C, D and E) used in vaccinated farms were included for genotyping. All the vaccine strains were genotyped from its corresponding commercial product, except for the strain coming from the vaccine E, which was directly genotyped from a bacterial culture (kindly provided by manufacturer E), due to the impossibility of being directly amplified from the vaccine product. Moreover, the two reference strains, strain 11 (ATCC® 25095™) and strain J (ATCC® 25934™), were included in the study as controls. In order to test the reference strain in the same conditions as the strains detected in lung samples, different lung tissue portions negative to rt-PCR were spiked with each RF culture in vitro and subsequently processed as slaughterhouse lung portions.

5.2.4. DNA extraction and *M. hyopneumoniae* detection

Approximately 1 cm^3 including affected and healthy lung tissue from slaughtered pigs or the lung portion spiked with *M. hyopneumoniae* reference strain was homogenized in plastic tubes with 600 µL of PBS and glass beads. These lung homogenates were disrupted using TissueLyser (Qiagen GmbH, Germany) by shaking for 10 min and centrifuged at 11,000 g for 1 min. After centrifugation, 200 µL of tissue supernatant was collected for DNA extraction. Likewise, 200 µL of each vaccine product was directly used for extraction.

DNA extraction from tissue supernatant, vaccines and *M. hyopneumoniae* culture (vaccine E strain) was performed by MagMax™ DNA Multi-Sample Kit (Life Technologies, USA) according to the manufacturer's instructions on the BioSprint 96 workstation (Qiagen GmbH, Germany). Two different positive extraction controls were utilized in each extraction: 1) a lung tissue portion spiked with *M. hyopneumoniae* strain 11 (ATCC®25095™) was added to each extraction plate; and 2) a commercial internal positive control (Xeno™, included in qPCR Master Mix kit, VetMax™-Plus, Life Technologies, USA) was added to every tissue sample. Negative controls (PBS) were also included to assess potential contamination during extraction.

Extracted DNA was tested by a commercial rt-PCR for *M. hyopneumoniae* detection: VetMax™-Plus qPCR Master Mix (Life Technologies, USA) with VetMax™ *M. hyopneumoniae* Reagents (Life Technologies, USA) according to the manufacturer's instructions. A positive DNA control for amplification of *M. hyopneumoniae* (VetMax™ *M. hyopneumoniae* Controls) was also included in the rt-PCR procedure. All rt-PCR runs were carried out in ABIPRISM® 7500 machine (Applied Biosystems, Singapore). The rt-PCR threshold was set at 10% of the maximum fluorescence value of the commercial DNA positive control. Samples with Ct values lower than 40 were considered positive. Only samples with *M. hyopneumoniae* positive rt-PCR with Ct values ≤30 were selected to be characterized by sequencing of different loci.

5.2.5. Characterization of *M. hyopneumoniae* strains

Characterization of *M. hyopneumoniae* strains was based on the VNTR count of four different loci related with the adhesion: P97 (RR1), P146 (RR3), H1 (complete loci) and H5 (RR2; Vranckx *et al.,* 2011). For the genotyping assay, the four loci were individually amplified in a final volume of 50 µL. Reaction mixtures contained 1X PCR Buffer, 1.5 mM MgCl2, 0.2 mM of each deoxynucleotide

triphosphate, 0.4 µM of each primer, 1.5 U of GoTaq® G2 Flexi DNA Polymerase (Promega, Madison, USA), and finally, 6 µL of extracted DNA diluted 1:10. Cycling conditions were 4 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 53°C and 30 s at 72°C; then, a final extension step of 7 min at 72°C was performed.

The PCR products from each locus were analyzed by electrophoresis on 2% agarose gel in Tris-Acetate-EDTA (TAE)-buffer and stained with ethidium bromide. Afterwards, products were purified by ExoSAP-IT® (Isogen Life Science, The Netherlands) according to manufacturer's instructions and sequenced using ABI PRISM 3130xl (Applied Biosystems, Singapore) genetic analyzer.

5.2.6. Data analysis

Nucleotide sequences were aligned to obtain the consensus sequence obtained using FingerPrinting II Informatix software (Applied Maths, Saint-Martens-Latem, Belgium). The translation of consensus sequences to protein sequences was performed using the Expasy web tool (http://web.expasy.org/translate). Subsequently, VNTR counts per locus were performed, according to the following amino acid repetitions: AAKP[EV] for P97, S for P146, QTTQ(KD) for H1 and Q for H5 (Vranckx *et al.,* 2011). All translated sequences obtained for each loci were first aligned using MUSCLE (v3.8.31) and, afterwards, concatenated to obtain all the loci per sample. Such analysis was performed in two different fashions, considering 4 loci, P97-P146-H1-H5 (Vranckx *et al.,* 2011; Michiels *et al.,* 2017a), or 2 loci, P97-P146 (Dos Santos *et al.,* 2015; Galina-Pantoja. *et al.,* 2016; Takeuti *et al.,* 2017a). Afterwards, a TP was defined for each different combination of VNTR when 4 (TP4) or 2 loci (TP2) were considered. Minimum spanning trees, constructed considering the origin and type of the sample (vaccinated and non-vaccinated farms, reference strains and vaccine strains)

together with the TP detected, were performed in PHYLOViZ 2.0 (Ribeiro-Gonçalves *et al.,* 2016).

5.2.7. Statistical analyses

Mean of continuous variables (EP-index and mean of Ct rt-PCR values) between non-vaccinated and vaccinated farms was compared by unpaired t-test. Additionally, one-way analysis of variance (ANOVA) was used to compare the lung lesion score of the three selected lungs per herd between vaccinated and non-vaccinated farms. All statistical analyses were performed using GraphPad Prism 6.07 software (GraphPad software Inc., San Diego, USA). The significance level was set to $p<0.05$.

5.3. Results

5.3.1. Lung lesion scoring

A total of 3,069 lungs were scored with an average (min. – max.) number of lungs evaluated per batch of 153 (93 – 212). The EP-index of each batch is shown in Tables 5-1. The mean of EP-index (mean ± standard deviation [SD]) in non-vaccinated farms (3.8±1.9) was numerically higher, but not significantly different, than that of vaccinated farms (2.2±1.3). When only the three selected pigs showing the highest lesion scores per batch were considered, the mean of lung lesion score was numerically higher in non-vaccinated farms than in vaccinated farms (7.2±5.3 and 5.8±3.9, respectively).

Table 5-1. Farm data, percentage (%) of lung lesions with cranio-ventral pulmonary consolidation (CVPC) and enzootic pneumonia (EP)-index ± standard deviation (SD) per farm.

Farm batch	Non-vaccinated farms										Vaccinated farms									
	NV1	**NV2**	**NV3**	**NV4**	**NV5**	**NV6**	**NV7**	**NV8**	**NV9**	**NV10**	**V1**	**V2**	**V3**	**V4**	**V5**	**V6**	**V7**	**V8**	**V9**	**V10**
Vaccine[a]	-	-	-	-	-	-	-	-	-	-	D	D	E	E	C	C	A	A	B	B
Breeding origin[b]	a	b	c	d	e	f	a	a	g	h	i	i	j	k	l	m	n	o	p	q
No. lungs	100	93	169	207	212	155	136	203	115	139	102	115	110	128	174	171	203	175	196	166
% lungs with CVPC	83	83	76	74	50	72	22	80	80	91	60	79	20	57	65	85	80	57	64	25
EP-index[c]	5.5	4.2	4.4	1.9	1.3	3.5	0.9	4.1	4.7	7.1	2.4	3.4	0.1	0.8	2.7	3.4	3.7	3.1	2.5	0.3
Mean % of lungs with CVPC	71.1±19.3										59.2±20.6									
Mean EP±SD	3.8±1.9										2.2±1.3									

[a] Capital letters (A, B, C, D and E) represent commercial vaccines against *M. hyopneumoniae* available in the Spanish market; [b]a–q: Different breeding origin farms; CVPC: cranio-ventral pulmonary consolidation; [c]EP-index: Enzootic pneumonia index was calculated by the Ceva Lung Program (CLP).

5.3.2. *Mycoplasma hyopneumoniae* detection by rt-PCR

From the 60 (30 from vaccinated and 30 from non-vaccinated farms) tested lung samples, 46 (76.7%, 25 from non-vaccinated and 21 from vaccinated herds) were positive by rt-PCR (Tables 5-2a and Table 5-2b). However, there were 4 farms (V3, V4, V10 and NV4) with all tested samples negative to rt-PCR and one farm (NV5) with only one rt-PCR positive sample. The Ct mean value (±SD) of rt-PCR positive lungs from vaccinated farms (25.5±2.9) was slightly higher than that of non-vaccinated farms (24.5±3.8), although this difference was not statistically significant. The rt-PCR Ct values for the five commercial vaccines against *M. hyopneumoniae* (A, B, C, D and E) and reference strains are detailed in Table 5-3.

Table 5-2a. Enzootic pneumonia lesion score, mean rt-PCR Ct value, counts of variable number of tandem repeats (VNTR) and typing profiles (TP) using 4 and 2 loci from the three non-vaccinated (NV) sampled animals per batch.

Origin	Sample ID	Lung lesion score	rt-PCR (Ct)	VNTR				Typing profile[b]	
				P97	P146	H1	H5	4 loci	2 loci
NV1	L1a	13	22.7	6*	25	6	18*	TP4-1	TP2-1
	L1b	12	22.0	6*	25	5	18*	TP4-2	TP2-1
	L1c	9	21.9	6*	25	6	16	TP4-3	TP2-1
NV2	L2a	14	23.9	10	29	5	15*	TP4-4	TP2-2
	L2b	6	24.7	12	17*	7	15*	TP4-5	TP2-3
	L2c	22	23.6	8	35	-	12*	-	TP2-4
NV3	L3a	18	18.6	11*	13*	12	15*	TP4-6	TP2-5
	L3b	7	23.4	11*	13*	12	15*	TP4-6	TP2-5
	L3c	8	19.7	9*	13*	10*	17	TP4-7	TP2-6
NV4	L4a	5	Und	-	-	-	-	-	-
	L4b	3	Und	-	-	-	-	-	-
	L4c	1	Und	-	-	-	-	-	-
NV5	L5a	1	37.2	-	-	-	-	-	-
	L5b	2	Und	-	-	-	-	-	-
	L5c	3	Und	-	-	-	-	-	-
NV6	L6a	3	22.6	9	15*	18	13*	TP4-8	TP2-7
	L6b	9	22.3	11	-	5	14	-	-
	L6c	7	23.4	2	-	5	-	-	-
NV7	L7a	7	26.1	6*	25	6	-	-	TP2-1
	L7b	7	25.2	6*	25	6	18*	TP4-1	TP2-1
	L7c	4	25.7	6*	25	6	-	-	TP2-1
NV8	L8a	5	23.6	6*	25	-	-	-	TP2-1
	L8b	5	24.2	6*	25	6	13	TP4-9	TP2-1
	L8c	7	23.9	6*	25	6	17	TP4-10	TP2-1
NV9	L9a	7	24.2	10*	13	4	13*	TP4-11	TP2-8
	L9b	4	29.8	10*	13	4	13*	TP4-11	TP2-8
	L9c	17	28.8	10*	13	4	13*	TP4-11	TP2-8
NV10	L10a	2	20.8	12	13	4	16	TP4-12	TP2-9
	L10b	1	30.3	6*	25	6	18*	TP4-1	TP2-1
	L10c	6	23.6	6*	25	6	18*	TP4-1	TP2-1

[a]EP-index: Enzootic pneumonia index calculated by Ceva Lung Program (CLP); [b]Typeable profile was defined for each different combination of VNTR when 4 or 2 loci were considered; *The VNTR of this locus was different from the one previously described; Und: non detected.

Table 5-2b. Enzootic pneumonia lesion score, mean rt-PCR Ct value, counts of variable number of tandem repeats (VNTR) and typing profiles (TP) using 4 and 2 loci from the three vaccinated (V) sampled animals per batch.

Origin	Sample ID	Lung lesion score	rt-PCR (Ct)	VNTR				Typing profile[b]	
				P97	P146	H1	H5	4 loci	2 loci
V1	Lv1a	12	24.6	9*	13*	6	27	TP4-13	TP2-6
	Lv1b	8	29.7	-	-	-	-	-	-
	Lv1c	10	26.1	9*	13*	-	-	-	TP2-6
V2	Lv2a	3	23.8	9*	13*	5	12*	TP4-14	TP2-6
	Lv2b	4	23.8	9*	13*	5	16*	TP4-15	TP2-6
	Lv2c	2	24.7	9*	13*	5	16	TP4-15	TP2-6
V3	Lv3a	2	Und	-	-	-	-	-	-
	Lv3b	2	Und	-	-	-	-	-	-
	Lv3c	1	Und	-	-	-	-	-	-
V4	Lv4a	3	Und	-	-	-	-	-	-
	Lv4b	1	Und	-	-	-	-	-	-
	Lv4c	1	Und	-	-	-	-	-	-
V5	Lv5a	10	24.6	10*	19	5	15*	TP4-16	TP2-10
	Lv5b	10	24.7	10*	19	5	13*	TP4-17	TP2-10
	Lv5c	4	23.3	10*	19	2	15*	TP4-18	TP2-10
V6	Lv6a	6	25.8	2	30	7	13*	TP4-19	TP2-11
	Lv6b	10	27.4	-	-	-	-	-	-
	Lv6c	4	23.2	2	29	7	14	TP4-20	TP2-12
V7	Lv7a	6	25.2	8	45	7	21*	TP4-21	TP2-13
	Lv7b	16	27.3	8	46	-	-	-	TP2-14
	Lv7c	7	24.9	8	46	11	11	TP4-22	TP2-14
V8	Lv8a	7	31.2	-	-	-	-	-	-
	Lv8b	9	28.3	8*	17	12	16*	TP4-23	TP2-15
	Lv8c	6	32.3	-	-	-	-	-	-
V9	Lv9a	10	22.3	9*	30	6	7*	TP4-24	TP2-16
	Lv9b	7	21.9	8	-	-	-	-	-
	Lv9c	8	21.3	8	30	6	-	-	TP2-17
V10	Lv10a	2	Und	-	-	-	-	-	-
	Lv10b	1	Und	-	-	-	-	-	-
	Lv10c	2	Und	-	-	-	-	-	-

[a]EP-index: Enzootic pneumonia index calculated by Ceva Lung Program (CLP); [b]Typeable profile was defined for each different combination of VNTR when 4 or 2 loci were considered; *The VNTR of this locus was different from the one previously described; Und: non detected.

Table 5-3. Information, values of rt-PCR and VNTR counting and typing profile of strains detected in the tested vaccines and reference strains.

Origin	Strain	ID	rt-PCR (Ct)	VNTR				Typing profile[a]	
				P97	P146	H1	H5	4 loci	2 loci
Vaccine A	J	VA	16.3	9	7	11	22*	TP4-25	TP2-18
Vaccine B	11	VB	35.5	-	21	-	13*	-	-
Vaccine C	NL-1042	VC	26.8	12	19	17	16*	TP4-26	TP2-19
Vaccine D	P-5722-3	VD	25.6	3	19	17	16*	TP4-27	TP2-20
Vaccine E	BA-2940-99	VE	39.3	-	-	-	-	-	-
		Culture	30.2	9	15	3	12*	TP4-28	TP2-7
RF	-	S11	21.3	14*	21	10	14*	TP4-29	TP2-21
RF	-	SJ	20.2	9*	14	11	25*	TP4-30	TP2-22

RF: reference strain; Ct: Cycle threshold; [a]Typeable profile was defined for each different combination of VNTR when 4 or 2 loci were considered; *The VNTR of this locus was different from the one previously described.

5.3.3. PCR amplification and number of VNTR

From the 46 samples that resulted positive by rt-PCR, the 43 (93.5%) having a Ct≤30 were selected to be genotyped. Although the 4 loci were amplified in 43 samples, in only 31 (72.1%) the 4 loci were sequenced appropriately and, therefore, the samples were typeable. However, when only 2 loci (P97 and P146) were considered, 38 (38/43, 88.4%) samples were typeable. Variations with respect to the amino acid sequence of the VNTR previously described were detect (i.e. TTKP [EV] instead of AAKP[VE]). Such repetition was counted but marked with a star (*) in the final number of repetitions of such loci (Table 5-2a and Table 5-2b).

While only one TP per sample was detected, one to three TP per farm were identified. Considering non-vaccinated and vaccinated farms, different VNTR were identified for each locus, i.e. 7 for P97, 10 for P146, 9 for H1, and 11 for

H5. The number of repetitions detected in each locus ranged between 2 to 12 for P97, 13 to 46 for P146, 2 to 18 for H1 and 13 to 27 for H5.

Considering the four loci, 24 different *M. hyopneumoniae* TP were identified: 12 in non-vaccinated and 12 in vaccinated farms (Table 5-2a and 5-2b). In contrast, when only 2 loci (P97 and P146) were considered, 17 different TP were detected, 9 TP in non-vaccinated and 9 in vaccinated farms, because TP2-6 was present in non-vaccinated and vaccinated farms (Tables 5-2a and 5-2b).

Regarding vaccine strains, vaccine B was considered non-typeable in all four genes due to the failure in the amplification and sequencing of loci P97 and H5. Thus, in the five vaccine strains, a total of four different TP were identified using either four or two loci (Table 5-3). As expected, detected TP in vaccinated and non-vaccinated pigs were different from the tested VS either using four or two loci (expect in the case of vaccine E and one of the pigs from farm NV6, which had the same TP when only two loci were considered). Likewise, in the *M. hyopneumoniae* RF, one TP was identified in each culture, being different from the TPs detected in vaccinated pigs and vaccine strains, regardless the number of loci considered for the analysis. The minimal spanning trees depicting the genetic similarity of the detected TP according to four loci (A) and two loci (B) are shown in Figure 5-1

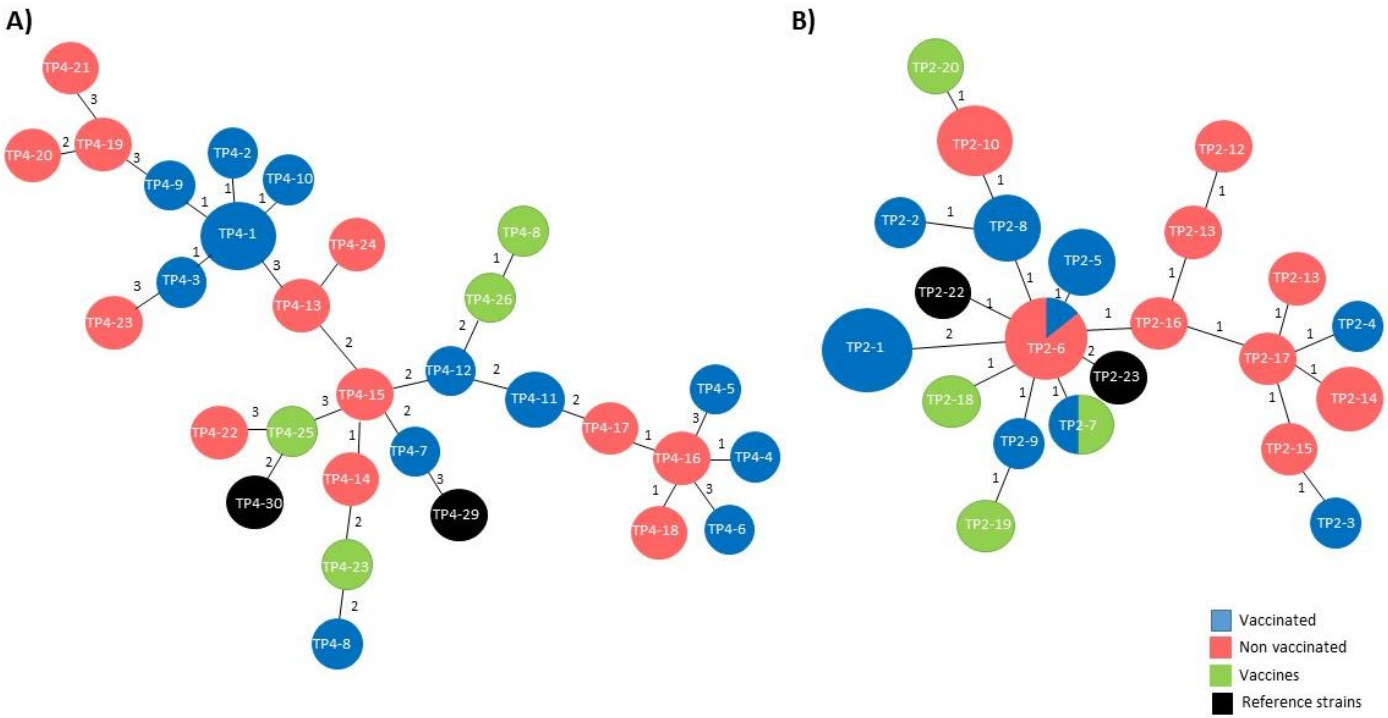

Figure 5-1. Minimum spanning tree showing the different *M. hyopneumoniae* variants profiles detected according to four (A) and two (B) loci. Each circle represents one typing profile of *M. hyopneumoniae*. The size of the circle is proportional with the number of samples harboring each typing profile. Absolute distances among typing profiles detected are represented by link label

5.4. Discussion

This study attempted to describe the *M. hyopneumoniae* genetic variability in lungs showing EP compatible lesions from various non-vaccinated and vaccinated Spanish farms. In addition, variability was compared with the strain of the vaccine used in each of the vaccinated farms, as well as with two reference strain. To reach the goals, previously described regions from four loci related with adhesion (Vranckx *et al.,* 2011) were sequenced, VNTR were counted and used to define the TP of each strain and the number of TP per farm.

Although the assessment of the vaccine efficacy was not included in the objective of the present study, the differences in lung score between vaccinated and non-vaccinated farms were calculated. Generally, lungs from non-vaccinated farms showed a numerically higher EP-index (more severe lesions) than vaccinated farms. The fact that the differences between vaccinated and non-vaccinated farms were not statistically significant might be explained by the different disease status of evaluated farms, the different vaccines used, the sample size, as well as the potential failure of inefficiency of vaccines. Interestingly, vaccinated farms with vaccine B (V10) or with vaccine E (V3 and V4) in which *M. hyopneumoniae* was not detected in the tested samples, showed the lowest EP-index. This finding would be in favor of the previously suggested capability of vaccines to decrease the bacterial load implying a lung lesion reduction (Vranckx *et al.,* 2012a; Woolley *et al.,* 2012; Michiels *et al.,* 2017b). However, similar results (no *M. hyopneumoniae* detection by rt-PCR) were detected in non-vaccinated farms NV4 and NV5. This can be explained by the fact that the lesions were on a resolution phase or were caused by other respiratory pathogens (i.e. swine influenza virus; Maes *et al.,* 2008). Therefore, considering that, in this study the presence of other CVPC-causing pathogens was not investigated, no definitive conclusions regarding the effect of the

vaccine on lung lesions reduction can be reached in the farms where *M. hyopneumoniae* was not detected.

In the present study, only one *M. hyopneumoniae* TP per sampled lung was able to be detected by Sanger sequencing. This result would differ from those previous studies, in which MLVA has been used, and in which co-infections with more than one *M. hyopneumoniae* strain (or TP) at pig level were described (Nathues *et al.,* 2011a; Vranckx *et al.,* 2012b; Charlebois *et al.,* 2014; Michiels *et al.,* 2017a). These divergent results could be derived from intrinsic limitations of the different techniques. While the observation of different peaks in MLVA informs on the presence of different strains, Sanger sequencing most probably identifies the predominant one (Vranckx *et al.,* 2011, 2012b, Michiels *et al.,* 2017a). Indeed, the effect of multiple strain infection at the level of lung lesion is still controversy. Whereas Michiels et al. (2017a) linked the number of different strains detected with the severity of lesions, other authors have not observed such association (Vranckx *et al.,* 2012b; Charlebois *et al.,* 2014).

The use of different genotyping techniques influences the number of TP profiles obtained and, thus, the conclusions on *M. hyopneumoniae* genetic variability. In Sanger sequences the number of VNTR is visually and directly counted from each sequence per locus allowing a clear definition of the obtained TP (unique combination of loci; Falde *et al.,* 2018). On the contrary, in MLVA, the number of VNTR is estimated from the height of the peak. Therefore, in cases of multiple infections, multiple peaks per loci would be obtained, and the exact number of VNTR for each strain would not properly ascertained.

An interesting conclusion from the data is that the number of TP obtained varied according to the number of loci used: the higher the number of tested loci, the higher the heterogeneity, which is especially evident in the minimum spanning tree. This finding would explain the results obtained in previous studies in terms

of genetic diversity. While high genetic diversity was detected at inter and intra farm level when four loci were considered (Nathues *et al.,* 2011; Tamiozzo *et al.,* 2015; Michiels *et al.,* 2017a), a limited variability has been detected when only two loci were studied, specially within the same herd (Charlebois *et al.,* 2014; Dos Santos *et al.,* 2015; Galina-Pantoja *et al.,* 2016). In the present study, genotyping with two loci allowed to detect the same TP in pigs from farms with the same breeding origin. Although this finding suggests that the sows could be the origin of infection (Sibila *et al.,* 2007), when 4 loci were used, such link was not found. Thus, the slightly difference on TPs using 4 loci could indicate TPs might vary independently during nursery and fattening period.

The comparison of VNTR between *M. hyopneumoniae* field samples, vaccines used in each farm, and RF strains revealed different TP. Interestingly, vaccine A had a different TP compared to that of TF type strain J, from which is originated. This difference could be explained by the effect of serial bacterium passages and/or the inactivation process in the case of vaccine manufacturing. Likewise, in agreement with previous studies, a low similarity between field strains and VS was also found (Charlebois *et al.,* 2014; Tamiozzo *et al.,* 2015). In fact, TPs detected in vaccinated farms were different from the vaccine strain of corresponding vaccine used in each vaccinated farm. It is not known whether this difference among field and vaccine TPs implies differences at antigenic level. If this would be the case, these differences might potentially explain the variable effect of vaccination observed under field conditions (Maes *et al.,* 2008). However, a previous study did not detect differences on protective efficacy using homologous or heterologous strains as bacterins in experimentally inoculated pigs (Villareal *et al.,* 2012).

Another important point to be considered for *M. hyopneumoniae* genotyping is locus selection. In the present study, four loci previously used in the literature

were selected (Vranckx *et al.,* 2011). Among them, the loci most frequently used are the P97 and P146 (Kuhnert *et al.,* 2011; Nathues *et al.,* 2011a; Charlebois *et al.,* 2014; Dos Santos *et al.,* 2015; Galina-Pantoja *et al.,* 2016; Takeuti *et al.,* 2017a), which encode adhesins involved in binding to cilia (Minion *et al.,* 2000; Bogema *et al.,* 2012). The variability and ranges of VNTR for these loci were in agreement with the previously described in Spanish field isolates (Dos Santos *et al.,* 2015), showing greater variability in P146 than in P97. On the contrary, in other countries such as Brazil, Mexico and United States, P97 has shown more variability than P146, although the ranges of P146 of Brazilian isolates were similar to the Spanish samples (Dos Santos *et al.,* 2015). The other two selected loci, H1 and H5, encode for hypothetical proteins related to adhesion (Vranckx *et al.,* 2011; Tamiozzo *et al.,* 2015; Michiels *et al.,* 2017a). The use of H1 and H5, from which previous information on VNTR was not available, resulted in a high number of non-typeable samples. The lack of success for sequencing has been previously associated with potential *M. hyopneumoniae* mutations in primer-binding sites or with insufficient DNA quantity or quality (Kuhnert *et al.,* 2011; Vranckx *et al.,* 2011; Tamiozzo *et al.,* 2015).

In summary, a high inter-farm *M. hyopneumoniae* genetic variability in slaughtered pigs from vaccinated and non-vaccinated Spanish farms was detected. Interestingly, detected TP in vaccinated farms were different from the strain of the corresponding vaccine used either four or two loci. Likewise, the analysis using two loci showed that pigs from farms with the same breeding origin harbored the same *M. hyopneumoniae* TP, but this link was not observed if four loci were considered. *M. hyopneumoniae* diversity at intra-farm level was limited and the number of TP detected per farm varied according to the number of the loci considered.

CHAPTER 6

General discussion

Respiratory diseases are highly prevalent in swine and represent an economically significant concern for the pig industry worldwide. Among them, EP and PRDC, in which *M. hyopneumoniae* plays an important role, mainly affect growing and fattening pigs. Although several control and elimination programs have been used to mitigate the impact associated to *M. hyopneumoniae* infections (Holst *et al.,* 2015; Maes *et al.,* 2018), the concern regarding this pathogen still continues. Nowadays, a successful control of *M. hyopneumoniae* infections within herds has been proposed as an integral approach including optimization of management and biosecurity practices, housing conditions, acclimation strategies, vaccination programs and antimicrobial treatments (Maes *et al.,* 2018). In the present thesis, knowledge on different aspects related mainly to acclimation strategies (*Chapters 3* and *4*), vaccination programs and genetic variability (*Chapters 4* and *5*) has been generated to control *M. hyopneumoniae* infection.

The severity of *M. hyopneumoniae* induced lesions in growing and finishing pigs has been linked to the infection prevalence in weaning piglets (Fano *et al.,* 2007; Sibila *et al.,* 2007b). Since newborn piglets are *M. hyopneumoniae* free, it is considered that the dam is the primary source of infection for the offspring (Pieters and Fano, 2016). Although *M. hyopneumoniae* can be excreted by young and older sows, it is thought that the young ones are the main shedders (Calsamiglia and Pijoan, 2000; Boonsoongnern *et al.,* 2012). Thus, gilts are currently considered as a key point for controlling subsequent *M. hyopneumoniae* infections at weaning, and consequently, the lung lesions development at later production stages (Pieters and Fano, 2016). Accordingly, taking into account the high replacement rates in commercial farms (Nathues *et al.,* 2013; Pieters *et al.,* 2014), gilts represent a major risk that may compromise the sow herd stability and underlines the necessity of an appropriate

acclimation strategy focused on getting non-shedding, immunized gilts at first farrowing (Pieters and Fano, 2016).

Although different strategies are being used for *M. hyopneumoniae* gilt acclimation worldwide, information about those practices and its efficacy is minimal in the literature. Before the starting of the present PhD investigations, only two non-peer reviewed studies describing the acclimation strategies used in the USA (Fano and Payne, 2015) and Mexico (Centeno *et al.,* 2016) did exist, and no data regarding European practices were readily available. In order to expand the knowledge about the current acclimation strategies, a survey was designed and distributed among veterinarians from different European countries in order to describe the *M. hyopneumoniae* gilt acclimation strategies carried out in Europe (*Chapter 3*).

The survey was designed to obtain information on different aspects related to the acclimation period such as the health status of recipient herd and the incoming replacement, as well as the type of replacement, strategies used for acclimation and verification of those practices, among others. The fact that all but one of the farms evaluated the *M. hyopneumoniae* health status *(Chapter 3)* suggests that this respiratory pathogen is still a significant concern for European swine producers, as previously described by Holtkamp *et al.* (2007) in the USA. Across the years, several clinical and laboratory methods have been used to assess *M. hyopneumoniae* presence within farms (Pieters *et al.,* 2017), being clinical signs and lung lesions associated to *M. hyopneumoniae* the most common monitoring strategies identified by this survey. However, the assessment of *M. hyopneumoniae* status using these non-specific methods can only provide a tentative diagnosis. Indeed, *M. hyopneumoniae*-associated coughing is not possible to be distinguished from other major pathogens such as SIV (Maes *et al.,* 2018). Similarly, suggestive lung lesions of *M.*

hyopneumoniae infection can also be caused by other pathogens such as SIV or *P. multocida* (Sibila *et al.,* 2009; Maes *et al.,* 2018). Therefore, further confirmation of *M. hyopneumoniae* involvement with laboratory methods (pathogen detection and/or presence of antibodies against it) needs to be implemented (Maes *et al.,* 2018; Garza-Moreno *et al.,* 2018). Once the health status of the recipient farm is really known, the incoming replacement status should also be assessed in order to verify its health status prior entrance and acclimation.

Data from *Chapter 3* showed that most European participant farms introduced external replacement (as in the studied farm of *Chapter 4*), and most of these replacements were supposed to be seropositive to *M. hyopneumoniae* on arrival. However, a minority of them verified the *M. hyopneumoniae* serological and shedding status of replacement at arrival (*Chapter 3)*. Although seropositivity is not necessarily correlated with shedding (Calsamiglia and Pijoan, 2000), the introduction of gilts with unknown *M. hyopneumoniae* shedding status into a recipient herd might also increase the likelihood of entrance of this respiratory bacterium and/or new strains (Maes *et al.,* 2008; Ramirez *et al.,* 2012). Therefore, this practice can lead to farm destabilization and a potential outbreaks appearance (Pieters *et al.,* 2009; Nathues *et al.,* 2013).

The exact explanation for farm destabilization is unknown. Although controversial, one study has suggested that different strains within farms may be correlated with a more severe disease presentation (Michiels *et al.,* 2017a). Among others factors, the genetic diversity detected within recipient herds could be potentially explained by the evidence of several *M. hyopneumoniae* TPs circulating within incoming replacement batches (Stakenborg *et al.,* 2006; Nathues *et al.,* 2011a; Tamiozzo *et al.,* 2015). In addition, a recently published study showed that different *M. hyopneumoniae* variants could be circulating in self-replacement batches as well (Takeuti *et al.,* 2017a). In the same line, the

two own replacement batches used for the farm screening in *Chapter 4* indicated the existence of multiple (although overall highly similar) *M. hyopneumoniae* TPs. Therefore, both own and external replacements might be a source of transmission depending on their infection status. For example, the close genetic profiles between the detected TPs within own and external replacement in *Chapter* 4 suggested that the source of infection for the studied external gilts was the own replacement once in the productive line (after 10 wpe). On the other hand, variability can also be generated by the potential mutation and recombination among existing strains of the farm, as has been previously reported for *M. hyopneumoniae* (Galina-Pantoja *et al.,* 2016) and other Mollicutes (Razin *et al.,* 1998).

The scenario described in *Chapter 4*, in which the health status of the incoming replacements (negative) and recipient herd (positive) was different and several TPs were circulating within replacement batch, suggests that the risk of outbreak may exist. However, since clinical signs (coughing) were not recorded in the assessed replacement batch, such association could not be established. Interestingly, results from this study also showed that gilt vaccination reduced the *M. hyopneumoniae* infectious pressure and the bacterium variability. Such scenario into the studied batch might have reduced the likelihood of clinical outbreak.

The second part of the survey (*Chapter 3)* evaluated *M. hyopneumoniae* gilt acclimation strategies performed by the participants in Europe. The most common strategy was vaccination, alone and/or combined with natural exposure to infected sows and/or pigs, being in agreement with similar studies performed in the USA and Mexico (Fano and Payne, 2015; Centeno *et al.,* 2016).

However, only a minority of those participants who performed gilt acclimation verified the effect of acclimation on replacements and/or its piglets. The use of systematic vaccination may be explained by the known effect on reduction the impact of this bacterium within incoming replacement and recipient herd (*Chapter 4*). Additionally, gilt vaccination would probably contribute to reduce the antibiotic usage and new generation of antibiotic resistance against the pathogen.

In general terms, there are a number of potential vaccination approaches (alone or in combination) to control *M. hyopneumoniae* within herds:

- Piglet vaccination. The efficacy of vaccination in piglets in terms of reduction of clinical signs and/or lesions observed has already been observed in several experimental and field studies (Del Pozo Sacristán *et al.,* 2014; Michiels *et al.,* 2017b; Maes et al., 2018). Although commercial vaccines against *M. hyopneumoniae* do not prevent the infection, vaccination produces a decrease in *M. hyopneumoniae* load (Woolley *et al.,* 2013), transmission ratio (Meyns *et al.,* 2004) and, consequently, the infectious pressure of the farms. Additionally, vaccination against *M. hyopneumoniae* enhances performance parameters such as ADWG and feed conversion ratio (Pallarés *et al.,* 2001). Thus, its usage together with other abovementioned management practices seems to be economically justified (Maes *et al.,* 2003).
- Sow vaccination. Since piglets are *M. hyopneumoniae* free at birth, sow vaccination programs at different timings of gestation have been also evaluated in order to reduce the vertical transmission (sow-to-piglet) and potentiate the transference of maternally derived immunity to the offspring (Martelli *et al.,* 2006). Previous studies concluded that sow vaccination does not protect the offspring against *M. hyopneumoniae*

infection, but the proportion of infected piglets at weaning was numerically reduced (Ruiz *et al.,* 2003; Sibila *et al.,* 2008). This aspect implies that sow vaccination may reduce the infectious pressure into farms, basically at the sow-piglet transmission level.

- Gilt vaccination. Gilts and young parity sows are considered the main shedders of *M. hyopneumoniae* to their progeny (Pieters and Fano, 2016). The use of gilt vaccination using multiple doses has been addressed by few studies (Alfonso *et al.,* 2004; Yeske, 2007; *Chapter 3*). The objective of this practice is to decrease the proportion of non-shedding gilts at first farrowing and to maximize the immunity in gilts and their piglets. To further evaluate its benefits, a field study (*Chapter* 4) was conducted to compare different gilt vaccination schedules based on multi-dose programs.

As described in *Chapter 4,* different gilt vaccination programs (0, 2 and 4 vaccine doses) were compared to evaluate their effects on *M. hyopneumoniae* colonization and humoral immunity in 1) the replacements from its arrival to one-week prior farrowing, and in 2) six piglets coming from those gilts at weaning. This study provided novel results showing that vaccination with four or two doses at the beginning of the acclimation period could reduce the proportion of shedding gilts during the whole acclimation period and first gestation. Although it is well known that vaccination against *M. hyopneumoniae* does not prevent the infection (Villarreal *et al.,* 2011; Maes *et al.,* 2018), this difference found might be explained by its effect reducing the number of *M. hyopneumoniae* organisms in the respiratory tract (Vranckx *et al.,* 2012b) and, consequently, the infection pressure into the herd (Sibila *et al.,* 2007). The fact that shedding gilts, particularly the non-vaccinated ones, were not detected at any subsequent time point would confirm the overall reduction of such infectious pressure into the studied batch. However, further and intermediate samplings would have provided a wider overview of the *M. hyopneumoniae*

infection dynamics in the three groups. Since no antimicrobials were administered to gilts throughout the study, the differences on shedding observed between vaccinated and non-vaccinated were potentially attributable to vaccination. Furthermore, since the risk of potential re-infections (Maes *et al.,* 2008; Villarreal *et al.,* 2009) and *M. hyopneumoniae* strain mutation and/or recombination (Galina-Pantoja *et al.,* 2016) could exist within herds, monitoring the gilts during more reproductive cycles would have been interesting in order to know the effect of gilt vaccination on such parameters along time.

Curiously enough, all piglets from the three studied groups of *Chapter 4* were *M. hyopneumoniae* rt-PCR negative. This result was expected since all gilts included in the study were rt-PCR negative prior farrowing. However, it cannot be discarded that the bacterial load in gilts and piglets was reduced until becoming undetectable by the used laboratory techniques. Therefore, and considering slaughtered-age animals of previous batches of this farm had EP-like lesions (data not shown), it would have been interesting to evaluate the *M. hyopneumoniae* infection dynamics of piglets coming from vaccinated and non-vaccinated gilts during subsequent production stages. In addition, pigs were routinely vaccinated against *M. hyopneumoniae* at weaning in this farm. Thus, it would have been also interesting to investigate if the global vaccination approach including gilts, sows and piglets could have reduced vertical and horizontal transmission and, consequently, decreased the EP-like lesions at slaughterhouses. In all cases, however, it would be important to assess the potential effect of maternally derived immunity on piglet vaccine efficacy.

Different types of samples have been proposed for an early detection of this bacterium (Fablet *et al.,* 2010; Vangroenweghe *et al.,* 2015a, 2015b; Pieters *et al.,* 2017). The laryngeal swab is considered to offer a high sensitivity in the early stages of the infection (Pieters *et al.,* 2017). For this reason, it was decided to

use laryngeal swabs in both gilts and piglets (*Chapter 4*). However, other authors have proposed that broncho-alveolar or trachea-bronchial lavages may also display high sensitivity (Vangroenweghe *et al.,* 2015a). In any case, there is a lack of consensus on which sample shows the highest sensitivity at different ages or stages of infection.

Another important effect of gilt vaccination against *M. hyopneumoniae* during the acclimation period was the significantly higher ELISA antibody values observed in vaccinated groups compared to the non-vaccinated one during almost the whole study period (*Chapter 4*). In contrast, no difference between vaccinated and non-vaccinated gilts was observed in terms of number of seropositive gilts after the acclimation period. However, statistically significant differences in terms of PI were observed among all three treatment groups. This situation could be explained by the following hypotheses:

- The lack of differences on the number of seropositive gilts might be explained by the fact that all vaccinated and non-vaccinated had probably seroconverted due to *M. hyopneumoniae* vaccination and/or infection. Interestingly, the fact that non-vaccinated gilts were seropositive would confirm the circulation of *M. hyopneumoniae* within the herd. Therefore, samplings just after acclimation (10 wpe) might have been helpful to describe more accurately the pattern of *M. hyopneumoniae* seroconversion in such population.

- The lowest homogeneity on antibody levels (PI values) in the non-vaccinated group may be explained by the fact that the serological response to *M. hyopneumoniae* natural infection is more variable in terms of levels of antibodies (Morris *et al.,* 1995) than those induced by vaccination.

In addition to the situation commented above, differences on antibody levels in terms of PI values between vaccinated groups with four or two doses were also detected after acclimation. This finding can probably be explained by the greater "booster" effect induced by those two extra doses received. Unfortunately, since commercial ELISA methods are not able to differentiate between antibodies elicited by *M. hyopneumoniae* vaccination or infection, the booster effect elicited by the infection in vaccinated gilts cannot be discarded. Thus, it is difficult to make a definite conclusion regarding the correlation between the high level of antibodies (low PI) observed in vaccinated groups and the exclusive origin of antibodies due to vaccination.

High number of seropositive sows to *M. hyopneumoniae* has been correlated with high number of seropositive piglets after colostrum intake (Martelli *et al.,* 2006; Sibila *et al.,* 2008; Bandrick *et al.,* 2011). Based on serological results *(Chapter 4)*, vaccination of gilts led to a significantly higher seropositivity as well as lower PI ELISA values in piglets coming from vaccinated gilts with four and two doses compared to those coming from non-vaccinated ones. Although some piglets from the non-vaccinated group were also seropositive, it is reasonable to think that differences observed in terms of PI between piglets coming from different groups are mainly due to a higher transfer of maternally derived antibodies from vaccinated gilts to piglets via colostrum (Martelli *et al.,* 2006; Bandrick *et al.,* 2011). Interestingly, the lack of cross-fostering practices between groups with different treatments would confirm that the origin of antibodies detected in seropositive piglets from non-vaccinated gilts was the colostrum containing maternal antibodies elicited by the infection. In contrast, the fact that some piglets coming from vaccinated gilts were seronegative could be explained by a poor colostrum intake (Quesnel, 2011) and/or the decay of maternal antibodies (Morris *et al.,* 1995). Further demonstration of these assumptions would be testing antibodies against *M. hyopneumoniae* directly in

the colostrum. Indeed, a previous study correlated antibody levels in colostrum with the antibody levels detected in piglet serum (Jenvey *et al.,* 2015).

Besides all mentioned effects associated to gilt *M. hyopneumoniae* vaccination, the evaluation of the cellular immune response in gilts and the transference of maternally derived cell-immunity to piglets would have been further interesting points to ascertain. In fact, few studies have been published regarding this matter in vaccinated sows (Bandrick *et al.,* 2008, 2014) and no information is available in gilts at the acclimation period. In addition, the fact that no significant differences were detected between vaccination schedules with four and two doses (*Chapter* 4) suggests the need to study the effect of gilt vaccination using only one dose.

Despite gilt vaccination is considered as the main strategy worldwide (Fano and Payne, 2015; Centeno *et al.,* 2016; *Chapter* 3), other gilt acclimation strategies such as the natural exposure to *M. hyopneumoniae* were also identified by the survey. Natural exposure to the bacterium is usually performed by direct contact with infected pigs and/or culled sows (*Chapter 3*). The disadvantage of natural exposure is the intermittent *M. hyopneumoniae* shedding by infected pigs (Roos *et al.,* 2016; Takeuti *et al.,* 2017a), suggesting a lack of consistent exposure. Thus, the accurate identification of shedder pigs/sows as well as the adequate ratio infected/naïve animals and time of exposure are required to reach a successful process (Roos *et al.,* 2016). Recently, the difficulties to reach a natural infection during acclimation have led to attempt a controlled exposure by lung homogenate and/or aerosol containing the bacterium (Fano and Payne, 2015; Centeno *et al.,* 2016; Sponheim, 2017). However, the uncertain efficacy and safety of both exposure methods lead to consider the vaccination as the most feasible and effective tool for gilt immunization against *M. hyopneumoniae.* Further research is needed to investigate the efficacy and safety of such

exposure methods as well as its effect in combination with vaccination strategies.

Another element that has been suggested playing a potential role for *M. hyopneumoniae* monitoring and control within herds is the variability of this bacterium (Sibila *et al.,* 2009; Maes *et al.,* 2018). In particular, the genetic variability of *M. hyopneumoniae* has been demonstrated in several published studies, but its importance is not fully understood. In the present Thesis (*Chapters 4 and 5*), this genetic variability was assessed using two different approaches. In *Chapter 4*, the characterization of *M. hyopneumoniae* was based on counting the VNTR of three loci (P97, P146 and H1) in laryngeal swabs positive by *M. hyopneumoniae* rt-PCR (*Chapter 4*). In parallel, in *Chapter 5*, such variability was assessed using two (P97 and P146) and those three plus and additional one (P97, P146, H1 and H5) in lung tissue samples.

In *Chapter 5*, lung tissue samples from slaughtered pigs with different vaccination status were collected for *M. hyopneumoniae* detection and characterization. This study represents the first time comparing *M. hyopneumoniae* genetic variability in non-vaccinated and vaccinated pigs, as well as in the vaccines used in the corresponding farms. Under the conditions of this study, high inter-farm and limited intra-farm variability was detected. Overall, TPs were different among non-vaccinated and vaccinated farms using two or four loci, except those with the same breeding origin farm. This novel finding suggests that TPs obtained in fattening farms, using two loci, might come from the breeding herd. However, such association was not found using four loci. Interestingly, variability depended on the number of studied loci and, thus, the higher the number of different TPs, the higher the genetic variability. Since the analysis of *M. hyopneumoniae* by four loci implies a wider region of bacterium genome evaluation, the likelihood to find some differences in the *M.*

hyopneumoniae genome increases. This assumption could be reinforced by some recent studies in which high intra-farm heterogeneity was detected using four loci (Tamiozzo *et al.,* 2015; Michiels *et al.,* 2017a) compared to the limited variability observed with two studied loci (Charlebois *et al.,* 2014; Galina-Pantoja *et al.,* 2016). On the contrary, other studies also detected high variability using two loci (Dos Santos *et al.,* 2015; Takeuti *et al.,* 2017a; Felde *et al.,* 2018).

Interestingly, the TPs detected in the vaccinated farms and the corresponding bacterin used in each farm were also different (*Chapter 5)*. This lack of genetic similarity for the studied genes might potentially explain the variable vaccination effect observed under field conditions (Maes *et al.,* 2008). Indeed, the partial efficacy of vaccines has been suggested to be linked to the diversity among field and vaccine strains (Minion, 2002; Assunçao *et al.,* 2005). In contrast, a previous experimental study did not detect differences on vaccination responses using homologous or heterologous *M. hyopneumoniae* strains as vaccines (Villareal *et al.,* 2012). Therefore, at present, it is difficult to conclude solid evidence about vaccine efficacy in respect the circulating *M. hyopneumoniae* strain or strains in a given farm.

Although the technique most commonly utilized in the literature has been MLVA (Dos Santos *et al.,* 2015; Galina-Pantoja *et al.,* 2016; Takeuti *et al.,* 2017a; Michiels *et al.,* 2017a), in this Thesis, conventional sequencing was performed to characterize *M. hyopneumoniae.* Both techniques are based on the detection of VNTR in different loci. Despite this, one of the advantages of conventional sequencing over the MLVA is its allowance for exact counting of the VNTR, identifying potential minimal changes in the number of repeats between strains (*Chapter 5*). However, the importance of those minimal variations is still unknown and further research is needed to ascertain their impact on *M. hyopneumoniae* variability. In contrast, MLVA seems to be able to detect the

presence of multiple strains in a sample (Nathues *et al.,* 2011a; Michiels *et al.,* 2017a), whereas using conventional sequencing only one strain per sample was detected in this Thesis (*Chapter 4 and 5)*. Such difference may be explained by the different discrimination power of both techniques and the type of sample used. While laryngeal swabs (*Chapter 4*) and lung tissue (*Chapter 5*) taken in this Thesis represent limited location areas of the respiratory tract, BALF could cover all respiratory tract (Nathues *et al.,* 2011a; Michiels *et al.,* 2017a).

In addition to the type of samples used, the contradictory results among published studies about *M. hyopneumoniae* variability could be also consequence of the lack of consensus on the characterization process. Recently, Michiels et al. (2017a) defined a strain as unique combination of VNTR according to studied loci using MLVA. Nevertheless, no information is available regarding to the most suitable technique for a successful and accurate characterization of *M. hyopneumoniae* variability, the type and number of loci required nor the criteria for discrimination between strains, among others. Therefore, results from *Chapter 5* may serve as basis for discussion that may lead to consensus on some of these variables.

In summary, the results of this Thesis have a very practical application for the swine veterinarians since gilt acclimation was identified as a widely used strategy to control *M. hyopneumoniae* in Europe. In particular, gilt vaccination using multiple doses of commercial vaccines against this bacterium was identified as the most utilized strategy to acclimate gilts (*Chapter 3*). In alignment to this finding, the second study (*Chapter 4)* supports the use of gilt vaccination during the acclimation period, since it reduced bacterium shedding and elicited high and long-term humoral immune response in gilts as well as ensuring maternally derived immunity transfer to piglets (measured at weaning). Nevertheless, since obtained results showed slight differences

between vaccination programs using four or two doses, those two extra doses might not be justified. Moreover, this Thesis described a high genetic variability of *M. hyopneumoniae* into replacement batches and breeding herd (*Chapter 4*) as well as in slaughtered pigs showing CVPC coming from non-vaccinated and vaccinated farms and the commercial vaccines used in these vaccinated farms (*Chapter 5*). However, the variability seems to be partially dependent on the number and type of loci considered for the analysis.

CHAPTER 7

Conclusions

1. Gilt acclimation strategies against *M. hyopneumoniae* are frequently used by European swine producers for controlling respiratory diseases associated to this pathogen. However, the verification of the effect of those acclimation strategies is rarely performed in Europe.

2. The most common strategy used by European swine producers to acclimate gilts for *M. hyopneumoniae* is vaccination, by means of one or two doses.

3. Gilt vaccination against *M. hyopneumoniae* using four or two doses during the acclimation period reduces the gilt shedding at the acclimation unit, and presumably, the infectious pressure into breeding herd. However, no significant differences in terms of gilt shedding between vaccination protocols using two or four doses are observed.

4. Immunization of gilts with a *M. hyopneumoniae* commercial vaccine using multi-dose protocols at acclimation period increases the number of seropositive gilts at farrowing compared to a non-vaccinated group. In addition, the humoral immune response in vaccinated gilts is of longer duration and more homogeneous in terms of PI values compared to non-vaccinated ones.

5. The percentage of seropositive piglets born from vaccinated gilts using four or two doses is higher compared to those coming from non-vaccinated ones. Similarly, maternally derived antibody levels of piglets coming from vaccinated gilts is higher than those coming from non-vaccinated gilts.

6. The number of tested loci used for gene characterization of *M. hyopneumoniae* can influence the detected genetic variability.

7. High number of TPs in gilts from two breeding origins as well as in the slaughtered pigs showing CVPC (coming from non-vaccinated and vaccinated farms) demonstrates substantial genetic diversity in *M. hyopneumoniae.*

8. *Mycoplasma hyopneumoniae* TPs detected in animals showing CVPC coming from vaccinated farms tested are different from the strain of the corresponding vaccine used.

CHAPTER 8

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