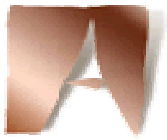

Universitat Autònoma de Barcelona

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Bellaterra, Juny 2004

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Memòria presentada per optar al grau de doctora en veterinària
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Certifiquen:

Que la tesis doctoral titulada **"Epidemiological features of natural *Mycoplasma hyopneumoniae* infection in pigs in Spain",** de la que és autora la llicenciada en Biologia, **Marina Sibila Vidal**, s'ha realitzat als laboratoris de la Unitat d'histologia i Anatomia patològica de la facultat de Veterinària de la Universitat Autònoma de Barcelona sota la nostra direcció.

I perquè així consti, a tots els efectes, firmem el present certificat a Bellaterra, 21 de Juny del 2004

Maria Calsamiglia Costa
Directora de Tesis

Joaquim Segalés i Coma
Tutor de la Tesis

El treball presentat en aquesta Tesis, forma part de les investigacions realitzades en el Centre de Recerca en Sanitat Animal (CReSA)

LITERATURE REVIEW

1 Etiologic agent:

1.1 Mycoplasmas and Porcine Mycoplasmas

Mycoplasma hyopneumoniae (M. hyopneumoniae) is a member of the Class Mollicutes which lacks of cell wall *(mollis*, soft; *cutis*, skin), Family Mycoplasmataceae (sterol required for growth), Genus Mycoplasma (Tully et al., 1996). Mollicutes are phylogenetically related to the Gram-positive bacteria.

Nowadays, Mycoplasmas are considered the simplest self-replicating organisms known. Despite their genetic simplicity, the members of the Class Mollicutes are considered as major animal and plant pathogens world-wide. The minimal characteristics to differentiate Mycoplasmas from other eubacteria are: permanent lack of cell wall, tendency to form fried egg colonies on solid media, pass through membrane filters (450-220 nm pore size), low genome G+C content (23-40 mol%) and small genome size (*Mycoplasma hyopneumoniae* =1070 bp) (Minion et al., 2002a). With the smallest genomes known for self-living organism their number of genes approaches the minimal set of genes required for life. Because of their low number of genes they have limited metabolic capabilities and they are well adapted to or dependant on their hosts (Garnier et al., 2001). The extremely low guanine + cytosine (G+C) imposes further restrictions on the amount of genetic information available to Mycoplasma cells, leading to the complex nutritional requirements and the parasitic or saprophytic mode of life. The Mycoplasma cell is bound by a plasma membrane and the enclosed cytoplasm contains ribosomes and a circular double-stranded DNA molecule (Razin et al., 1998). The major antigenic determinants described in these wall-less organisms are membrane proteins,

glycolipids and lipoglicans found in the cell membrane (Razin et al., 1998).

Although some Mycoplasmas are commensals and belong to the normal flora of their hosts, most species are pathogens. Many Mollicutes are inhabitants of the mucous membranes of the respiratory, urogenital or gastrointestinal tract of vertebrates (Tully et al., 1996). Moreover, arthropods and plants are also possible hosts of mycoplasma infections (Razin et al., 1998).

Besides *M. hyopneumoniae*, there are two other mycoplasmal species that can cause disease in swine: *M. hyorhinis* inhabits the upper respiratory tract (nasal cavity) and can occasionally cause poliserositis and arthritis in young pigs; *M. hyosynoviae* inhabits the nasal cavity and pharynx of pigs and can cause non-suppurative arthritis without polyserosits in grow-finishing pigs (Whittlestone et al., 1979). On the other hand, *M. flocculare*, appears to be a common inhabitant of the porcine lung and nasal cavities of pigs but is essentially non-pathogenic (Amstrong et al., 1981). Friis (1973, cited in Ross et al., 1999) presented evidence that *M. flocculare* produced lymphocytic infiltration in the nasal mucosa and peribronchial areas of gnobiotics pigs inoculated with this organism. However, there is still no evidence that these findings are of significance in naturally occurring respiratory disease. Although, a recent study suggested that *M.hyopneumoniae* may not be the only Mycoplasma important in respiratory disease in swine (Strait et al., 2003).

Several other species of Mollicutes are infrequently found in the porcine respiratory tract and are of questionable pathogenecity: these include *M. sualvi* and *M. hyopharyngis* and several species of acholeoplasmas (Ross, 1999).

Among all porcine mycoplasmas, *M. hyorhinis* and *M. flocculare* are the most commonly studied in relation with *M.hyopneumoniae*. Cross-

reactions in serologic techniques, as well as *M.hyopneumoniae* culture overgrown with these two mycoplasmas have been extensively described (Nicolet et al., 1980, Feld et al., 1992).

M. *flocculare* has many morphological, growth and antigenic similarities to *M. hyopneumoniae* (Ross, 1999). Results of DNA-DNA hybridization studies have indicated that there is some relation between *M. hyopneumoniae* and *M. flocculare* (10-11%); in contrast, *M. hyopneumoniae* and *M flocculare* only cross-hybridize with nonporcine Mycoplasmas at levels about 3% (Stemke et al., 1985).

Because the rRNA genes are among the most conserved genetic elements, comparisons of these sequences have become an important phylogenetic tool. Comparison of these genes in different Mycoplasma species have shown that *M.hyopneumoniae*, *M. flocculare* and *M. hyorhinis* are very closely related, particularly the first two (Stemke et al., 1994a). The homology between *M. hyopneumoniae* and *M. flocculare* 16S ribosomal sequence is 95,6% (Stemke et al., 1992). The genome of *M.hyopneumoniae* was found to be larger (1070 compared to 890 kb) than *M. flocculare* (Robertson et al., 1990). Presumably, pathogenicity of *M. hyopneumoniae* is facilitated by the absent genes in *M. flocculare*. An example could be the gene for ciliary adhesin (adh), wich is believed to be a virulence factor in *M.hyopneumoniae* (Hsu and Minion, 1998) and was not detected by hybridization in *M flocculare* nor in *M. hyorhinis* (Wilton et al., 1998).

1.2 Mycoplasma hyopneumoniae

1.2.1 Enzootic Pneumonia history

Initially, it was thought that the chronic pneumonia observed in growing and fattening pigs was caused by a virus and consequently termed as virus pneumonia (Betts, 1952, Whittlestone, 1979). The basis

for this idea was the transmission of the disease to pigs with bacteria-free filtrates and because penicillin and sulfamides did not affect the infectivity of affected lung material (Whittlestone, 1979). Some years later, evidences that lead to the identification of a Mycoplasma-related organism as the etiologic agent were: the efficacy of tetracycline antibiotics against the disease, the size of the causal agent (0.2-0.45 μm); the Giemsa staining; and the growth of the agent in media free of living cells clearly demonstrating that it was a nonviral microorganism (Whittlestone, 1979). Mare and Switzer (1965) obtained the first isolate of the microorganism and identified it as *M. hyopneumoniae* (strain 11 or VP119). The same year, Goodwin et al. (1965) induced EP to pigs inoculated with another isolated, which was named of *Mycoplasma suipneumoniae (strain J).* To reach a consensus, the Academy of Taxonomy of Mycoplasmatales, decided to designate a neotype strain and proposed the strain J (*M. suipneumoniae*) as the neotype strain of *M. hyopneumoniae.*

1.2.2 Main Immunogens

A better characterization of putative *M hyopneumoniae* antigens would help in detecting virulence factors, in pathogenecity studies, in serological differentiation of porcine Mycoplasmas, and finally in developing efficaceous vaccines. Up to date, several immunogens have been described:

A 54kDa protein located within the cell membrane with cytopathic effect was described (Geary and Walczak, 1985, Ross and Young , 1993). Debey and Ross, showed that the attachment of *M. hyopneumoniae* to ciliated epithelium was necessary to induce cytopathic effect in tracheal organ cultures from newborn piglets (Ross and Young, 1993).

Another well studied antigen is a cytosolic 36kDa protein (p36). This protein was cloned and expressed (Strasser et al., 1991), and afterwards characterized (Haldiman et al., 1993) as lactate dehydrogenase (LDH). This cytosolic protein induces an early immune response in naturally and experimentally infected pigs, is apparently highly conserved among different *M.hyopneumoniae* strains and does not cross-react with other porcine Mycoplasmas (Strasser et al., 1991). Frey et al., (1994) used an LDH expressed in *E. coli* for the development of a specific LDH-ELISA. Anti-LDH titres persist until 21 weeks post-infection, in contrast to the antibody titres against membrane proteins which decreased at 12 weeks post-infection. On the contrary, Caron et al., (2000b) found no correlation of clinical and pathological findings with an IIF specific test using p36 as antigen.

Another antigen used to develop new serodiagnostic tests was the ABC transporter ATP-binding protein (Blanchard et al., 1996, Subramaniam et al. 2000).

It has been proposed that cytoadherence of *M.hyopneumoniae* to porcine cilia might be a mutifactorial process. At the moment two adhesin proteins have been postulated: a 97kDa protein, named p97 (Zhang et al., 1995) and a 110kDa protein, named p110 (Chen et al., 1998). (See pathogenesis). Induction of humoral immune response after p97 recombinant vaccines is controversial. King et al., (1997) and Shimoji et al., (2003) were not able to induce serum antibody production, while Chen et al., (2001) observed a remarkable IgG responses in immunized pigs with a p97 RR1 vaccine. However, Shimoji et al., (2003), suggest that intranasal vaccination of YS-19 strain could prime a cell-mediated immune response against p97.

Other *M.hyopneumoniae* putative immunogens are: a 46kDa (p46) surface antigen (Futo et al., 1995) and a 65Kda (p65) lipoprotein, and a 74.5 kDa heat shock protein (hsp) (Brooks and Faulds 1989).

Heat shock proteins have been proposed as good immunogen to vaccine development. Scherm et al., (2002), identified and sequenced the Hsp60 gene of several mycoplasma species (including *M.hyopneumoniae*) and confirmed that the Mycoplasmal Hsp60 is immunogenic in natural infections.

Ross and Young (1993) identified strain specific immunogens of *M.hyopneumoniae* pathogenic strain 232 and non-pathogenic strain J. Strain 232 antigens were immunoblotted with convalescent serum previously adsorbed with either strain 232 or strain J whole cell. Results of this study revealed that there were 6 putative specific antigens abundant in the strain 232, which were not detected in strain J. These 6 antigens were: 145kDa, 97kDa, 86 kDa, 75kDa, 60kDa, and 46kDa.

1.2.3 *M.hyopneumoniae* strains

Since the designation of strain J, a non-virulent strain, as the neotype strain of *M.hyopneumoniae,* several studies have looked for differences between field strains. Ro and Ross (1983) demonstrated by growth-inhibition and metabolic-inhibition tests some heterogeneicity among the strains they tested. Moreover, Frey et al. (1992) found by enzyme restriction digestion run in a field inversion gel electrophoresis (FIGE) that chromosomes of *M.hyopneumoniae* strains were quite different. In 1996, (Artiushin and Minion, 1996) found differences in RAPD (randomly amplified polymorphic DNA) band DNA pattern even within isolates from the same geographical location.

In 1998, Wilton et al., showed that the number of tandem amino acid repeats in R1 of ciliar-adhesin p97 varied consistently between strains.

Moreover, intraespecies variation among field USA isolates by total protein profile, glycoprotein profile, and size difference in the amplified PCR product of p97 adhesin repeat region R1 has been also described (Lin, 2001). PCR amplifications of R1 region of seven commercial vaccines produced also different size of amplicons (Lin, 2001).

Although genetic heterogeneity between field strains had been extensively reported, it had not been related with virulence differences. In 2003, Vicca et al. analyzed also by RAPD six *M.hyopneumoniae* field isolates and linked it to genetic and virulence differences. Based on clinical and pathological parameters, the isolates tested were classified as highly, moderately or non-virulent isolates. Moreover, a 5000pb RAPD band was found in the highly and moderately virulent isolates but not in the avirulent ones. Recently, differences in virulence factors between field isolates were studied in a challenge trial. In that study, differences in percentage of lung affection were observed among the groups inoculated with different field isolates (Strait et al, 2004).

Some species of pathogenic mycoplasmas (*M. hyorhinis, M. bovis, M. gallisepticum*) have genetic machinery for altering their surface characteristics (antigenic variation). It remains to be determined whether these mechanisms are also characteristic of *M.hyopneumoniae* and to what extent they may explain apparent genetic and antigenic differences between strains (Desrosiers 2001).

2 The disease: Enzootic Pneumonia and PRDC

Mycoplasma hyopneumoniae is the principal etiologic agent of Mycoplasmal pneumonia (MPS) or Enzootic Pneumonia (EP) in pigs. EP is a heterogeneous disease that results from combined infctions with *M.hyopneumoniae* and one or more opportunistic, secondary bacterial pathogens such as *Pasteurella multocida*, *Mycoplasma hyorhinis, Streptococcus suis, Haemophilus parasuis, Bordetella bronchiseptica, Actinomyces pyogenes* and *Actinobacillus pleuroneumonia* (Done et al., 1997). The two most frequent association are *M.hyopneumonia* with *A. pleuropneumoniae* (Yagishaji et al., 1984), and with *P. Multocida* (Ciprián et al., 1988)

Nowadays, EP is found in all pig producing countries. In fact, abattoir surveys in many countries have demonstrated high percentages of EP seropositive pigs (Falk and Lium, 1991, Wallgren et al., 1993, Wallgren et al., 1994) and EP lung lesions (Falk and Lium, 1991, Lium and Falk, 1991). In Spain, an abattoir studyshowed a 96% of slaughter pigs had lung lesions, from which a 68.8% were compatible with EP characteristics lung lesions (Cubero, 2000). These results, lead to consider *M. hyopneumoniae* the most economically significant bacterial respiratory pathogen.

In the last decade, a new respiratory syndrome named as Porcine respiratory disease complex (PRDC) emerged as a serious health problem in most of pig producing countries. This respiratory complex includes both viral and bacterial agents: Porcine reproductive and respiratory syndrome virus (PRRSV), *Mycoplasma hyopneumoniae*, swine Influenza virus (SIV), *Pasteurella multocida, Actinobacillus pleuropneumoniae, Haemophilus parasuis, Streptococcus suis*, Porcine circovirus type 2 (PCV2) and Aujeszky's disease virus (ADV) (Thacker, 2001a).

Pneumonia in pigs with PRDC is due to a combination of both viral and bacterial agents, being PRRSV and *Mycoplasma hyopneumoniae* the main causative agents (Thacker et al., 1999).

PRDC is characterized by slow growth, decreased feed efficiency, anorexia, fever, cough, and dyspnea in finishing pigs (Thacker, 2001a). PRDC typically occurs at 14 to 20 weeks of age, being also referred as the "18 week wall". It has been described in all kinds of management and facilities schemes, including intensive production systems.

2.1 **Clinical presentation**

Enzootic pneumonia is characterized by a high morbidity and low mortality. Although pigs of all ages are susceptible to be infected with *M. hyopneumoniae* (Piffer and Ross 1984, Kobisch et al., 1993). EP is usually not observed in animals younger than 6 weeks of age (Ross, 1999). The rarity of disease in pigs under 6 weeks of age suggests that transmission from sow to suckling pigs is inefficient. This inefficiency may be due to little shedding from sows or the relative protection of pigs afforded by colostral immunity (Stevenson et al., 1998). From 6 weeks to 3 months of age, EP is infrequently observed. Finally, the peak prevalence of EP occurs later, at market weight (Done, 1997).

Severity of symptoms depends on the intensity of infection, presence of secondary infections and enviromental conditions (Ross et al., 1999, Leon et al., 2001). When Mycoplasma infection is not complicated with secondary pathogens, the disease can take a subclinic course (no coughing) or mild clinical signs consisting on chronic nonproductive cough and reduced rate of gain and feed efficiency (Desrosiers 2001, Maes, 1996). When infection with secondary pathogens occurs, other symptoms such as laboured breathing and fever, and even death may occur (Kobisch, 2000).

Under field conditions, EP incubation period is very variable. Coughing is not constant and is generally noticeable 2 to 6 weeks after infection and ceases after 2 months post infection (Kobisch et al., 1993, Maes 1996, Done 1997, Sorensen et al., 1997). However, considerable variation may occur. In incubation period cases, development of clinical signs may take up to 16 months (Piffer and Ross; 1984, Done 1997).

Inoculation studies have shown that the incubation period and the development of clinical signs are dose-dependant (Ross, 1999, Stevenson et al., 1998). At high doses, clinical pneumonia may develop following an incubation period of 11 days. At moderate doses, clinical pneumonia develops after a prolonged incubation period of up to 4-6 weeks. And at low doses, only subclinical (no coughing) infection develops (Stevenson et al., 1998).

Although in field cases is unknown, a similar dose-dependency is expected. This dependency has also been linked to a certain percentages of pigs being infected (Calsamiglia et al., 1999b). High percentages of infected pigs lead to a faster spread in the nurseries and earlier disease outbreak (Pijoan, 1995).

Quantification of pathogen's DNA present in clinically and subclinically infected pigs would help to elucidate the necessary dose to elicit lung lesions and the consequent cough.

2.2 Gross lesions

Mycoplasmal pneumonia is a catarrhal bronchopneumonia with a cranioventral distribution involving cranial, middle and intermediate lobes as well as cranioventral portion of caudal lobes (Taylor et la.,1996). The intermediate, the left cardiac and the left apical lobes are affected less frequently. The diaphragmatic lobes are rarely affected. This distribution

may reflect the airflow patterns, as the right apical lobe receives a stem bronchus directly from the trachea (Livigston et la., 1972, Maes, 1996).

In early stages of infection gross lesions are characterized by purple to gray areas of consolidation. The lung is more collapsed than in control lungs and when the lesion is incised, the consistency is meaty but not excessively firm (Christensen et al.,1999). In the acute phase of the disease, the lobules are swollen and the airways contain copious amounts of stick white exudates (Armstrong et al., 1983). If samples are taken at this stage, large numbers of Mycoplasma cells will be found. In the chronic phase, lesions are contracted rather than swollen and contain the causative agent in low numbers (Armstrong et al.,1983, Amanfu et la., 1984, Livingston et la., 1972). Livingston (1972) described that in uncomplicated Mycoplasma infection, gross lesions will run a course of 5 to 6 weeks, after which lesions start to regress.

When secondary bacterial infections occur, gross lesions have a more grayish color and are firmer due to formation of fibrous tissue, leading to a purulent bronchopneumonia (Christensen et al., 1999). Correlation between onset of clinical symptoms (coughing) and pneumonic lesions has been described (Kobisch et al., 1993). In this study, macroscopic lesions and coughing were seen after 1 and 2 weeks post infection, respectively. After 7-9 weeks post infection, lesions recovered and coughing disappeared.

In experimental inoculations with pure *M. hyopneumoniae*, there is rarely more than 20% of lung affection. However, in field cases, complications by secondary invaders lead to more extended lesions (up to 80-90% of lung involvement) (Done, 1997).

2.3 Microscopic lesions

In early stages of the disease, loss of cilia, exfoliation and small accumulations of neutrophils in lumina around airways can be seen. As the disease progresses, there are increased numbers of lymphoctes in peribronchial, peribronchiolar and perivascular spaces (Ross, 1999). Acute cases may show minimal lymphoid hyperplasia and a predominantly neutrophilic alveolar exudate (Taylor et al., 1996). In chronic stage of the disease, there is lymphoid hyperplasia or cuffing and the consequent thickening of the interalveolar septa. In recovering lesions, collapsed alveoli, alveolar emphysema and lymphoid nodules can be seen (Ross, 1999). Lymphoid hyperplasia itself may be related to clinical signs, because pressure from aggregates of lymphoid tissue may obliterate the lumen of the softwalled bronchiole and cause collapse of surrounding alveoli (Bakersville 1981).

The overall chronology in experimental inoculations would be the following: two to four weeks post inoculation (PI), macroscopic lesions are maximal and cuffing appears, at 8 weeks pI macroscopic lesions start healing and if no secondary infections occur only few macroscopic lesions remain at 10 weeks pI. Approximately at 4 months pI, cuffing begins to disappear (cited in Maes, 1996).

2.4 Seroconversion

Time of seroconversion depends on the infection pressure present, the management factors established in the farm and also the serological assay used (Maes, 1996).

In intratracheal inoculation experiments, serum antibodies were first detected in some pigs at 2 –4 weeks PI, reaching the peak of antibody titres after 11-12 weeks PI (Piffer et al., 1984, Suter et al., 1985, Messier and Ross, 1990, Sheldrake et al., 1990, Kobisch et al., 1993, Le Potier, et

al.,1994, Djordjevic et al., 1994). However, not all animals seroconverted at the same time. In most of these studies, it took some weeks to detect serum antibodies in all tested pigs. In aerosol infected pigs, Sorensen et al., (1997) reported seroconversion as early as 8 days PI, but it took a further 5 weeks for all animals to become positive. In conditions more similar to natural infections, seroconversion is delayed. Armstrong et al., (1983), reported that intranasal exposed pigs seroconverted earlier and developed higher titres than contact-exposure pigs.

In natural conditions, time to seroconversion appears to be more delayed. Thus seroconversion occurs mainly in the growing-to-finishing units (8-24 weeks of age), after 6-9 weeks post infection (Lam et al., 1972, Sheldrake et al., 1990, Yagihashi et al. 1993, Djordjevic et al., 1994, Morris et al. 1995, Sitjar et al., 1996, Andreasen et al. 2000, Leon et al., 2001, Vicca et al., 2002). In a field study where clinically infected herds were compared with subclinically infected herds, differences in the seroconversion patterns were observed (Vicca et al., 2002). At 15 and 18 weeks of age, percentage of seropositive pigs was significantly higher in the clinically than in the subclinically infected herds. While seroconversion in clinically infected herds was observed between 12 and 15 weeks of age, seroconversion in subclinical herds occurred between 15 and 18 weeks of age.

Different degrees of correlation between time of seroconversion and gross lung lesions have been reported (Falk and Lium 1991, Yagihashi et la., 1993, Morris et al, 1995, Sitjar et al., 1996, Leon et al,. 2001). Time elapsed between onset of clinical signs or pneumonic lesions to seroconversion seems to be increased in naturally infected pigs compared to experimentally infected pigs. A delay of approximately 8 weeks between peak pneumonia and seroconversion has been described (Suter 1985, Young and Ross, 1987, Sitjar et la., 1996). This delay was

attributed to the poorly invasive character of Mycoplasma and the consequent detection of IgG antibodies by the serological test (Sitjar et la., 1996).

Seroconversion has been correlated not only with onset of pneumonic lesions but also with the extent of these lesions (Kobisch et al., 1993, Sitjar et al., 1996). Andreasen et al., (2001b) was in agreement with Sitjar et. al. (1996), reporting that pigs seroconverting to *M.hyopneumoniae* close to slaughter expressed the largest extent of Mycoplasma-like catarrhal pneumonia.

Relation of seroconversion with average daily gain is controversial. Wallgren et al., 1998 reported that pigs seroconverting early in life tended to have a decreased weight gain. On the contrary, others found that pneumonia occurring later in life has the largest impact on daily weight gain. (Christensen et al., 1999, Sitjar et al., 1996). And finally, no significant differences between *M.hyopneumoniae* seronegative or sepositive pigs with weight gain was reported by Morris et al., (1995) and Andreasen et al, (2001a).

2.5 **Experimental Inoculations**

Reproduction of EP in laboratory animals has been difficult. Administration of cultured bacteria or lung homogenates via intranasal (Armstrong et al., 1983, Kobisch et al., 1987) or intratracheal (Suter et al., 1985, Messier et al., 1990, Sherldrake et al., 1990, Djordjevic et al., 1997, Kurth et al., 2002) routes has been used to reproduce te disease (Suter et al., 1985, Messier et al., 1990, Sherldrake et al., 1990, Strasser et al., 1992, Kobisch et al., 1993, Djordjevic et al., 1997, Calsamiglia et al., 1999a, Thacker et al., 2000a, Thacker et al., 2000b, Ruiz et al., 2002, Thacker et al., 2002). However, these methods have the disadvantage that animals need to be sedated, and that the dose administered is difficult to

measure (Czaja et al., 2002). Moreover, intratracheally administration may induce an inflammatory response in the inoculated tissue (Czaja et al., 2002). Another alternative is intranasal or intratracheal administration of 10^5-10^9 color changing units (CCU) of in-vitro cultured organism. The main drawback of these type of inoculations is that they are dose-dependant, and very high doses are needed to achieve clinical disease (Stevenson et al., 1998). Finally, aerosol administration of an in-vitro cultured *M.hyopneumoniae* (Sorensen et al., 1997, Czaja et al., 2002) has also been described. Cazja et al., (2002) reported that small aerosol inoculation doses are very effective in producing EP lung lesions, probably because the aerosol deposits the bacteria directly on their site of colonization.

3 Diagnosis

Combination of the different diagnostic tools along with clinical evaluation of the pigs will ensure an accurate diagnostic of the health status of the herd.

3.1 Lesion detection

3.1.1 Gross Lesions

Scoring systems to evaluate respiratory disease based on gross pathological lung lesions have been described by several authors (Morrison et al., 1985, Lium and Falk 1991, Hannan et al., 1982, Hurnik et al., 1993,). Since most of these scoring methods are based on a visual subjective estimation of the proportion of lung affected, it may evolve to possible errors (Davies et al., 1995). Thirty is the number of lungs that has been suggested to be inspected in order to reliably estimate the prevalence and severity of pneumonia on a herd level (Davies et al., 1995).

Although gross lesions are suggestive of enzootic pneumonia, they are not pathognomonic, as other organisms, such as Swine influenza virus (SIV) might produce similar lesions (Ross, 1999, Done et al., 1991, Thacker et al., 2001c). Also, Fuentes and Pijoan (1987) were able to induce lesions indistinguishable from that caused by *M. hyopneumoniae* by co-infecting pigs with pseudorabies virus and *P. multocida*.

Moreover, lesion resolution may lead to a false negative diagnosis of early mycoplasmosis in slaughter checks (Livingston et al., 1972, Sorensen et al., 1997).

Hence, the subjectivity of the scoring method, regression of lung lesions and the non-specificity of these lesions make the diagnosis based solely on gross lung lesions a poor indicator of lifetime pneumonia. Armstrong et al. (1984), recommended that the final diagnosis regarding infection of an individual animal should be based on a laboratory follow-up in combination with clinical and pathological findings.

3.1.2 Microscopic lesions

Microscopic lung lesions present in animals infected with *M. hyopneumoniae* have been classified based on their characteristics and severity (Livingston et al., 1972, Morris et al., 1995, Calsamiglia et al., 2000a). Calsamiglia et al., 2000a suggested a modification of other two criteria previously described, grading the lesions from 0-4. No lesions were scored 0. Lesions characterized by inflammation different from categories 3 and 4 (such as interstitial pneumonia or purulent bronchopneumonia) were given a score 1. Light to moderate cellular infiltrates (macrophages ad lymphocytes) with mild cellular exudate into the airways were graded 2. Scores 1 and 2 were considered non-diagnostic of *M.hyopneumoniae* infection. Lesions with perivascular and peribronchiolar lymphoplasmaytic hyperplasia, pneumocytic type II

hyperplasia and vacuolation, edema fluid in the alveolar spaces with neutophils, macrophages and plasma cells were graded 3. When these latter lesions were present together with peribronchial and perivascular lymphoid nodules, they were graded 4. Scores 3 and 4 were considered to be typical of *M. hyopneumoniae* infection. However, they are not pathognomic of *M. hyopneumoniae*. Swine Influenza Virus microscopic lesions consist of epithelial disruption in the bronchioles and interstitial pneumonia. Mild to peribronchiolar and perivascular lymphocytic infiltration occurs at all levels of the airways (Thacker et al., 2001c)

Although, presence of *M.hyopneumoniae* specific microscopic lesions is considered diagnostic of *M. hyopneumoniae* infection, detection of the pathogen in animals without lesions has also been described (Sorensen et al., 1997, Calsamiglia et al., 2000a). Therefore, diagnostic based solely on microscopic lesions may mislead animals in early or in recovering phases of the infection.

3.2 Agent detection: Microbiological Culture

Cultivation is considered as the “gold standard” technique to a definitive diagnosis of *M.hyopneumoniae* infection (Sorensen et al., 1997). Sorensen et al. (1997), compared culture with IF, ELISA and PCR and found that culture was the most sensitive technique at late stages of the disease (when fewer Mycoplasmas were present). However, it is used in few laboratories due to the difficulty, time required for isolation of this pathogen in field samples (usually 7-10 days) and because cultures are frequently overgrown by *M. hyorhinis* and *M. flocculare* (Maes, 1996).

3.3 Antigen Detection: Immunofluorescents (IFA), Immunohistochemestry (IHC)

These techniques are considered specific to detect *M.hyopneumoniae* on lung tissue smears. The main drawback of these techniques is that diagnosis is done once the animals have been slaughtered and that the sensitivity of the assay is easily compromised if tissues are not handled properly. In IFA, the tissue is embedded with polyethylene glycol and afterwards frozen. In frozen tissue sections, microestructures are most frequently broken and difficult to recognize, and the use of polyclonal antisera may result in non-specific detection of other closely related Mycoplasmas (Cheikh Saad Bouh et al., 2003). A positive IFA test is associated with the acute-stage of pneumonia, where high numbers of Mycoplasmal cells are present (Ross, 1999). Amanfu et al. (1984) showed that after an experimental inoculation, 10e4 Mycoplasmas per gram of lung tissue were necessary to obtain an IFA positive result. In consequence, the disadvantage of this technique is that only acutely infected pigs, harboring a high amount of bacteria, would give a positive signal. Therefore, low sensitivity, difficulty in obtaining specific-antibodies, and artifacts due to freeze procedures has limited the use of IFA in diagnostic laboratories.

Immunohistochemestry (IHC) uses lung tissue in formalin-fixed parafin-embbeded tissue blocks. IHC is thought to be sensitive, specific and allows the pathologist to observe the presence of the targeted antigen and the typical lesions attributed to a particular diseas (Halbur, 1997). Use of monoclonal and policlonal antibodies in IHC has been also used to detect *M.hyopneumoniae* antigens and to determine cellular populations present in lungs lesions of *M.hyopneumoniae* infected pigs (Sarradell et al., 2003, Rodríguez et al., 2004). Moreover, enzimatic staining with

peroxidase reactions (Immunoperoxidase, (IP)) in sections of formalin-fixed paraffin-embedded porcine lungs has been also used to diagnose *M. hyopneumoniae* in naturally and experimentally infected pigs (Doster et al., 1988). In 2003, Cheikh Saad Bouh (2003) developed and compared an IP (paraffin-embedded lung tissue) versus IFA (frozen tissue), both techniques against the p46 and p65 membrane proteins. The advantage of IP labeling techniques versus IFA is that the pathological lesions and the cell types involved in the inflammatory process can be both visualized, and therefore antigen detection and microscopic lesions related (Cheihk Saad Bouh et al., 2003).

3.4 DNA detection:

3.4.1 *in situ* hibridization (ISH)

Detection of nucleic acid in formalin-fixed paraffin-embedded tissues by ISH has also been reported (Kwon et al., 1999, Kwon et al., 2002). ISH is used to describe the specific location of *M. hyopneumoniae* in the respiratory cells of affected tissues (Kwon et al., 1999). Hybridization signals have been seen in the bronchial and bronchiolar epithelium, alveolar and interstitial macrophages, and type I pneumocytes of *M. hyopneumoniae* naturally infected pigs. The main drawback of ISH is that lung samples from dead pigs are needed and that is a time-consuming technique. Therefore, ISH is a very interesting technique to perform pathogenesis studies but when a rapid diagnostic in live animals is required, it is not adequate.

3.4.2 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a technique that allows the amplification of the DNA fragment flanked by two known sequences. Detection of the pathogen's DNA has some advantages over other

diagnostic techniques: the infectious agent is detected rather than the immune response, this test is quicker than culture, there is less interference with the treatment using antibiotics, it is very sensitive and specific, it can be performed in live or dead animals and from diverse kind of samples.

As all diagnostic techniques, PCR has some disadvantages: a higher risk of contamination due to its high sensitivity, it cannot be associated with lesion detection as in IHC or ISH, and since only a small fragment of the DNA is amplified, no further bacterial characterization can be performed, such as antibiograms or serotypeing, Also, the technique amplifies DNA from live and dead organisms, which can make interpretation of positive results difficult (Calsamiglia et al., 1999, Ruiz et al., 2002a, 2002b). Conventional PCR are qualitative techniques, since it only informs about the presence of the microorganism but not about the load.

In 1991, the first PCR test to specifically amplify *M.hyopneumoniae* was described (Harasawa et al., 1991). Since then, different PCR tests have been reported: single PCR (Harasawa et al., 1991, Artiushin et al., 1993, Mattson et a., 1995, Blanchard et al., 1996, Sorensen et al., 1997, Baumaister et al., 1998, Caron et al., 2000a), multiplex PCR (Caron et al., 2000a), nPCR (Stärk et al., 1998, Calsamiglia et al., 1999a, Verdin et al., 2000a, Kurth et al., 2002) and PCR with an internal control (Verdin et al., 2000b, Kurth et al., 2002).

The sensitivity of PCR tests has improved since the first described assay. Currently, some of the above mentioned nPCR are able to detect one *M. hyopneumoniae* cell per assay (Stärk et al., 1998, Verdin et al. 2000a, Kurth et al., 2002).

A great variety of samples have been used to detect *M.hyopneumoniae* DNA from infected pigs: nasal swabs (Mattson et al. 1995, Sorensen et

al., 1997, Calsamiglia et al., 1999a, 1999b, 2000b, Caron et al., 2000a, Ohlinger et al., 2000, Verdin et al., 2000a, 2000b, Batista et al., 2002, Bosch et al., 2000, Kurth et al., 2002, Ruiz et al., 2002a, 2002b, 2002c, 2002d, 2003, Vicca et al., 2002), bronchial swabs (Sorensen et al., 1997, Calsamiglia et al. 2000a, Caron et al., 2000a, Kurth et al., 2002), tracheal swabs (Kurth et al., 2000, Ruiz et al., 2002a) tonsillar swabs (Kurth et al., 2002), tracheobronchial washings (Mattson et al. 1995, Blanchard et al., 1996 Verdin et al., 2000a, 2000b) broncho-alveolar fluids (Baumesiter et al., 1998, Kurth et al., 2002) lung tissue homogenates (Mattson et al. 1995, Caron et al., 2000a, Rautiainen and Wallgren, 2001), lung tissue (Thongkumkoon et al., 2000, Hipple et al., 2002), and filtered air from infected barns (Stärk et al., 1998, Fano et al., 2003).

Most of the respiratory samples listed above have the disadvantage that they are taken once the animal has been sacrificed (bronchial swab, bronchoalveolar fluids and lung tissue) or they are difficult or laborious to take in live animals (tonsillar and tracheal swabs). On the contrary, nasal swabs can be obtained from live or dead pigs and is easy to sample. Although detection of *M.hyopneumoniae* from nasal swabs confers several advantages, several points need to be considered when its results are interpreted. The fact that the DNA detected in nasal swabs by nPCR may belong to viable and nonviable cells, arises the question whether these animals are infected or only eliminating dead bacteria (Calsamiglia et al., 1999a). On the other hand, colonization of ciliated epithelium has only been described in the lower respiratory tract cells (Mebus and Underdahl, 1977), and not in the epithelium of the nasal cavities. In fact, cultivation of *M.hyopneumoniae* from nasal swabs is extremely difficult, supporting the idea that levels of the pathogen in nose are very low (Mattson et al., 1995, Sorensen et al., 1997). Therefore, remains unclear if a positive nPCR result obtained from nasal samples reflects the

colonization of lower respiratory tract and consequent macroscopic and microscopic lung lesion development. In fact, Calsamiglia et al., (2000a) found that nPCR detection in bronchi (bronchial swab) corralated very well with histopathology lesions characteristic of *M. hyopneumoniae*.

Another point of interest regarding nasal swab detection is for how long the animal would be nPCR positive. Ruiz et al. (2002a), described a ihigh an intermittent shedding of *M.hyopneumoniae* along the disease course in experimentally infected pigs by SEM and nPCR. According to the author, shortly after infection, nasal swabs are positive due to few Mycoplasma cells attached to the cilia. These cells are presumably dead cells being cleared by the mucus escalator. Following this initial period, Mycoplasma cells are firmly attached, there is little mucus clearing, leading to a negative nPCR nasal swab. And finally a later proliferation of Mycoplasmas cells forming microcolonies, will probably result in the late positive nasal swab. In another study, Ruiz et al., 2002c, reported the nPCR detection in nasal swabs from 72 naturally infected pigs from two different groups, for a period of 7 weeks. A 55 and 65% of total populations were positive by nPCR during nursery period, being most of the pigs positive at one sampling time.

In order to test the most adequate sampling sites for *M. hyopneumoniae* detection, Kurth et al., (2002), tested different samples (nasal swabs, tracheobronchial brush, tracheobronchial swabs, tonsillar swab, and bronchoalveolar lavage fluid) from experimentally infected pigs. According to the author, the most reliable sites to detect *M.hyopneumoniae*, determined for experimentally infected pigs, were BAL fluid and tracheobronchial brushes. With these samples a nPCR is not required since there is sufficient load of organisms to be detected in a single PCR. On the contrary, if nasal swabs are sampled, a nPCR is needed. Moreover, failure in detection of *M.hyopneumoniae* in tonsillar

swabs was also described. It should be taken into account that the different samples tested in this work, came from different challenge experiments, and not from natural infection cases. This can be important when trying to elucidate the natural colonization sites and their significance in disease.

Although PCR techniques to diagnose *M.hyopneumoniae* infection described so far are very sensitive and very specific, a very important information is missing: the bacterial load present in a given sample. A novel quantitative PCR test for *M. hyopneumoniae* that uses a temperature cycler and an ELISA reader has been described (Minion, 2002b), and our group is currently developing a TaqMan PCR assay for *M.hyopneumoniae* quantification.

3.5 Antibody detection techniques: complement fixation test (CF), enzyme-linked immunosorbent assay (ELISA)

Serological test are among the most commonly used strategies for practitioners to evaluate health status of pig herds. Detection of antibodies against *M.hyopneumoniae* may be done by complement fixation test (CF) assay and the most frequently used, enzyme-linked immunosorbent assay (ELISA).

CF is based on complement consumption by antigen-antibody complexes present in serum samples. Although IgM and IgG are capable of fixing the complement, the former do it more efficiently. Therefore, higher CF titers suggest high IgM levels, corresponding to a recent exposure (Halbur, 1997). This fact may lead to false negative results in later stages of infection, while the microorganism can be still isolated in the lungs. (Roberts et al., 1970, Armstrong et al., 1983, Bereiter et al., 1990). Moreover, cross-reactions with *M. flocculare* and *M. hyorhinis* have been also described (Roberts et al., 1970). Therefore, due to

sensitivity and specificity problems, CF is not frequently used to test *M.hyopneumoniae* serologic response.

Nowadays, ELISA tests are the most widely used serologic techniques used to detect antibodies against *M.hyopneumoniae*. The main advantages of ELISA test versus other serological test are the following: a large number of samples can be tested automatically and concomitantly, all classes of immunoglobulins can be detected, gives quantitatively measurable results, is very sensitive and is not expensive (Calsamiglia et al., 1999).

The first developed ELISA test was an indirect ELISA with crude antigen coating the plastic wells (Ross, 1999). Use of the crude antigen solubilized with SDS lead to cross-reactions with *M. flocculare* and *M. hyorhinis*. Nicolet et al., (1980), showed that solubilizing the antigen with Tween 20 detergent enhanced the test specificity. However, cross-reaction with *M. flocculare* was still not eliminated. Finally, Bereiter et al.,(1990) established an OD cut-point to distinguish *M. flocculare* cross-reactivity from a *M.hyopneumoniae* specific antibody response.

Some years later, test specificity was determined by the detector antibody rather than the antigen. Feld et al. (1992), developed a blocking or competitive ELISA (c-ELISA) using a monoclonal antibody (MAb) against the 74kDa protein. Using MAb against a particular protein, the specificity was enhanced, even when incompletely purified antigen was used.

Blocking ELISA using MAb against the 40kDa membrane protein (Le Potier et al.,1994), 43kDa protein (Djorjevic et al., 1994) and LDH-ELISA. (Frey et al., 1994) have also been described.

Sensitivity and specificity of the different available ELISA tests have been compared in several studies (Le Potier et al., 1994, Levonen et al., 1999, Erlandson et al., 2002, Chitik et al., 2000, Strait et al., 2004).

Results from these studies are controversial and lead to confusing conclusions. According to Pijoan et al. (1994), while Tween-ELISA is very suitable for serum profiling because has a greater sensitivity and lower price, c-ELISA has a higher specificity and therefore is suitable for negative high-health herds monitoring.

A non-invasive method for antibody detection is using colostrum samples which may contain locally produced IgA and IgG and IgM transferred from serum. Several authors have described and approved the colostrum sampling, highlighting its utility for regular herd monitoring and in reducing cross-reactions with common inhabitants of the pigs' respiratory tract (Levonen et al., 1994 Rautiainen et al. 1998, 2000). The main drawback is that collection of colostrum should be done after farrowing and therefore constant monitoring of the sows is required (Stärk et al., 2000). If sampling is performed immediately after farrowing, positive ELISA test results are observed earlier and at higher frequency than in serum among naturally infected pigs (Yagihashi et al., 1993, Morris et al., 1994, Stärk et al., 2000, Leon et al., 2001).

4 Pathogenesis

4.1 Adherence and Ciliostasis

M.hyopneumoniae binds specifically to the sulfated glycolipid receptor on the cilia of swine respiratory epithelia (Zhang et al., 1994a). Three glycolipids were identified as adherence receptors, named La, Lb, LC, being the latter one the strongest binding site (Zhang et al., 1994a). This specific adherence has been confirmed by *in vivo* and *in vitro* studies (Livingston et al., 1972, Mebus and Underdahl, 1977, Zielinski et al., 1990a, Zielinski et al., 1990b, Debey et al., 1992, Blanchard et al., 1992, , Zielinski and Ross, 1993, Zhang et al., 1994b, Debey and Ross 1994,

Young et al., 2000). This pathogen does not colonize any other cell type of the upper respiratory tract (Mebus and Underdahl, 1977, Kobisch et al., 2000).

M. flocculare is able to adhere to epithelial cells, suggesting that receptors for this non-pathogenic Mycoplasma are also present, but are different from the receptors of *M.hyopneumoanie* (Young et al., 2000). Moreover, the different ability of the pathogenic versus non-pathogenic *M.hyopneumoniae* strains to adhere to cilia (i,e: Strain J), suggests that adherence to the cilia is associated with the induction of Mycoplasma pneumonia (Young et al., 2000). In fact, failure to induce disease with Strain J was first attributed to the decrease in pathogenecity after high-passages in vitro (Zielisnki et al., 1990a), but it was finally correlated with the low adherence activity of this strain (Zhang et al., 1995).

- The organism does not penetrate the airway epithelium. Early in the infection process, it forms micro colonies at the tip of the cilia, later on in the interciliary space and sometimes in contact with the microvilli (Jacques et al., 1992, Blanchard et al., 1992,). As the infection progresses, there is a reduction of ciliary activity, a gradual loss of cilia, microcolony formation and accumulation of *M.hyopneumoniae* in adjacent cells. Finally, a complete loss of cilia and destruction and exfoliation of epithelial cells is observed (Jacques et al., 1992, Debey and Ross, 1994). Limited information is available on the mechanism which triggers the damage of cilia or active inflammation in the host lung after the attachment of the pathogen to the epithelial cells. It has been hypothesized that *M. hyopneumoniae* may increase the intracellular free Ca2+ of ciliated epithelial cells, which would serve as an intracellular signal to induce loss of cilia (Debey et al., 1993, Zhang et al., 1994, Park et al., 2002). Park et al., 2002, showed that neither *M.hyopneumoniae* strain J or *M. flocculare* could induce the increase intracellular calcium

Besides the reduced ciliary activity there is a hypersecretion and altered glycoprotein production in goblet cells, leading to the presence of viscous exudate in the airways (DeBey et al., 1992).

In 1995, a 97-kDa surface protein was described as a *M.hyopneumoniae* adhesin by immunoblot assay (Zhang et al.1995). Nowadays, it is known that p97 has two repetitive sequences named RR1and RR2 (Hsu et al., 1997) and that RR1 participates directly in the adhesion process (Hsu and Minion 1998). In fact eight units of R1 repeat sequence are needed for adherence to the cilia (Minion et al, 2000).

The incomplete inhibition of adhesion to cilia by the p97 monoclonal antibody (Hsu and Minion, 1998, Chen te al., 1998), the variability in the number of repeats in different *M.hyopneumoniae* field strains (Wilton et al. 1998) and that strain's J p97 binding epitope is fully functional (Hsu and Minion 1998), support the hypothesis that other adhesins may exist. In fact, a 110kDa protein (p110) was described by Chen (1998) as another putative *M.hyopneumoniae* adhesin. The existence of several adhesin proteins would increase the specificity of attachement.

4.2 **Evasion and modulation of host immune response**

The slow and ineffective immune response, measured by low levels of antibodies in the respiratory tract (Minion et al., 2002a), can be considered as another pathogenic mechanism. *M. hyopneumoniae* modulates the host immune response inducing immunpathogenic changes:

- Antigenic variation is a pathogenic mechanism, described also in other Mycoplasma species such as *M. bovis and M, gallisepticum* (Minion et al., 2002a). These antigenic variation is mainly based on the spontaneously variation in size of the repetitive sequence of lipoproteins, present in high quantities in Mycoplasma membranes (Minion et al.,

2002a). In *M. hyopneumoniae*, antigenic variation of adhesins has also been suggested as pathogenic mechanism used to evade the porcine immune system (Zhang et al., 1995).

- Phagocytosis by alveolar macrophages is the principal means by which pathogens are cleared from the lung. Regulation of the phagocytic activity of pulmonary alveolar macrophages in *M.hyopneumoniae* infection has been observed. While infection with *M. hyopneumoniae* stimulated the alveolar macrophages functions, when infection was complicated with secondary pathogens (i.e *A. pleuropneumoniae*) suppression of the phagocytic activity response occur (Carusso and Ross, 1990). This potential suppression of the phagocytic response may represent a predisposition of the host to secondary bacterial infections. (Carusso and Ross, 1990)

- *M. hyopneumoniae* induces macrophages and monocytes to produce proinflamatory cytokines including TNF-α, IL-1-α and β, and, IL6 in BALT of experimentally infected pigs (Asai et al., 1993, Asai et al., 1994,). The fact that TNF-α and IL-1 are activation factors of T lymphocytes, would explain their association with the development of pneumonic lesions. Recently, an increase of IL-2, IL-4 and TNF-α and, in lesser extent, of IL-1 (-α and β) and IL-6 were detected by immunohistochemical labeling in BALT of naturally infected pigs, which showed intense lymphoid hyperplasia (Rodríguez et al., 2004). The mechanism by which *M. hyopneumoniae* activates this process is still unknown, but it has been suggested that lipoproteins may be involved. Generally, inflammation is a proper response of the host's immune system to fight against infection. But in *M.hyopneumoniae* infection, lung lesions and specially lymphoreticular hyperplasia of the BALT may be a consequence of the increased expression of these proinflamatory

citokines (Rodríguez et al., 2004). Moreover, this inflammation appears to be an important factor in the potentiation of PRRSV-induced pneumonia (Thacker et al., 1999, Thanawongnuwech et al., 2000).

- Implication of the immune system in mycoplasma penumonia development is evidenced by the aggregation of lymphocytes and monocytes into the lung tissue. Accumulation of lymphocytes in lung lesions is probably caused by a specific (Roberts et al., 1973) and non-specific (Messier and Ross, 1991) stimulation of mitogenesis. Messier and Ross, (1991) suggested that the mitogenic factor responsible for the non-specific stimulation of lymphocytes may be found in *M.hyopneumoniae* membranes. On the other hand, Kishima et al. (1985), reported the supressive effect of *M.hyopneumoniae* on phytohemagluinin-induced transformation of swine lymphocytes. Cytopathic factors might be involved in the supression of lymphocyte transformation (Kishima and Ross, 1985).
- Debey et al., (1993) reported higher concentrations of calcium in neutrophils incubated with pathogenic strains of *M.hyopneumoniae* than in those incubated with a non-pathogenic strains. Recently, it has been hypothesized that this increase of free calcium would be an intracellular signal to induce loss of cilia (Debey et al., 1993, Zhang et al., 1994, Park et al., 2002).

5 Epidemiology

5.1 Transmission

M.hyopneumoniae, like other respiratory pathogens, is primarily transmitted through respiratory exudates and secretions that are spread in large droplets through direct contact or coughing/sneezing in close proximity (Stevenson et al., 1998). Morris et al. (1995), reported that direct contact was the only significant variable associated with

seroconversion of infected pigs. This “nose-to-nose contact” transmission and the consequent recirculation of the microorganism in a herd can occur by three different ways:

1) Transmission of *M.hyopneumoniae* from sow-to-piglet (vertical transmission) (Ross, 1999). Presumably, low parity sows or gilts have lower immunity and excrete more microorganisms than other sows (Maes, 1996), although Calsamiglia et al. (2000b), obtained opposite results. According to Morris et al. (1995), pigs in direct contact with seropositive gilts, were seven times more likely to seroconvert than those in only indirect contact;

2) Horizontal transmission among pen mates, especially after piglets are pooled together;

3) Transmission from older pigs to younger pigs in finishing units with continuous flow production systems (Sheldrake et al., 1990, Ross et al., 1999). The critical period for *M.hyopneumoniae* transmission of seems to be around the beginning of the finishing period, when pigs have low concentrations of antibodies against this agent (Sheldrake et al., 1990, Clark et al., 1991, Leon et al., 2001).

Recently, different colonization patterns among pigs sired by different boars have been suggested (Ruiz et al., 2002a). These findings would introduce a possible genetic effect on *M.hyopneumoniae* susceptibility and transmission. However, the small sample size tested in this study, and the fact that the role of the sow was not studied makes further research on this topic necessary to reach a conclusion.

The main source of infection is believed to be the sub-clinically infected pigs (carrier pigs), although, airborne transmission has been also proposed (Desrosiers, 2004). Nowadays, airborne transmission is gaining consistency as a very important route of transmission (Goodwin, 1985; Stärk et a., 1992, Thomsen and Jorsal 1992, Morris et la., 1995, Stärk et

al., 1998, Leon et al., 2001, Desrosiers 2004). Airborne particles containing the microorganism are generated by animals sneezing and coughing and in lower concentrations exhaled in the normal breath (Stärk, 1999). Goodwin (1985) suggested that aerosol transmission between farms could occur. In fact, the author found that the risk of a herd becoming infected with *M.hyopneumoniae* was inversely related to the proximity of other pigs. As a consequence, the minimum distance between pig farms to avoid air-bone transmission was determined to be less than 3 Km (Goodwin et la., 1985). Moreover, Zuang et al., (2002) found that the risk of infection was strongly associated with pig density and distance to the neighboring farms. In Denmark, Thomsen proposed airborne transmission and spread through trade as the two major sources of *M.hyopneumoniae* infections in Mycoplasma-free SPF herds (Jorsal and Thomsen, 1988, Thomsen et al, 1992). The authors also stated that the risk of being infected was greater when there was proximity to large infected herds rather than to a small herd. Another finding that supports airborne transmission is the seasonal pattern of *M.hyopneumoniae* infections and reinfections. Jorsal and Thomsen (1988), found also that the wind had an impact on infections, referred to as windborne transmission. The risk of infections increased in larger herds, and average daily temperature, as well as rainfall, were negatively associated with *M. hyopneumoniae* infections.

Recently, Fano et al. (2002), reported the infection of negative pigs placed in a trailer 6 meters away from *M.hyopneumoniae* intratracheally inoculated pigs. Finally, Cardona et al. (2003), detected *M. hyopneumoniae*'s DNA, in air at 1, 75 and 150 meters away from where the microorganism were aerolized.

Apart from sow-to-pig, pig-to-pig and airborne transmission, indirect transmission through fomites has also been suggested (Goodwin et al.,

1985). Batista et al., (2002) investigated the role of personnel as mechanical vectors of transmission. In this study where standard hygiene protocols were used, *M.hyopneumoniae* was not transmitted to naive pigs by personnel who were in a weekly contact with infected pigs.

Although all these possibilities of *M.hyopneumoniae* transmission exist, it is proposed that its spread is slow and quite inefficient since infection among pen mates does not always take place (Morris et al., 1995, Stevenson et al., 1998, Torremorell et al., 2000).

5.2 Environmental and management factors:

Clinical signs, severity of lung lesions and economic impact of EP are influenced by other factors a part from *M. hyopneumoniae* infection. These factors may affect the presence of the pathogen or may affect the susceptibility of an individual animal and consequently the establishment of the infection. (Stärk et al., 2000).

Following are the main factors with an important effect on EP development:

- Purchase policy: purchase of pigs or gilts, number of pigs purchased and multisource purchase policy seem to be important risk factors. Fattening herds which buy large number of pig have the greatest risk of infection (Done et al., 1991, Thomsen et al., 1992, Maes, 1996, Stärk et al,. 2000).
- Production system: all-in/all-out strategies imply a routinely cleaning and disinfecting before entering new pigs in the barn. These systems ensure a more clean and stable environment and therefore a lower infectious pressure (Done et al., 1991, Klark et al., 1991a, Hurnik et al., 1994, Stärk et al,. 2000, Maes et al., 2000). Moreover, separation of production sites (2 or 3- site

systems) or medicated eraly weaning strategies decrease the chance of vertical transmission (Harris et al., 1988).

- Herd Size: increasing herd size, increases the risk of infection. (Thomsen et al., 1992)
- Management factors influencing airborne particles: stocking density, ventilation, air volume per pig, use of bedding, manure disposal and feeding system (Hurnik et al., 1994, Stärk et al., 2000).
- Climatic factors. In autumn and winter infections and reinfections of *M.hyopneumoniae* were are more likely to occur (Goodwin et la., 1985, Jorsal and Thomsen, 1988, Särk et al., 1992). This seasonal pattern is likely due to the more favorable conditions for survival of the microorganism. *M.hyopneumoniae* survives for at least 31 days in water at temperatures of 2 to 7º C (Goodwin et la., 1985). Although low temperature and high indoors humidity are associated with peak of pneumonia prevalence's, high humidity outdoors may help in reducing pneumonia indoors, by increasing the sedimentation of particles (Done et al., 1991).
- Other risk factors described are: dust, amonia, carbon dioxide and presence of other microbes (Donham, 1991). Amonia, reduces the ciliary activity and therefore enhances the attachment of respiratory pathogens.

Overall, paying attention to the environmental and management conditions cited above, EP prevalence at a herd level could be reduced (Yagihashi et al., 1993, Christensen et al., 1999). However, application of good management conditions does not imply absence of *M.hyopneumoniae* infection and vice versa. Vicca et al., (2002) compared the *M.hyopneumoniae* infection pattern in five subclinically infected farms with poor housing and management conditions with five clinically

infected farms with good housing and management conditions. In this study it was concluded that additional factors different from housing and management conditions, such as strain differences, may determine the infection pattern and clinical course of EP.

5.3 Herd Monitoring:

For many years, seroprofiles have been one of the most used strategies by practitioners to test health herd status. Seroprofiles provide information of levels and duration of maternal antibodies, time of seroconversion and the moment of seroconversion, but not on the time of infection. Frequently, time of infection in live animals has been deduced from time of seroconversion (Sheldrake 1990, Kobisch et al., 1993, Le Potier et al., 1994, Morris et al., 1995, Sitjar et al., 1996, Sorensen 1997, Leon et al, 2001). In viral or other bacterial systemic agents, seroconversion occurs in a more predictible time period after infection, and therfore the extrapolation seroconversion-moment of infection is accurate. Since seroconversion of *M. hyopneumoniae* in the field occurs 2 to 9 weeks after infection (Morris et al., 1995, Sitjar et al., 1996, Sorensen 1997, Leon et al, 2001), seroprofiles in combination with bacterioprofiles (by nPCR from nasal swabs) have been proposed and is widely used (Calsamiglia et la, 1999b, Calsamiglia et al, 2000b, Bosch et al., 2000, Ohlinger et al., 2000, Batista et al., 2002, Vicca et al, 2002)

Using this combination of techniques provides useful information on *M.hyopneumoniae* infections dynamics and prevalence in vaccinated and non vaccinated infected herds (Calsamiglia et al., 1999b), in clinically and subclinically infected herds (Vicca et al., 2002), and in herds dually co-infected with PRRSV (Bosch et al., 2000). Moreover transmission from sow to piglet (Calsamiglia et al., 2000b, Bosch et al., 2000, Ruiz et al., 2002a, Ruiz et al., 2003) and by personnel (Batista et

al., 2002) has been also studied. An important limitation of these studies are that they are transversal studies rather than longitudinal. In crossectional studies, the profile obtained does not reflect the infection dynamics in a group of animals, but represents a compositions of infection status of different age-groups at one point in time. To better understand infection, transmission and appearance of disease, longitudinal studies should be performed.

6 **Immunity**

Protective immunity is transferred from sow to piglets via colostrum (Wallgren et al., 1998). *M.hyopneumoniae* serum antibody levels in sows decrease from 4 weeks ante partum to partus, leading the sows more susceptible to be infected at the time of farrowing. *M. hyopneumoniae* antibody levels in colostrum are comparable to that in sow's serum 4 weeks ante partum (Wallgren et al., 1998, Rautianen and Wallgren, 2001).

Older parity sows are supposed to transmit less pathogen to their offspring than young parity sows (Goodwin et al., 1965), probably because older sows have been exposed longer time to the pathogen and have had time to develop immunity towards *M. hyopneumoniae*. However, controversial results have been published describing the immunity of older parity sows. Morris et al, 1994. found higher antibody concentrations in older sows, explaining the positive association between parity with duration of maternal antibodies; and Rautiainen et al., 2000, reported that older sows (parity >5) in endemically infected herds, were three times more likely to have antibodies to *M.hyopneumoniae* in their colostrums than younger sows. On the contrary, Calsamiglia et al. (2000b), found that older parity sows (third to seventh) had the potential to spread the microorganisms to their piglets (were nPCR positive in

nasal swabs), and also found an inverse relationship between parity and sow antibody titres. Finally, Rautiainen and Wallgren (2001) no association between the antibody level in colostrum and the age of the sows.

Humoral response to *M.hyopneumoniae* varies between sows and the antibody level of serum of the offspring is related to the antibody level of sow colostrum (Wallgren et la., 1998, Rautianen and Wallgren, 2001). If piglets receive sufficient quantity of antibodies to *M.hyopneumoniae* in colostrum, they appear to be protected from *M.hyopneumoniae* for up to 14 days of age (Rautiainen and Wallgren , 2001). In fact, the median half life of serum antibodies was established by Morris et al (1994) as 15.8 days. The persistence of these antibodies was related to the initial antibody concentration, persisting much longer if there was a high maternal antibody transfer to the piglet (Yagishahi et al., 1993, Leon et al., 2001).

The role of humoral and cell-mediated immune response against *M.hyopneumoniae* infection and the mechanism that confers protection against this infection are still under debate. While some authors have reported evidence that serum antibodies are involved in the partial protection against *M.hyopneumoniae* infection (Lam and Switzer 1971, Suter et al., 1985), others have reported the contrary: a lack of correlation of serum antibody titers with protection against experimental challenge (Djordejevic et al.,1997, Thacker et al., 2000b). An autoimmune response elicited by *M. hyopneumoniae* infection has been proposed to explain the correlation of antibodies and lung lesions (Baumgartner and Nicolet, 1984). Thacker et al., (1998) found that neither blood lymphocyte proliferative response nor serum antibody titers correlated with protection on an individual pig basis, but that group antibody titers were related to group percentage pneumonic tissue. Moreover, Suter et al., (1985)

observed a maximum antibody response during the establishment of the lesions and Messier et al., (1990) highlighted the coincidence on time of local antibody response, resolution of pneumonic lesions and decrease in number of microorganisms. Sarradell et al., 2003, determined the cellular populations present in lung and especially in BALT. Hyperplasia observed in EP field cases consisted of macrophages, dendritic cells, T and B lymphocytes, and IgG^+ and IgA^+ plasma cells, suggesting that local humoral immunity appears to play an important role in *M.hyopneumoniae* infection.

Because *M. hyopneumoniae* colonizes the respiratory epithelium in a non invasive way, induction of secretory IgA may play a role in the defense against the infection. In fact, Sheldrake et al., (1993) and Sarradell et al., (2003) supported the idea that protection is mainly based on cell- mediated immunity and local secretion of IgA. In another experimental challenge,protection was attributed to interferon-gamma-secreting T lymphocytes and antibodies present at the mucosal sites (Thacker et al., 2000b). On the contrary, an experimental challenge where T-cell response was supressed by thymectomy, a decreased severity of microscopic pneumonic lesions and an increase of *M. hyopneumoniae* dissemination in tissues was observed (Tajima et al., 1984).

The role of cytokines in the immune response against *M.hyopneumoniae*, seems also to be double. Firstly, expression of several cytokines (IL-1, IL-2, IL-4, IL-6, and TNFα) was higher in the BALT of infected tissues than in controls, indicating that these cytokines play an important role in lymphoreticular hyperplasia (Rodríguez et al., 2004). On the contrary, these cytokines, may contribute to the development of an adequate humoral and cellular immune response by activation of macrophages and lymphocyte (Rodríguez et al., 2004).

Since local humoral immune response seems to play an important role in protective immunity against *M.hyopneumoniae*, further research on specific *M.hyopneumoniae* IgA expression and production in naturally and infected pigs should be done.

7 Vaccination

Vaccination with commercial bacterins has become an important tool to control *M.hyopneumoniae* infections. These vaccines are usually inactivated preparations consisting on membrane or whole-cells adjuvant preparations (Thacker et al., 1997). It has been demonstrated that these vaccines do not prevent from colonization of the lungs but induce partial protection against development of gross lesions (Maes et al., 1999). The beneficial effect of vaccination include: improved daily gain, feed conversion ratio, shorter time to reach slaughter weight, reduced clinical signs, reduced of prevalence and severity of lung lesions and occasionally reduced mortality (Scheidt et al., 1994, Dohoo et al., 1996, Maes et al., 1999, Okada et al., 1999, Wallgren et al., 2000, Kyriakis et al., 2001, Pallarés et al., 2001, Dawson et al., 2002, Llopart et al., 2002, Moreau et al., 2004)

Although the exact mechanism of protection is not understood, it is suggested that both local mucosal humoral response and cell-mediated immune response play an important role (Thacker et al., 2000a). According to Thacker et al., (2000a) reduced pneumonia, elevated serum antibody level after challenge and elevated antibody in BAL fluid, provide evidence that a vaccine has induced an effective immune response. Okada et al., (2000a) suggested that vaccination contributes to a decreased TNF-α production, and therefore, inflammatory response in lung is diminished resulting in fewer lung lesions.

Different vaccination schedules have been applied depending on the type of herd, production systems and infection patterns. In farms were *M.hyopneumoniae* infections occur in the early production stages (e.g. continuous production systems), double vaccination of pigs in farrowing and in nursery units or one-shot vaccination at 3 weeks or older are usually administered. (Dohoo et al., 1996, Kyriakis et al., 2001, Dawson et al., 2002, Kuhn et al., 2004, Haesebrouck et al, 2004). Effect of two-shot versus one-shot schedules have been reported to be similar in several field trials (Reynaud et a., 1998) or better (Kyriakis et al., 2001, Ruiz et al, 2002c). In case of infection occurring during the finishing period (e.g. all-in/all-out or early weaning strategies), vaccination is advised later, at 10 weeks of age. (Wallgren et al., 2000, Haesebrouck et al, 2004). According to Maes et al., (2000b), single strategic vaccination is only useful when time of infection (seroconversion) is known and when pigs become infected some weeks after the start of the fattening period.

Cases of vaccine failure appear to be increasing in frequency. Different reasons have been proposed: improper injection techniques, antigenic differences between field strains and vaccine strains, presence of the disease at the moment of vaccination time, immunosupression due to stress, or co-infections at vaccination, short duration of immunity and interference of maternal antibodies in the immune response induced by the vaccine (Thacker et al., 2001b, Vicca et al., 2002).

It is known that there is passive transmission of antibodies from *M.hyopneumoniae* vaccinated sows to their offspring (Kobisch et al., 1987, Kristensen et al., 2004). According to Ruiz et al. (2003), vaccination of sows could be use to control *M.hyopneumoniae* infection, since the author showed that vaccinating sows reduced the prevalence of nPCR positive pigs at weaning.

Maternal antibody (MDA) reduced the severity of lesions induced by challenge, but they had no impact on the level of infection (Lam and Switzer 1971). Moreover, Thacker et al., (2000) reported that piglets with maternal immunity had less severe lung lesions after challenge than piglets from naïve sows. In an experimental challenge, a group of sows, from a *M.hyopneumoniae* free herd, were vaccinated prior to farrowing to induce high levels of MDA (Thacker et al., 2001). In that study, there was no difference in the level of protection provided by vaccination between pigs from vaccinated and unvaccinated sows. It has been suggested that titre of maternal antibodies at the time of piglet vaccination may play a more important role than the age of pigs (Jayappa et al., 2001, Hodgings et al. 2004). In fact, Hodgings et al (2004) described active immune response induced by vaccination in pigs as young as 2 weeks of age, in spite of moderate titres of maternal antibodies.

Protective effect of other vaccination strategies have been studied in experimental challenges. Lung lesion reduction was reported by vaccinating with an inactivated vaccine prepared from culture supernatant of *M.hyopneumoniae* (Okada et al., 2000a, 200b). In order to elicit a local humoral immune response, oral immunizations have also been tested, obtaining different degrees of protection (Fagan et al,. 2001, Lin et al., 2003). Also several recombinant vaccines have been constructed. Fagan et al, (1996) reported a reduction of lung lesions and higher average daily gain, by vaccinating pigs with a recombinant vaccines that included nrdF gene. Only minimal protection against lung lesions was provided by a Mhp1 protein fused to glutathione S-transferase (King et al., 1997). Chen et al., (2001) achieved a marked increase of IgG against a recombinant chimera containing the RR1 repeat region of the p97 adhesin and pseudomonas exotoxin. Afterwards, p97 was expressed in an attenuated *Erysipelothrix rhusiopathiae* YS-19 recombinant protein (Shimoji et al.,

2003). In latter study, although serum antibody production was not observed, a higher stimulation index of peripheral blood mononuclear cells in vaccinated pigs was achieved (Shimoji et al., 2003). Chen et al., 2003, reported the use of the heat shock protein antigen p42 as a DNA vaccine which was able to induce both TH1 and TH2 immune response. Finally, preliminary studies of a life vaccine using avirulent *M.hyopneumoniae* strains have also been described. Utrera et al., (2002), produced a temperature mutant of *M.hyopneumoniae* and evaluated its application as a live vaccine in susceptible pigs. Reduction in severity of pneumonic lesions and improved on ADG levels were observed in intranasal and intramuscular vaccinated pigs.

Differences in virulence within field strains may be involved in the efficiency of *M.hyopneumoniae* vaccines. From an economic point of view, further studies searching for antigenic and virulence differences between *M.hyopneumoniae* field strains should be carried out to design better vaccines against the disease.

AIMS

The main objective of this study was to better understand the dynamics of *M.. hyopneumoniae* infection by serumprofiles and bacterioprofiles at different respiratory sites and correlate them to macroscopic and microscopic lung lesions in naturally infected pigs. This main objective has been divided in three sub objectives:

1. To determine the involvement of *M.hyopneumoniae* in respiratory outbreaks in herds of pigs with serumprofiles and bacterium profiles; and to determine if the dynamics of *M.hyopneumoniae* infection differed between 3-sites versus 1-or-2 site production systems.
2. To determine whether there is a correlation between *M.hyopneumoniae* detection in two upper respiratory sites (nasal cavity and tonsil) and its correlation in bronchi, as well as the presence of microscopic lesions.
3. To study longitudinally *M.hyopneumoniae* infection in three respiratory sampling sites (nasal, tonsil and bronchi) in naturally infected pigs at different ages and correlate this detection with seroconversion, macroscopic and microscopic EP compatible lung lesions.

Chapter 1

Dynamics of *Mycoplasma hyopneumoniae* infection in 12 farms with different production systems

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Dynamics of *Mycoplasma hyopneumoniae* infection in 12 farms with different production systems

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Abstract

This study had 2 objectives: 1) to determine the involvement of *Mycoplasma hyopneumoniae* in respiratory outbreaks in herds of pigs, with the use of a nested polymerase chain reaction (nPCR) and an enzyme-linked immunosorbent assay (ELISA); and 2) to determine if the dynamics of *M. hyopneumoniae* infection differ between 3-site versus 1- or 2-site production systems (in which at least farrowing/gestation and nursery pigs are on the same site). Animals of different ages from 12 Spanish farms with respiratory problems were randomly sampled. Blood samples and nasal swabs were collected in a single farm visit, and ELISA and nPCR tests, respectively, were performed. All the farms demonstrated *M. hyopneumoniae*. According to the proportions of infected animals and the appearance of clinical signs in the different age groups, the farms were divided into 2 groups: farms in which *M. hyopneumoniae* probably played an important role in the observed respiratory outbreak and farms in which *M. hyopneumoniae* was not the main agent involved in the outbreak. Although seroconversion occurred in most herds in the finishing units, the number of seropositive pigs in the first group of farms was greater than the number in the second group. Statistically significant differences ($P < 0.0001$) between farms with a 1- or 2-site production system versus those with a 3-site production system were detected in nPCR results but not in rates of seroconversion. The farm effect also had a great influence on both controlled parameters: the pathogen's DNA and antibody detection. Thus, although *M. hyopneumoniae* was present in all the studied farms, there were significant differences in the infection dynamics and clinical implications according to the type of production system, and *M. hyopneumoniae* colonization and seroconversion were greatly influenced by the effect of the individual farm.

Résumé

(Traduit par Docteur Serge Messier)

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Introduction

Mycoplasma hyopneumoniae is the primary agent of enzootic pneumonia (EP) in pigs. This chronic worldwide disease causes important economic losses. The use of just 1 diagnostic technique [culture, serology, or polymerase chain reaction (PCR)] to implement prevention programs and to perform epidemiologic studies could be inappropriate owing to limitations inherent in each technique. A definitive diagnosis should involve the combination of some of these diagnostic tools (1). The combination of serology [enzyme-linked immunosorbent assay (ELISA)] and nested PCR (nPCR) in nasal swabs has provided new and useful information about *M. hyopneumoniae* infection and dynamics (1). The detection of microorganisms in nasal swabs provides accurate information about the time of infection (2,3), whereas serology indicates the time of seroconversion, which is variable in mycoplasma infection in swine (1).

Different studies have shown that the clinical outcome of *M. hyopneumoniae* infection is dependent on environmental and management factors. The latter include high replacement rates (4), multiple-origin nursery or fattening stock (5), continuous-flow practices (6), and high animal density, all these factors having a great impact on disease outcome (4). In contrast, all-in/all-out procedures in all production stages can reduce the prevalence and severity of EP (7). Modern swine production systems include 3 sites, for the 3 stages of production (farrowing/gestation, nursery, and fattening) (8), which implies a further step in breaking transmission between different age groups (9).

The objectives of this study were: 1) to determine the involvement of *Mycoplasma hyopneumoniae* in respiratory outbreaks in herds of pigs, with the use of nPCR and ELISA; and 2) to determine if the dynamics of *M. hyopneumoniae* infection differ between 3-site versus 1- or 2-site production systems (in which at least farrowing/gestation and nursery pigs are on the same site).

Materials and methods

Herds

Twelve Spanish herds with clinical respiratory symptoms (coughing) in the nursery or fattening stage, or both stages, were included in the study. Of the 12 farms, 7 had more than 2000 sows, 3 had 1000 to 2000 sows, and 2 had about 700 sows (Table I). Of the 12 farms, 5 were using *M. hyopneumoniae* vaccination (Stellamune, Pfizer), 8 were using a specific medication against the microorganism at the time of sampling, and 1 farm was not vaccinating or medicating.

Samples

Samples were taken from different age groups during a single visit, starting with suckling piglets and then sampling at different weekly intervals depending on the available groups in each farm. Sampled animals were selected randomly. The age range was 1 to 28 wk. Nasal swabs (Culturette; Becton Dickinson, Le Pont de Claix, France) were obtained by swabbing the mucosa of both nostrils, reaching deeply into the turbinates. Blood samples were collected from the jugular vein into tubes without anticoagulant (5-mL Venoject; Terumo Europe, Madrid, Spain). Samples were individually identified, refrigerated, and delivered by express mail to the laboratory, where they were processed.

DNA extraction

The nasal swabs were resuspended in 1000 μL of sterile phosphate-buffered saline (PBS) and vigorously vortexed; 400 μL of the suspension was used for DNA extraction. The samples were processed with a DNA extraction kit (Nucleospin Blood; Macherey-Nagel GmbH & Co KG, Düren, Germany), according to the manufacturer's instructions. To test for contamination during the extraction procedure, we included a negative control, using PBS as the extraction substrate, in each group of processed samples.

Polymerase chain reaction

The 4 stages of the PCR process (DNA extraction, mix preparation, DNA amplification, and electrophoresis) were conducted in 4 different rooms to minimize contamination. We performed nPCR using primers and conditions previously described (3). The specificity of this technique had previously been assessed with the use of DNA of different mycoplasmas and acholeplasmas, as well as other bacterial species found in the respiratory tract of swine, of which only *M. hyopneumoniae* was amplified (3). The nPCR has a sensitivity of 80 mycoplasmal cells (3).

Two sets of primers from the 16S ribosomal gene of *M. hyopneumoniae* were used. Briefly, 2.5 μL of the DNA preparation was used as PCR templates in the first reaction and 0.5 μL in the second reaction. Amplification was performed in a final volume of 25 μL. The reaction mixture consisted of 200 nM of each primer, 0.2 mM of each nucleotide (Amersham–Pharmacia Biotech, Spain), 1 × PCR buffer (Ecogen, Barcelona, Spain), 4 mM $MgCl_2$ (Ecogen), and 1 U of *Taq* DNA polymerase (Ecogen).

The 2 reactions were performed in a thermocycler (GeneAmp PCR system 9700; Applied Biosystems, Spain) under the same conditions: 30 cycles, denaturation at 94°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 30 s. In each run, we included with every 4 test samples a negative control consisting of millipore water, as well as a positive control in the last position (DNA extracted from a pure culture of *M. hyopneumoniae*). If 1 negative control was PCR-positive, all positive samples were tested again.

The amplified products (300 base pairs) were run in a 2% agarose gel with 0.05 mg/mL of ethidium bromide and then visualized and pictured (Bio-1d V.96; Vilber Lourmat, Marine la Vallee, France).

Enzyme linked immunosorbent assay

Blood samples were centrifuged at 760 × *g* for 10 min at 4°C; the serum was used for the ELISA technique. Antibodies against *M. hyopneumoniae* were assayed with a monoclonal blocking ELISA (DAKO *M. hyopneumoniae*, ELISA kit; DAKO A/S, Glostrup, Denmark). The sensitivity of the monoclonal antibody used (mAb17, 74 kDa) has been shown to be 10^3 CCU in the 20 *M. hyopneumoniae* strains tested (10). No reactions of the mAb 17 were observed with 10^5 CCU of *M. flocculare*, *M. hyorhinis*, *M. hyosynoviae*, or *M. dispar*.

The inhibition percentage (IP) was calculated considering the optical density (OD) of each sample as well as the negative control. Classification of individual animals on the basis of IP values was as

Table I. Main characteristics of the 12 farms tested for the presence of *Mycoplasma hyopneumoniae*

	Production system (number of sows)[1]		Vaccination against *M. hyopneumoniae*[2]		Medication against *M. hyopneumoniae*		
Farm number	1 or 2 sites	3 sites	Yes/No	Age (wk)	Yes/No	Stage	Other respiratory pathogens detected
1		4000	No		Yes	F	PRRSV
2		2400 + 1000	Yes	1–3	Yes	N, F	SIV
3		6000	No		Yes	N, F	PCV2
4		750	No		Yes	N, F	PRRSV, App, PCV2
5		2600	No		No		PRRSV, SIV, App
6		7000	Yes	1–3	No		PRRSV, PCV2, App, *P. multocida*
7		5250	Yes	1–3	No		PRRSV
8	1500		Yes	3–6	Yes		PRRSV, App
9	600		Yes	1–3	No		PRRSV, ADV, SIV
10	1500		No		Yes	F	App, *H. parasuis*
11	2400		No		Yes		PRRSV, PCV2, ADV
12	1064		No		Yes	N, F	PRRSV, ADV, App, *H. parasuis*, *P. multocida*

N — nursery; F — finishing; PRRSV — porcine reproductive and respiratory syndrome virus; SIV — swine influenza virus; PCV2 — porcine circovirus type 2; App — *Actinobacillus pleuropneumoniae*; *P.* — *Pasteurella*; ADV — Aujeszky's disease virus; *H.* — *Haemophilus*

follows: IP $<$ 30%, negative; IP $>$ 50%, positive; IP $>$ 30% and $<$ 50%, suspicious. The percentage was obtained from the following formula: % IP = (mean negative value OD − sample OD) ÷ (mean negative value OD).

On farms 4, 5, 7, and 9, where other respiratory pathogens were suspected to be present, we also performed serologic tests for antibodies against porcine respiratory and reproductive syndrome virus (PRRSV) (Porcine Respiratory and Reproductive Syndrome virus Antibody test kit; IDEXX Europe, BV, The Netherlands), Aujeszky's disease virus (Pseudorabies Virus gpI Antibody Test Kit; IDEXX Europe), swine influenza virus (SIV) (Civtest suis Influenza; Hipra, Spain), and *Actinobacillus pleuropneumoniae* (Civtest suis App.; Hipra).

Other techniques

Immunohistochemistry was also used to detect SIV antigen (on farm 2) and in situ hybridization to detect porcine circovirus type 2 (11) (on farms 3 and 5, where high mortality rates and poor performance in the finishing pigs were observed).

Statistical analysis

To study the relationships between variables, we performed a descriptive univariate test using analysis of variance and chi-squared tests. To analyze the influence of different variables on *M. hyopneumoniae* infection in 1-site or 2-site versus 3-site production systems, we used 2 multivariate linear mixed models, measuring infection by *M. hyopneumoniae* DNA detection in the first model and by antibody detection in the second model. The factors included in both models were age, production system (3-site versus 1- or 2-site), vaccination, medication, and farm (random effect). The farm effect included factors that were not controlled in the study, such as stocking densities, farm localization, genetics, weather, nutrition, and concomitant infections, among others. Vaccination and medication were nested within *farm*. All analyses were based on type III sums of squares.

To study the dynamics of *M. hyopneumoniae* infection in 1- or 2-site ($n = 5$) versus 3-site ($n = 7$) production systems, we tested the differences in least square means (LSMeans). To compare the prevalence of *M. hyopneumoniae* antibodies in the 12 tested farms, we performed a chi-squared test with the ELISA results. The level of significance was set to P values lower than 0.05.

The statistical analysis was performed with the use of the computer software (SAS System for Windows, version 8.0; SAS Institute Inc., Cary, North Carolina, USA).

Results

Respiratory outbreak by nPCR and serology results

A total of 704 nasal swabs and 706 serum samples were processed. Both techniques detected *M. hyopneumoniae* infection on all 12 farms, but at different percentages. Table II presents the percentages of positive pigs with each test in the 5 age groups. The 12 farms were divided into 2 groups according to the *M. hyopneumoniae* nPCR results and the appearance of clinical signs: A) farms in which the proportion of nPCR-positive pigs was zero or very low when coughing was observed and remained low ($<$ 30%) throughout the older groups (farms 2, 3, 4, 5, 7, and 12); and B) farms in which the proportion of nPCR-positive pigs was high when coughing was observed and increased in the older groups (farms 1, 6, 8, 9, 10, and 11).

In group A, the microorganism's genome was detected in a low proportion of the tested nursery pigs (0 to 15.8%) and in growers and finishers (0 to 30%). An exception was farm 7, where 62.5 % of weaning pigs (3 wk of age) were already colonized, although the proportion decreased and remained low (10% to 30%) in the nursery and fattening pigs. Further serologic analyses indicated concurrent infections with other major respiratory pathogens when respiratory signs were observed. An outbreak of postweaning multisystemic wasting syndrome was diagnosed in farm 3, starting in late-nursery pigs. In farm 5, antibodies to a low-virulence strain of *A. pleuropneumoniae* appeared at 15 wk of age. In farm 12, antibodies against PRRSV appeared at 10 wk of age.

Table II. Percentages of pigs positive for *M. hyopneumoniae* by nested polymerase chain reaction (nPCR) or enzyme-linked immunosorbent assay (ELISA) by farm and age

	% positive by nPCR; age (wk)					% positive by ELISA; age (wk)				
Farm no.	1–5	6–10	11–15	16–20	21–25+	1–5	6–10	11–15	16–20	21–25+
2	10 (0.0)	10 (0.0)	20 (5.0)	10 (0.0)	10 (30.0)	10 (10.0)	10 (60.0)	20 (35.0)	10 (10.0)	10 (15.0)
3	10 (0.0)	30 (0.0)	10 (0.0)	10 (20.0)		10 (20.0)	30 (0.0)	10 (0.0)	10 (35.0)	
4		10 (10.0)	30 (6.7)	10 (30.0)			10 (0.0)	30 (6.7)	10 (30.0)	9 (33.3)
5		19 (15.8)	10 (0.0)	10 (20.0)	10 (30.0)		19 (0.0)	10 (0.0)	10 (0.0)	10 (35.0)
7	16 (62.5)	20 (10.0)		20 (10.0)	10 (30.0)	16 (12.5)	20 (0.0)	10 (0.0)	10 (0.0)	10 (65.0)
12	10 (0.0)	20 (0.0)	10 (30.0)	20 (10.0)		10 (50.0)	18 (11.1)	8 (62.5)	18 (27.8)	
1	27 (0.0)	19 (0.0)	8 (12.5)	5 (40.0)	9 (55.6)	27 (40.7)	19 (10.5)	8 (12.5)	5 (80.0)	9 (66.7)
6		10 (10.0)	20 (5.0)	20 (20.0)	10 (70.0)		10 (20.0)	20 (10.0)	20 (25.0)	10 (100.0)
8	10 (20.0)	20 (45.0)	10 (50.0)	10 (20.0)	10 (60.0)	10 (0.0)	30 (0.0)	10 (10.0)	10 (20.0)	10 (30.0)
9		20 (15.0)	10 (10.0)	10 (40.0)	8 (37.5)		20 (15.0)	10 (20.0)	10 (30.0)	8 (87.5)
10		19 (15.8)	10 (50.0)	9 (66.7)	9 (77.8)		19 (42.1)	10 (20.0)	9 (33.3)	9 (33.3)
11	20 (20.0)	20 (10.0)	27 (29.6)	9 (55.6)		20 (0.0)	20 (0.0)	29 (0.0)	10 (30.0)	5 (60.0)

Note: Shading indicates occurrence of clinical signs (coughing)

In all the farms of group B, colonization occurred several weeks earlier than the appearance of clinical signs, and the proportion of nPCR-positive animals markedly increased when the clinical signs appeared.

Seroconversion occurred in all the finishing units. However, the proportions of seropositive pigs in group A (17.4%) and group B (23.4%) were significantly different [$P < 0.05$, odds ratio = 0.69 (95% confidence interval, 0.47 to 1.01)].

Results for farms with 1- or 2-site versus 3-site production systems

The univariate analysis showed that the age of the tested animals had a statistically significant effect on the nPCR and ELISA results (Table III). Medication had no significant effect on nPCR or ELISA results, but vaccination had a significant effect on nPCR detection of *M. hyopneumoniae* (Table IV).

The multivariate model showed that all the variables analyzed had a significant effect on nPCR detection of *M. hyopneumoniae* (Table V). The farm was the variable with the greatest effect, followed by the type of production system. Vaccinated and medicated pigs reared in 3-site production systems had the lowest probability of being nPCR-positive (5%) and vaccinated and medicated pigs reared in 1- or 2-site productions systems the greatest probability (59.52%).

On the other hand, detection of antibodies against *M. hyopneumoniae* was significantly influenced by only farm, medication, and age (Table V). As in the nPCR analyses, the probability of having antibodies against *M. hyopneumoniae* increased with age.

Infection with *M. hyopneumoniae* was first detected by nPCR at different ages and in different proportions among the tested farms. Although not statistically significant, piglets were already colonized in the nurseries in higher proportions in the 1- or 2-site farms (4 out of 5) than in the 3-site farms (4 out of 7). In addition, in farms with 1- or 2-site systems, the percentage of infected pigs increased progressively with age, whereas in farms with 3-site production systems, the percentage decreased with age in the nurseries and abruptly increased in the fattening pigs (Figure 1). However, the probability of detecting the pathogen's DNA in nasal swabs increased with age in both types of farm ($P < 0.0351$).

Seroconversion was observed rather late in the finishing period in both types of farm (Figure 2). In fact, no significant differences in

Table III. Total numbers and percentages of positive pigs in the different age groups

Test	No. (and %) positive; age (wk)					*P* value in univariate model
	1–5	6–10	11–15	16–20	21–25+	
nPCR	103 (15.53)	217 (11.06)	165 (16.36)	143 (23.78)	76 (48.68)	< 0.0001
ELISA	103 (20.39)	215 (10.70)	165 (13.33)	142 (23.24)	81 (56.79)	< 0.0001

Table IV. Total numbers and percentages of positive pigs among those medicated or vaccinated

Test	Medicated			Vaccinated		
	No	Yes	*P* value[a]	No	Yes	*P* value[a]
nPCR	223 (21.97)	481 (18.50)	0.2812	410 (16.83)	294 (23.47)	0.0292
ELISA	223 (21.52)	483 (20.08)	0.6594	412 (18.93)	294 (22.79)	0.2116

[a] In univariate model

Table V. Type 3 GEE analysis of nPCR and ELISA results

Variable	nPCR			ELISA		
	DF	Chi-squared	Pr > chi-squared	DF	Chi-squared	Pr > chi-squared
Age	1	4.44	0.0351	1	8.49	0.0036
Production system	1	13838.6	< 0.0001	1	0.22	0.6403
Vaccination	1	9.34	0.0022	1	1.67	0.1963
Medication	1	26.72	< 0.0001	1	4.85	0.0276
Farm effect	8	6.06e14	< 0.0001	8	7.18e12	< 0.0001

DF — degrees of freedom

time of seroconversion between 1- or 2-site systems and 3-site systems were found (Table V). However, in farms 1 and 3 (3-site systems) and farm 12 (1-site system), seroconversion occurred at the same time when the microorganism was first detected by nPCR.

Discussion

In this study, the presence of *M. hyopneumoniae* in 12 Spanish farms with respiratory problems was assessed. The results indicated the presence of the organism on all the farms, although with different infection dynamics, proportions of infected animals, and clinical implications.

In the farms in which the proportion of animals nPCR-positive for *M. hyopneumoniae* remained low or was absent in the age groups in which coughing was recorded, the pathogen was probably not playing a central role in the clinical respiratory problems observed. The other respiratory agents detected in the affected groups may have triggered the respiratory outbreak.

In the farms in which the proportions of infected pigs were high and increasing when clinical signs occurred, *M. hyopneumoniae* probably played an important role in the respiratory outbreak, although other agents were also concomitantly found.

As previously described (1,12), in most farms, colonization occurred several weeks before clinical signs appeared. It seems that a certain, unknown percentage of animals must be infected to elicit the onset of clinical signs (1). In farm 7, however, a high percentage of weaning piglets were infected without showing clinical signs. A possible explanation is the protective effect of maternal immunity (13), which is still present in 3-wk-old piglets, as indicated by the presence of antibodies. Another possible explanation is that the tested animals were still in the incubation period. Moreover, the proportion of colonized piglets decreased with age on this farm. The low stocking densities could explain the decrease in colonization observed in fattening pigs (4,14), since the fatteners sampled were selected replacement gilts.

Seroconversion occurred on all farms in the finishing stages, independent of mycoplasma involvement in the respiratory process, time of colonization, or production system. Therefore, in contrast to nPCR, the ELISA test performed was useful in determining the presence of *M. hyopneumoniae* but did not indicate if *M. hyopneumoniae* was involved in the clinical signs. These results support the idea that the time of seroconversion is highly variable; it may depend on infectious dose (15) and on the onset and severity of pneumonic lesions (16).

Although the microorganism circulated in all the farms, lower proportions of positive animals were detected than in a study conducted in the midwestern United States (1). The percentage of infected animals was lower even when *M. hyopneumoniae* seemed to play a central role in the respiratory process. Possible explanations for this finding include the wider use of antibiotics in feed in the Spanish herds tested compared with the US herds used in the previous study.

Significant differences were found between nPCR results from the 2 categories of production system studied. The fact that nursery colonization was greater in the farms with a 1- or 2-site system could support the idea that the use of multiple isolated sites decreases the

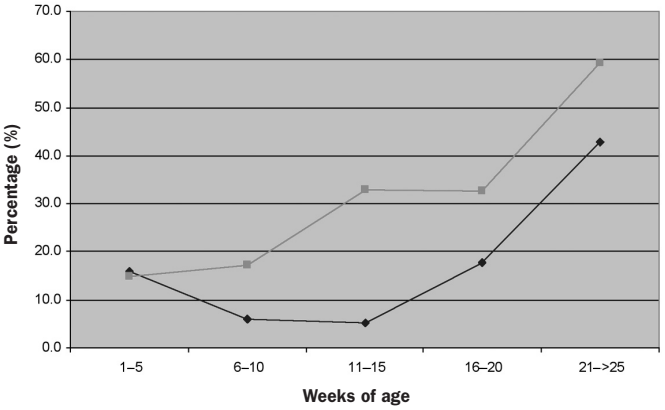

Figure 1: Mean percentage of animals positive for *Mycoplasma hyopneumoniae* infection by nested polymerase chain reaction in farms with a 1- or 2-site production system ( ) versus those with a 3-site ( ) system.

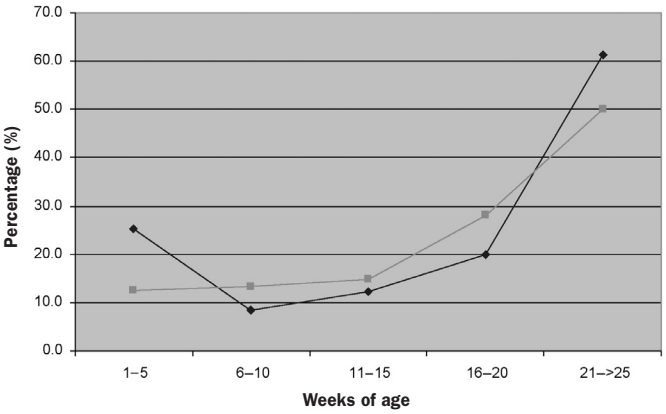

Figure 2: Mean percentage of positive animals by enzyme-linked immunosorbent assay in the same farms.

chance of vertical transmission of disease (8). The evolution of infection observed in the 1- or 2-site systems suggests a progressive transmission of the microorganism, whereas in the 3-site systems the appearance of the microorganism was more abrupt. This difference is probably a consequence of the interruption of horizontal transmission by the physical separation of the pigs in a 3-site system. The particular infection dynamics observed in the 3-site systems may, in part, explain the new presentation of respiratory outbreaks in modern swine production systems: acute and affecting late-finishing pigs (17).

Besides the observed difference in infection dynamics in the 2 systems, overall an animal reared in a 1- or 2-site production system had a greater probability of being colonized by *M. hyopneumoniae* than an animal reared in a 3-site production system.

Vaccination and medication strategies significantly lowered the percentage of infected animals as determined by nPCR. However, the number of farms with 1 or 2 sites versus 3 sites with and without treatment was insufficient to properly quantify these effects.

In conclusion, this study showed that 1) *M. hyopneumoniae* was present in all the studied farms, although with different infection dynamics and clinical implications; 2) the dynamics of *M. hyopneumoniae* infection differed significantly between the farms with a 1- or 2-site system and those with a 3-site system; and 3) *M. hyopneumoniae* colonization and seroconversion were greatly influenced by the effect of each individual farm.

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Chapter 2

Association between *Mycoplasma hyopneumoniae* at different respiratory sites and presence of histopathological lung lesions detection

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Association between *Mycoplasma hyopneumoniae* at different respiratory sites and presence of histopathological lung lesionsdetection

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Mycoplasma hyopneumoniae (*M.hyopneumoniae*) is the primary etiologic agent of swine enzootic pneumonia (EP), a chronic respiratory disease worldwide distributed. Culture of the microorganism is considered the gold standard technique to diagnose *M.hyopneumoniae* infection in pigs (Ross 1999, Desrosiers 2001). However, the fastidious and slow growth of the pathogen makes the implementation of this technique difficult in diagnostic laboratories. Therefore, EP diagnostics is usually based on macroscopic lesion observation in slaughterchecks and serology (Sitjar and others 1996, Desrosiers 2001). In the past few years, due to their specificity, sensitivity, and easiness, several polymerase chain reaction (PCR) techniques have been developed to detect *M. hyopneumoniae* DNA in live and dead pigs. Since the first descriptions of *M. hyopneumoniae* nested PCR (nPCR) (Stärk and others 1998; Calsamiglia and others 1999a), the technique has been used to detect this *M. hyopneumoniae* from air samples (Stärk and others 1998), nasal, tonsillar and bronchial sites (Mattson and others 1995, Blanchard and others 1996, Sørensen and others 1997, Calsamiglia and others 1999a, b, 2000a, b, Caron and others 2000, Verdin and others 2000a, b, Kurth and others 2002, Ruiz and others 2002, Vicca and others 2002).

Monitoring *M. hyopneumoniae* infection by nPCR from nasal swabs alone or together with serology has been described under field conditions (Calsamiglia and others 1999a, b, 2000a,b, Vicca and others 2002) or in experimentally challenged pigs (Calsamiglia and others 1999a, Kurth and others 2002).

However, it is still unknown whether detection of *M.hyopneumoniae* DNA in nasal cavities is indicative of the presence of the microorganism in bronchi (pathogen's main site of infection) or of the presence of EP characteristic lung lesions. Also, it is not known if nasal cavities are the most suitable detection site in the upper respiratory tract. Thus, the objective of the present study was to correlate *M. hyopneumoniae* detection by nPCR in two upper-respiratory sites (nasal cavity and tonsil), with its detection in bronchi as well as the presence of characteristic EP microscopic lesions. This information will enable better making recommendations for proper monitoring *M. hyopneumoniae* infection in live pigs.

In total, 195 pigs were included in this study. From these, 155 pigs ranging from 1 weeks to 3 months of age, showing different clinical signs (mainly, respiratory signs, growth retardation, diarrhoea and central nervous symptoms), were submitted for postmortem examination to the Pathology Diagnostic Service of the Veterinary School of Barcelona. The remaining 40 pigs were selected from a Spanish slaughterhouse, based on the presence of compatible EP macroscopic lung lesions. Nasal, tonsillar and bronchial swabs (Culturette TM, Becton Dickinson, Sparks, USA) and lung samples were collected from each animal. Nasal and tonsillar swabs were obtained during the necropsy or while the animals were hanging by the hind legs in the production chain at slaughterhouse. Nasal swabs were obtained by swabbing the nasal mucosa of both nostrils, reaching deeply into the turbinates, while tonsil swabs were obtained by swabbing the surface of the tonsil thoroughly but gently. Bronchial swabs and lung samples were obtained from the lungs already separated from the carcase, from pneumonic areas, when present, and from the cranial and middle lobes when not present.

Histopathological slides from lung samples (apical, middle and cranial portion of the diaphragmatic lobes) were examined using a light microscope and scored in a blind fashion. Histopathological lesions were graded from 0 to 4, following the previously described criteria (Livingston and others 1972, Calsamiglia and others 2000a), where 0 to 2 scores were classified as non-diagnostic EP microscopic lesions and 3 to 4 scores were considered compatible EP microscopic lesions.

DNA extraction and nPCR for *M. hyopneumoniae* amplification were performed as previously described (Calsamiglia and others 1999a).

Statistical analysis was performed using Chi-square tests (EpiInfo 6, Version 6.04b, World Health Organization. Genova, Switzerland). To analyse the relationship between nPCR results and lung lesions, animals were divided with regard to the presence of non-diagnostic (0-2 histopathological score) or diagnostic (3-4 histopathological scoring) EP microscopic lung lesions. Odds ratio (OR) and its confidence interval from the significant p-values were calculated. The kappa test (Analyse-It Software) was performed to measure the correlation between nPCR results at different respiratory sites and microscopic lung lesions

The findings of the nPCR and EP microscopic lesions are summarized in table 1. Overall, 91 out of 195 (46,6 %) pigs had at least one swab positive by nPCR, whereas only 49 pigs (25,1%) presented EP compatible microscopic lung lesions. From these 49 pigs (9 submitted to the veterinary school), 34 had a positive nPCR in the three tested sites, whereas 12 were nPCR positive in 1 or 2 locations (Table 2). The remaining 3 pigs were nPCR negative to all tested swabs. Of the 104 (53,3%) animals negative by nPCR in the three examined sites, 101 did not present EP compatible lung lesion. A statistically significant relationship ($p<0.05$) between the number of *M. hyopneumoniae* nPCR positive swabs and presence of EP compatible microscopic lung lesions was found.

When detection of *M. hyopneumoniae* genome in the three different respiratory sites was analysed, a significant relationship ($p<0.05$) and a substantial agreement between the nasal (K=0.76) and tonsil (K=0.70) with bronchial swabs were found. The chance of finding a positive bronchial sample was almost 58 times greater (OR=58.02, [22.04-158.69]) in an animal with a positive nasal swab than in an animal with a negative nPCR nasal swab, and 38 times (OR=37. 83, [15.19 -97.13]) greater when the tonsillar swab was positive. Both upper-respiratory sites (nasal and tonsillar swabs) were also significantly related ($p<0.05$, K=0.74, OR =56.42 [20.96-158.36]) between them. Separately, each detection site (tonsillar, nasal, or bronchial swabs) was significantly correlated ($p<0.05$) and showed a moderate association (k=0.44, k=0.55 and k=0.57, respectively) with presence of EP compatible microscopic lung lesions. The chance of finding these lesions in a pig was greater when the bronchial swab was nPCR positive (OR=31.35 [10.72-98.6]), followed by nPCR positive nasal swab (OR=24,54 [8.98-70.73]) and finally a nPCR positive tonsillar swab (OR=9.48 [4.26-21.44]).

The results obtained in this study indicated that detection of *M. hyopneumoniae* in nasal swabs is a good indicator of its presence in bronchi, the main site of action of the pathogen. Although both upper respiratory locations (nasal and tonsil) were substantially corralated with *M. hyopneumoniae* detection in bronchi, the microorganism was more readily detected in nasal (76 pigs) than in tonsillar samples (69 pigs). These results and the fact that nasal swabbing is easier than tonsil sampling, indicate that nasal swabs are the most suitable of the two upper respiratory sites tested for *M. hyopneumoniae* detection in live animals.

The results of our study are in disagreement with a recently published work (Kurth and others 2002) where nasal and tonsillar swabs were not reliable indicators of *M. hyopneumoniae* in an experimentally-

induced infection. However, in that study, where animals were intratracheally challenged, comparison of the three different detection sites was not carried out on the same animal. The sampling method, different route of infection, dose, conditions of exposure and management conditions of challenged pigs versus farm animals could explain the divergent results. More in agreement with our findings, Ruiz and others (2002) reported detection of the pathogen's DNA in high percentage of nasal and tracheal swabs up to 4 weeks post-inoculation in intratracheally-challenged pigs.

The correlation between *M. hyopneumoniae* nPCR detection in bronchi and presence of EP microscopic lung lesion has been already described (Calsamiglia and others 2000a). Surprisingly, detection of *M. hyopneumoniae* in both upper respiratory sites and the presence of EP characteristic lesions gave the same moderate correlation as that between *M.hyopneumoniae* in bronchial swabs and the presence of EP lesions. These results further support that detection of *M. hyopneumoniae* in upper respiratory sites could be used as an indicator of the presence of EP characteristic microscopic lung lesions in alive pigs.

In summary, this study showed that detection of *M. hyopneumoniae* in upper respiratory sites is in agreement with its detection in bronchi; the three respiratory sites, are good indicators of presence of EP microscopic lung lesions, although bronchial and nasal swabs are the best choices; andthe more nPCR positive respiratory sites, the higher association with presence of EP compatible microscopic lung lesions.

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Table 1: nPCR results in pigs for the detection of *M hyopneumoniae* in nasal, bronchial and tonsil swabs and lung lesion scores graded from 0 to 4, where 0 to 2 is calssified as non-diagnostic of enzootic pneumonia (EP) and 3 to 4 is considered compatible with EP microscopic lung lesions

nPCR Results			**Microscopic EP lung lesion**		
Nasal cavity	**Bronchi**	**Tonsil**	**Non-diagnostic (0-2 score)**	**Diagnostic (3-4 score)**	**Total (%)**
+	**-**	**-**	5	0	**5 (2.6)**
-	**+**	**-**	4	3	**7 (3.6)**
-	**-**	**+**	4	0	**4 (2.0)**
+	**+**	**-**	3	7	**10 (5.1)**
-	**+**	**+**	4	0	**4 (2.0)**
+	**-**	**+**	4	2	**6 (3.1)**
+	**+**	**+**	21	34	**55 (28.2)**
-	**-**	**-**	101	3	**104 (53.3)**
Total			**146**	**49**	**195 (100.0)**

Table 2: Number of positive swabs by nPCR correlated with non-diagnostic (0-2 histopathological score) or diagnostic (3-4 histopathological score) of microscopic EP compatible microscopic lung lesions in tested pigs.

	Microscopic EP compatible microscopic lung lesions					
Nº of positive swabs by nPCR	**Non-diagnostic (0-2 score) %**		**Diagnostic (3-4 score) %**		**Total (%)**	
0	101	97.1	3	2.9	104	53.3
1	13	81.3	3	18.7	16	8.2
2	11	55	9	45	20	10.2
3	21	38.2	34	61.8	55	28.2
Total	**146**	**74.9**	**49**	**25.1**	**195**	**100**

Chapter 3

Correlation between localization of *Mycoplasma hyopneumoniae* in respiratory airways with macroscopic and microscopic lung lesions in a longitudinal study

CORRELATION BETWEEN LOCALIZATION OF *Mycoplasma hyopneumoniae* IN RESPIRATORY AIRWAYS WITH MACROSCOPIC AND MICROSCOPIC LUNG LESIONS IN A LONGITUDINAL STUDY

M. Sibila, M. Calsamiglia, M. Nofrarías, S. López-Soria ,J. Segalés, P. Riera, D. Llopart, C. Artigas

INTRODUCTION

Mycoplasma hyopneumoniae (*M. hyopneumoniae)* is the etiologic agent of Enzootic Pneumonia (EP) of pigs and is also thought to play a central role in the porcine respiratory disease complex (PRDC) (Thacker et al., 2001a). Nowadays, *M.hyopneumoniae* infection dynamics in field trials is tested by serumprofiles with combination of bacterioprofiles (by nPCR from nasal swabs) (Calsamiglia et al., 1999b, Bosch et al., 2002, Ohlinger et al., 2002, Vicca et al., 2002, Sibila et al., 2004a). The main drawback of these studies is that they are transversal rather than longitudinal; in other words, the profiles obtained represent the infectious status of pigs of different ages and different batches, but they do not reflect the evolution of infection through the life time of a group of pigs.

M.hyopneumoniae bacterioprofiles have been performed with the amplification of the agent's DNA directly from nasal swabs, providing information on the timing of nasal colonization by the pathogen. Although this information is very useful, care must be taken in the proper interpretation of the results (Calsamiglia et al., 1999a). Since the PCR technique allows detection of viable and nonviable Mycoplasma cells, it is not known if positive results in nasal cavities indicate a true infection and/or presence of EP macroscopic and microscopic compatible lesions

(Calsamiglia et al., 2000). This question has been addressed in a few studies. Firstly, a good correlation between detection of the pathogen's DNA in bronchial swabs and presence of characteristic EP microscopic lung lesions was observed (Calsamiglia et al., 2000); moreover, in a recent study, three respiratory sites (nasal cavity, tonsil and bronchi), were shown to be correlated with the presence of EP-compatible microscopic lung lesions, being bronchi and nasal cavity the best choices (Sibila et al., 2004b). However, both studies (Calsamiglia et al., 2000, Sibila et al., 2004b) were performed using pigs received in veterinary diagnostic laboratories, from different farms, ages and health status. No information on the chronology of infection in the different respiratory sites and its correlation with the onset of lung macroscopic and microscopic lesions at the herd level have been generated.

The objectives of the present work were to study longitudinally the detection of *M. hyopneumoniae* at different respiratory tract levels and their correlation with seroconversion, macroscopic and microscopic EP characteristic lesions.

MATERIALS AND METHODS

Farm and assessment of *M.hyopneumoniae*-related disease

A one-site 240-sow, farrow-to-finish herd with continuous flow production system was selected to perform this study. The farm had respiratory disease in the fattening stage. To confirm *M. hyopneumoniae*–related disease, 34 animals were analysed at slaughter, by taking nasal swabs, blood samples and gross lung evaluation. A 100% and 64% of the tested pigs were positive by nPCR and serology, respectively. Mean macroscopic lung score was 9,16 based on the classification by Hannan et

al., 1982. The results of these analyses confirmed the presence of pneumonia problems associated with by *M.hyopneumoniae* in the farm.

Animals and Samples

One hundred and ninety five pigs from three farrowing batches were included in this study. Two groups were born in September 2002 (A, n=58 pigs; B, n=77 pigs) and the third one in April 2003 (C, n=60 pigs). Piglets included in the study were ear-tagged. From 3 weeks onward, necropsies of randomly selected pigs were performed. A mean of six pigs were necropsied at 3, 6, 9, and 12 weeks of age, and a mean of nine pigs were necropsied at 15, 18 and 22 weeks of age. The remaining pigs were slaughtered at 25 –28 weeks of age.

Pigs between 3 and 22 weeks were euthanized with an intravenous overdose of sodium pentobarbital. Blood samples (5ml Venoject, Terumo Europe, Madrid, Spain) from the jugular vein were taken immediately after. Afterwards pigs were necropsied and lung samples were taken, as well as nasal, tonsillar and bronchial swabs (Culturette TM, Becton Dickinson, Sparks, USA).

EP compatible macroscopic lung lesions were registered on a lungs' diagram with a maximum score of 35 points (Hannan et al., 1982). Once the lung lesions were recorded, bronchial swabs were taken, and lung sample from pneumonic areas, if present, and from the cranial and middle lobes when not present were fixed by immersion in buffered formalin.

Swab samples and blood were individually identified, refrigerated and transported to the laboratory, where they were processed immediately thereafter.

ELISA Test

Blood samples were centrifuged at 760 g during 10 minutes at 4ºC and the obtained sera were tested by a monoclonal blocking ELISA (CIVTEST SUIS MYCOPLASMA HYOPNEUMONIAE, Laboratorios HIPRA. Girona, Spain).

The inhibition percentage (IP) was calculated taking into account the optical densities (OD) of each sample as well as the negative control. Classification of individual animals, based on IP values, was as follows: IP< 30%, negative; IP> 50%, positive; IP >30% and < 50%, doubtful. This percentage was obtained from the following formula:

$$\% \text{ IP} = \frac{\text{Mean Negative value OD- sample OD}}{\text{Mean Negative value OD}}$$

DNA Extraction

Swabs were resuspended in 1ml of sterile PBS and vigorously vortexed. Four hundred microliters of the suspension were used for DNA extraction. DNA was extracted using a commercial kit (Nucleospin® Blood, Macherey-Nagel GmbH & Co KG Düren, Germany) and the samples were processed according to the manufacturer's instructions. In order to test for contaminations during the extraction procedure, a negative control was included using PBS as extraction substrate in each group of processed samples.

nPCR

Each stage of the PCR process (DNA extractions, mix preparations, DNA amplifications and electrophoresis) was carried out in four different rooms to minimize contaminations. A nested polymerase chain reaction (nPCR) was performed using primers and conditions previously described (Calsamiglia et al., 1999a). Each PCR reaction contained several negative

controls, consisting of millipore water, included every four test samples, as well as a positive control in the last position (DNA extracted from a pure culture of *M. hyopneumoniae*). If one negative control was PCR positive, all positive samples were repeated. The amplified products (300 bp) were run in a 2% agarose gel with 0.05 mg/ml of ethidium bromide, and then visualized and pictured (Bio-1d V.96, Vilber Lourmat, France).

Macroscopic lung lesion score

The extent of lung lesions observed at post mortem examination was drawn onto a lung's diagram (Hannan et al., 1982). Each lobe was arbitrary allotted a maximum possible score of 5. The pneumonic area of each lobe was then assessed and expressed as a fraction of 5 to give the pneumonic score per lobe. The maximum total score possible per lung was 35.

Microscopic lung lesion score

Histopathologic slides from lung samples (apical, middle and cranial portion of the diaphragmatic lobes) were examined using a light microscope and scored in a blind fashion. Histopathologic lesions were graded from 0 to 4, following previously described criteria (Calsamiglia et al., 2000). Absence of lesions was scored 0. Lesions characterized by inflammation different from categories 3 and 4 (such as interstitial pneumonia or purulent bronchopneumonia) were scored as 1 . Light to moderate cellular infiltrates (macrophages and lymphocytes) with mild cellular exudates into the airways were graded 2. Scores 1 and 2 were considered non-diagnostic *of M. hyopneumoniae.* Lesions with perivascular and peribronchiolar lymphoplasmacytic hyperplasia, pneumocytic type II hyperplasia and vacuolation, edema fluid in the alveolar spaces with neutrophils, macrophages and plasma cells were

scored 3. Lesions with the characteristics of grade three, together with peribronchial and perivascular lymphoid follicles were scored 4. Scores 3 and 4 were considered highly compatible with lesions of *M. hyopneumoniae* infection.

Statistical analysis.

Chi-square analysis was used to correlate detection of *M.hyopneumoniae* in the three respiratory sites among them as well as with macroscopic and microscopic score lung lesions. To analyse the relationship between nPCR results and lung lesions, animals were divided regarding presence of non-diagnostic (0-2 Histopathologic score) or diagnostic (3-4 Histopathologic scoring) EP microscopic lung lesions. Odds ratio (OR) and its confidence interval from the significant p-values were given. The kappa test was performed to measure the agreement between nPCR results and microscopic lung lesions from different respiratory sites. Statistical analyses were performed with SAS system for windows version 8.0 (SAS Institute Inc, Cary, North Carolina, USA).

RESULTS

Overall, *M.hyopneumoniae* was detected in greater percentages in bronchial swabs (52%), than tonsillar (31%) and nasal (22%) swabs. A significant relationship ($p<0.001$) but a fair concordance between tonsillar and nasal swab nPCR results (K=0.335, OR= 5.48 [2.50-12.13]) and bronchial and nasal swab results (K=0.246, OR=12.23 [5,08-30,47]) were observed. On the other hand, tonsillar and bronchial swab nPCR results were significantly related and had a moderate agreement ($p<0.001$,K= 0.439) between them.

Moreover, bronchial swab PCR results were significantly related ($p<0.001$) and had a moderate agreement (K= 0.416, OR= 6.88 (3.39-

14.10)) with microscopic scores 3-4 (microscopic lesions compatible with EP). On the contrary, tonsillar and nasal swabs results were in slight agreement (K=0.197, OR= 3.33 (1.55-7.28) p=0.006, and K=0.212 (0.92-5.00) p=0.55, respectively) with presence of EP compatible microscopic lung lesions.

When the number of nPCR positive respiratory sites in the same animal was analysed, some significant relationships were observed. The number of positive nPCR swabs (one, two or three of them) was greater in older age groups ($p<0.001$). Also, as the number of nPCR positive swabs increased, more pigs showed microscopic lesions compatible with EP ($p<0.001$) (table 1). Hence, percentage of pigs with microscopic lung lesions compatible with EP also increased with the age ($p<0.001$). Mean gross lung lesions were significantly lower when any of the tested swabs were positive ($p<0.001$).

Statistically significant differences ($p<0.001$, OR=7,56 (2,84-20,85)) were also observed when the number of pigs showing macroscopic and microscopic lesions were compared. There were 50 (25,6%) pigs that did not show any macroscopic lesions, although microscopic lesion with score 1-4 were observed. From these 50 pigs, 31 (62%) had a microscopic 3-4 score.

When the number of positive swabs were analysed by group, it was observed that group C had statistically significant ($p<0.005$) greater number of pigs with two or three respiratory sites nPCR positive than group A and group B (Table 1).

Different dynamics of infection at different respiratory levels were observed in the three groups of animals tested. In group A, *M. hyopneumoniae* infection was first detected at 15 weeks of age, being the detection rate in bronchial swabs always in higher percentages than in other respiratory sites tested (Fig 1). While the rate of *M. hyopneumoniae*

tonsillar detection increased over time, percentages of pigs with nPCR positive nasal swabs were intermittent. Seroconversion occurred at 15 weeks of age coinciding with the first detection of the pathogen by nPCR. On the contrary, gross and EP-compatible microscopic lesions (scores 3-4) appeared at 12 and 9 weeks earlier, respectively, than pathogen's detection (Figs. 4 and 5).

In group B, *M.hyopneumoniae* DNA was detected in a tonsillar swab from a 3-week-old pig (out of 9) (Fig 2). At 15 weeks seroconversion was detected, which coincided with the detection of *M.hyopneumoniae* DNA in the three respiratory sites and with the increase of the mean of macroscopic lung lesions. The number of positive nPCR results increased over time, being almost always greater in higher percentages in bronchial swabs. As in group A, percentages of detection in nasal swabs were very variable from one week to the following sampling. Macroscopic lesions appeared at 15 weeks of age reaching a maximum at 18 and 22 weeks of age (Fig 4). EP-compatible microscopic lesions appeared at 6 weeks of age and increased until 12 weeks of age (Fig 5). At 15 weeks of age, although a 66,6% and 16,6% of bronchial and tonsillar swabs were PCR positive, none of the tested pigs showed EP-compatible microscopic lesion (scored 3-4) . At 18 weeks of age, microscopic lesion scores 3-4 reappeared with increasing percentages of affected pigs until slaughter age.

In group C, overall percentages of *M. hyopneumoaniae* detection by nPCR throughout time were greater than in groups A and B (Fig 3). *M.hyopneumoniae* was detected initially observed in a bronchial swab at 3 weeks of age. In the following samplings (6 weeks of age), the pathogen was also detected in tonsillar swabs. From 12 weeks onwards, nasal swabs became positive, being always in lower percentages than tonsillar and bronchial swabs. In this group of pigs, seroconversion

occurred at 9 weeks of age. Macroscopic lung lesions appeared at 3 weeks of age and were discontinuously found in the successive sampling times weeks, peaking at 15 weeks of age. EP-compatible microscopic lung lesions appeared at 6 weeks of age and in increasing percentages thereafter.

DISCUSSION

Overall, *M.hyopneumoniae* was detected in greater percentages in bronchi, followed by tonsil and finally in nasal cavity in all three groups. As in previous studies, bronchial swabs were better predictors of EP-compatible microscopic lung lesions than swabs from upper respiratory sites (Calsamiglia et al., 2000, Sibila et al., 2004b). Moreover, a better agreement between *M.hyopneumoniae* detection in bronchi and tonsils than in bronchi and nasal cavities was found. Therefore, in the animals tested in this study, tonsils seemed the best tested upper respiratory site to predict *M. hyopneumoniae* infection at the bronchial level. These results are in partial disagreement with the ones obtained in an experimental challenge, where detection of *M.hyopneumoniae* in different sampling sites was compared (Kurth et al. 2002). In this latter study, nPCR detection in nasal and tonsillar swabs was very low (15.3% and 0%, respectively) compared to the detection in bronchial swabs (83.3%). It should be taken into account, that samples tested in that study came from different animals and that these pigs were intratracheally inoculated. The route of inoculation, differences in nPCR conditions and sensitivity, number of tested animals and origin of the samples, may contribute to explain the variable rate of low detection observed in upper respiratory sites among different studies.

The results obtained in the present work, are in disagreement with a recent published study (Sibila et al., 2004b), where 195 pigs received at a

Pathological diagnostic laboratory were used. In that study, nasal swabs were better correlated with nPCR *M.hyopneumoniae* detection in bronchial swabs than tonsillar swabs. Hence, the relative proportion of nasal detection was lower in this field study than in the study using pigs received at the diagnostic laboratory. A possible explanation for these findings could be the stress due to transportation of the animals to the laboratory prior to necropsy. An increase of *M.hyopneumoniae* shedding in nasal cavities due to stressfull situations has been proposed (Ruiz et al., 2003). This hypothesis is also supported by challenge studies, where clinical disease and pathogen loads were potentiated in dexamethasone coinoculated pigs versus single inoculated pigs with the pathogen (Vecht et al., 1989; Narita et al., 1985). On the other hand, a significant proportion of pigs received at the diagnostic service were already dead. The change in microbial distribution some hours after the animal's death could also partially explain the observed differences. A wider distribution of bacteria after the animal's death has been described for *Streptococcus suis* (Rovira, personal communication). On the contrary, most of the samples tested in the present study, were taken at the farm. Only the samples at slaughter were from pigs that suffered stress from transportation. It remains unexplained why only the relative proportion of nasal swabs was different in both studies, whereas the correlation between tonsillar and bronchial swabs remained similar.

Another interesting finding related with nasal swab detection was that percentages varied from one week to the following sampling. This intermittent detection has been already described in a field trial (Ruiz et al., 2002a) and vaccination challenge trial (Ruiz et al., 2002b). According to the authors, shedding of *M. hyopneumoniae* in nasal swab was intermittent, depending on the stage of infection at the time of sampling.

On the other hand, bronchi had, as expected, the best correlation with microscopic lung lesions compatible with EP, coinciding with previously reported results (Calsamiglia et al., 2000, Sibila et al., 2004b). On the contrary, a slight correlation between both nasal and tonsillar swabs and EP characteristic microscopic lesions was observed. All these results reflect that the main site of multiplication and action of *M.hyopneumoniae* is the bronchi. However, the higher the number of nPCR positive swabs, the higher the probability of having EP-compatible lesions..

Relationship between macroscopic and microscopic lung lesions was difficult to assess. While the macroscopic lesion score reflect the extent of lesion, the microscopic score measures the severity and specificity of the sampled lung fragment, which included a portion of lesion, if grossly observed. There were some lung samples that showed EP-compatible histopathologic lesions without any macroscopic lesions. In those cases, microscopic lesions were probably too localized and of minimal extension that they were not seen macroscopically.

Results obtained from bacteriumprofiles, serumprofiles and lung lesion analyses, suggest that different dynamics of infection among groups of the same farm can be seen. *M. hyopneumoniae* detection at the different respiratory levels suggested that groups A and B suffered from *M. hyopneumoniae* infection at mid-late finishing periods. Moreover presence of EP-compatible microscopic lesions, prior to the detection of the microorganism in any of the tested sites (groups A and B), suggest that these lesions might be caused by other pathogens different from *M. hyopneumoniae*. In fact, gross histopathologic lesions caused by Swine influenza virus (SIV) infections are very difficult to distinguish from *M. hyopneumoniae* induced lesions (Thacker et al., 2001b). Moreover, seroconversion against SIV was detected in slaughtered pigs of these

groups (data not shown), indicating that this virus had circulated among the pigs during their production period.

In these two groups of pigs (A and B), seroconversion coincided with a sudden increase of *M. hyopneumoniae* detection in bronchus, which was associated with an increased mean macroscopic lung lesion score and with highest percentage of lungs with EP- compatible microscopic lesions. These results would support two previously suggested hypotheses: 1) a critical mass of infected pigs should be necessary to elicit clinical signs and seroconversion (Calsamiglia et al., 1999b) and 2) severity of infection may affect magnitude and time of seroconversion (Sitjar et al., 1996).

In group C, early colonization (3-6 weeks of age) in tonsil and bronchi was observed. This early detection coincided with appearance of microscopic lesions (6 weeks of age) but not with an increase of mean macroscopic lung score nor seroconversion. As in groups A and B, a clear seroconversion occurred when a high proportion of pigs was infected. Only in that stage (where high percentages of bronchi were positive, and microscopic and macroscopic lesions were observed), the microorganism was detected in nasal swabs.

In conclusion, the chronology of *M. hyopneumoniae* infection at different levels of the respiratory tract in the three groups of naturally infected pigs has been described. *M. hyopneumoniae* was detected in greater percentages in bronchi followed by tonsil and finally in nasal cavities. In this study, *M.hyopneumoniae* detection in tonsil was better correlated with its presence in bronchi than in nasal cavity. Nasal swab detection of *M.hyopneumoaniae* might be useful to detect severe infections, when macroscopic lesions are already present. Moreover, this study has shown that differences in dynamics of infection within the same farm may exist.

Therefore, the assumption that dynamics of infection is relatively static in each farm, is probably not too acurate. Variability of infection dynamics within groups of the same farm, difficults the prediction of dynamics of infection in the following groups and therefore the implemention of medication/vaccination strategies.

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Table 1: Number of nPCR positive swabs in the three tested groups

Nº of nPCR Positive swabs	GROUP			TOTAL
	A	B	C	
0	31	31	18	80
1	14	26	10	50
2	9	14	18	41
3	4	6	14	24
Total	58	77	60	195

Figure 1: Percentage of seropositive pigs (line) and nPCR positive nasal (white), tonsillar (black), and bronchial (grey) swabs by ages in group A.

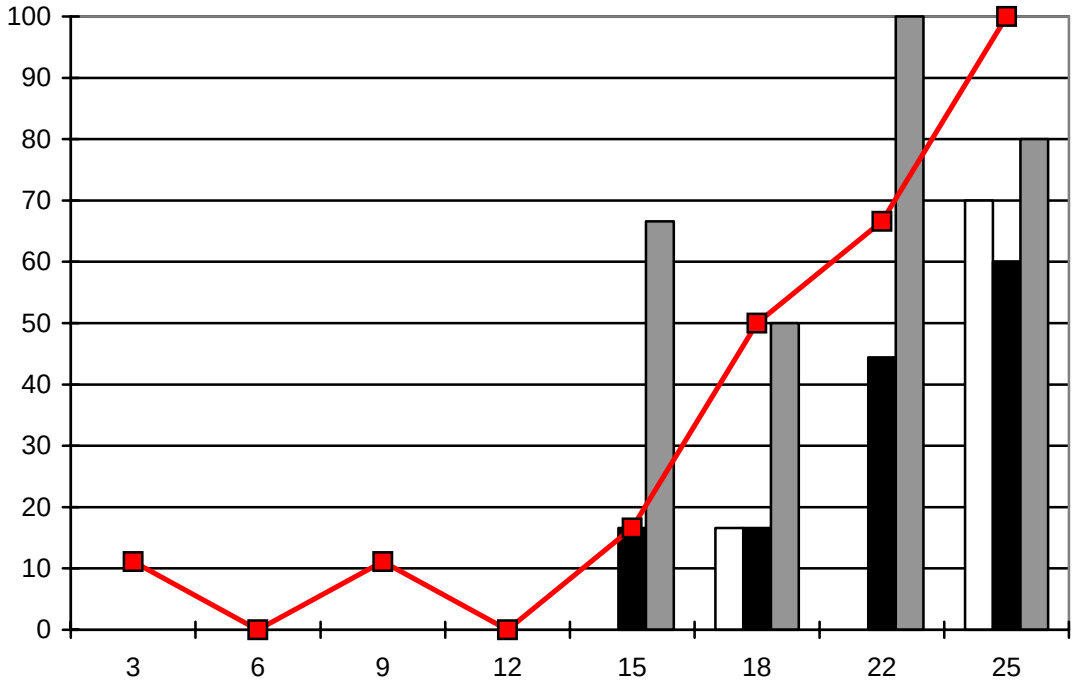


Figure 2: Percentage of seropositive pigs (line) and nPCR positive nasal (white), tonsillar (black) and, bronchial (grey) swabs by age in group B.

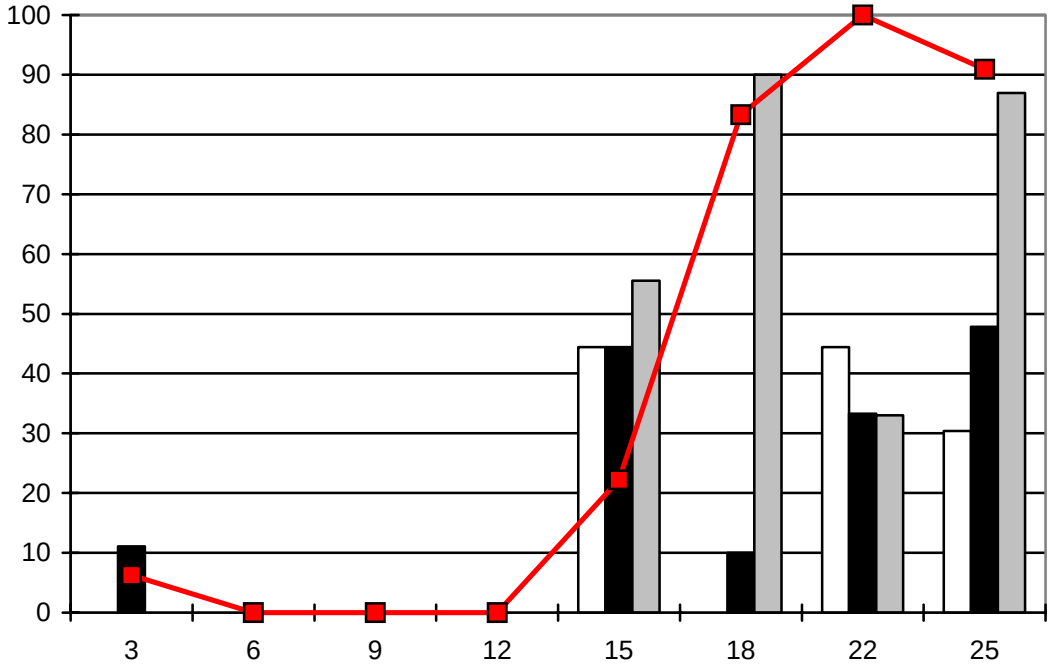


Figure 3: Percentage of seropositive pigs (line) and nPCR positive nasal (white), tonsillar (black), and bronchial (grey) swabs by age in group C.

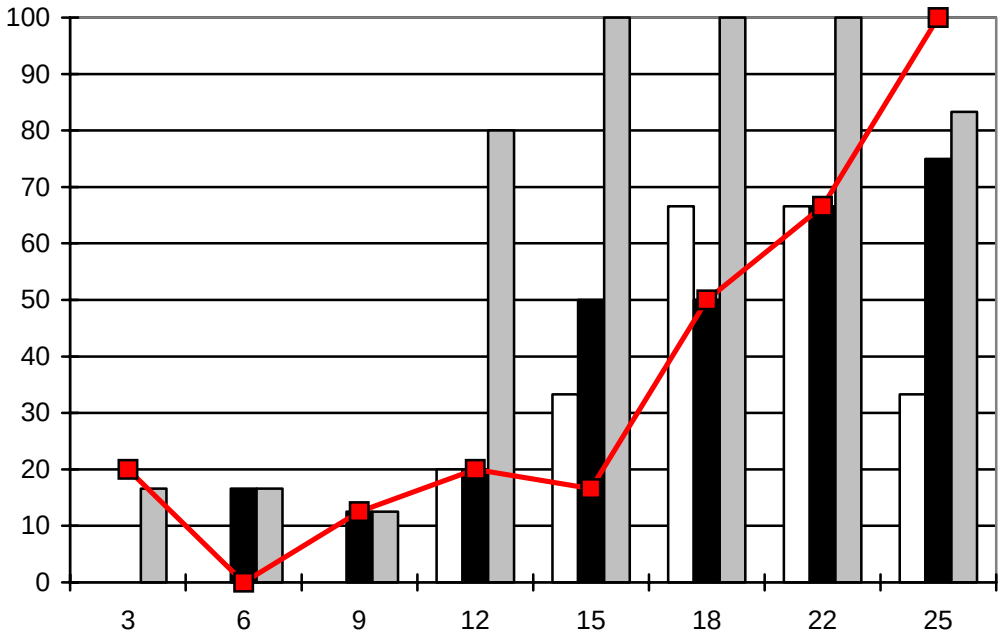


Figure 4: Mean macrosopic lung lesions score in group A (black), group B (white) and group C (grey) by age

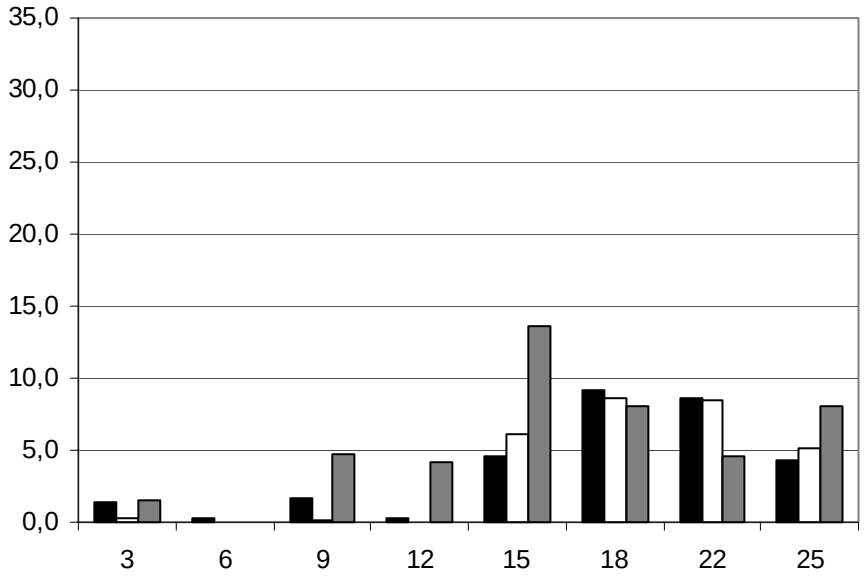
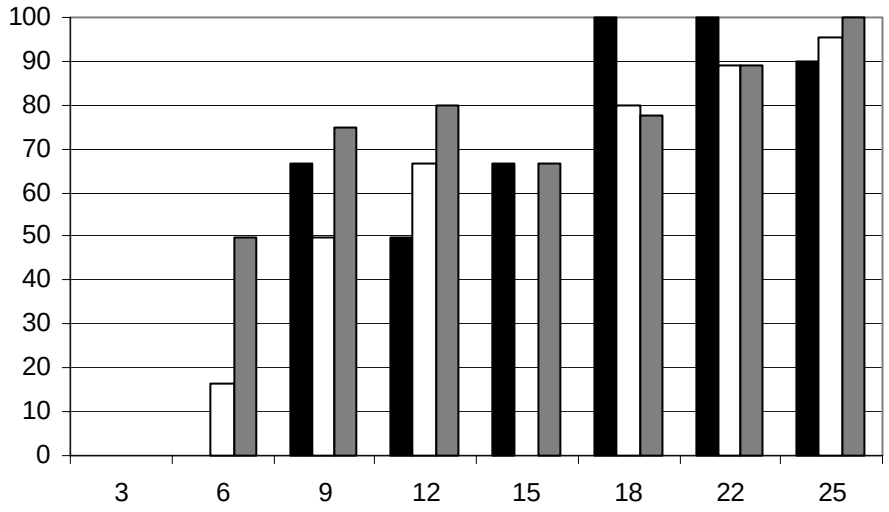


Figure 5: Percentage of microscopic lung lesions compatibles with Enzootic pneumonia in group A (black), group B (white) and group C (grey) by age



GENERAL DISCUSSION

In this section, a general discussion on the conclusions obtained in each study, their contributions and their limitations on the knowledge on *M.hyopneumoniae* infection will be addressed.

In order to control a disease, it is critical to understand its transmission and circulation of the causative agent within a herd and factors that are involved in the manifestation of the clinical outcome. The first step in studying a disease is to diagnose it; in case of infectious disease, this point is initially based on the detection of the etiological agent. In the case of *M. hyopneumoniae*, bacteriological culture is difficult and time-consuming, and for these reasons molecular tools have been developed and used in upper respiratory tract samples to monitor the presence of agent in field studies. The research presented in this Thesis assess the dynamics of infection of *M. hyopneumoniae* at a herd level and its relationship with production systems used in a transversal field trial (Chapter 1), and with EP-compatible microscopic and macroscopic lesions and/or disease in a longitudinal study (Chapter 3). Moreover, correlation of *M.hyopneumoniae* detection in upper respiratory site with detection in bronchi, and with EP microscopic and macroscopic lung lesions at individual pig level has been also addressed (Chapter 2). Relationships between detection of *M.hyopneumoniae* in upper and lower respiratory sites needs to be clarified in order to interpret herd monitoring results using nPCR from upper respiratory site samples.

The studies on infection dynamics show that the factor with greatest effect on *M. hyopneumoniae* circulation pattern was the farm (Chapter 1). Even within a farm (Chapter 3), significant differences in the dynamics of infection between different batches were observed, which

were also related with different degrees and timings of *M. hyopneumoniae* –related lesions appearance.

Moreover, the type of production system (1/2-site versus 3-site herds) is another factor that influences *M.hyopneumoniae* infection dynamics. In modern swine production, at approximately 18-20 weeks of age, pigs appear to experience severe outbreaks, resulting in elevated morbidity and even increased mortality (Dee, 1996). This clinical condition has been defined and characterized as porcine respiratory disease complex (PRDC), where *M. hyopneumoniae* plays a major role (Thacker et al., 1999). PRDC appears to be closely associated with multisite production systems (Dee, 1996). Results of our field trial in regards *M.hyopneumoniae*, involving 12 Spanish herds clearly support this idea. *M. hyopneumoanie* colonization in nurseries was lower and occurred later in time in 3-site versus in 1/2-site production systems. Moreover, an abrupt increase in the proportion of infected animals, associated with late respiratory outbreaks was seen in the multisite production systems.

Besides the influence of production systems, involvement of *M. hyopneumoniae* in respiratory disease observed in these farms was analysed. Although *M. hyopneumoniae* infection was detected in all farms, it was not always involved in the respiratory disease. An important contribution of this work is that, although seroconversion and *M. hyopneumoniae* infection were detected in all herds, clinical and subclinical infections were described. This is important for decision making on the control strategies to implement at the farm. Serumprofiles were not discriminative to determine the involvement of *M. hyopneumoniae* in a clinical outbreak. Bacteriumprofiles were more useful in determining *M. hyopneumoniae* involvement in respiratory disease, since an increase of the proportion of infected pigs (based on

detection of *M. hyopneumoniae* in nasal swabs) was clearly related with appearance of the clinical outbreak. The idea that a certain percentage of infected pigs is necessary to induce disease is supported in both field studies (Chapers 1 and 3), together with other recently published studies (Vicca et al., 2002). Although not discriminative, the proportion of seropositive pigs was also greater in clinically than in subclinically infected herds.

So far, there are three crossectional studies from different countries, USA (Calsamiglia et al., 1999b), Belgium (Vicca et al., 2002) and Spain (Chapter 1) in which infection dynamics was studied using serumprofiles in combination with bacteriumprofiles. Within the *M. hyopneumoniae* clinically affected farms, proportions of infected pigs when the outbreak occurred varied between the three studies (USA: 70-100%, Spain: 10-70%, Belgium: 8-24%). These differences in proportions may be explained by: differences in sensitivity, use of antibiotics, different genetic lines, presence of co-infections, number of farms tested, and inherent characteristics of each farm.

An important limitation of the abovementioned studies is that they are transversal rather than longitudinal. Therefore, the information obtained reflects the *M. hyopneumoniae* infection status of a different group of animals in a point in time rather than the evolution of infection throughout life time of a particular group of pigs.

Dynamics of serocoversion against *M. hyopneumoniae* was another issue addressed in both, transversal and longitudinal field trials. Several authors have described the time of seroconversion under field conditions as slow, delayed and very variable (Morris et al. 1995, Sitjar et al., 1996, Calsamiglia et a., 1999b). In the two field trials described in this Thesis, time of seroconversion was also variable. Results obtained in Chapter 3 indicate that seroconversion was more related to detection of *M.*

hyopneumoniae in bronchial swabs rather than in nasal or tonsillar swabs, and to the detection of a higher percentage of pigs with a positive nPCR bronchial swab. Although the relation with proportions of pigs infected pigs at bronchi level, seroconversion was still variable with respect to the age in which these proportion increase.

In chapter 1, information regarding vaccination and medication strategies conducted in the tested farms was provided by the farmer. These factors were, therefore, considered as non-controlled variables. However, they were tested in a univariate and multivariate statistical model. The first model showed that vaccination against *M.hyopneumoniae* had a significant effect on pathogen's nPCR detection. On the contrary, when the effect of all variables was analysed in the multivariate analysis, both vaccination and medication had a significant effect on lowering the nPCR results. These results are in agreement with a *M. hyopneumoniae* vaccination field trial, where prevalence of nPCR-positive piglets at weaning was lower in groups of vaccinated sows (Ruiz et al., 2003). This reduction could be due to lower levels of shedding in vaccinated sows and/or an increased maternal immunity, and therefore lower levels of nasal *M. hyopneumoniae* colonization in the offspring. However, the effect of vaccination and medication strategies on colonization (nPCR) at the sow and piglets level should be studied in more detail.

Results from Chapter 1 and Chapter 3 highlighted that early colonization of *M.hyopneumoniae* occurs at different levels of the respiratory tract. This is the first time that *M. hyopneumoniae* infection is detected at the lung level (bronchial swabs) in such an early stage (3 weeks of age).

Another objective aimed in this Thesis was to assess the significance of detection *M.hyopneumoniae* in nasal swabs. Our initial

hypothesis was that nasal swabs are adequate sampling sites to monitor *M. hyopneumoniae* infection in live pigs. Chapters 2 and 3 addressed this point; the first study, using animals received at the *Servei de Diagnòstic Veterinari at the Veterinary school of the Universitat Autònoma de Barcelona*, and the latter one in a longitudinal study carried in a farm suffering from mycoplasma-related disease. As expected, in both studies bronchial swabs were the best predictors of presence of microscopic and gross EP lesions coinciding with previously published studies (Calsamiglia et al., 2000). However, to sample bronchial swabs from live animals is not possible. Surprisingly, the detection in nasal swabs and the level of prediction was different in Chapters 2 and 3. The relative proportion of nasal detection was lower in the field study than in the study using pigs received at the diagnostic laboratory. An increase of *M.hyopneumoniae* shedding in nasal cavities due to stressful situations (such as transport) or due to increase multiplication of bacteria after the animal's death have been proposed as potential explanations for a larger proportion nPCR positive nasal samples obtained in Chapter 2.

Another interesting finding in Chapter 3 is the discontinuous detection of *M. hyopneumoniae* in nasal swabs. Ruiz et al,. (2002a) reported a high but discontinuous pattern of nasal and tracheal detection in an challenge study. Moreover, in a longitudinal field study, where nursery pigs were sampled 7 times during the nursery period, most of the pigs were positive only in one sampling (Ruiz et al., 2002c). Data from other analyses performed in our group (data not shown) demonstrated that detection in nasal swabs throughout the live of pigs is discontinuous: a maximum of three positive nasal samplings out of nine were detected in the same animal. Moreover, these three positive swabs were not always detected in consecutive samplings. From the results of these previously published studies (Ruiz et al., 2002a, Ruiz et al., 2002c) and the work

performed by our group, it can be proposed that *M.hyopneumoniae* nasal swab detection by nPCR is not as frequent and constant as previously thought, and that detection of *M. hyopneumoniae* in nasal swabs becomes more constant when microscopic and macroscopic lesions exist.

Although tonsillar sampling in live animals is more laborious than nasal samplings, it is possibly more adequate to monitor *M. hyopneumoniae* detection at a farm level. Tonsillar swabs are currently being used to detect other swine pathogens such as *Actinabacillus pleuropneumoniae* (Chiers, 2004). This respiratory pathogen, usually attaches to the cells of the terminal bronchioli and alveolar epithelial cells, but it is also detected in upper respiratory sites (Chiers, 2004). Studies using electronic microscopy have shown that, in carrier pigs, the pathogen can reside in tonsillar crypts for long periods without developing the disease. Therefore, tonsillar sampling is considered a useful site for detection of *A. pleuropneumoniae* sublinically infected pigs. Shedding of this pathogen in nasal secretions only takes place at the time of acute lung infection (Chiers, 2004). Taking into account these observations, studies addressing the interactions of *M.hyopneumoniae* with cells of both upper respiratory sites will help in interpreting its detection in tonsil and nasal cavities.

In Chapters 2 and 3, analysis of microscopic lesions was performed. Results of these studies and from previous works (Calsamiglia et al., 2000), showed that when bronchial swabs are positive the probability to show EP-compatible microscopic lesions in the animal is high. However, presence of EP-compatible lesions does not always imply *M. hyopneumoniae* detection in bronchi. In Chapter 3, EP-compatible microscopic lesions appeared in pigs from groups A and B before *M.hyopneumoniae* was detected in bronchial swabs. Swine influenza virus (SIV) is known to cause microscopic lesions compatible with the

defined scores 3 and 4 (EP-compatible microscopic lesions). SIV antibodies were detected in slaughter pigs, indicating that infection with this agent occurred in those groups of pigs.

One limitation of the longitudinal study presented in Chapter 3 was that clinical signs (such as coughing) were not properly recorded. In that study, clinical signs were only recorded when observed on the sampling day. Therefore, the time of appearance and number of animals affected are unknown.

A complementary and informative approach to *M.hyopneumoniae* dynamics of infection should be the knowledge on bacterial loads. Quantification of Mycoplasma cells will help to relate the proportion of infected animals and individual bacterial loads, and better define the concept “pressure of infection” used in these studies. Also, chronology of infection at the different respiratory levels will be better interpreted and presumably more easily related with microscopic and macroscopic lesions and disease. Therefore, an interesting future research line on *M.hyopneumoniae* would be the use of a quantitative PCR method in order to assess these latter points.

CONCLUSIONS

1. A certain percentage of *M. hyopneumoniae* infected pigs seems to be necessary to induce clinical signs. Thus, *M.hyopneumoniae* clinically infected herds have greater percentages of nPCR positive pigs than subclinically infected herds without a clinical outbreak.
2. The Production system type, may affect *M.hyopneumoniae* colonization and infection dynamics: 3- site production systems had later colonization than 1/2-site systems, and a more abrupt increase of infected pigs was observed in the finishers, which was associated with disease.
3. *M. hyopneumoniae* infection dynamics is greatly influenced by characteristics inherent to each farm.
4. Seroconversion in field situations is very variable and is related with certain percentage of infected pigs, mainly at the bronchi levels, to be elicited.
5. Although combination of serumprofiles with bacteriumprofiles provide very useful information on *M. hyopneumoniae* infection dynamics, differences between different batches of the same farm occur, and therefore its application for decision making on vaccination/medication programs in subsequent groups is not so reliable.
6. *M. hyopneumoniae* nasal swab detection was not so frequent and constant as previously thought. In the longitudinal study, it was shown that nasal swab detection becomes more reliable when microscopic and macroscopic already exist.
7. Tonsillar swabs are an alternative sampling site for monitoring *M. hyopneumoniae* infection in field studies. Detection of *M.hyopneumoniae* in tonsillar swabs was observed even in

farrowing and nursery pigs, previously to the appearance of microscopic and macroscopic lung lesions.

8. Detection of *M.hyopneumoniae* in bronchial swabs was greater than in nasal and tonsillar swabs, and is the best predictor of EP-compatible microscopic lesions. Early colonization was also detected using bronchial swabs.

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