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The science behind *Mycoplasma hyopneumoniae* infections and diagnostics

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Introduction

Mycoplasma hyopneumoniae (*M hyopneumoniae*) is the primary etiological agent of Enzootic Pneumonia (Mare and Switzer, 1965; Goodwin et al., 1965), a chronic respiratory condition that affects pigs of all ages (Ross, 1999) and has almost worldwide distribution. The detrimental effect of *M hyopneumoniae* infection can be accounted for by the direct action of the pathogen on the respiratory tract, but most importantly by the predisposition of the pigs to other respiratory infections of viral and bacterial origin (Maes et al., 2008). In sum, *M hyopneumoniae* infection can severely affect pig performance causing great losses for the swine industry and big concerns related to the animal's well-being.

Infection with *M hyopneumoniae* are highly prevalent, can be transmitted by pig-to-pig contact or through the air, and once it had gained entry to a farm, it becomes a long term problem. Several strategies can be applied in order to control *M hyopneumoniae* infections, and eradication from swine farms, and even countries, is achievable (Stark, 2007). However, diagnostics of *M hyopneumoniae* in field settings continues to be a difficult process for the swine practitioner and diagnostician. This document summarizes several key points in the infection dynamics of *M hyopneumoniae*.

M hyopneumoniae infection dynamics and diagnostic implications

Whether natural or experimentally induced, the hallmarks of *M hyopneumoniae* infection are the lag time to show clinical signs and the subsequent long duration of infection before clearance from the pig's respiratory system.

M hyopneumoniae is recognized as a swine respiratory pathogen, unlike other members of the Mycoplasma genera identified in pigs (e.g. *M flocculare*), that is not linked with disease (Thacker et al., 2006). *M hyopneumoniae* is a small bacterium that lacks several important metabolic pathways, which makes it an obligatory parasite of the pig. Mycoplasmas have special growth requirements and a very slow multiplication rate. A doubling time of 7 hours seems to be the average for *M hyopneumoniae* strains under ideal

culturing conditions, and this time could even be longer depending on the virulence of the isolate (Meyns et al., 2007). However, bacterial isolation is considered the "gold-standard" for *M hyopneumoniae* diagnostics, but is not attempted on a routine basis, mostly due to the low sensitivity of the technique, the requirement of specialized media, the length of time it takes to complete the process, and the high rate of contamination with other bacteria (i.e. *M hyorhinis*). Therefore, bacterial culture is pursued under specific circumstances and only a handful of diagnostic laboratories perform it.

M hyopneumoniae isolates have been classified into three virulence groups: highly virulent isolates, moderately virulent isolates and low virulent isolates based on clinical and pathological parameters (Vicca et al., 2003). More recently, bacteriological and molecular typing techniques have allowed confirming differences among isolates (Meyns et al., 2007; Stakenborg et al., 2007; Vranckx et al., 2011).

Pigs become infected with *M hyopneumoniae* through the respiratory route, by either direct contact with infected pigs (e.g. sow-to-piglet transmission; Calsamiglia and Pijoan, 2000), or by inhaling the microorganism in the air coming from infected barns (Fano et al., 2005; Otake et al., 2010). Once infected, a pig can act as a long term infection amplifier, by shedding microorganisms for up to 7 months after inoculation (Pieters et al., 2009). Within-pen transmission tends to occur rather slowly but effectively, as demonstrated by a low, but sustained R_0 of 1.16 (Meyns et al., 2004). Even in vaccinated groups, transmission will take place with no significant difference compared with non-vaccinated animals, under experimental settings (Meyns et al., 2006; Pieters et al., 2010) and in field conditions (Villareal et al., 2011).

The course of *M hyopneumoniae* infection is divided in two phases, acute and chronic, depending on the presentation of clinical signs (dry cough). The acute phase is considered to start with the infection and subsequent development of cough, and to end with the disappearance of cough. The chronic phase refers to the time when the pig is still infected but not coughing. The chronic phase of *M hyopneumoniae* infection is unusually long and the

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microorganism persists in the respiratory tract of the pig in the presence of a detectable immune response (i.e. antibodies to *M hyopneumoniae*).

The typical dry cough that accompanies Enzootic Pneumonia does not develop immediately after infection. At least 10-16 days appear to be a minimum for a cough to be heard in a group of experimentally inoculated animals (Ross, 1999). Then, coughing will be apparent in animals for a period of up to 60-70 days post infection, until it ceases (Fano et al., 2005; Pieters et al., 2009). It is important to note that coughing alone cannot be used as a solely indicator of disease, as cough is not a pathognomonic sign of infection. Also, it is worth saying that the lack of cough doesn't translate into lack of infectious potential, as *M hyopneumoniae* genetic material has been detected in nasal swabs from experimentally infected animals during the incubation period, before cough appears (Pieters and Pijoan, 2006) and experimentally infected animals are able to transmit the pathogen to naïve sentinels several months after cough has disappeared in the group of pigs (Pieters et al., 2009).

Antibodies for *M hyopneumoniae* are not developed in the experimentally infected pig until after 2 to 6 weeks, and this process varies depending on the route of infection, and the infectious dose (Fano et al., 2005; Marois et al., 2010), respectively. In Fano's study, a comparison of seeder, direct contact and indirect contact pigs showed a seroconversion delay in the mentioned order for the experimental groups. Also, intra-tracheally infected animals may remain seropositive for up to 9 months (last time experimentally evaluated), even when pigs are no longer infectious (Pieters et al., 2009).

M hyopneumoniae is a respiratory contained pathogen under normal conditions, and does not produce systemic infection. However, antibody detection by ELISA is performed in serum samples. Little correlation exists between circulating antibodies and disease protection, and a positive ELISA result is considered an indicator of antigen exposure, which can be due to infection or vaccination. Nowadays, no commercial ELISA test differentiates vaccinated from unvaccinated animals. The use of an ELISA test for detection of secretory Ig-A in broncho-alveolar lavage fluids (BALF) have been published (Feng et al., 2010). The authors have suggested that detecting SIgA in BALF allowed them to differentiate vaccinated from infected animals, using field case samples.

Another means to identify the presence and quantify the effect of *M hyopneumoniae* in a herd is by scoring lung lesions (Pointon, 1999). Macroscopic lung lesions may develop in experimentally infected animals as early as 1 week after infection (Underdahl et al., 1980) and tend to heal with time, even when pigs remain infected and

capable of transmitting the microorganism to naïve animals (Pieters et al., 2009). Nevertheless, the lung lesions scoring technique is subjective by nature and implies a post-mortem testing. It is worth noting that lung lesions are not pathognomonic and should be confirmed with microscopic tissue evaluation and detection of *M hyopneumoniae* in the lungs.

Molecular based assays (e.g. PCR) constitute fast and reliable ways for detection of *M hyopneumoniae* genetic material from a wide array of clinical specimens, including swabs collected along the respiratory tract (from nasal cavities to bronchia), BALF and lung tissue. However, the sensitivity of the assay varies according to the type of sample specimen used for extraction of the genetic material. Investigations have consistently found that the highest sensitivity is achieved by using bronchial swabs, BALF or lung tissue samples (Sibila et al., 2004; Fablet et al., 2010), meaning that a post-mortem testing is usually required. Another source of sensitivity variation is the time between infection and sample collection, as the acute and chronic phases of infection have different shedding patterns. For example, a nasal swab collected from a pig that has been infected during 1.5 months (acute phase) has a higher sensitivity than the same type of sample collected 6 months after infection. And bronchial swab PCR is highly sensitive during both phases of infection.

During the last few decades, a body of evidence has been generated that supports the hypothesis of *M hyopneumoniae* strain heterogeneity (Frey et al., 1992; Vicca et al., 2003; Calus et al., 2007; Vranckx et al., 2011), and one limitation encountered with PCR based tests is the potential lack of detection of certain *M hyopneumoniae* isolates. Comparisons studies have identified tests that based on primer design include the most conserved areas of the genome, and are therefore capable of detecting positive samples from multiple isolates (Strait et al., 2008).

The ability to quantify the bacterial load in clinical samples has also been developed and validated for *M hyopneumoniae* PCR (Dubosson et al., 2004; Marois et al., 2010). Most recently, isothermal tests, for example, Loop-mediated isothermal amplification (Rovira et al., 2008), have been developed for a less expensive, yet sensitive, *M hyopneumoniae* antigen detection, that can be adapted to less equipped diagnostic laboratories around the world.

An issue still latent with PCR based detection is the inability to differentiate between viable and dead cells in the sample specimen, as a small piece of genetic material from dead cells can trigger the exponential amplification of the PCR and render a positive result. Ways to circumvent this issue are based on transmission experiments or bioassays, using naïve pigs, which can be costly, time consuming and require the use of isolation facilities.

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Conclusions and implications

M hyopneumoniae infection is characterized for having a slow onset and a long persistence. These two features have an important effect on the clinical presentation, bacterial shedding, and detection of the pathogen and its lesions. A clear understanding of *M hyopneumoniae* infection dynamics constitutes the basis for proper sample collection and interpretation of diagnostic tests' results.

Surveillance of *M hyopneumoniae* infection under field conditions can be a stressful and uncertain process for the swine practitioner and diagnostician, as no clear course of action have been published to lead the best path to follow. It is necessary to unify the criteria, as to when and where collect samples from and how to evaluate them, in order to establish guidelines for diagnosis, as it has been done for other swine diseases.

More than 45 years after its recognition as a porcine pathogen, *M hyopneumoniae* still remains an important disease causing agent present in a high percentage of swine operations around the world. Perhaps, it has been *M hyopneumoniae*'s elusive nature what has made it possible for this pathogen to persist in swine herds for such long times. Newer technologies and information that could help unraveling the problem of *M hyopneumoniae* infection are being published and applied in different areas of science. For example, using molecular tools to investigate heterogeneity among isolates, or identifying missing metabolic pathways of this bacterium to improve bacteriological methods. It is up to the swine industry, the practitioners and the scientific community to work hand-and-hand in order to come up with a more profound understanding of the ecology of this pathogen that would translate into more effective means to detect and eventually control *M hyopneumoniae*.

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