Review

Improving *Mycoplasma hyopneumoniae* diagnostic capabilities by harnessing the infection dynamics

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Abstract

Mycoplasma hyopneumoniae remains one of the most problematic bacterial pathogens for pig production. Despite an abundance of observational and laboratory testing capabilities for this organism, diagnostic interpretation of test results can be challenging and ambiguous. This is partly explained by the chronic nature of *M. hyopneumoniae* infection and its tropism for lower respiratory tract epithelium, which affects diagnostic sensitivities associated with sampling location and stage of infection. A thorough knowledge of the available tools for routine *M. hyopneumoniae* diagnostic testing, together with a detailed understanding of infection dynamics, are essential for optimizing sampling strategies and providing confidence in the diagnostic process. This study reviewed known information on sampling and diagnostic tools for *M. hyopneumoniae* and summarized literature reports of the dynamics of key infection outcomes, including clinical signs, lung lesions, pathogen detection, and humoral immune responses. Such knowledge will assist a better understanding of the performance of different diagnostic approaches at various stages of infection.

Keywords: Infection dynamics; Lung lesions; *Mycoplasma hyopneumoniae*; PCR; Serology

Introduction

Several bacteria can colonize the porcine respiratory tract, among which *Mycoplasma hyopneumoniae* is a major pathogen (Pieters and Maes, 2019). This bacterium significantly reduces the efficiency of clearance by the mucociliary apparatus (Underdahl et al., 1980; DeBey et al., 1992; DeBey and Ross, 1994), and modulates and/or eventually evades the host immune response (Leal-Zimmer et al., 2020). Altogether it offers a favorable environment for the establishment and proliferation of upper respiratory commensal bacteria and/or other microorganisms as secondary pathogens, resulting in either enzootic pneumonia (EP) or in the porcine respiratory disease complex (Pieters and Maes, 2019).

This review aimed to improve the diagnosis of *M. hyopneumoniae* infection and its associated disease by providing a background of knowledge covering state-of-the-art diagnostic and sampling tools as well as insights into infection dynamics. Accurate diagnosis of *M. hyopneumoniae* impacts the decision-making process and thus the outcome of herd-based strategies to control or eliminate disease. Understanding *M. hyopneumoniae* infection dynamics and collecting appropriate specimens at specific times are key to obtaining reliable diagnostic results. Information regarding the dynamics of key infection outcomes, such as coughing, lung lesions, pathogen detection by PCR, and the humoral immune response obtained from experimental infections has been summarized (Fig. 1). Experimental studies are ideal for describing the bacterium's infection dynamics as conditions are usually standardized. However, extrapolation of results to natural field infection should be performed carefully, as outcomes here might be affected by several conditions that are probably altered at the experimental level.

Clinical and pathological aspects of *Mycoplasma hyopneumoniae* infection

In herds endemically infected with *M. hyopneumoniae*, respiratory disease usually manifests as a high morbidity and low mortality pneumonia in growing pigs (Pieters and Maes, 2019). A dry, hacking cough commonly appears as the only obvious sign of disease (Straw et al., 1989), which can be coupled with reduced growth (Pointon et al., 1985; Straw et al., 1989; Buddle and O'Hara, 2005; Ferraz et al., 2020) and increased feed conversion ratios (Pallarés et al., 2001; Donkó et al., 2005). In uncomplicated cases, a variable proportion of animals can remain subclinically infected with no evidence of coughing or pulmonary lesions (Regula et al., 2000; Fano et al., 2005).

However in naïve pig populations, *M. hyopneumoniae* infection may be associated with disease in pigs of all ages, and more severe and acute clinical signs, including dyspnea, pyrexia, anorexia, lethargy, and death (Goodwin, 1984; Wallgren, 1998; Bargen, 2004). The onset, duration, and severity of clinical signs and pneumonic lesions may also be influenced by the circulating strain of *M. hyopneumoniae* (Vicca et al., 2003; Villarreal et al., 2011; Woolley et al., 2012), the coexistence of other respiratory pathogens (Saade et al., 2020), the genetics of the pigs (Borjigin et al., 2016), and the environmental conditions (Buddle and O'Hara, 2005; Michiels et al., 2015).

Coughing

Coughing assessment

Coughing is the only *in vivo* parameter for practical assessment of the clinical severity of EP, and as a quantitative measurement has been used to support the diagnosis of *M. hyopneumoniae* infection at the group level (Sørensen et al., 1997; Leon et al., 2001; Nathues et al., 2012). Notwithstanding, coughing is a non-specific clinical manifestation that can be

caused by a variety of infectious agents and be influenced by co-infections (Nathues et al., 2012). Several scoring systems to measure coughing in pigs have been described (Halbur et al., 1996; Leon et al., 2001; Mombarg et al., 2002; Nathues et al., 2012). Coughing indexes measure coughing bouts during a certain period of evaluation, performed at the room, pen, or individual level. Audio-based sensor systems for cough recording at the barn level are a potential tool for early EP detection. Polson et al. (2018) described detection of respiratory disease episodes by cough monitors 3 to 5 days earlier than detection by farm personnel. Additionally, automatic recordings of coughing events have the potential to remotely differentiate the etiology of clinical respiratory episodes. For example, differences in cough patterns were observed between influenza A virus (IAV) and *M. hyopneumoniae* (Polson et al., 2018; Spronk et al., 2019). To date, audio devices have been mainly employed among fattening pigs, so procedures for evaluating coughing in all infection scenarios are still missing.

Coughing dynamics

Onset of coughing can be variable and intermittent, occurring within 1-3 weeks post-infection in experimental settings (Fig. 1). With natural infection, clinical disease onset is less predictable as it is challenging to pinpoint when *M. hyopneumoniae* exposure occurs within a pig population (Morris et al., 1995; Leon et al., 2001). After onset, coughing gradually increases in the population over time, typically reaching a peak at 3-5 weeks post-challenge (Sørensen et al., 1997; Vicca et al., 2003; Arsenakis et al., 2016). In a recent field study with one pen of 10 seeder pigs in a population of 1,250 six-week-old contact pigs, cough monitors recorded the first respiratory distress alert 7.9 weeks post-inoculation with the highest average respiratory distress index at 13.6 weeks post-inoculation (Clavijo et al., 2021). Coughing gradually disappears and inoculated pigs commonly cease coughing between 8 and 14 weeks

after onset (Sørensen et al., 1997; Fano et al., 2005; Pieters et al., 2009; Sponheim et al., 2020). The average duration of coughing in a *M. hyopneumoniae* infected individual remains uncertain, with Morris et al. (1995) reporting a duration of coughing ranging from 3 to 66 days under field conditions.

Pathological examination

Lung lesion assessment

Gross pathology and histopathology provide visual evidence of lesions compatible with *M. hyopneumoniae* infection. Characteristic lung lesions induced by *M. hyopneumoniae* show red-tan-gray tissue discoloration, collapse, and rubbery firmness, mainly affecting the cranioventral regions of the lungs in a lobular pattern (Caswell and Williams, 2016), and commonly referred to as cranioventral pulmonary consolidation (CVPC)(Fraile et al., 2010). Several macroscopic scoring systems have been described (Garcia-Morante et al., 2016). Studies using different lung scoring methods are difficult to compare, adding biases and impreciseness to an already subjective method (Steinmann et al., 2014; Garcia-Morante et al., 2016). Software-based scoring systems using image analysis (Sibila et al., 2014) and artificial intelligence-based technologies (Trachtman et al., 2020) are approaches to reduce subjectivity and inter-observer bias, whereas other image diagnostic techniques, such as computed tomography, can assess the development of lung lesions *intra vitam* (Pósa et al., 2013). In any case, implementation of these methods in field or slaughterhouse settings has not yet been reported.

The histologic evaluation of lung sections is more sensitive than the macroscopic counterpart (Underdahl et al., 1980; Chae et al., 2020; Sibila et al., 2020). Microscopically, CVPC corresponds with a pattern of bronchointerstitial pneumonia (BIP), whose severity can

be assessed employing different scoring systems (Livingston et al., 1972; Morris et al., 1995; Calsamiglia et al., 2000; Woolley et al., 2012). The percentage of lung area occupied by air, determined using automatic image analysis methods, has also been employed to evaluate BIP severity (Vicca et al., 2003; Michiels et al., 2017). One of the possible pitfalls of many scoring systems is when severity is related only to the extension of bronchus-associated lymphoid tissue (BALT) hyperplasia. Hyperplasia of BALT is intrinsically related with the chronicity of the lesions, and often it remains residual once the cellular exudate has been cleared. Henceforth, high microscopic scores should be characterized not only by extensive peribronchiolar and perivascular lymphoid hyperplasia, but also by abundant inflammatory infiltrate (Woolley et al., 2012).

Lung lesions dynamics

Lung lesions associated with mycoplasmal pneumonia are naturally chronic. Pneumonia may start developing one week after infection (Underdahl et al., 1980; Kobisch et al., 1993; Lorenzo et al., 2006) and increase progressively until reaching maximal extension and severity by 4 weeks post-infection (Figure 1)(Garcia-Morante et al., 2017b). After peaking, macroscopic lung lesions may persist until week 8 post-infection and decrease gradually thereafter (Kobisch et al., 1993; Sørensen et al., 1997). If there are no further complications, mycoplasmal pneumonia is generally resolved by week 12 post-infection, when the remaining gross lesions may consist of interlobular scarring with tissue retraction (Kobisch et al., 1993; Sørensen et al., 1997). However, studies that have followed pigs for long periods after *M. hyopneumoniae* artificial inoculation have reported CVPC in about 40% of the inoculated pigs by 13 weeks post-inoculation (Pieters et al., 2009) and some degree of pneumonia up to 26 weeks post-infection (Fano et al., 2005).

Laboratory diagnostic tools for *Mycoplasma hyopneumoniae*

A summary of current *M. hyopneumoniae* diagnostic assays, the principles they are based on, and the relative diagnostic sensitivity by sample type are presented in Table 1.

Isolation and culture

Culture has traditionally been the definitive specific test for *M. hyopneumoniae* and is widely referred to as the "gold standard" for diagnosis (Thacker, 2004; Sibila et al., 2009; Chae et al., 2020). However, *M. hyopneumoniae* is one of the most fastidious mycoplasmas to isolate (Goodwin and Hurrell, 1970; Hwang et al., 2010; Cook et al., 2016). It is estimated that the *M. hyopneumoniae* cell uses 84% of total ATP production for non-growth associated maintenance and only 16% for growth in culture (Kamminga et al., 2017). In addition, the medium used to grow *M. hyopneumoniae* is easily overgrown by faster-growing mycoplasmas, such as *M. hyorhinis* and *M. flocculare* (Kobisch and Friis, 1996). Therefore, failure to isolate *M. hyopneumoniae* should never be used to exclude its presence (Thacker, 2004; Sibila et al., 2009; Chae et al., 2020), and calls the current gold standard method into question.

Isolation is generally achieved from a suspension of pneumonic tissue in a highly enriched liquid medium. Tonsillar, nasal, tracheal, and bronchial swabs, and bronchoalveolar lavage fluid (BALF) have been also used for isolation purposes (Baumeister et al., 1998; Otagiri et al., 2005; Marois et al., 2007). Occasionally, isolation from inner organs and tissues such as liver, spleen, kidneys, and bronchial lymph nodes has also been reported (Tajima et al., 1984; Yagihashi et al., 1984; Le Carrou et al., 2006; Marois et al., 2007). In field cases of EP, the isolation rate has varied from 13 to 56% for individual cases and 18 to 75% for herds (Goodwin et al., 1968; Goodwin and Hurrell, 1970; Otagiri et al., 2005). In a study using BALF, only 1 of 40 pigs with chronic pneumonia was culture positive (Baumeister et al.,

1998). Charlebois et al. (2014) isolated *M. hyopneumoniae* in pure culture from 1/160 lungs with gross EP lesions and a further 9/160 in mixed culture with *M. hyorhinis.* Isolation rates are usually higher in experimental settings, with rates of up to 100% reported in artificially inoculated pigs (Sørensen et al., 1997; Otagiri et al., 2005; Marois et al., 2007).

Despite its limitations, cultural isolation of *M. hyopneumoniae* is still necessary for generating of isolate collections to perform different types of studies (e.g., whole genome sequencing [WGS]), for maintaining strain stocks used in experimental disease models and autogenous vaccine development, and to assess minimum inhibitory concentrations (MICs) of antimicrobial compounds. Moreover, bacteriological culture remains the only method available to date to assess *M. hyopneumoniae* viability. Therefore, continuous efforts towards optimized culture media conditions for *M. hyopneumoniae* are needed. It has been proposed that 10.9% w/v fresh yeast extract, 15% v/v horse serum, and 31.5% v/v porcine serum are ideal concentrations for the optimal growth of *M. hyopneumoniae* in culture (Hwang et al., 2010). More recently, an optimized solid medium for selection of *M. hyopneumoniae* has been reported (Cook et al., 2016). The same authors also found the incorporation of kanamycin into the agar medium selectively inhibited the growth of *M. hyorhinis*, while *M. hyopneumoniae* was able to grow (Cook et al., 2016). Lastly, a metabolic model predicted that pyruvate addition into culture media increases the *in vitro* growth rate of *M. hyopneumoniae* (Kamminga et al., 2017).

Visualization of antigen and nucleic acid in tissue

Both immunohistochemistry (IHC) and immunofluorescence (IF) assays detect *M. hyopneumoniae* antigen in respiratory tissue sections by using specific labelled antibodies. *In situ* hybridization (ISH) using a probe targeting a DNA or RNA-specific region can achieve

higher specificity and sensitivity compared to IF and IHC tests (Freeman et al., 1984; Bölske et al., 1987). Since these techniques enable a direct correlation of the presence of typical lesions and *M. hyopneumoniae*, they can provide convincing evidence for the causal role played by *M. hyopneumoniae* in observed lesions. However, they can only be performed *post-mortem* and only a small area of the lung is normally examined, increasing the risk of a false-negative result.

Immunofluorescence has mainly been applied under experimental settings as frozen sample collection may be problematic in the field and the accuracy of this technique is highly reliant on sample quality (Cheikh Saad Bouh et al., 2003; Sibila et al., 2009). In contrast, IHC and ISH are similar but more practical techniques for field-collected samples, as they can be performed on formalin-fixed, paraffin-embedded tissues, avoiding the need for fresh or frozen materials. These techniques have been mainly reported at an experimental level (Kwon et al., 2002; Sarradell et al., 2003; Redondo et al., 2009), and have been largely replaced by polymerase chain reaction (PCR) technology, which overcomes most of their diagnostic limitations.

Molecular tools for diagnosis and characterization

PCR-based methods

Several PCR techniques have been developed for detection of the *M. hyopneumoniae* genome in clinical specimens (Sibila et al., 2009; Chae et al., 2020). Conventional PCR assays using a single set of primers appeared insensitive for accurate detection of *M. hyopneumoniae*. Thus, nested PCR assays arose as highly sensitive alternatives and became commonly used in diagnostic laboratories (Stemke, 1997; Stärk et al., 1998; Calsamiglia et al., 1999b; Verdin et al., 2000; Kurth et al., 2002). Nonetheless, such extremely sensitive assays,

able to detect the equivalent of one bacterial organism in the sample (Kurth et al., 2002), can lead to problems related to contamination and false positive reactions.

Real-time PCR assays have become the preferred technique in diagnostic and research settings for *M. hyopneumoniae* detection in the most recent decade. This technique renders highly accurate results and enables high throughputs with less laborious procedures and improved quality control. Moreover, real-time PCR provides a semi- or quantitative value for the amount of genetic material detected, which can be used to estimate the relative bacterial load in a sample (Marois et al., 2010; Fourour et al., 2018).

The diagnostic sensitivity of PCR detection for *M. hyopneumoniae* will vary based on the material of the collection swab (Takeuti et al., 2017b), the DNA extraction method (Vangroenweghe et al., 2015a; Nascimento et al., 2019), the sample type, and the stage of infection (Fablet et al., 2010; Vangroenweghe et al., 2015a; Pieters et al., 2017; Sponehim et al., 2020; Clavijo et al., 2021). Takeuti et al. (2017b) found nylon-flocked swabs had greater absorption capacity and detection rates when compared to rayon-bud swabs, while Vangroenweghe et al. (2015a) found better detection of *M. hyopneumoniae* with a total nucleic acid extraction kit compared with a viral RNA extraction kit, based on cycle threshold (Ct) values and detection rates. A 15 times higher probability of PCR positivity using phenol-chloroform extraction method compared to a commercial kit has also been shown (Nascimento et al., 2019).

Using PCR, *M. hyopneumoniae* DNA has been detected in various *ante-mortem* samples, including airway lavages (e.g., tracheobronchial lavage) and swabs (e.g., nasal and

laryngeal swabs), oral fluids, and tracheal secretions. The terms tracheobronchial (Fablet et al., 2010; Vangroenweghe et al., 2015b) or tracheal (Clavijo et al., 2021) swab, and deep tracheal catheter (Betlach et al., 2020; Sponheim et al., 2020) are used indistinctly in the literature to describe specimens collected from tracheal secretions. *Mycoplasma hyopneumoniae* has also been detected *post-mortem* in bronchial swabs, lung tissue, and BALF (Baumeister et al., 1998; Kurth et al., 2002; Moorkamp et al., 2010; Pieters et al., 2017). In general, samples obtained *in vivo* from the upper respiratory tract secretions offer lower sensitivity than those obtained *post-mortem* from the lower respiratory tract (Otagiri et al., 2005; Fablet et al., 2010; Sievers et al., 2015; Pieters et al., 2017; Betlach et al., 2020).

Several studies have shown tracheal secretions provide the highest diagnostic sensitivity for *ante-mortem* detection of *M. hyopneumoniae* in both experimental and naturally-infected pigs (Fablet et al., 2010; Vangroenweghe et al., 2015a; Betlach et al., 2020; Sponheim et al., 2020; Clavijo et al., 2021), followed by laryngeal swabs, and then nasal swabs (Pieters et al., 2017; Moiso et al., 2020). Positive PCR results have also been obtained from aggregated samples, such as oral fluids (Hernandez-Garcia et al., 2017; Pieters et al., 2017; Clavijo et al., 2021) and processing fluids (Vilalta et al., 2019, 2020). Due to the ease of collection and its non-invasiveness, the use of oral fluids for pathogen surveillance and monitoring has been of growing interest. However, extremely low sensitivity as well as limited and inconsistent detection of *M. hyopneumoniae* has been reported from oral fluids, especially during the early stages of infection (Hernandez-Garcia et al., 2017; Pieters et al., 2017; Betlach et al., 2020; Clavijo et al., 2021). In addition, PCR inhibitors have been described in this sample type, which can result in false negative results (Ochert et al., 1994; Chittick et al., 2011; Schrader et al., 2012). The use of processing fluids for assessing *M.*

285 *hyopneumoniae* infection in breeding herds has not been validated, as the detected genetic
286 material may be due to environmental contamination (Vilalta et al., 2019, 2020).
287 PCR detection dynamics
288 The first positive PCR detection of *M. hyopneumoniae* in respiratory secretions
289 appears to occur within 4-5 days of infection (Fig. 1; Calsamiglia et al., 1999b; Pieters et al.,
290 2017). Pigs may then remain persistently infected for several months, with estimates of 100%,
291 78% and 61% of experimentally-infected pigs being PCR positive in necropsy samples at 3, 6
292 and 7.5 months post-infection, respectively (Fano et al., 2005; Pieters et al., 2009). In addition,
293 *M. hyopneumoniae* detection patterns can be intermittent (Roos et al., 2016; Takeuti et al.,
294 2017a), affecting diagnostic sensitivity during the infected period. Persistence and clearance
295 may depend on several aspects, including the bacterial strain evaluated. In any case, Pieters et
296 al. (2009) pointed out that complete clearance of *M. hyopneumoniae* infection (based on PCR)
297 may occur between 7.5 and 9 months post-infection.
298 Molecular typing techniques
299 Different methods used to characterize and classify *M. hyopneumoniae* at the genomic
300 level have been reviewed by Betlach et al. (2019). In the event of an outbreak or eradication
301 failure, the application of *M. hyopneumoniae* genomic characterization can provide insight
302 into pathogen transmission and control. Recent improvements to sequencing technologies
303 with higher speed and output-to-cost ratios render WGS valuable for thorough
304 characterization of *M. hyopneumoniae* isolates. Indeed, a total of 23 genome assemblies are
305 available for *M. hyopneumoniae* to date, of which 11 are complete genomes[1]. All of these are
306 derived from cultivated isolates, as WGS from genetic material extracted directly from
307 clinical specimens has not yet been reported. Added to this major limiting factor, processing

[1] See: National Center for Biotechnology Information Genome Tool. https://www.ncbi.nlm.nih.gov/genome/190. (Accessed 05 April 2021).

cost and advanced computational requirements for analysis and interpretation still favor other typing methods for *M. hyopneumoniae* characterization purposes over WGS, both at field and experimental levels.

Serological tests

Specific-antibody assessment

Detection of *M. hyopneumoniae*-specific antibodies is currently mainly performed by enzyme-linked immunosorbent assay (ELISA). This serologic test is designed to detect passively (maternal) or actively (infection and/or vaccination) acquired antibodies in serum or, if modified and validated, in colostrum, BALF, oral fluids, or other body fluids (Morris et al., 1994; Okada et al., 2000; Rautiainen et al., 2000; Pieters et al., 2017). Various commercial *M. hyopneumoniae* ELISAs are currently available (Chae et al., 2020; Poeta Silva et al., 2020). These have been compared in several studies and, overall, serum antibodies are barely detected during the early stages of *M. hyopneumoniae* infection.

An indirect blocking ELISA (Thermo Scientific™ Oxoid™ *Mycoplasma hyponeumoniae* Detection Kit) showed the earliest onset of antibody detection compared to standard indirect ELISAs (Ameri-Mahabadi et al., 2005; Erlandson et al., 2005; Fano et al., 2012; Gomes-Neto et al., 2014; Poeta Silva et al., 2020). On the other hand, antibodies to *M. flocculare* have been reported to cross-react with *M. hyopneumoniae* (Freeman et al., 1984; Bereiter et al., 1990), and cross-reactivity may occur with *M. flocculare* and *M. hyosynoviae* in some commercial assays (Gomes-Neto et al. 2014). Poeta Silva et al. (2020) reported false-positive rates of six *M. hyopneumoniae* antibody ELISAs by testing pigs inoculated with *M. flocculare*, *M. hyorhinis*, and *M. hyosynoviae*. No significant difference in the misclassification rate was detected among BioChek, IDEXX, Hipra, and Oxoid ELISAs,

whereas significantly higher misclassification rates were observed in Eurofins Ingenasa and IDvet ELISAs (Poeta Silva et al., 2020).

The high prevalence of vaccination to control *M. hyopneumoniae* infection has implications for diagnosis, as commercial ELISA kits measuring IgG antibodies cannot differentiate infected from vaccinated pigs or pigs with maternally derived antibodies (MDA)(Meens et al., 2010). For this purpose, the P97R1 and Mhp366 immunogenic proteins, up-regulated or solely expressed during infection, have been proposed as potential candidates to be used in DIVA (differentiating infected from vaccinated animals) assays (Meens et al., 2010; Feng et al., 2014; Ding et al., 2019). Similarly, since IgA antibodies of the upper respiratory tract are detected in challenged pigs, but not in vaccinated pigs or pigs with MDA (Djordjevic et al., 1997; Bai et al., 2018), a secretory IgA-ELISA performed from nasal swab samples has been proposed as a DIVA assay and a tool for the early diagnosis of *M. hyopneumoniae* infection (Feng et al., 2010; Bai et al., 2018). To date, procedures for proposed DIVA tests in field scenarios have not yet been standardized and research is needed to evaluate their value in diagnosis.

Serum antibodies dynamics

The induction of systemic humoral immune response by *M. hyopneumoniae* is slow, inconsistent among pigs and ELISA tests, and the time elapsed between infection and seroconversion is highly variable (Sibila et al., 2009; Chae et al., 2020; Poeta Silva et al., 2020). Under experimental settings, seroconversion onset as early as 7 to 9 days post-infection has been reported at the individual animal level (Sheldrake et al., 1990; Sørensen et al., 1997). However, the onset of seroconversion at 2-3 weeks post-infection is often reported (Nicolet et al., 1980; Piffer et al., 1984; Bereiter et al., 1990; Gomes-Neto et al., 2014; Poeta

356 Silva et al., 2020) and it is not until week 4 to 9 post-infection that all infected animals have
357 detectable serum antibodies (Fig. 1; Sørensen et al., 1997; Fano et al., 2005; Pieters et al.,
358 2009; Poeta Silva et al., 2020). Since infection may occur 1-6 weeks before seroconversion is
359 detected in experimentally infected pigs (Sheldrake et al., 1990; Kobisch et al., 1993; Morris
360 et al., 1995; Sørensen et al., 1997; Poeta Silva et al., 2020), and this variability is likely to be
361 even greater under natural conditions, it can be erroneous to determine time of infection from
362 serology. Furthermore, increased levels of serum antibodies have been described when *M.*
363 *hyopneumoniae* is in co-infection with the porcine reproductive and respiratory syndrome
364 virus (Thacker et al., 1999), porcine circovirus 2 (Opriessnig et al., 2004) or IAV (Thacker et
365 al., 2001). Although this putative synergism is not always observed (Sibila et al., 2012), it
366 could also affect serological results under field conditions.
367
368 The dynamics of antibody responses measured by ELISA are variable. Optical density
369 values or titers have been described to peak about 7-10 weeks post-infection, and decline
370 gradually thereafter (Bereiter et al., 1990; Sheldrake et al., 1990; Kobisch et al., 1993; Okada
371 et al., 2005), or develop slowly until peaking at 17-19 weeks post-infection (Armstrong et al.,
372 1983; Fano et al., 2012; Gomes-Neto et al., 2014). In some cases, *M. hyopneumoniae*-specific
373 antibodies have shown to persist for at least a year post-inoculation (Bruggmann et al., 1977;
374 Armstrong et al., 1983; Bereiter et al., 1990).
375
376 The proportion of pigs seroconverting after vaccination, as well as their antibody
377 concentrations has varied depending on the vaccine composition, administration route,
378 vaccination strategy, and pig infection status (Thacker et al., 1998; Calsamiglia et al., 1999a;
379 Maes et al., 1999; Martelli et al., 2006). However, serum antibodies are usually detected at 2-

4 weeks after a two-dose vaccination and may remain detectable for weeks to months (Maes et al., 2021). In the absence of natural infections that boost the immune system, antibody levels may decrease below detectable limits 1-3 months after vaccination (Calsamiglia et al., 1999a; Maes et al., 1999). In any case, the serological profiles in vaccinated pigs that are housed for long periods on a farm (e.g., late-finishing or breeding pigs) are poorly described.

Important considerations in the diagnosis of *Mycoplasma hyopneumoniae* infection and associated disease

The process of assessing *M. hyopneumoniae* involvement through clinical and/or gross pathological examination is complex and lacks specificity, as there is usually a polymicrobial component that can mask the underlying clinicopathological features or even mimic them. Hence, accurate diagnosis of *M. hyopneumoniae* infection is best achieved when clinical and pathological observations are aligned with appropriate laboratory test results (Pieters and Maes, 2019; Chae et al., 2020).

Because bronchial and bronchiolar epithelia are major sites of *M. hyopneumoniae* replication (Underdahl et al., 1980; DeBey et al., 1992; Kwon et al., 2002), PCR tests on *post-mortem* samples from these sites (i.e., bronchial swabs, BALF, and lung tissue) yield the highest diagnostic sensitivity (Fablet et al., 2010; Sievers et al., 2015; Pieters et al., 2017; Betlach et al., 2020). Importantly, *M. hyopneumoniae* can be detected across different anatomical lung sections, regardless of evident macroscopic lung lesions (McMahon et al., 2020; Tonni et al., 2021). Lung tissue collection also allows *M. hyopneumoniae* visualization by means of IF, IHC or ISH. The detection of antigen or nucleic acid linked with

403 histopathologic changes can confirm the significance of *M. hyopneumoniae* in disease, which
404 might not be achieved as effectively by other laboratory methods.
405
406 When *post-mortem* samples are not available, collection of tracheal secretions, either
407 by means of lavages, swabs or catheters, is considered the most sensitive approach for direct
408 detection of *M. hyopneumoniae* by PCR in live pigs (Kurth et al., 2002; Fablet et al., 2010;
409 Vangroenweghe et al., 2015a; Betlach et al., 2020; Sponheim et al., 2020). Moreover, low Ct
410 values in tracheal secretions have been reported to correlate with greater and consistent
411 bacterial load throughout the whole lung (McMahon et al., 2020), suggesting that this *ante-*
412 *mortem* sample might be representative of infection at the lung level. During the early stages
413 of infection, detection from nasal swabs has shown the lowest sensitivity compared to other
414 more invasive sample types, both under experimental (Kurth et al., 2002; Marois et al., 2007;
415 Pieters et al., 2017) and field conditions (Fablet et al., 2010; Vangroenweghe et al., 2015a;
416 Moiso et al., 2020). However, from a practical point of view, it may be valid at a group level
417 to use a higher number of less invasive samples with a lower sensitivity to achieve a
418 satisfactory diagnostic sensitivity.
419
420 By using PCR, it is possible to identify *M. hyopneumoniae* infected pigs prior to the
421 observation of clinical signs, and gross and histopathological lesions, and before
422 seroconversion occurs (Chae et al., 2020). This is of paramount importance in surveillance
423 programs of naïve pig populations, in which *M. hyopneumoniae* early detection is critical. In
424 such scenarios, the likelihood of missing a *M. hyopneumoniae* introduction is high if, for
425 instance, seroconversion assessment by ELISA or PCR detection in oral fluids is chosen as
426 the diagnostic approach (Betlach et al., 2020; Clavijo et al., 2021). Besides, non-clinical,

427 serologically negative carriers of *M. hyopneumoniae* have been described (Pieters et al., 2009),
428 and genomic detection by PCR provides the only possible approach to detect such animals.
429
430 Nevertheless, PCR-based techniques detect DNA derived from live and/or dead
431 bacteria and, hence, cannot be robustly used to assess *M. hyopneumoniae* viability (Calus et
432 al., 2010; Garcia-Morante et al., 2018). In other fields, a combination of real-time PCR with
433 propidium monoazide (PMA) treatment has been investigated for specific monitoring of
434 viable target bacteria (Yáñez et al., 2011; Yokomachi and Yaguchi, 2012; Van
435 Frankenhuyzen et al., 2013). While low Ct values are often a sign of active infection, high Ct
436 values remain as one of the main concerns for pig veterinarians and producers, as unequivocal
437 evidence of active infection is usually unattainable.
438
439 Although advances in veterinary diagnostics have been made and limitations of
440 serology are well-known, there is still a large dependence on ELISA-based serological assays
441 for field diagnosis of *M. hyopneumoniae* infections. In several studies in non-vaccinated pigs,
442 *M. hyopneumoniae*-specific antibodies are associated with disease occurrence, coughing and
443 lung lesions (Kobisch et al., 1993; Morris et al., 1995; Sørensen et al., 1997; Leon et al., 2001;
444 Garcia-Morante et al., 2017). As coughing may begin at about the same time as
445 seroconversion and increase in parallel with the increasing proportion of seropositive pigs
446 (Fig. 1), its presence may be a good indicator to detect seroconversion. In advanced infection
447 stages when most of the pig population may have seroconverted, ELISA might be even more
448 reliable than PCR, as *M. hyopneumoniae* shedding might follow an intermittent pattern
449 influencing PCR diagnostic sensitivity (Roos et al., 2016; Takeuti et al., 2017a).
450

The eventual benefits of ELISA over PCR apply only to non-vaccinated populations or pigs without detectable vaccine-derived antibodies anymore, as none of the commercially available ELISA tests can be used to differentiate infected from vaccinated animals. The development of DIVA marker vaccines has the potential to bypass the abovementioned problem, but it is uncertain if these technologies are yet under development for commercial applications.

Conclusions

While veterinarians rely strongly on diagnostic results to guide health and management decisions, awareness of both the available diagnostic tools and *M. hyopneumoniae* infection dynamics will aid the collection of the best specimens at an appropriate time, promoting reliable results and diagnoses made with a higher level of confidence. Although clinicopathological findings pose a great diagnostic challenge due to the myriad etiologies of respiratory disease, PCR can detect a high proportion of *M. hyopneumoniae* infected animals in the early and very late stages of the infection and provide accurate information on infection dynamics. Tracheal secretions are the *in vivo* sample of choice with the highest diagnostic sensitivity for *M. hyopneumoniae* detection by PCR. One drawback of PCR techniques is that they do not confirm the presence of viable organisms, which may limit interpretation of a positive result. Because serological responses to vaccination and time frame from infection to seroconversion can be highly variable, and infection and vaccination responses are mostly indistinguishable, serological information can be misleading and confuse correct judgements, in particular if used to predict *M. hyopneumoniae* infection on an individual basis.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Table 1. Description of current *M. hyopneumoniae* diagnostic assays and relative diagnostic sensitivity by sample type. Relative diagnostic sensitivity is based on comparisons between sample types within the same assay type, from very low (-) to very high (++++). Only clinical specimens that have been compared in the literature are included, although more sample types may exist per assay type.

Diagnostic category	Assay	Principle	Sample	Relative diagnostic sensitivity	References
Detection of viable bacteria	Bacterial culture	Isolation of *M. hyopneumoniae*	Nasal swab	+	(Marois et al., 2007)
			Tonsillar swab	++	
			Lung tissue	+/++	
			Tracheobronchial swab	+++	
			Tracheobronchial lavage	+++	
Detection of bacterial antigens	Immunofluorescence	Detection of *M. hyopneumoniae* antigens using antibodies in tissue	Frozen lung tissue	No comparison available	
	Immunohistochemistry	Detection of *M. hyopneumoniae* antigens using antibodies in tissue	Fixed lung tissue		
Detection of bacterial nucleic acid	*In situ* hybridization	Detection of *M. hyopneumoniae*-specific genome regions using complementary probes in tissue	Fixed lung tissue	No comparison available	
	Standard PCR	Primer specific amplification of *M. hyopneumoniae* nucleic acid	Multiple sample type	No comparison available	
	Nested PCR	Two primer specific amplifications of *M. hyopneumoniae* nucleic acid, using internal specific primers complementary to the first amplification nucleotide sequence	Nasal swab	+	(Kurth et al., 2002; Sibila et al., 2004; Marois et al., 2007, 2010; Fablet et al., 2010)
			Oro-pharyngeal swab	++	(Fablet et al., 2010)
			Tonsillar swab	++	(Sibila et al., 2004; Marois et al., 2007, 2010)
			Lung tissue	++	(Kurth et al., 2002)

			Tracheobronchial swab	+++	(Kurth et al., 2002; Marois et al., 2007, 2010; Fablet et al., 2010)
			Tracheobronchial lavage	+++	(Kurth et al., 2002; Marois et al., 2007; Fablet et al., 2010)
			Bronchial swab	+++	(Sibila et al., 2004)
	Real-time PCR	Primer specific amplification and fluorescent probe-based detection of *M. hyopneumoniae* nucleic acid	Oral fluids	-/+	(Pieters et al., 2017)
			Nasal swab	+	(Marois et al., 2010; Pieters et al., 2017)
			Tonsillar swab	++	(Marois et al., 2010)
			Tracheobronchial lavage	++	(Pieters et al., 2017)
			Lung tissue	++	(Marois et al., 2010)
			Laryngeal swab	+++	(Sievers et al., 2015; Pieters et al., 2017; Betlach et al., 2020; Sponheim et al., 2020)
			Tracheobronchial swab	+/+++	(Marois et al., 2010; Sievers et al., 2015)
			Deep-tracheal catheter	+/+++	(Betlach et al., 2020; Sponheim et al., 2020)
			Bronchial swab	++++	(Sievers et al., 2015; Betlach et al., 2020)
Detection of host antibody responses	Indirect ELISA	Two-step detection of serum antibodies with coated-antigen and anti-swine secondary antibody	Serum	+/++	(Ameri-Mahabadi et al., 2005; Erlandson et al., 2005; Fano et al., 2012; Gomes-Neto et al., 2014; Pieters et al., 2017)
	Indirect blocking ELISA	Serum and competitive reference antibodies compete for limited amount of antigen coated on the plate	Serum	+++	(Ameri-Mahabadi et al., 2005; Erlandson et al., 2005; Fano et al., 2012; Gomes-Neto et al., 2014; Pieters et al., 2017)

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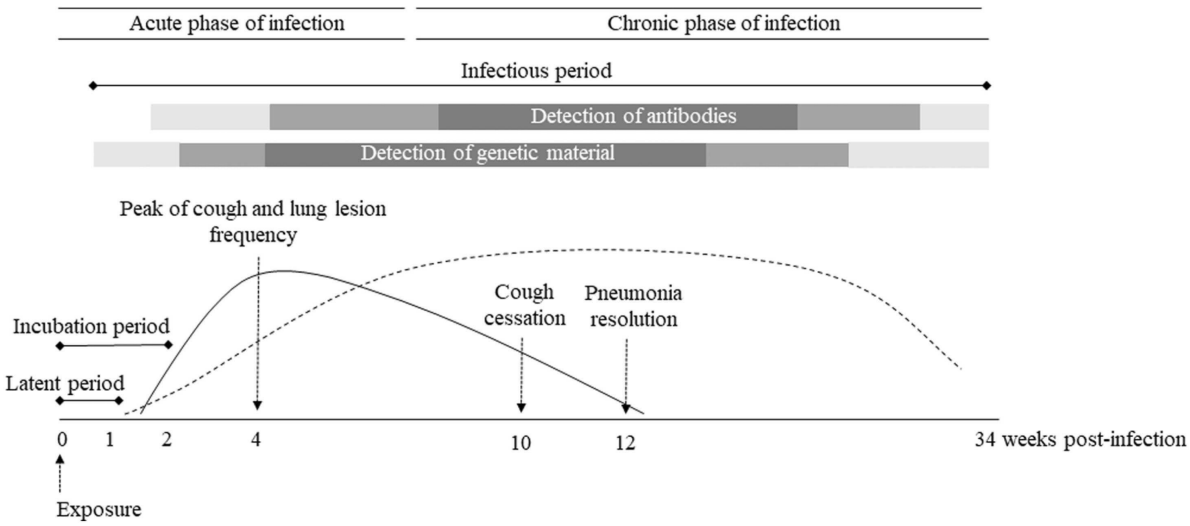
Figure legends

Fig. 1. Schematic overview of coughing, gross lung lesions and antibody dynamics, and detection of *M. hyopneumoniae* by PCR in clinical samples of pigs after experimental infection. Latent period: proposed time interval between infection and infectiousness (to transmission/infection of other pigs). Incubation period: proposed time elapsed between infection and appearance of clinical signs (onset of disease). The solid line represents coughing and lung lesion dynamics whereas the dotted line depicts *M. hyopneumoniae*-specific antibody dynamics. Gray shading symbolizes the likelihood of genetic material or antibody detection for *M. hyopneumoniae*, with darkest shade having the highest likelihood. Onset and duration of each parameter is proposed based on summarized information described in the literature.



- This review updates *M. hyopneumoniae* infection diagnosis and dynamics.
- Understanding *M. hyopneumoniae* infection dynamics is critical for diagnosis.
- Final diagnosis is based on clinical observations aligned with laboratory results.
- Laboratory tests are useful for detection of asymptomatic carriers.