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Review Article

Early Detection of *Mycoplasma hyopneumoniae* in Pigs under Field Conditions Using Tracheo-bronchial Swab Sampling

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Abstract

Tracheo-bronchial swab (TBS) sampling is a rapid, reliable and animal-friendly diagnostic sampling of the respiratory tract for detection of *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*). This mini-review gives an overview of different diagnostic approaches for *M. hyopneumoniae* from clinical signs, necropsy, microscopy to detection of the pathogen by immunohistochemistry, culture and PCR techniques. Subsequently, development, validation and obtained results are described using TBS for the early detection of *M. hyopneumoniae* in pigs with clinical symptoms of respiratory distress from 2 weeks of age onwards. Future perspectives on the application of TBS in diagnostic concepts, epidemiology and gilt adaptions protocols are also discussed.

Keywords: *Mycoplasma hyopneumoniae, Swab sampling*

Introdcution

Diagnostic approach for *Mycoplasma hyopneumoniae*

Diagnostic approach towards *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*), a major pathogen in PRDC with a large economic impact on profitability of modern pig production, remains a difficult issue under practical conditions [1-3]. Throughout the years, a broad variety of diagnostic methods have been developed and evaluated for diagnosis of *M. hyopneumoniae* under field conditions. These diagnostic methods range from assessment of clinical signs of coughing and a coughing index [4-9], macroscopic and microscopic lesions at necropsy, including immunohistochemical [10,11] and immunofluorescent identification of *M. hyopneumoniae* in lung tissue samples [12,13]. Additionally, different lung scoring systems at slaughter have been developed [14]. Laboratory diagnostics include serology from complement fixation tests to ELISA based on several specific adhesion factors [15-17] and molecular identification of the pathogen in several sample types, such as lung tissue, nasal swabs (NS) [18-20], laryngeal swabs (LS), broncho-alveolar lavage fluid (BALF) [21,22] and tracheo-bronchial swabs (TBS) [22-24]. However, under field conditions and for standard monitoring purposes, swine veterinarians and routine diagnostic laboratories have limited their approach mostly to clinical signs, macroscopic and microscopic evaluation upon necropsy, including lung lesion scoring at slaughter and serological monitoring. Clinical signs and lung lesions can only give a tentative diagnosis, which needs further confirmation with laboratory tests. Recently, the use of mobile systems (SOMO; SoundTalks NV, Leuven, Belgium) for cough recording at barn level with subsequent analysis of coughing patterns have been developed and validated under field conditions [2]. This innovative tool might support early *M. hyopneumoniae* diagnosis, although it still remains difficult to specifically differentiate coughing by *M. hyopneumoniae* from other major pathogens involved in PRDC, such as IAV-S, PRRSV and A. pleuropneumoniae. Therefore, even with a positive indication on clinical *M. hyopneumoniae*-indicative coughing, confirmation through pathogen identification remains crucial for further implementation of treatment or preventive measures. The early detection of *M. hyopneumoniae* through necropsy or lung lesion scoring systems at slaughter also remains under discussion. The lesions, namely purple to grey consolidated areas affecting predominantly the apical and middle lobes and eventually the cranial part of the diaphragmatic lobes, identified in both necropsy or slaughterhouse assessment are suggestive, but not pathognomonic for *M. hyopneumoniae* infection [2]. Other pathogens such as IAV-S or *P. multocida* should be considered as most probable differential diagnoses [1]. Moreover, since animals are dead or slaughtered at the moment of the diagnosis, the definition of 'early' detection cannot really be applied and curative or preventive measures will only have an effect on the next batches of animals within the same production system. For several decades, therefore, serological tests – more specifically ELISA – have been used to detect and monitor *M. hyopneumoniae* at herd level. Early studies have shown a serious delay between the initial clinical signs of coughing and the first detection of *M. hyopneumoniae* using ELISA of about 22 days [25]. This delay in seroconversion has recently been reconfirmed for two commercially available ELISA tests, demonstrating a minimum interval of 21 days before the first

incomplete seroconversion could be detected in both ELISA tests [3]. Moreover, substantial differences exist between different ELISA tests towards their reactivity following initial seroconversion [3,17] in naive animals. Therefore, serological monitoring can neither be considered as a tool for early *M. hyopneumoniae* detection under field conditions. Nevertheless, for routine farm monitoring and follow-up on changes in *M. hyopneumoniae* infection pattern due to adaptations of management or vaccination strategy, serological tests have clearly shown their value [26].

Tracheo-bronchial swab sampling – development and validation

Taking these considerations into account, a reliable and early detection of *M. hyopneumoniae* in living animals under field conditions is urgently needed in order to be able to confirm an upcoming infection as early as possible. This is crucial under modern pig farming conditions to efficiently apply curative and preventive measures to omit further spread of the pathogen within the farm. Especially lactating gilts with an active *M. hyopneumoniae* infection present in the farrowing room imply a major risk for further transmission of *M. hyopneumoniae* to their offspring, resulting in a fairly high number of *M. hyopneumoniae*-positive piglets at weaning [18-20,23,27-31]. However, until recently, most *M. hyopneumoniae* prevalence studies in piglets and sows have been conducted using a rather easily applicable sampling technique, namely NS [18-20,27-28,31]. More recently, others have applied BALF or lung tissue sampling to study *M. hyopneumoniae* prevalence around weaning age [29,30] (Table 1).

Tracheo-bronchial swab (TBS) sampling technique has been developed as a diagnostic tool to screen pigs for respiratory pathogens, such as *M. hyopneumoniae*, through sampling of live pigs without anesthesia at the level of the trachea-bronchial split [9,23]. A study comparing different sampling techniques for *M. hyopneumoniae* recovery in piglets has demonstrated that NS have 3.89 times less sensitivity in recovering *M. hyopneumoniae* from infected piglets as compared to the TBS technique, with LS and BALF in an intermediate position (1.39 and 1.09 times less sensitivity compared to TBS, respectively) [23]. Moreover, the amount of DNA material recovered from TBS samples was higher as compared to other diagnostic techniques in live pigs [32-34]. Recent research into welfare aspects of respiratory tract sampling demonstrated no additional stress when sampling pigs by TBS as compared to NS [35].

Tracheo-bronchial swab sampling – technical aspects

In order to perform TBS sampling in a comfortable way, fixation of the piglet using a rope and positioning the head in a hyperextensive position is key for success. The hyperextension is important to gain easy access to the glottis to subsequently go down the trachea. A mouth opener is positioned and the catheter is passed through the mouth down to the pharyngeal region, where a slight resistance might be observed at the level of the glottis. When the piglet stops screaming, the glottis will open for inhalation and the catheter can be moved further down the trachea to the level of the trachea-bronchial split. There, the catheter tip is turned around to collect as much mucus as possible. After retraction of the catheter, the tip (max. 10 cm) is collected in a sterile transport tube (10 mL) with 1 mL of sterile physiological saline (0.9% NaCl) and transported to the analytic laboratory [33,36] (Figure 1).

A systematic quality check can be performed during the live sampling procedure to assess for correct sampling location [37].

Tracheo-bronchial swab sampling – high detection through optimal DNA recovery

To further elaborate on the TBS sampling, performance of TBS was compared to NS and bronchial swabs (BS), BALF and lung tissue samples in relation to detection of *M. hyopneumoniae* [33]. Recovery of *M. hyopneumoniae* was overall highest (59.3%) in TBS, whereas other sampling techniques such as NS had a limited recovery rate (6.25%). All three post-mortem sampling techniques – BALF, lung tissue samples and BS – had a good recovery (46.7 to 51.0%), though as already stated earlier, for monitoring purposes, sampling techniques in live animals should be preferred. Although BALF can also be performed

Table 1: Studies of *M. hyopneumoniae* prevalence at various ages (expressed as % *M. hyopneumoniae*-positive piglets) using different sampling techniques.

Piglet age (weeks)	Prevalence (%) *M. hyopneumoniae*	Sampling technique	References
3	7.7-9.6	NS	Calsamiglia and Pijoan, 2000 [18]
3	2.6-13.2	NS	Ruiz et al., 2003 [27]
1-3	0.5-5.5	NS	Sibila et al., 2007 [19]
6-9	2.0-9.0	NS	Sibila et al., 2007 [28]
3	0.0-51.3	NS	Fano et al., 2007 [29]
3	10.6	BALF	Moorkamp et al., 2009 [29]
6	12.3	BALF	Moorkamp et al., 2009 [29]
2	2.0	Lung	Nathues et al., 2010 [30]
4-10	9.3	Lung	Nathues et al., 2010 [30]
3	10.7	NS	Villarreal et al., 2010 [31]
4	14.1	TBS	Fablet et al., 2012 [24]
2.5-3	3.9	NS	Nathues et al., 2013 [20]
2	1.1	TBS	Vangroenweghe et al., 2015a [33]
3-5	7.1	TBS	Vangroenweghe et al., 2015b [36]
6-11	10.9	TBS	Vangroenweghe et al., 2015b [36]
NS, nasal swab; BALF, broncho-alveolar lavage fluid; TBS, tracheo-bronchial swab.			

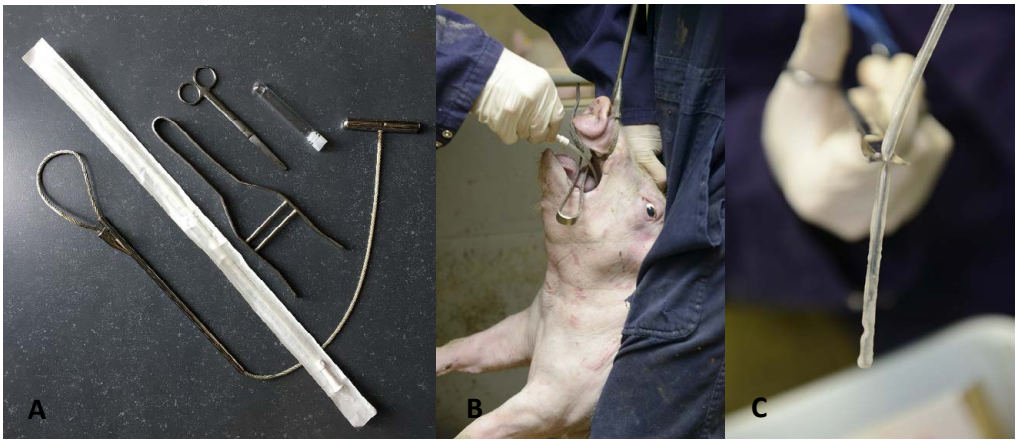

Figure 1: A. Set of materials needed for TBS sampling (from left under to right up), including nasal rope, sample catheter, mouth opener, scissors and a 10 mL transportation tube containing 1 mL of sterile physiological saline (0.9% NaCl) solution; **B.** Optimal piglet fixation and presentation to obtain a TBS sample; **C.** Sample catheter with mucus on the tip ready to cut in the transportation tube.

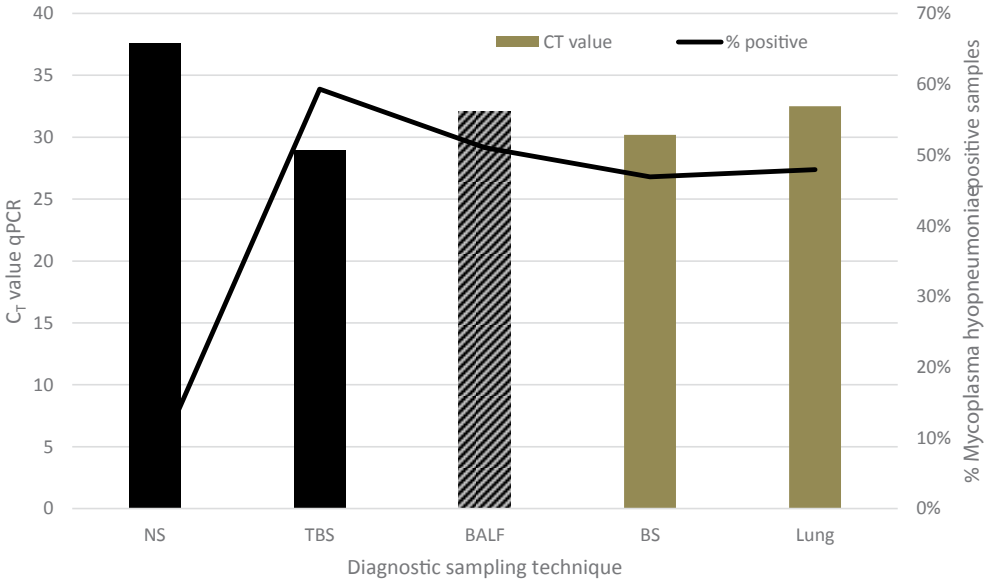

Figure 2: Comparison among different diagnostic sampling techniques combined with qPCR detection for *M. hyopneumoniae* in relation to estimated amount of DNA material (expressed as C_T value) and percentage of positively detected animals. Black bars indicate live sampling techniques grey bars indicate sampling techniques in dead animals. NS – nasal swab; TBS – trachea-bronchial swab; BALF – broncho-alveolar lavage fluid; BS – bronchial swab; Lung – lung tissue sample (adapted from Vangroenweghe et al., 2015a).

in live animals, to our opinion, BALF sampling technique has several disadvantages for live piglet sampling as compared to TBS. There is need to sedate animals before the procedure and a more rigid catheter is needed for the intervention, which might harm the tubular structure of trachea or larger bronchi. A major concern is the variability in recovered fluid volume from the lavage in different piglets, which might interfere with the detection limit of the *M. hyopneumoniae* qPCR used in these samples. Evaluation of the level of recovery of *M. hyopneumoniae* (based on the C_T values in the qPCR) for the live piglet sampling techniques, TBS samples resulted in the lowest C_T values, whereas NS had a significantly lower yield in the qPCR. Only BS samples revealed a comparable recovery to TBS with lung tissue samples and BALF obtaining intermediate results (Figure 2).

However, these samples – except for BALF – can only be taken in dead animals and are therefore not suitable for the intended purpose of live monitoring to detect *M. hyopneumoniae* at an early stage. Another field study on fattening pigs with clinical signs typical for *M. hyopneumoniae* compared three sampling techniques – TBS, LS and the pharyngeal spoon technique – for their detection of *M. hyopneumoniae* [32,34]. Whereas the LS collects mucus at the level of the larynx, using a speculum and a long dry cotton swab, the pharyngeal spoon technique collects mucus just cranial to the laryngeal region, using an elongated spoon. In this study, fattening pigs sampled with all three techniques were positive for *M. hyopneumoniae*. However, TBS consistently had the lowest C_T value, indicating the highest amount of collected genetic material (DNA) from the pathogens present at the level of the respiratory tract (Figure 3).

Tracheo-bronchial swab sampling and early detection of *M. hyopneumoniae*

Early detection of *M. hyopneumoniae* infection is essential in case of SPF breeding herds. In two recent case reports, TBS-qPCR

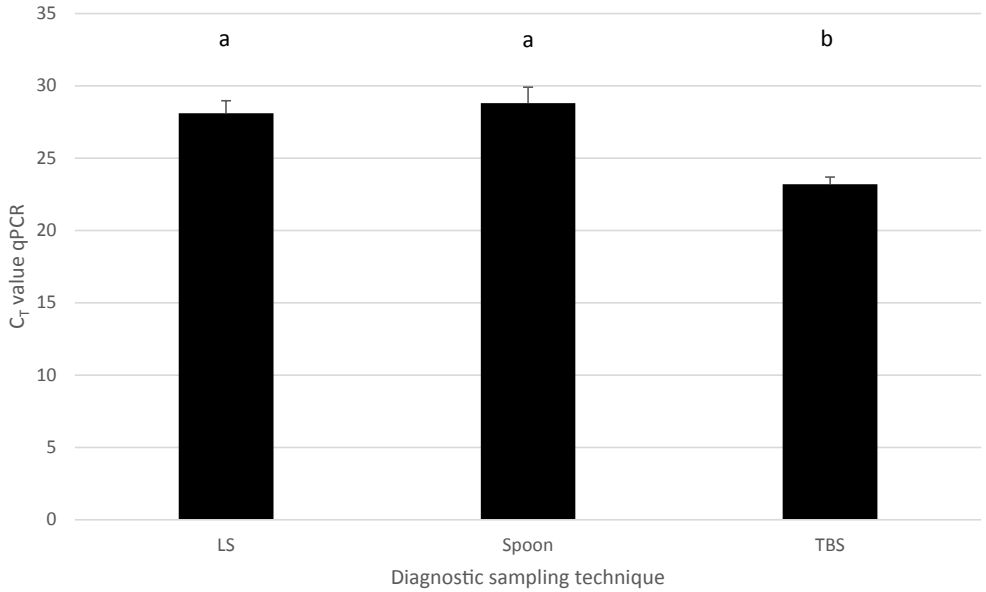

Figure 3: Comparison among different diagnostic sampling techniques combined with qPCR detection for *M. hyopneumoniae* in relation to estimated amount of DNA material (expressed as C_T value ± SEM). Different letters in superscript indicate significant differences ($P = 0.046$). LS – laryngeal swab; Spoon – deep pharyngeal spoon technique; TBS – tracheo-bronchial swab (adapted from Betlach et al., 2018 and Vangroenweghe et al., 2018).

has been demonstrated to detect *M. hyopneumoniae* at the moment where serology and lung lesions scoring still remained negative. Therefore, TBS should be the preferred option to screen a farm for *M. hyopneumoniae*-free certification and to capture early introduction of *M. hyopneumoniae* [38,39].

Tracheo-bronchial swabs sampling: future perspectives and applications

1. *Mycoplasma hyopneumoniae* diagnosis should be further elaborated, especially towards the optimization of sampling protocols for specific field conditions, such as early detection of the pathogen, certification of freedom of disease or other diagnostic approaches. Nowadays, it becomes more and more important to have a rapid and reliable diagnosis and therefore PCR tests should be available wherever possible and needed. Recent evolutions towards on-farm test applications (LAMP and helicase-dependent amplification technology [40] and on-site PCR kits) should be further developed and validated in order to have immediate results. This would help to determine an efficient treatment, which could result in less antibiotic use as compared to treatment of animals already suffering from *M. hyopneumoniae* in a more chronic stage of the disease.

2. In the near future, technology already applied in clinical and food microbiology will become readily available for use in veterinary diagnostic laboratories. Besides automation in the form of sample-to-result instrumentation for qPCR assay, which reduces labor and limits the risk for contamination during manipulation, multiplex tests are now available that enable single specimens to be evaluated for the presence of multiple pathogens associated with various clinical syndromes. Digital PCR and next-generation sequencing will push the landscape of molecular diagnostics further, allowing for analysis of complex, polymicrobial specimens and enabling accurate quantification of organisms present at very low levels (< 0.01% of the microbial consortium) in a specimen [40]. Another promising technique is Matrix-Assisted Laser Desorption Ionization-Time to Flight MS (MALDI-TOF), which enables the identification of bacteria and other microorganisms by non-fragmenting or 'soft ionization' techniques [40]. If these technologies become available for *M. hyopneumoniae* diagnostics, faster and more accurate diagnosis can be performed, especially if combined with a sampling technique that provides a high yield of pathogenic material, such as TBS.

3. TBS combined with improved isolation methods – such as automation of primary processing and plating, coupled with initial culture examination aided by high-resolution optics [40] – could help to improve collection of field strains to further monitor antimicrobial sensitivity (MycoPath; [41]) which in turn would assist in reducing ineffective treatments against *M. hyopneumoniae* in case of antimicrobial resistance.

4. *Mycoplasma hyopneumoniae* epidemiology remains a challenging domain of research, which still undergoes interesting new evolutions, especially within the field of early gilt exposure to *M. hyopneumoniae* in order to prevent excretion during lactation which might affect her piglets' *M. hyopneumoniae* infection status. Recently, several studies have been performed to evaluate the optimal transmission of *M. hyopneumoniae* from infected to naive gilts through direct contact [42,43] or using more challenging techniques such as intra-tracheal inoculation [43] or even aerosol applications of lung homogenate [43,44]. In these cases, *M. hyopneumoniae* transmission success is crucial for future stability of on-farm *M. hyopneumoniae* infection status (Figure 4).

Figure 4: Gilt M. hyopneumoniae exposure timeline based on the 254 days of M. hyopneumoniae detection from initial infection onwards [45] (adapted from [43]).

Table 2: Overview of *M. hyopneumoniae* diagnostic sampling options for specific practical situations, combined with the average interval (expressed in days) between infection moment and first possible detection moment, the positivity at first detection (expressed as % *M. hyopneumoniae*-positive samples) and estimated amount of DNA material collected with each specific diagnostic sampling option. Interval infection *vs.* positivity was based on Pieters *et al.*, 2017.

Diagnostic sampling option and detection technique	Status *M. hyopneumoniae* end of fattening period	Early *M. hyopneumoniae* infection	Acute outbreak of *M. hyopneumoniae*	Interval infection – positivity (days/% positive) *M. hyopneumoniae*	Estimated amount of DNA material collected
Nasal swab - PCR	-	±	±	5 d (14%)	+
BALF - PCR	+	++	++	5 d (19%)	++
TBS - PCR	++	+++	+++	5 d (80%)	+++
Oral fluids - PCR	+	-	-	9 - 28 d (67%)*	+
Serology - ELISA	++	-	-	21 d (12%)	N/A
Lung lesions scoring	++	-	-	N/A	N/A

*Results obtained at pen level.
N/A – not available.

5. Under these conditions, TBS could be applied to check for inoculation success in the earliest possible way. Interestingly, the above stated inoculation methods are still under development and evaluation concerning the inoculum dose, the number of subsequent exposure events and the efficacy to transmit *M. hyopneumoniae* to all animals exposed [45,46]. Currently, no standard protocol is available and therefore, additional research should be carried out to determine the minimal exposure in order to obtain colonization of the respiratory tract. For these purposes, TBS could be applied as an early detection tool to assess inoculation success.

6. Besides the deliberate or accidental exposure of gilts to *M. hyopneumoniae*, checking the actual *M. hyopneumoniae* status in farms is also of major importance. A recent survey on *M. hyopneumoniae* gilt introduction in conventional farms revealed major room for improvement, especially towards increasing the knowledge on the *M. hyopneumoniae* status of replacement gilts at the moment of arrival into the quarantine/ adaptation facilities [47]. Again, early *M. hyopneumoniae* detection is crucial in order to adapt the preventive measures to the current health status at gilt delivery. In case of *M. hyopneumoniae*-positive gilts, curative treatment might be considered, whereas in case of a *M. hyopneumoniae*-negative status, correct vaccination with a *M. hyopneumoniae* vaccine might be considered to rapidly boost the gilt's immunity before exposure at introduction into the existing conventional *M. hyopneumoniae*-positive sow population [48].

7. Finally, as piglet infection status for *M. hyopneumoniae* at weaning is a leading indicator towards the percentage of lung lesions at slaughter [28], it still remains of major importance to monitor the *M. hyopneumoniae* infection status of piglets on a regular basis. Especially since it has been clearly demonstrated that between-batch variability might be high and unpredictable based on the previous batch [28]. Within these monitoring schedules focused on early detection of *M. hyopneumoniae*, TBS could play a predominant role in the near future. Knowledge on the 'information' gap between *M. hyopneumoniae* detection using serology and TBS is also key in case of SPF certification programs for breeding herds producing *M. hyopneumoniae*-free gilts, in order to guarantee continuous delivery of *M. hyopneumoniae*-free reproduction gilts to end-customers or for their internal replacement [36-37].

Conclusion

Early diagnosis of *M. hyopneumoniae* in young piglets and at first clinical symptoms can be easily performed using TBS sampling. A comprehensive overview of the different diagnostic options and their potential for early diagnosis are given in Table 2. Different diagnostic scenarios are given: *M. hyopneumoniae* status at the end of the fattening period, detection of early *M. hyopneumoniae* infection in piglets and in case of acute respiratory disease. Tracheo-bronchial swab sampling has the potential to both detect *M. hyopneumoniae* at an early stage in life and during infection, especially due to the collection of a sufficient high amount of pathogen material from the respiratory tract during sampling. This confirms TBS combined with qPCR as the preferred method for *M. hyopneumoniae* diagnostics.

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