RESEARCH REPORT

SWINE HEALTH

Title: Early detection of *Mycoplasma hyopneumoniae* infections in live pigs: Comparison of current methods and development of new assays – **NPB #12-047**

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Industry summary:

Detection of *M. hyopneumoniae* infections in live pigs is difficult for the producer, the field veterinarian and the diagnostician. This study was designed and performed with the objective to compare various samples types and diagnostic assays for their sensitivity to detect pigs infected with *M. hyopneumoniae* during the first 4 weeks after experimental infection. All testing and comparisons was performed in a side-by-side fashion. Twenty three 8-week old *M. hyopneumoniae* free pigs were employed in this investigation. Two pigs were housed in an experimental room and served as non-infected controls. A group of 21 pigs were divided into three experimental rooms (7 pigs per room), were intra-tracheally inoculated with a strain of *M. hyopneumoniae* of moderate virulence, and were sampled a total of 7 times during 4 weeks, being 0, 2, 5, 9, 14, 21 and 28 days after inoculation. Blood samples, nasal swabs, laryngeal swabs, trachea-bronchial lavages and oral fluids were collected every time pigs were sampled. Bronchial swabs and lung lavages were collected at the last day of sample collection, after euthanasia. Also, lung lesions were blindly scored in all pigs after euthanasia. Control pigs remained negative to *M. hyopneumoniae* throughout the study. Genetic material from *M. hyopneumoniae* was detected in various percentages of the experimentally infected pigs on days 5, 9, 14, 21 and 28 after experimental infection by means of nasal swabs, laryngeal swabs and trachea-bronchial lavages. *M. hyopneumoniae* was detected in oral fluids obtained from 2 of the 3 rooms housing infected pigs at 9 and 28 days after infection. Commercial ELISA assays detected experimentally infected pigs at days 21 and 28 after infection. The sensitivity of the various commercial ELISA assays to detect *M. hyopneumoniae* antibodies used in this investigation was similar. IgM was detected in experimentally infected pigs at 9, 14 and 21 days after infection. IgA was detected in trachea-bronchial lavages at 28 days after infection, but was not detected in oral fluids at any sampling point. *M. hyopneumoniae* specific proteins in serum were only observed in a small proportion of pigs at 28 days after infection. In summary, the results of this study suggest that the combination of PCR testing in laryngeal swabs was the most sensitive tool for *M. hyopneumoniae* detection in live pigs during the first 4 weeks after infection, followed by tracheo-bronchial lavages, nasal swabs and oral fluids.

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Keywords:

Mycoplasma hyopneumoniae, in vivo, real-time PCR, ELISA, laryngeal swabs, diagnostics

Scientific abstract:

In vivo detection of *M. hyopneumoniae* infected animals constitutes a difficult diagnostic task. The location of the pathogen in the lower sections of the respiratory tract makes it inaccessible for direct pathogen detection, and the shedding in the upper sections of the respiratory tract seem to be late and non-continuous. Moreover, specific antibody detection is of slow onset and sometimes unspecific, as represented for the detection of false positive animals and presumably *M. hyopneumoniae* free herds. Various sample types and testing methods have been developed over time in an effort to achieve early diagnosis of *M. hyopneumoniae* infections. However, comparisons have been performed under different conditions which make it difficult to establish the most accurate diagnostic tool. Therefore, the objectives of this study were to identify the most accurate current diagnostic tools for early detection of *M. hyopneumoniae*, through a side-by-side comparison. And to develop alternatives to current diagnostic procedures for early detection of *M. hyopneumoniae* in live pigs. Twenty three 8-week old pigs obtained from a source negative to *M. hyopneumoniae* and other major pathogens were employed in this investigation. Pigs were randomly allocated to 2 experimental groups. One group of 2 non- infected pigs considered as a negative control and a group of 21 experimentally infected pigs. Pigs in the control group were intra-tracheally inoculated with 10mL of sterile Friis medium, while pigs in experimentally infected group were inoculated with 10mL of Friis medium containing $1x10^5$ CCU/mL of *M. hyopneumoniae* strain 232 from lung homogenate. All pigs were sampled at 0, 2, 5, 9, 14, 21, and 28 days post inoculation (dpi) by means of nasal swabs, laryngeal swabs, trachea-bronchial lavages, oral fluids and blood samples. Bronchial swabs and lung lavages were collected after euthanasia on day 28 dpi. Gross and microscopic lesions were blindly scored in all animals after euthanasia. Negative control pigs were negative to *M. hyopneumoniae* by all tests and at all sampling dates. All pigs in the experimentally infected group were detected positive to *M. hyopneumoniae* by various sample types and at various dpi. *M. hyopneumoniae* was not detected in the experimentally inoculated pigs at 0 and 2 dpi. PCR detection of *M. hyopneumoniae* was achieved with greater sensitivity by means of laryngeal swabs, followed by trachea-bronchial lavages, nasal swabs and oral fluids. Antibody detection using commercial ELISA kits was observed at 21 and 28 dpi, with no statistical significance among the tests used. *M. hyopneumoniae* specific IgM was detected as early as 9 dpi in a small percentage of experimentally infected pigs, increasing at 14 dpi and decaying at 21 and 28 dpi. IgA antibodies were not detected in oral fluids at any point after infection, but were detected in trachea-bronchial lavages at 28 dpi. C reactive protein detection was similar between pigs of the infected and non-infected groups. Specific protein detection by Western blot showed an undifferentiated pattern in most pigs, regardless of their experimental group, with a small proportion of pigs showing specific bands attributable to a *M. hyopneumoniae* specific response. Under the conditions of this study, detection of *M. hyopneumoniae* after experimental infection was achieved earlier and with greater sensitivity by using laryngeal swabs in combination with real-time PCR testing, compared to the other samples and assays used in this investigation.

Introduction:

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) infections continue to be a major problem for the swine industry worldwide (Sibila et al., 2009). The detrimental effect of this bacterium in swine populations is not only due to the pneumonia and the associated decreased performance caused by the pathogen itself, but also by the animal's predisposition to secondary respiratory infections of bacterial and viral origin. Therefore, *M. hyopneumoniae* infections cause significant losses to swine producers each year.

Disease control, typically attempted through vaccination and/or medication of growing pigs, is expensive and does not consistently work in all herds (Maes et al., 2008). For these reasons, during the last few years many veterinarians and producers have chosen to completely eliminate *M. hyopneumoniae* from their herds (Stark, 2007; Schneider, 2008; Yeske, 2010). This has resulted in an increased number of *M. hyopneumoniae*-free herds. Continuous serological surveillance of negative herds by serum ELISA is a good strategy that most negative herds have in place. However, these ELISA-based surveillance protocols have two main limitations:

- Current ELISAs lack the ability to detect early infections. Pigs do not become ELISA-positive until 14-21 days post-infection, under experimental conditions. Therefore, there is a window of time in which animals can be infected and test negative. During this time, *M. hyopneumoniae* infection can be detected in the lower respiratory tract by PCR, however this usually requires euthanizing the animal. Other methods that do not require euthanizing the animal have low sensitivity, as is the case for nasal swab PCR, or have not been fully validated (tracheobronchial swab, oral fluids). Methods to detect *M. hyopneumoniae* in tracheobronchial samples (lavages or swabs) have been recently described (Marois et al., 2007; Fablet et al., 2010). However, obtaining these samples requires some expertise. On the other hand, *M. hyopneumoniae* can be detected in oral fluids, which is an easy sample to collect. However, information on the sensitivity of oral fluids PCR for acutely infected pigs is currently lacking.

- Current ELISAs produce false positives and no good confirmatory test is available. Usually another ELISA is used as a confirmatory test, which does not completely clear the uncertainty created by false positives. Current ELISAs are good screening tests, with approximately 98.5% specificity (1.5% false positive rate). This specificity is considered good for a screening test and is comparable to other tests such as PRRSV ELISA in pigs or HIV ELISA in humans. Typically, in surveillance protocols for negative populations, samples testing positive for the screening test are further tested with a highly specific, confirmatory test. For example, PRRSV ELISA positive results in pigs are confirmed by IFA, and HIV ELISA positive results in humans are confirmed by Western Blot. The problem in *M. hyopneumoniae* surveillance protocols is that there is no good confirmatory test available. In many instances, the question of whether the original positive result was a false positive or the true early manifestation of a recent infection of the herd remains unanswered. In replacement breeding stock populations confirmation of the status of a population following questionable positive ELISA results often requires costly euthanasia of high value animals for tissue submissions. In addition, accurate detection of an early infection to prevent downstream spread is even more important.

The lack of tools to efficiently detect *M. hyopneumoniae* early infections in live pigs, further complicates this scenario. *M. hyopneumoniae* diagnosis in live pigs, and especially during early stages of infection is very challenging. Therefore, there is a growing need for surveillance tools that complement ELISA tests to demonstrate that populations remain negative overtime. In response to this need, several sampling and diagnostic tools to be used in live pigs have been proposed, for example, oral fluids, tracheal swabs, and tracheo-bronchial lavages. However, some of these techniques have not been properly evaluated and they have not been compared side by side.

In this investigation we propose a side by side comparison of sampling and testing tools currently available for detection of *M. hyopneumoniae* during the early phase of infection, namely: 1. Detection of IgG in serum by two ELISA kits: HerdCheck® *M. hyopneumoniae* antibody test kit (IDEXX Laboratories) and OXOID (formerly Dako) kit (Feld et al., 1992). 2. Detection of genetic material by PCR (VetMAX™ Life Technologies Inc.; Strait et al., 2008) in nasal and tracheal swabs, oral fluids and tracheobronchial lavage fluid (TBLF), and 3. Detection of specific *M. hyopneumoniae* proteins by Western Blot as described by Ameri et al., (2006), in serum samples from ELISA positive animals.

Also, we propose the use of three alternative procedures: 1. Modified ELISA for detection of IgA antibodies in oral fluids and TBLF. A recent publication by Feng et al., (2010) showed that specific anti-*M. hyopneumoniae* IgA was detected in field samples from herds naturally infected with M. hyopneumoniae. 2. Modified ELISA for detection of IgM in oral fluids, TBLF and serum. We propose the use of this modified ELISA for IgM detection instead of IgG, as IgM is the first antibody to appear after antigen exposure, and 3. Detection of porcine C reactive protein in serum by ELISA. C reactive protein is a positive acute phase protein that can be detected in serum during inflammatory processes. Recent data from Parra et al., (2006) showed an association of high levels of C reactive protein in pigs from herds naturally infected with *M. hyopneumoniae*. Moreover, preliminary (unpublished) data from our research group has shown a sustained increase of C reactive protein detection in serum samples in conventional pigs from day 5 until day 21 (last time measured) after experimental infection with *M. hyopneumoniae*.

Our goal is to compare the current and alternative tools and to prepare guidelines for diagnosis of *M. hyopneumoniae* infections during the early stages of infections. We are convinced that this approach will improve the likelihood of early detection and confirmation of *M. hyopneumoniae* infection and will generate important information that will aid diagnosis.

Objectives:

The overall objective of this investigation was to improve the diagnostic tools available to detect *M. hyopneumoniae* infections. Therefore, three aims were proposed to achieve this objective:

1. To identify the most accurate current diagnostic tools for early detection of *M. hyopneumoniae*, through a side-by-side comparison.

2. To develop alternatives to current diagnostic procedures for early detection of *M. hyopneumoniae* in live pigs.

3. To generate guidelines for identification of *M. hyopneumoniae* infected animals during early phases of infection based on the information obtained from the previous two objectives.

Materials and methods:

Pigs and housing: Twenty three 8-week old conventional pigs were obtained from a source known to be negative to major swine pathogens, including *M. hyopneumoniae* and PRRSV. Pigs were housed in the Isolation Units at the St. Paul Campus, of the University of Minnesota. Strict biosecurity measures were maintained in order to avoid animal contamination with other pathogens.

Experimental design: Pigs were ear-tagged at arrival and divided in two groups of 21 and 2 pigs that were housed separately. Each pig in the group of 21 pigs was inoculated with *M. hyopneumoniae* 7 days after arrival. Pigs inoculated with *M. hyopneumoniae* were housed in 3 rooms of 7 pigs each. The remaining two pigs were inoculated with un-inoculated Friis medium and served negative controls. However, samples from these two pigs, collected in the same manner as for the inoculated pigs, were used to control for contamination during sample processing and testing. Each pig was sampled at 0, 2, 5, 9, 14, 21 and 28 days post-inoculation (dpi). At each sampling time, serum, nasal swab, laryngeal swab, TBLF and oral fluids were collected. All animals were euthanized at 28 dpi and *M. hyopneumoniae* infection was assessed by gross and hystopathological lesions and by bronchial swab PCR.

M. hyopneumoniae experimental infection: Twenty pigs were intra-tracheally inoculated with 10 ml of a lung homogenate containing 1 x 10^5 CCU/ml of *M. hyopneumoniae* strain 232. Strain 232 has been fully sequenced (Minion et

al., 2004) and constitutes a U.S. reference strain of moderate virulence. Pigs in the control group were sham inoculated with Friis media.

Sample size calculation: The sample size of 10 pigs for the inoculated group was sufficient to identify significant differences between the proportion of positive samples obtained with different tests, assuming expected proportions of 0.5 and 0.9, confidence level of 95% and power of 80%.

Animal sampling: Pigs were sampled a total of 8 times during the course of this investigation. Sampling dates will be: 0, 2, 5, 9, 14, 21 and 28 dpi. The following samples were collected each sampling date: (except for bronchial swabs, which were only obtained after euthanasia).

1. Blood, obtained by venipuncture and collected in BD Serum Vacutainers® (Becton Dickinson and Company, Franklin Lakes, NJ, USA).

2. Nasal swabs: Sterile swabs (BBL™ CultureSwab™, Sparks, MD, USA) were used. The applicator was inserted deep into each nostril and rotated clock-wise and counter clock-wise.

3. Laryngeal swabs: The larynx was swabbed with a sterile swab (BBL™ CultureSwab™, Sparks, MD, USA). A snare and a mouth gag were used for pig restrain, while a laryngoscope was used to guide the introduction of the swab in the larynx.

4. Tracheo-bronchial lavage fluid (TBLF): Were collected by tracheal aspiration as described by Pommier and Abiven (1993). Briefly, 10 mL of saline solution were introduced into the trachea with a sterile catheter and immediately aspirated.

5. Oral fluids: Room based oral fluids were collected, as described by Prickett and Zimmerman (2010). Briefly, a 70-cm length of 3-strand twisted rope was untwisted and suspended at shoulder height from a metal bar in the room. The rope was left in the room so that the pigs could chew on it for 20-30 min.

6. Bronchial swabs: Were obtained as previously described by Pieters et al., (2009). Briefly, sterile swabs (BBL™ CultureSwab™, Sparks, MD, USA) were rotated in the bronchia and then moved up and down. Bronchia were exposed for sampling purposes using disinfected scissors and forceps.

- Sample processing:

1. Blood samples were separated to obtain serum that was aliquoted and prepared for the following testing:

- IDEXX and Oxoid ELISA assays: Samples were submitted to the Veterinary Diagnostic Laboratory of the University of Minnesota (VDL).

- Western blot: SDS-polyacrylamide gel electrophoresis (PAGE) were performed as described by Laemmli (1970). *M. hyopneumoniae* was cultured in modified Friis media at 37°C. Culture was centrifuged and pelleted cells were washed twice with PBS, sonicated for 5 min, and heated for 3 min in boiling water with sample buffer (0.06 M Tris-HCl [pH 6.8], 3% SDS, 5% mercaptoethanol, 10% glycerol, 0.01% bromophenol blue). Fifty micrograms of whole-cell lysate protein, as determined by the Bradford protein assay, were loaded in each lane of 7.5 or 10.0% acrylamide gel. Then, vertical electrophoresis was performed at 25 mA for 4 to 6 h. Proteins separated in gels were transferred by semidry electrophoresis onto nitrocellulose membranes with a Polyblot transfer system (American Bionetics, Hayward, CA) in a discontinuous transfer buffer, following manufacturer's instructions. The transfer was performed at 450 mA for 30 min. Nonidet P-40. Membrane was washed 3 times with PBS-T and was allowed to react for 1 hour with individual serum samples diluted 1:40 in 1% BSA in PBS-T. The membrane was washed with PBS-T 3 times for 5 minutes each, and

horseradish peroxidase (HRP)-conjugated goat antiswine IgG (H + L)e diluted 1:1,000 in PBS-T was used to detect the serum antibodies that had bound to the antigens. After incubation for 1 hour, the membranes was washed again twice for 5 minutes with PBS (pH 7.2) to remove the detergent. The 3,39-diaminobenzidine tetra-hydrochloride liquid substrate system was used to develop the color reaction.

- C reactive protein: C-reactive protein in serum was measured using the PHASE® (Tridelta Development Ltd, Maynooth, Ireland) ELISA assay kit following the manufacturer's instructions.

- Modified ELISA for IgM: Commercial *M. hyopneumoniae* specific ELISA kit for detection of IgG was modified for detection of IgM in serum by using a conjugate for an anti-pig IgM (conjugate was purchased from Kirkegaard and Perry Laboratories, Inc).

All serum samples were processed right after collection and aliquots were stored at -80°C until analysis, for the assays that were not performed immediately.

2. Nasal, laryngeal and bronchial swabs were submitted to the VDL for DNA extraction and PCR testing.

3. TBLF: Suspensions were centrifuged at 5000 x g for 30 min and the pellet was suspended in 2 ml of PBS. Suspended pellet was submitted to the VDL for DNA extraction and PCR testing. The supernatant was stored at -80°C until processed for IgM and IgA detection.

4. Oral fluids: The rope was placed inside a plastic storage bag, and the oral fluids were wrung out. The bag was sealed, and a bottom corner of the bag was cut off with scissors to pour the contents into a 50-ml conical centrifuge tube. Tubes were centrifuged at 2,272 X g for 30 min. Centrifuged oral fluids were submitted to the VDL for DNA extraction and *M. hyopneumoniae* PCR testing. An aliquot of the oral fluids was stored -80°C until processed for IgM and IgA detection.

DNA from nasal, laryngeal and bronchial swabs, BALF and oral fluids was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). Extracted DNA was assayed for genetic material detection by Real Time PCR VetMAX™ (Life Technologies Corporation, Carlsbad, CA, USA) with *M. hyopneumoniae* specific reagents and controls.

After euthanasia, macroscopic lung lesion evaluations were performed as a single blind study. Lung lesions were scored based on the percentage of lung covered in lesions suggestive of *M. hyopneumoniae* infection, as previously described (Pointon, 1999). Microscopic lung lesion evaluation were done using the fixed lung tissues collected at euthanasia. Samples were fixed in 10% buffered formalin, embedded in paraffin, sectioned onto slides and stained with hematoxilin/eosin for evaluation through an optic microscope.

- Data analysis: The percentage of positive pigs for each diagnostic procedure was compared with McNemar tests for paired samples at each sampling time.

Results:

Successful *M. hyopneumoniae* infection

Effective infection of the experimentally inoculated animals was demonstrated by parallel interpretation of diagnostic tests including bronchial swab PCR and scoring microscopic lung lesions suggestive of *M. hyopneumoniae* infection. Strict biosecurity practices were applied during the study and lack of cross-contamination was confirmed by the *M. hyopneumoniae* free status in the negative control pigs throughout the study.

Detection of *M. hyopneumoniae* by real-time PCR

The presence of *M. hyopneumoniae* genetic material was evaluated by means of real-time PCR in nasal swabs, laryngeal swabs, tracheo-bronchial lavages, and oral fluids in samples collected at 0, 2, 5, 9, 14, 21 and 28 dpi. Negative control pigs remained negative for *M. hyopneumoniae* detection throughout the study. The genetic material detection in the experimentally infected pigs is shown in Figure 1. All pigs in the experimentally infected group were negative for *M. hyopneumoniae* at 0 and 2 dpi. PCR detection of *M. hyopneumoniae* occurred for the first time at 5 dpi in 81% of laryngeal swabs, 19% of trachea-bronchial lavages and 14% of the nasal swabs. Detection of *M. hyopneumoniae* by PCR in laryngeal swabs was greater than any other sample type at all dpi when positive pigs were detected. Significant differences in the percentage of pigs detected positive to *M. hyopneumoniae* by laryngeal swabs compared to nasal swabs were obtained at 5, 9, 14, and 21 dpi (*p*-value 0.001 or less). Laryngeal swabs were statistically more sensitive than trachea-bronchial lavages at 5, 9 and 28 dpi (*p*-value <0.05). *M. hyopneumoniae* genetic material was detected in a greater percentage in trachea-bronchial lavages compared to nasal swabs at 5, 9, 14, 21 and 28 dpi, being statistically significant from 9 dpi onwards (*p*-value <0.05). Detection of *M. hyopneumoniae* DNA in nasal swabs showed the lowest sensitivity compared to laryngeal swabs and trachea-bronchial lavages, until it increased at 28 dpi. Detection of *M. hyopneumoniae* DNA in oral fluids was observed in 2/3 rooms at 9 and 28 dpi.

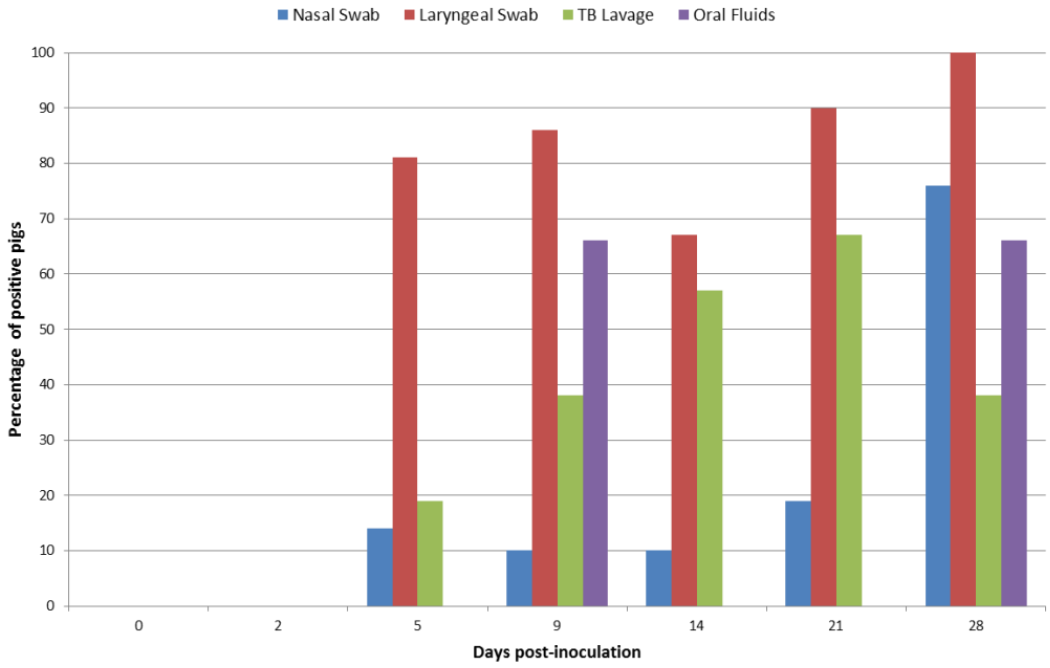

Figure 1. Detection of *M. hyopneumoniae* DNA by PCR in samples collected in vivo during 28 dpi. A total of 21 experimentally infected pigs are represented in this figure.

Detection of *M. hyopneumoniae* specific antibodies in serum:

Antibodies to *M. hyopneumoniae* were assayed in serum samples from all pigs in the study. Negative control pigs were detected as negative throughout the study. A proportion on the experimentally infected pigs seroconverted to *M. hyopneumoniae* after inoculation. Specific antibodies in serum were detected by an ELISA for IgM and commercially available ELISA kits for IgG detection. Seroconversion results in the experimentally infected pigs are shown in Figure 2. IgM antibodies to *M. hyopneumoniae* were detected in pigs at 9 dpi, peaked at 14 dpi and waned at 21 and 28 dpi. IgG antibodies were detected for the first time at 21 dpi and showed an increase in the number of pigs seroconverting at 28 dpi. Three different commercial ELISA assays for IgG were used in this investigation, namely Idexx, Oxoid and Biochek. Seroconversion results from all 3 ELISA assays were similar (*p*-value >0.05)

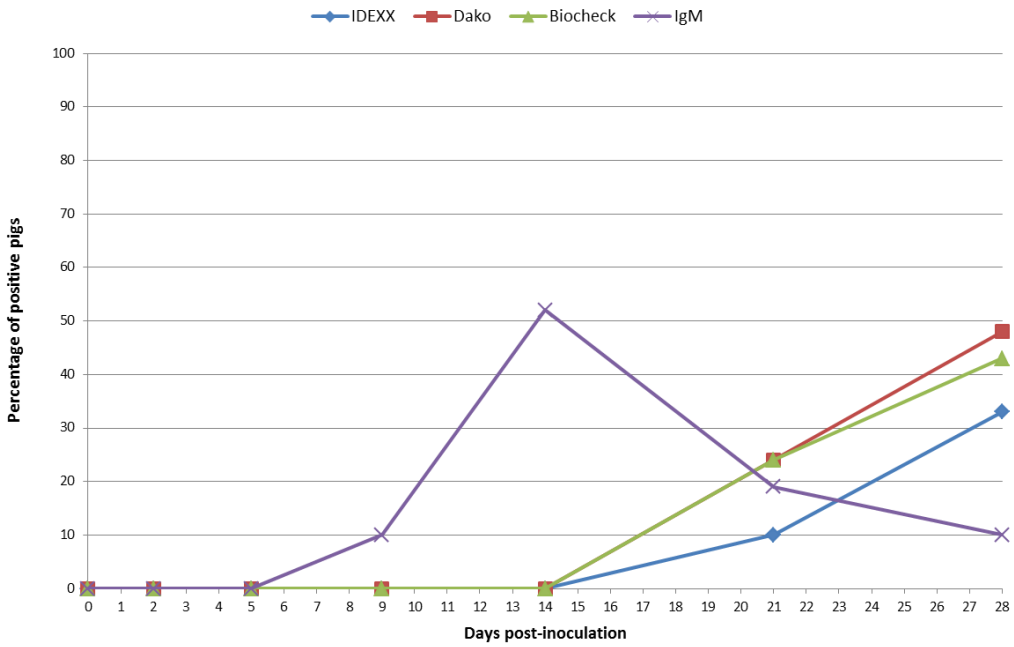

Figure 2. *M. hyopneumoniae* specific antibodies were detected in the experimentally infected pigs by various ELISA assays. IgM was detected by modifying the Idexx platform. IgG was detected with 3 different commercial ELISA kits.

Detection of *M. hyopneumoniae* specific antibodies in various fluids:

M. hyopneumoniae specific antibodies were assayed in fluids other than serum, specifically in oral fluids and trachea-bronchial lavages at 0, 2, 5, 9, 14, 21 and 28 dpi, and in lung lavage post-mortem (28 dpi; Figure 3). IgA antibodies for *M. hyopneumoniae* were not detected in oral fluids at any sampling point, while IgA was detected in trachea-bronchial lavages at 28 dpi. IgA and IgG positive pigs were detected by means on assaying the lung lavage post mortem.

Detection of acute phase proteins:

A commercially available ELISA kit was used to detect C reactive protein in the serum of pigs in this investigation. Results of C reactive protein detection for all experimental groups is shown in Figure 4. Briefly, the detection of C reactive protein in negative control pigs was similar to the one of experimentally infected pigs. Both groups of pigs had a similar baseline C reactive protein level, which increased after the inoculation, in both groups, regardless of the presence of the infectious material only in the experimentally infected group. The level of C reactive protein peaked during the first days after inoculation and was at baseline levels at 9 dpi. There were no statistical differences between the 2 groups at any sampling point.

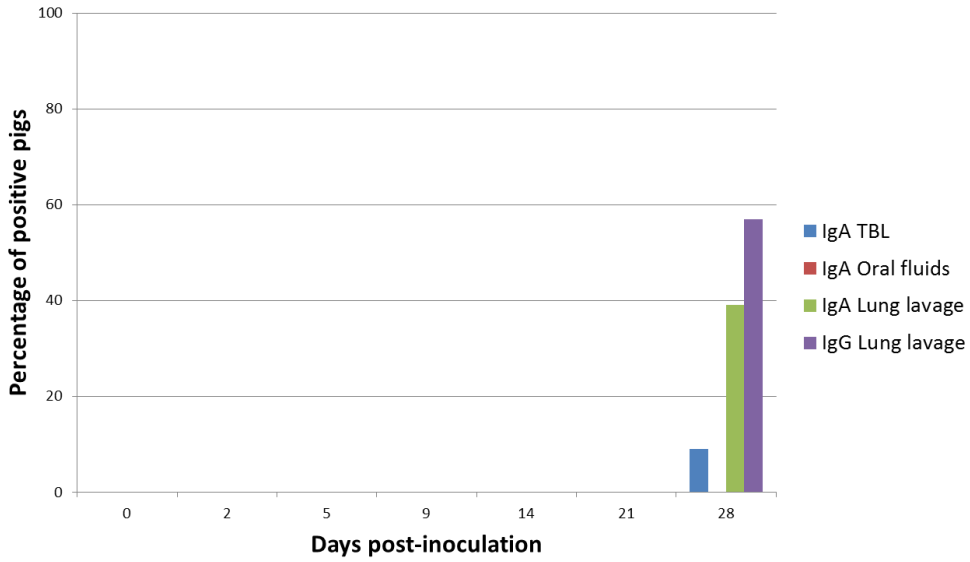

Figure 3. Detection of *M. hyopneumoniae* specific antibodies in sample fluids: oral fluids, trachea-bronchial lavages and lung lavage). IgA was detected by modifying the Tween 20 platform. IgG was detected by modifying the Idexx platform.

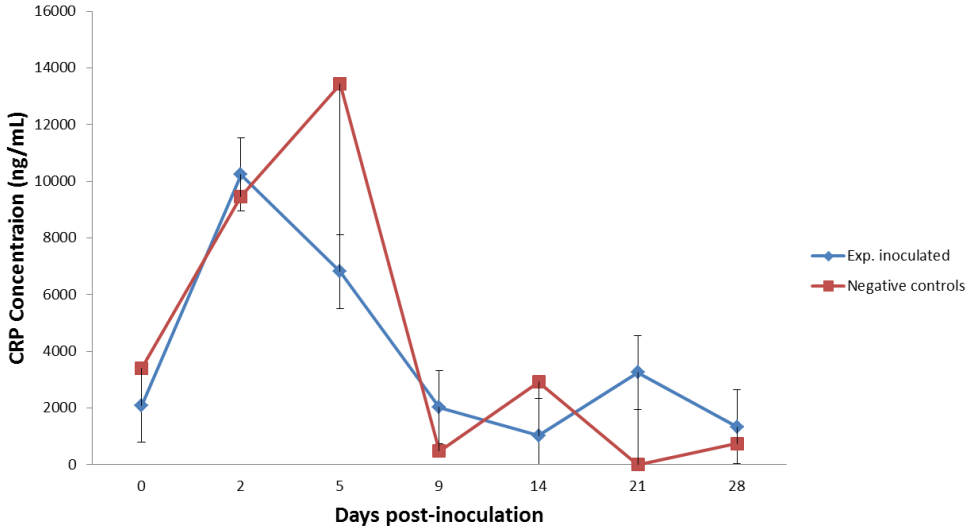

Figure 4. C reactive protein detection in pigs inoculated with sterile Friis medium and with Friis medium containing *M. hyopneumoniae* strain 232.

Detection of *M. hyopneumoniae* specific proteins in serum:

A *M. hyopneumoniae* protein pattern was constructed for each pig at each sampling point, using serum samples. Two of the 21 experimentally infected pigs showed different one band at 21 and 28 dpi. One pig showed 2 *M. hyopneumoniae* bands at the last 2 sampling points, as seen in Figure 5.

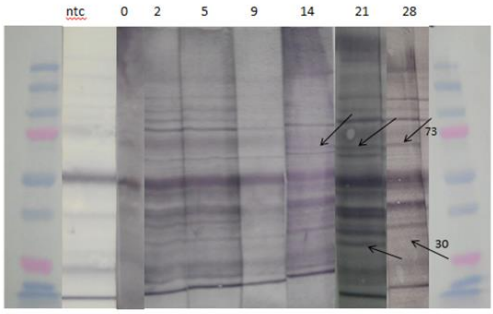

Figure 5. Band pattern of 1 experimentally infected pig in which 2 *M. hyopneumoniae* infection associated bands (73 and 30 KDa) were observed.

Discussion:

The objectives of this study were to compare side-by-side various samples types, to develop new assays for detection of *M. hyopneumoniae* during the early phase of infection and to generate guidelines for diagnostic of *M. hyopneumoniae* early after infection based on the results from the first 2 aims. A group of pigs obtained from a high health herd were used for this investigation. Two pigs were inoculated with sterile Friis medium and remained uninfected throughout the study, while 21 experimentally infected pigs were successfully infected with *M. hyopneumoniae*. Then, a sampling protocol of 7 sampling time points in a period of 28 days was applied to all the pigs in the study. During each sampling time a total of 4 samples were on an individual basis and 1 sample on a room basis. The individual samples were nasal swabs, laryngeal swabs, trachea-bronchial lavages and a blood sample. While the oral fluid samples were collected by room.

To our knowledge, this is the first time a side-by-side comparison of *M. hyopneumoniae* detection in various samples types is performed with such a frequent sampling protocol during the early phase of infection. Other studies have investigated the sensitivity of non-common samples types, for example, trachea-bronchial swabs for detection of *M. hyopneumoniae* in naturally infected pigs or with experimentally infected pigs using a wider interval in between samplings.

Under the conditions of this investigation, laryngeal swab PCRs was the most sensitive sample type for detection of *M. hyopneumoniae* after experimental infection. *M. hyopneumoniae* positive pigs were detected as early as 5 dpi. Laryngeal swabs can be collected in field under practical situations and only requiring the use of an extra person for sampling purposes. Laryngeal swabbing seems to be less invasive than other sample collection techniques, such as trachea-bronchial swabs, but still confers a superior sensitivity compared to other commonly collected samples, like nasal swabs.

In this study we validated the detection of *M. hyopneumoniae* in oral fluids using an experimental model. Oral fluid samples are commonly submitted to diagnostic laboratories for detection of multiple pathogens, and while their use can be important for other pathogens, it seemed like their value for detection of *M. hyopneumoniae* during the early phase of infection is very low.

Specific antibodies for *M. hyopneumoniae* are generated after infection; however, their development is slower compared to the multiplication of the pathogen in the respiratory tract of the pig and the potential to detect it by PCR as shown in this study. Therefore, *M. hyopneumoniae* DNA detection still constitutes a diagnostic tool of superior value for earlier detection.

In the past, detection of acute phase proteins have been proposed as biomarkers of disease quickly after the infection takes place. In this study, C reactive protein production was increased in pigs regardless of their infection status, therefore the lack of specificity on the C reactive protein response does not make it an ideal measurement to identify recently infected pigs.

In an attempt to achieve confirmation of *M. hyopneumoniae* infection in pigs by detecting specific proteins in their serum after experimental infection, we investigated the individuals pig protein band pattern. However, a differential protein pattern was only observed during the last days of infection and in a small proportion of pigs; the same pigs that evidenced the highest s/p values for IgG ELISA, which turns to be a long time for confirmation of *M. hyopneumoniae* infection in the few pigs in which it appeared.

It is important to note that a limitation of this investigation is the fact that pigs were experimentally infected using an intra-tracheal infection model. It may happen that the course of natural infection with *M. hyopneumoniae* could results in different sensitivities than the ones observed in this study. However, preliminary investigations of our group in field cases showed similar results.

In summary, this investigation has generated data that brings the possibilities for detection of early *M. hyopneumoniae* infections to a whole new level of knowledge, understanding and applicability in the daily diagnostic activities for respiratory diseases in swine populations.