RESEARCH REPORT

SWINE HEALTH

Title: Establishment of sensitivity and specificity levels of different sampling techniques for early detection of Mycoplasma hyopneumoniae and correlation between oral fluid PCR results and clinical signs. ***#18-133***

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Date Submitted: 9/18/2020

Industry Summary:
Mycoplasma hyopneumoniae (MHP) is the cause of enzootic pneumonia; a disease that costs the swine industry approximately $400 million annually. Although detection of MHP DNA by PCR from a tracheal swab is the preferred specimen for early detection, oral fluid samples are easily collected and offers prevalence assessment at the herd level. Therefore, the goal of this study was to evaluate the association between oral fluid or tracheal swab PCR results and clinical signs from animals of known MHP infection status. Additionally, the sensitivity of two PCR protocol were compared and a preliminary evaluation on the utility of air and water sampling to detect MHP DNA was conducted.

Six-week-old MHP-free pigs (n = 39) were randomized to 5 groups housed separately: (1) sham-inoculated, negative control (n = 3), (2) 1 MHP-inoculated pig + 8 susceptibles, (3) 3 MHP-inoculated + 6 susceptibles, (4) 6 MHP-inoculated + 3 susceptibles, and (5) 9 MHP-inoculated pigs, i.e., MHP prevalence differed by group. MHP pigs were inoculated intratracheally (10 ml) with a lung homogenate containing MHP 232. Tracheal swabs and individual cough scores were taken on -3, 3, 7, 10, 14, 21, 24, 28, 35, 38, 45, 52, and 59 days post inoculation (DPI). Oral fluids were daily collected. Water samples were taken once weekly. Air samples were collected three times weekly in Groups 1, 3 and 5. Samples were tested by PCR. Pigs were euthanized 59 DPI. Linear or logistic mixed regression were used to estimate the associations between PCR cycle threshold (Ct) values of tracheal samples and individual cough scores and between an oral fluid PCR result and the number of pigs coughing. Additionally, a subset of samples (tracheal swabs, oral fluids and water samples) were tested by two different PCR protocols. Logistic regression was used to assess difference between protocols on the proportion of PCR positive results from MHP-inoculated groups.

MHP was detected in 90% (18/20) of MHP-inoculated pigs at 3 DPI. MHP was first detected in oral fluids in group 2 and 5 at 8 DPI and was inconsistently detected in group 2 until 40 DPI but was detected consistently thereafter. MHP was continuously detected in oral fluids in group 4 and 5 as early as 15 and 12 DPI, respectively, and until the termination of the study in group 4. MHP DNA was detected in 6 or 7 air samples in groups 3 or 5, respectively, and 21 water samples at various time points throughout the study (5 to 6 samples per MHP-inoculated group). Coughing was first noted in group 3 at 10 DPI and was later detected in groups 2, 3, and 4 until 59 DPI. MHP was not detected by PCR in any sample type from the sham-inoculated, negative control group and cough was not observed in this group.

There was a significant inverse association between tracheal sample PCR Ct value and individual cough score meaning that a pig with a lower Ct value is more likely to be coughing. No significant association was found between oral fluid PCR positive result and number of pigs coughing. Statistical differences were observed in the detection rate of MHP DNA between the two PCR protocols on tracheal swabs, oral fluids and water samples. While tracheal swabs remain the gold standard for early detection of MHP infection, the data presented in this study suggests that when MHP prevalence is high, oral fluids, water samples, or air samples offer an easier alternative to monitor MHP at the herd level. Although not initially a focus of this project but as a direct result of it, an improved PCR protocol (increased sensitivity) has been put in place by the Iowa State University Veterinary Diagnostic Laboratory for the detection of MHP in samples such as oral fluids and water facilitating MHP herd level monitoring.

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Key Findings:

- Tracheal swabs remain the gold standard for early detection of *Mycoplasma hyopneumoniae* infection with 90% of inoculated animals testing positive by 3 days post-inoculation.
- When *Mycoplasma hyopneumoniae* prevalence is high, oral fluids offers an easier alternative to monitor *Mycoplasma hyopneumoniae* at the herd level.
- *Mycoplasma hyopneumoniae* DNA was detected in water samples at a similar detection frequency as oral fluids suggesting this sample type may also assist in the monitoring of *Mycoplasma hyopneumoniae*.
- An improved PCR protocol is now available at the ISU VDL for detection of MHP.

Keywords: *Mycoplasma hyopneumoniae,* tracheal swabs, water samples, oral fluids, air sampling

Scientific Abstract:

Mycoplasma hyopneumoniae (MHP) is the cause of enzootic pneumonia; a disease that costs the swine industry approximately $400 million annually. Although detection of MHP DNA by PCR from a tracheal swab is the preferred specimen for early detection, oral fluid samples are easily collected and offers prevalence assessment at the herd level. Therefore, the goal of this study was to evaluate the predictive association between oral fluid or tracheal swab PCR results and clinical signs from animals of known MHP infection status as this knowledge does not currently exist. Additionally, the sensitivity of two PCR protocol were compared and a preliminary evaluation on the utility of air and water sampling was conducted. Six-week-old MHP-free pigs (n = 39) were blocked by litter and randomized to 5 groups housed separately: (1) sham-inoculated, negative control (n = 3), (2) 1 MHP-inoculated pig + 8 susceptibles, (3) 3 MHP-inoculated + 6 susceptibles, (4) 6 MHP-inoculated + 3 susceptibles, and (5) 9 MHP-inoculated pigs, i.e., MHP prevalence differed by group. MHP pigs were inoculated intratracheally (10 ml) with a lung homogenate containing MHP 232. Tracheal swabs were taken on -3, 3, 7, 10, 14, 21, 24, 28, 35, 38, 45, 52, and 59 days post inoculation (DPI). Oral fluid were daily collected. Individual scores were taken by a blinded individual twice weekly. Water samples were taken once weekly. Air samples were collected three times weekly in Groups 1, 3 and 5. Samples were tested by two distinct PCR protocols. Pigs were euthanized 59 DPI. Linear mixed regression was used to estimate the association between PCR cycle threshold (Ct) values of tracheal samples and individual cough scores. The association between oral fluid PCR result and the number of pigs coughing was estimated with logistic mixed regression. Logistic regression was used to assess difference between protocols on the proportion of PCR positive results from MHP-inoculated groups. MHP was not detected by PCR in any sample type from the sham-inoculated, negative control group and cough was not observed in this group. MHP was detected in 90% (18/20) of MHP-inoculated pigs at 3 DPI. MHP

was first detected in oral fluids in groups 2 and 5 at 8 DPI and was inconsistently detected in group 2 until 40 DPI but was detected consistently thereafter. MHP was continuously detected in oral fluids in group 4 and 5 as earlier as 15 and 12 DPI, respectively, and until the termination of the study in group 4. MHP DNA was detected in 13 air samples and 21 water samples at various time points throughout the study. Coughing was first noted in group 3 at 10 DPI and was later detected in groups 2, 3, and 4 until 59 DPI. A significant inverse association between tracheal sample PCR Ct value and individual cough was found. No significant association was found between oral fluid PCR result and the number of pigs coughing. One of the PCR protocols offered the highest detection rate of MHP DNA in tracheal swabs, oral fluid and water samples. While tracheal swabs remain the gold standard for early detection of MHP infection, the data presented in this study suggests that when MHP prevalence is high, oral fluids, water samples and/or air samples offer an easier alternative to monitor MHP at the herd level. In addition, differences in the frequency of detection between two PCR protocols suggest opportunities for molecular testing optimization.

Introduction:

Mycoplasma hyopneumoniae (MHP) is the etiologic agent of porcine enzootic pneumonia (PEP), an infectious respiratory disease characterized by a non-productive cough, reduced daily weight gain and poor feed conversion. PEP is common in US swine herds: 20 of the largest swine producing companies (each one producing more than 150,000 pigs/year) reported that approximately 18% of the finishing pigs in their herd were affected by PEP. Estimates of PEP-related losses to the industry are $375 – 400 million annually. This loss is highest in finishers where the cost of PEP is estimated at $5.82 per pig.

Solutions for the prevention and/or control of MHP are limited. Vaccination and antimicrobial treatments to control MHP can be laborious, expensive, and frequently not effective in eliminating or reducing PEP, in large part due to its complex epidemiology. Further, the use of antimicrobials to prevent and treat disease in food animals is of increasing concern to consumers and the medical community, which has led to increased oversight of their use in pork production.

Regardless of the measures taken to ameliorate or control PEP, accurate diagnosis is necessary to design and apply effective treatment and effective monitoring is needed to fully evaluate the impact of control measures implemented in a population. New sampling techniques (e.g. oral fluids and tracheal swabs) allied with the development of new diagnostic tests have broadened the options available to field veterinarians and producers for herd surveillance and monitoring. However, consistent data characterizing the correlation between a positive sample and clinical disease, detection power, applicability, and the sensitivity and specificity of sample types at different known prevalence levels is unavailable. Establishing the most suitable sample for different levels of herd prevalence is critical in order to design and apply effective and feasible disease surveillance/control programs and consequently reduce losses incurred by PEP.

The goals of this project are 1) estimate the sensitivity and specificity of different sampling techniques for early detection of MHP at different prevalence levels and 2) evaluate the association between oral fluid or tracheal swab PCR results and clinical signs of PEP. Currently, several sample types and diagnostic tools have been used to detect MHP; however, consistent data concerning the best sample type for early detection of MHP and correlation between sample type (including oral fluids) and clinical signs are not available. To address this shortfall, our group will generate samples of known status and use them to determine the diagnostic test(s) and sample type(s) that best detect MHP early in the colonization phase, and characterize the correlation between oral fluids and clinical disease.

Objectives:

Objective 1: Estimate the diagnostic sensitivity and specificity of oral fluids and tracheal swabs for early detection of *M. hyopneumoniae*.

Aim 1. Generate a panel of tracheal swabs and oral fluids of precise and accurate known exposure and disease status. These samples are currently unavailable.

Aim 2. Estimate and compare the diagnostic performance (diagnostic sensitivity and diagnostic specificity) between of oral fluids and tracheal swabs for early detection of MHP. This information is not currently available.

Meeting these aims will provide guidance in the selection of antemortem diagnostic techniques, surveillance methods, and monitoring protocols. In addition, this information is pertinent to practitioners for best practices for vaccination or timing of antibiotic intervention, which will result in better respiratory disease management strategies for swine production systems, enhance production efficiency, animal well-being, and improve animal health.

Objective 2: Evaluate the predictive association between oral fluid or tracheal swab PCR results and clinical signs.

Meeting this objective will provide a means to assess the use of oral fluids in determining herd status, compare to other sample types and implement improved disease prevention and mitigation efforts.

Materials & Methods:
Six-week-old MHP-free pigs (n = 39) were blocked by litter and randomized to 5 groups housed separately: (1) sham-inoculated, negative control (n = 3), (2) 1 MHP-inoculated pig + 8 susceptibles, (3) 3 MHP-inoculated + 6 susceptibles, (4) 6 MHP-inoculated + 3 susceptibles, and (5) 9 MHP-inoculated pigs, i.e., MHP prevalence differed by group. MHP pigs were inoculated intratracheally (10 ml) with a lung homogenate containing MHP 232. Tracheal swabs were taken on -3, 3, 7, 10, 14, 21, 24, 28, 35, 38, 45, 52, and 59 days post inoculation (DPI). Oral fluids were collected daily. Individual scores were observed over 27 minute period by a blinded individual twice weekly. Water samples were taken once weekly. Air samples were collected three times weekly in Groups 1, 3 and 5. Samples were tested by PCR. A subset of samples were tested by two distinct PCR protocols. Pigs were euthanized 59 DPI.

Linear mixed regression was used to estimate the association between PCR cycle threshold (Ct) values of tracheal samples and individual cough scores, DPI and inoculation status were considered fixed effects, and pig was the random effect. Association between PCR result of oral fluid sample and number of pigs coughing was estimated with logistic mixed regression, DPI was considered fixed effect, and group as random effect. Logistic regression was used to assess difference between protocols on the proportion of PCR positive results from MHP-inoculated groups. Analyses were performed using R program version 3.5.2.

Results:
MHP was not detected by PCR in any sample type from the sham-inoculated, negative control group and cough was not observed in this group. A summary of PCR results by sample type, cough score and transmission is presented in Table 1. MHP was detected in 90% (18/20) of MHP-inoculated pigs at 3 DPI. MHP was first detected in oral fluids in groups 2 and 5 at 8 DPI and was inconsistently detected in group 2 until 40 DPI but was detected consistently thereafter. MHP was continuously detected in oral fluids in group 4 and 5 as early as 15 and 12 DPI, respectively, and until the termination of the study in group 4. MHP DNA was detected in 13 air samples and 21 water samples as early as 10 and 7 DPI, respectively, and at various time points throughout the study. Coughing was first noted in group 3 at 10 DPI and was later detected in groups 2, 3, and 4 until 59 DPI. A significant inverse association between tracheal sample PCR Ct value and individual cough score [exp(B) = 0.86; P = 0.02] was found. No significant association was found between oral fluid PCR result and number of pigs coughing. A comparison of the two PCR protocols by sample type is summarized in Table 2. One of the PCR protocols offered the highest detection rate of MHP DNA in tracheal samples, oral fluid, and water samples.

Table 1. MHP DNA detection by sample type and cough score in MHP-inoculated groups.

	Group (MHP inoculated: MHP non-inoculated)			
	2 (1 : 8)	3 (3 : 6)	4 (6 : 9)	5 (9 : 9)
Tracheal swab - Pig-to-pig[1]	DPI 14	DPI 10	DPI 7	NA[2]

- 100% detection	DPI 52	DPI 38	DPI 21	DPI 7
Oral fluid				
- First detection	DPI 8	DPI 9	DPI 14	DPI 8
- PCR positive	25 of 58	42 of 58	45 of 58	36 of 58
Water				
- First detection	DPI 21	DPI 14	DPI 21	DPI 7
- PCR positive	5 of 8	5 of 8	5 of 8	6 of 8
Air				
- First detection	ND[3]	DPI 10	ND	DPI 16
- PCR positive	ND[3]	6 of 26	ND	7 of 26
Sum of individual cough score	97	156	312	301

[1]First pig-to-pig transmission based on tracheal swab PCR positivity; [2]NA, Not applicable; [3]ND, Not done

Table 2. Comparison of two PCR protocols by sample type.

Sample type	Positive samples Old protocol	Positive samples New protocol	Total no. of samples that could be positive	% Increase
Tracheal swab	304	318	413	3%
Water	7	21	40	35%*
Oral fluid	109	148	236	17%*

*Statistically significant ($p < 0.05$)

Discussion:

MHP DNA was detected as early as 3 DPI and 8 DPI in tracheal swabs and oral fluid samples, respectively. MHP DNA was also detected in air and water samples suggesting these sample types may also assist in the monitoring of MHP. A statistically significant inverse association between tracheal sample PCR Ct value and cough score was observed. Suggesting that as the bacterial load increases clinical signs become apparent. No such association was found between the number of pigs coughing in a pen and oral fluids. While tracheal swabs remain the gold standard for early detection of MHP infection, the data presented in this study suggests that when MHP prevalence is high, oral fluids, water, or air samples offer easier alternatives to monitor MHP at the herd level. In addition, differences in the frequency of detection between two PCR protocols suggest opportunities for molecular testing optimization.