RESEARCH REPORT
SWINE HEALTH

Title: International collaboration to investigate the sensitivity and specificity of *Mycoplasma hyopneumoniae* PCR assays - **NPB#07-094**

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Industry summary: *Mycoplasma hyopneumoniae* (MHYO) remains a significant source of economic loss for today's swine industry. Reliable diagnosis of this organism continues to be a challenge as demonstrated by the difficultly in confirming the negative status of herds due to delay in seroconversion following infection. In addition, it has been found that pigs with low-level infection demonstrate an even greater variation in seroconversion. Alternatively, the use of polymerase chase reaction (PCR) has been shown to be a sensitive method to detect MHYO in pigs. Many PCR assays have been described, but some of the currently published PCR assays are not able to detect all isolates of MHYO due to genetic variation in the field. In order to identify one or more PCR assays that can be used diagnostically to successfully detect genetically diverse MHYO field isolates, a multi-site evaluation from labs specializing in MHYO research throughout the world was performed. Laboratories from 6 countries tested their panels of MHYO isolates against up to 19 different PCR assays targeting 6 different genes that included both gel-based and real-time tests. The results from this study identified four gel-based and two real-time PCR assays that were able to detect all of the isolates that were evaluated. The findings from this study will lead to better detection of MHYO in diagnostic specimens by reducing the potential for false negative results and improve our accuracy in identifying herds that are negative or positive for MHYO. These findings may also lead to more standardization in PCR tests offered among the various diagnostic laboratories.
I.
Scientific abstract: *Mycoplasma hyopneumoniae* (MHYO) remains a significant source of economic loss for today's swine industry. Reliable diagnosis of this organism continues to be a challenge as demonstrated by the difficultly in confirming the negative status of herds due to delay in seroconversion following infection. In addition, it has been found that pigs with low-level infection demonstrate an even greater variation in seroconversion. Alternatively, the use of polymerase chase reaction (PCR) has been shown to be a sensitive method to detect MHYO in pigs. Many PCR assays have been described, but some of the currently published PCR assays are not able to detect all isolates of MHYO due to genetic variation in the field. In order to identify one or more PCR assays that can be used diagnostically to successfully detect genetically diverse MHYO field isolates, a multi-site evaluation from labs specializing in MHYO research throughout the world was performed. Laboratories from 6 countries tested their panels of MHYO isolates against up to 19 different PCR assays targeting 6 different genes that included both gel-based and real-time tests. The results from this study identified four gel-based and two real-time PCR assays that were able to detect all of the isolates that were evaluated. The

findings from this study will lead to better detection of MHYO in diagnostic specimens by reducing the potential for false negative results and improve our accuracy in identifying herds that are negative or positive for MHYO. These findings may also lead to more standardization in PCR tests offered among the various diagnostic laboratories.

II. Introduction:

The polymerase chase reaction (PCR) has been shown to be a sensitive method to detect *Mycoplasma hyopneumoniae* (MHYO) in pigs. A number of assays have been described targeting different areas of the genome of MHYO. Of these, there is no single PCR assay that is commonly used by all laboratories. Even among the veterinary diagnostic laboratories across the United States there is no consensus on which assay to use. Recent studies have demonstrated the existence of genetic variation among MHYO isolates and it has been shown that some of the currently published PCR assays do not detect all isolates of MHYO. However the differences in detecting the various isolates throughout the world are unknown. This study sought to organize a collaborative study including laboratories around the world to evaluate the ability of commonly used MHYO PCR assays to accurately detect different MHYO isolates. The results of this study will provide important information to producers regarding MHYO diagnostic assays and improve our accuracy in identifying herds that are negative or positive for MHYO. This may also lead to more standardization in PCR tests offered among the various diagnostic laboratories.

III. Objectives:

1. Identify the list of PCR assays currently being employed including each laboratory's specific modifications and protocols. Assays for other species of mycoplasmas in swine will be included for specificity.
2. Test this panel of assays against each laboratory's panel of mycoplasma isolates to identify the most sensitive and specific PCR test(s).
3.

IV. Materials and methods:

In addition to the Thacker laboratory at Iowa State University, laboratories specializing in MHYO research from France, Denmark, Belgium, Spain, Switzerland and Australia collaborated to compile a list of currently used MHYO-specific PCR assays (1-11). The assays included both gel-based and real-time PCR protocols. The gel-based assays were adapted to a single mastermix rather than performed as published for ease of testing.

The following instructions were supplied with the panels for testing:
NOTE: an additional real-time PCR assay referred to as "S" was later added in a supplemental shipment.

Instructions for *Mycoplasma hyopneumoniae* PCR screen

Box contents:
For standard PCR:

- Primers labeled A-M forward (F) and reverse (R), except three assays (J, K, L) use the same reverse primer (J,K,L-R) for a total of 24 primer tubes.
- Control DNAs; 4 tubes each containing 200 ng of DNA.
- Buffers: MasterAmp E from EpiCentre
- Taq polymerase will be shipped directly from New England Biolabs (NEB).

For real time assays:
If you are performing real time assays, your package will include the ABI Universal PCR master mix kit. Primers labeled N-R forward (F) and reverse (R). Assay N has two reverse primers representing SNPs (N-R1, N-R2) and one forward primer (N-F) for a total of 11 primers. These need to be reconstituted as described below.

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You will need to supply water, tubes, cycler and DNA templates from field isolates.

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1. Primers and probes need to be reconstituted. (Probes will NOT be included in your kit if you are not performing real time PCR.). Primers need to be reconstituted with 100 µl of PCR grade water for a final concentration of 100 µM. This will be your stock solution. A ten-fold dilution needs to be made for a 10 µM working stock. Probes are reconstituted to the following volumes to bring them to 100 µM: N-P to 19 µl; O-P to 37 µl; P-P to 55 µl; Q-P to 33 µl; R-P to 33 µl.
2. Control DNAs need to be reconstituted in 20 µl PCR grade water to a final concentration of 10 ng per µl. The 232 template will be used for a ten-fold dilution series to determine the detection limits of your equipment.
3. Run the control 232 DNA template DNA dilution series (6 dilutions) with every primer set along with the three other control DNA templates at 1 ng per µl.
4. Standard PCR reactions for all primer pairs are set up as follows:

MasterAmp E buffer	12.5 µl
Primers each at 10 µM	1 µl each
NEB Taq polymerase	0.2 µl
Water	9.3 µl
DNA template	1 µl

Not every laboratory was equipped to perform both the gel-based and real-time assays so the isolates included in the study were not all tested against every assay in the panel.

V. Results:

Each of the laboratories reported their results in Excel sheets that were provided. Information included was: Isolate identification, positive or negative results for each assay, and the sensitivity for each assay based on DNA standards that were included.

Seven laboratories from the United States, France, Denmark, Belgium, Spain, Switzerland and Australia collectively tested 186 MHYO isolates by up to 19 different PCR assays.

Of the PCR assays tested, four gel-based assays detected all of the isolates tested. Three target the 16S ribosomal RNA gene (1) and the other targets the mhp165 gene of MHYO (3,9). In addition, two real-time assays detected all of the isolates tested against them. One targeted the mhp165 gene and the other targeted the mhp183 gene (9).

VI. Discussion:

Previous reports of false negatives by some MHYO-specific PCR assays due to genetic variation have been described (2,9). Therefore, this study sought to test currently used PCR assays for MHYO against an international collection of MHYO isolates in order to identify the most robust test(s) available for diagnostic use. Four gel-based and two real-time PCR assays were found to detect each of the isolates against which they were tested. Three veterinary diagnostic laboratories in the United States have adopted new PCR assays for the detection of MHYO based on these results. This should lead to better uniformity in testing across diagnostic laboratories and the occurrence of fewer false negative results. Continued testing should occur to further confirm the ability of these assays to detect all field isolates of MHYO.

References

1. Calsamiglia M, et al. 1999. J. Vet. Diag. Invest. 11:246-251.
2. Dubosson CR, et al. 2004. Vet. Microbiol. 102:55-65.
3. Fraser T, et al. 1999. Abstr. p. 36, 42nd annual mtg Am. Assoc. Vet. Lab. Diag.
4. Kurth KT, et al. 2002. J. Vet. Diag. Invest. 14 :463-469
5. Sorensen V, et al. 1997. Vet. Microbiol. 54:23-34.
6. Stakenborg T, et al. 2006. Vet. Res. Commun. 30 :239-247.
7. Stark KD, et al. 1998. Appl. Environ. Microbiol. 64:543-548.
8. Stemke GW, et al. 1994. Am. J. Vet. Res. 55:81-84.
9. Strait E, et al. 2008. J. Clin. Micro. 46: 2491-2498.
10. Verdin E, et al. 2000. Vet. Microbiol. 76: 31-40.
11. Unpublished, in-house assay Iowa State University, Veterinary Diagnostic Laboratory.