RESEARCH REPORT
SWINE HEALTH

Title: Development of improved diagnostic PCR assays for *Mycoplasma hyopneumoniae*-**NPB# 01-135**

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Abstract

The diagnosis of pneumonia caused by *Mycoplasma hyopneumoniae* remains frustrating. To increase our ability to detect *M. hyopneumoniae,* we modified our present *M. hyopneumoniae* PCR assay to enable us to quantify the number of organisms in a sample and to allow the detection of the organism in formalin fixed and paraffin embedded lung tissue samples. An ELISA-based *M. hyopneumoniae* PCR assay utilizing phenylboronic acid- saclicylhydroxamic acid, biotinylated primers and strepavidin-alkaline phosphatase conjugation was used to quantify *M. hyopneumoniae* DNA. Known amounts of *M. hyopneumoniae* DNA was used to develop a standard curve and known and unknown samples of *M. hyopneumoniae* were assayed. In addition, the quantitiy of DNA obtained from cultured organisms was successfully compared to titration of cultured organisms, which is currently considered the gold standard. Detection of *M. hyopneumoniae* by PCR was successful using fixed lung tissues collected at necropsy from pigs experimentally infected with *M. hyopneumoniae* . Lung tissues were placed in formalin for various lengths of time followed by DNA extraction and subjected to PCR. The results correlated to the PCR results obtained from fresh tissues. *M. hyopneumoniae* DNA was detected by PCR in all experimentally infected lung tissues after 168 hours of fixation in formalin. In order to evaluate the PCR assay under field conditions, lung tissue samples from 7 pigs presented to the Iowa State Veterinary Diagnostic Laboratory (ISUVDL) with respiratory disease were also assayed. Three lung samples were *M. hyopneumoniae* PCR positive using fresh samples, and 2 remained positive following 24 hours of formalin fixation. Paraffin-embedded lung tissues from experimentally infected pigs were processed, DNA extracted and subjected to PCR. Preliminary results indicate that DNA for *M. hyopneumoniae* can be successfully extracted from paraffin embedded samples and detected by PCR.

Introduction

M. hyopneumoniae is the causative pathogen of enzootic pneumonia in pigs. In conjunction with other bacteria and viruses, *M. hyopneumoniae* plays an important role in the porcine respiratory disease complex (PRDC). Although by itself, low levels of *M. hyopneumoniae* may induce minimal disease, a significant impact on herd health has been observed in conjunction with other respiratory pathogens. In order to accurately measure both the level of infection and the effectiveness of control measures within swine herds, new diagnostic procedures are required.

Currently accurate detection and diagnosis of *M. hyopneumoniae*-induced disease is difficult. Culture of the organism is slow and difficult, often times taking up to 8 weeks[1]. Serology remains a poor diagnostic option due to the long time period between infection and seroconversion and the inability to determine vaccine-induced antibodies from antibodies produced following natural infection. The development of PCR assays specific to *M. hyopneumoniae* has greatly enhanced our ability to detect the presence of the organism[2,3] However, current procedures do not readily lend themselves to the detection of the organism in samples collected under field conditions. The development of a PCR to detect *M. hyopneumoniae* in formalin fixed and paraffin embedded lung tissue samples enables us to more readily detect the organisms from samples collected under field conditions. Currently diagnosis and quantification of *M. hyopneumoniae* is limited to relatively imprecise methods such as lung scoring and titration procedures in culture. Improved diagnostic assays that allow for the quantification of the number of organisms present are critically needed in order to more accurately determine the level of *M. hyopneumoniae* infection within a herd.

Objective

The objective of this study was to modify our present PCR assay to enable the quantification of *M. hyopneumoniae* organisms and to accurately detect the presence of *M. hyopneumoniae* DNA in formalin fixed and paraffin embedded lung tissue. Specifically we sought to develop an ELISA based PCR assay for *M. hyopneumoniae* to detect and quantify the organism present.

Procedures

Protocol for Quantitative PCR for *Mycoplasma hyopneumoniae*

M. hyopneumoniae DNA was extracted from bronchial alveolar lavage fluid collected from pigs experimentally infected with *M. hyopneumoniae*. PCR primers were biotinylated and a ratio of modified dUTP labeled with phenyldiboronic acid (PDBA) and dTTP were used to label the PCR product so that an ELISA assay could be used for quantitation. Reaction mixes for the PCR reactions used were from a previous protocol[4]. The PCR product was then appropriately labeled for an ELISA test and placed in salichydroxamic acid (SHA)- modified plates (Prolinx) for assay. All standards and samples were run in triplicate. The PCR products were diluted with 20X SSC (1.5 M NaCl, 150 mM sodium citrate, pH 7.6) to a final concentration of 10X SSC and 100 uL of the standards and samples were put into the appropriate wells and incubated. The plates were washed and strepavidin-alkaline phosphatase (S-AP) was added. The substrate, 4-nitrophenyl phosphate, was added and the color change was read on a SPECTRAMAX 190 plate reader (Molecular Devices). The OD values were analyzed with the Softmax PRO program (Molecular Devices).

Protocol for *M. hyopneumoniae* PCR on DNA from Formalin Fixed Tissues:

Lung samples were collected at necropsy from pigs experimentally infected with *M. hyopneumoniae*. DNA samples were collected from either fresh or tissues that had been fixed in formalin for 24, 72, 120, and 168 hours. The formalin fixed samples were

rinsed in PBS prior to processing. In order to determine the ability to detect the organism from pigs naturally infected with *M. hyopneumoniae,* lung tissue from 7 pigs presented to the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) with clinical disease consistent with respiratory disease. DNA was collected immediately or following 24 hours formalin fixation. DNA was extracted using the QIAmp DNA mini kit (Qiagen) following the kit directions. Approximately 25 mg of lung tissue was mechanically homogenized (TissueTearer, Biospec Products Inc.) in ATL buffer (Qiagen). Samples were incubated overnight with Proteinase K and DNA was extracted and eluted in 75 □Ls of AE buffer (Qiagen). DNA was assayed by nested PCR for *M. hyopneumoniae* using a previously described protocol[5]. The PCR product was run on a 1.8% agarose gel and viewed using a UV transilluminator and software (Spectronics Corporation).

Results

Quantitative PCR:

The optimal conditions for the biotinylated primers, streptavidin -alkaline phosphatase conjugation and incubation times for the quantitative PCR have been established. Using known amounts of *M. hyopneumoniae* DNA, a standard curve was determined. *M. hyopneumoniae* DNA obtained from a number of samples, including nasal swabs, lung tissue, bronchial swabs and bronchoalveolar lavage fluid was quantified using the PCR assay. In addition, *M. hyopneumoniae* organisms from culture have been diluted and assayed to determine the number of organisms based on the quantitative PCR. We are currently in the process of correlating the quantity of *M. hyopneumoniae* DNA levels obtained from the bronchoalveolar lavage fluid from experimentally infected pigs to titration in Friis mycoplasma media, which is currently considered the “gold standard” for quantifying organisms

Formalin Fixed Tissues:

Lung tissues from all experimentally infected pigs were PCR positive for *M. hyopneumoniae* organism immediately following necropsy (fresh) and remained positive following fixation in formalin for up to 168 hours (Fig. 1).

Lung tissues from 3 of the 7 pigs submitted to the ISUVDL were positive for *M. hyopneumoniae* by PCR immediately following collection. Results from the ISU-VDL found no evidence of *M. hyopneumoniae* antigen using an immunohistochemistry assay, however various viruses and bacteria were isolated from the respiratory tract of all 7 pigs (Table 1). Two of the PCR positive samples remained positive after fixation in formalin for 24 hours (Fig. 2).

Preliminary PCR results from lung tissue from experimentally infected, formalin fixed lung tissues that had been processed into paraffin blocks remained positive by PCR assay (Fig. 3). Assays will be performed on tissues in paraffin blocks collected in the ISU-VDL as they become available. Currently, the samples assayed has been limited by the availability of paraffin blocks from appropriate cases.

Discussion

The development of PCR assays to diagnose and detect *M. hyopneumoniae* has greatly increased the ability to monitor disease status and control measures. Assays to quantify the number of *M. hyopneumoniae* organisms and detect DNA in formalin fixed tissues were successfully developed in this study. Correlation of number of *M. hyopneumoniae* organisms to disease will need to be determined in order to assess the importance of the quantitative PCR. However, use of this assay will aid in evaluating the ability of various intervention strategies to reduce the number of organisms in an animal. Current culture systems are slow, tedious and relatively inaccurate.

A PCR assay to detect *M. hyopneumoniae* in formalin fixed tissues has the potential to greatly increase the ability of producers and veterinarians to monitor disease at the herd level under field conditions. In addition, the ability to detect *M. hyopneumoniae* DNA in paraffin fixed tissues will enable pathologists to confirm the presence of *M. hyopneumoniae* in lung samples from animals with lesions suggestive of mycoplasmal pneumonia, thus improving our diagnostic capabilities. The use of these assays will potentially aid in determining the strategic placement of intervention strategies for control of mycoplasmal pneumonia and other respiratory disease.

The results of this project have been presented as a poster at the 17th Congress of the International Pig Veterinary Society meeting in Ames, IA, and were published as an abstract in the proceedings.

In addition, the final results of this study will be submitted for publication in the appropriate journal.

References

1. Ross R. Mycoplasmal diseases In: A. D. Leman, B. Straw and W. L. Mengeling, eds. *Diseases of Swine*. 7th ed. Ames, IA: Iowa State University Press, 1992;537-551.
2. Artiushin S, Stipkovits L, Minion FC. Development of polymerase chain reaction primers to detect *Mycoplasma hyopneumoniae*. *Molecular and Cellular Probes* 1993;7:381-385.
3. Calsamiglia M, Pijoan C, Trigo A. Application of a nested polymerase chain reaction assay to detect *Mycoplasma hyopneumoniae* from nasal swabs. *Journal of Veterinary Diagnostic Investigation* 1999;11:246-51.
4. Minion FC, Kurth K, Lovekamp M, et al. A novel quantitative polymerase chain reaction test for *Mycoplasma hyopneumoniae*. International Pig Veterinary Society 17th Congress 2002;77.
5. Artiushin S, Minion FC. Arbitrarily primed PCR analysis of *Mycoplasma hyopneumoniae* field isolates demonstrates genetic heterogeneity. *International Journal of Systematic Bacteriology* 1996;46:324-328.