

Title: Assessment of *Mycoplasma hyopneumoniae* strain variability and relationship to virulence - **NPB #02-126**

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Abstract: The objective of this proposal is to assess variability of low-passage, field strains of *Mycoplasma hyopneumoniae*. Five strains were evaluated in a pig challenge model by comparing lung lesion scores with virulent strain 11. In addition, we studied the feasibility of using microarrays and other molecular techniques to assess genetic differences in twelve field strains.

Introduction: *M. hyopneumoniae* infection of swine has been universally established as among the most important diseases in the swine industry. Economic losses attributed to mycoplasma pneumonia in the U.S. swine industry easily exceeds \$2 per pig or approximately \$200 million annually. Loss occurs as a consequence of reduced average daily gain and efficiency of feed utilization, prophylactic and therapeutic interventions, and mortality. Prevalence of lesions of enzootic pneumonia in swine at slaughter has been very high (>70%) for many years and does not seem to have abated over the past 40 years. During recent years, swine practitioners and diagnosticians have noted an increased occurrence of severe pneumonia, referred to as Porcine Respiratory Disease Complex (PRDC). *M. hyopneumoniae* is an integral component of PRDC in association with other swine pathogens such as PRRSV and SIV. In combination with other respiratory pathogens such as PRRSV, however, pneumonia due to the second pathogen is significantly enhanced. Vaccination against *M. hyopneumoniae* neither prevents colonization of the swine respiratory tract nor protects sufficiently against disease. In addition, vaccination does not obviate the potentiating role of *M. hyopneumoniae* in dual infection with other pathogens. In the absence of effective intervention strategies to reduce disease, improved vaccines are essential if we are to reduce economic losses due to mycoplasma pneumonia.

An important problem that has not received adequate study is the possibility of genetic variability in *M. hyopneumoniae* field strains and the impact that would have on virulence. Genetic variability can significantly alter disease progression and complicate control measures by changing the surface architecture of *M. hyopneumoniae* and its presentation to the host's immune response. The extent of genetic variability in *M. hyopneumoniae* is not known.

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The surface topography of several mycoplasma species is under intensive investigation, and it is clear from these studies that variation in surface lipoproteins creates a constantly changing antigenic surface. For *M. hyopneumoniae*, however, variation in lipoprotein antigens has not been observed nor has it been well studied. It is likely that other mechanisms are operative in this species to generate surface diversity. One of these mechanisms is likely to be genetic variation, which could impact immunological resistance and disease severity.

Objectives: The objective of this proposal was to assess variability of twelve low-passage, field strains of *Mycoplasma hyopneumoniae* using challenge studies and microarray technology. Five of the twelve strains were tested in a pig challenge model and lung lesion scores correlated with the differences between the field strains and virulent strain 11.

Materials & Methods:

Vaccination and challenge: Thirty five *M. hyopneumoniae*-free, male, castrated pigs were obtained at 8-12 days of age and randomly assigned to 6 groups of 7 pigs with stratification by weight. An additional four pigs were used as negative controls. Pigs were acclimated for 1 week prior to vaccination. *M. hyopneumoniae* (strain 232, an *in vivo* passaged derivative of strain 11) challenge inoculum was administered intratracheally to pigs at a dilution of 1:100. Field strains were grown *in vitro* (passage number less than 8) and administered intratracheally on three consecutive days. The inoculum consisted of 10 mls of log phase broth grown organisms. Color changing units were determined for each of the cultures.

Clinical evaluation: Pigs were evaluated daily for a period of 15 minutes for clinical signs including cough or behavioral changes. All pigs were weighed periodically and at necropsy to evaluate growth. Mycoplasma serum antibody levels were measured weekly and at necropsy.

Necropsy: Pigs were euthanized by pentobarbital overdose followed by exsanguination 28 days following challenge. Lung lavage was taken and mycoplasma-specific secretory antibody assessed by ELISA. Lung lesions were sketched on a standard diagram and assessed for the proportion of lung surface exhibiting lesions. Tissues were collected and processed for histopathological and immunohistochemical examination.

Statistics: Analysis of variance was used to detect significant differences among treatment groups. Fisher's protected LSD for mean comparisons was also used. A non-parametric ANOVA was used for non-normally distributed data or when group variances were dissimilar.

Construction of the microarray. Microarrays were constructed with purified PCR products obtained for each ORF of *M. hyopneumoniae* strain 232. The genome sequence of this strain was completed in the Minion laboratory. PCR primers to amplify each ORF were designed using Primer3 software that was modified to eliminate false priming and potential cross hybridization among the primers. Each PCR reaction was quality controlled by analysis on agarose gels and purified using a glass binding technique to remove the PCR primers, dNTPs and salts. PCR products were quantified by UV spectroscopy, dried, resuspended in spotting buffer and printed to Corning UltraGAPS substrates.

Genomic DNA was isolated from *M. hyopneumoniae* field strains, fragmented to 200-300 bp by nebulization and then labeled using the ULYSIS kit from Molecular Probes. Hybridizations were performed manually in AHC-1 hybridization chambers. Each probe was purified to remove unincorporated fluorescent label, dried, and

dissolved in hybridization solution (Corning). Strain 232 DNA was used as a control and compared to all of the field strains by differential fluorescence labeling. The hybridizations were performed overnight at 42°C. Slides were washed twice in wash buffer A (1X SSC, 0.2% SDS) for 5 minutes each at 25°C, then in wash buffer B (0.1X SSC, 0.2% SDS) for 5 minutes and then in 0.1X SSC for five minutes. The slides were air dried prior to scanning. Signal ratios of strain 232/field strain were determined for each spot and values >10 were considered to indicate absence of gene; values >3 and <10 were considered to have significant sequence differences.

Results: Challenge studies. The challenge studies were performed with four field strains and compared to the challenge strain 232. Strain 232 is the sequenced strain and is the challenge strain used in both the Minion and Thacker labs for animal disease studies. The results of the challenge studies are shown in Table 1 and Figure 1. Table 1 gives the average challenge color changing units (CCUs), a measure of the number of organisms in the challenge, the number of culture positive and PCR positive pigs at the time of necropsy. Figure 1 shows the lung lesion scores in both a Wisker and box plot and the bar graph of mean \pm standard deviation. Only strain F showed a significantly different lung lesion score; it was significantly different ($p < 0.05$) than strain 29024. Because of the variability in the animal studies (common with *M. hyopneumoniae* challenge models), we were unable to demonstrate statistical significance with the other strains although strains P and 27533 both had a higher mean lung lesion score than either 232 or F. Only four of the seven 29024-infected pigs were positive by culture, while all of the other mycoplasma-infected pigs were positive by culture. None of the negative control pigs were positive by either culture or PCR (data not shown).

Table 1. *M. hyopneumoniae* strains*

Strain	Average CCUs	Culture	PCR
232	10^{-4}	7/7	7/7
F	10^{-4}	7/7	7/7
P	10^{-4}	7/7	7/7
27533	10^{-7}	7/7	7/7
29024	10^{-7}	4/7	7/7

* average CCUs of inoculum, number of culture positive pigs by lung lavage, number of nested PCR positive pigs.

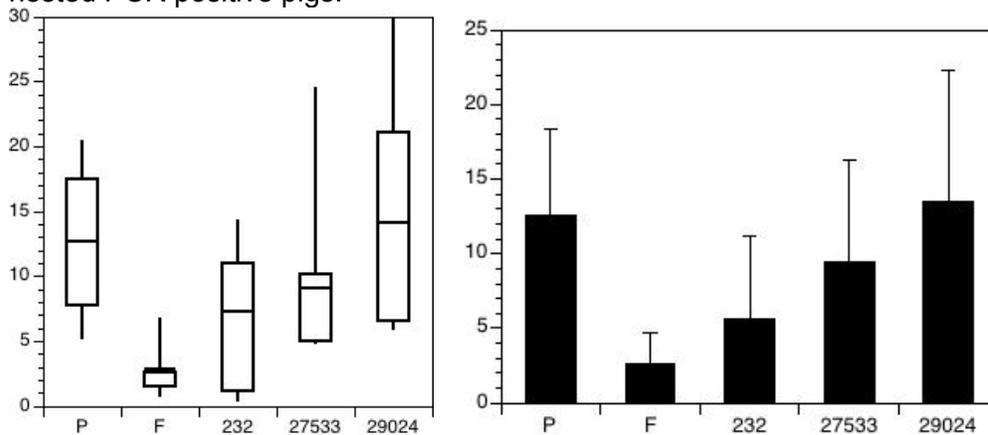


Figure 1. Group lung lesion scores. Left: Wisker and box plot. Data indicates mean (horizontal line), box of 75% (upper) and 25% (lower) of the values, and whiskers of the high and low values of each strain. Right: mean \pm standard deviation of the lung lesion scores. Only strain F had a significantly different score. It was significantly ($p < 0.05$) different from strain 29024 but not from the other strains.

Detection by PCR. As expected, all of the pigs were positive by nested PCR. During the course of these studies, we were developing a real time PCR (RT-PCR) system to quantitate numbers of organisms more quickly and reliably. The DNA target sequence was chosen within the nested PCR target (RT-PCR targets are generally small in comparison to other PCR targets), but two of the field strains failed detection by this assay (Figure 2). To understand this further, the product of the nested PCR outer primer pair was purified and sequenced. When the sequences were compared, differences in this region of the chromosome were noted between the strains as is shown in Figure 3. The Pustell DNA Matrix Plots of the DNA sequences of the field strains against strain 232 revealed that strains 232, 27533 and 29024 were homologous in this region of the genome, while strains F and P showed regions of non-homology by both Pustell Matrix and blast comparisons. The RT-PCR product target was in the region of non-homology (red horizontal bar).

To further explore genetic variation in the field strains, the R1 and R2 regions of the cilium adhesin were analyzed. These regions represent repetitive units that have been shown to vary in number between different *M. hyopneumoniae* strains. Analysis was performed by PCR using primers that flank the two regions and estimating the size of the PCR products by agarose gel. The results of this study are shown in Figure 4. Strain P was identical to 232 in both the R1 and R2 regions, but the other strains varied in size. Interestingly, strain 27533 showed multiple banding patterns indicating that the primer binding sites may have been duplicated at each end of the repeat regions.

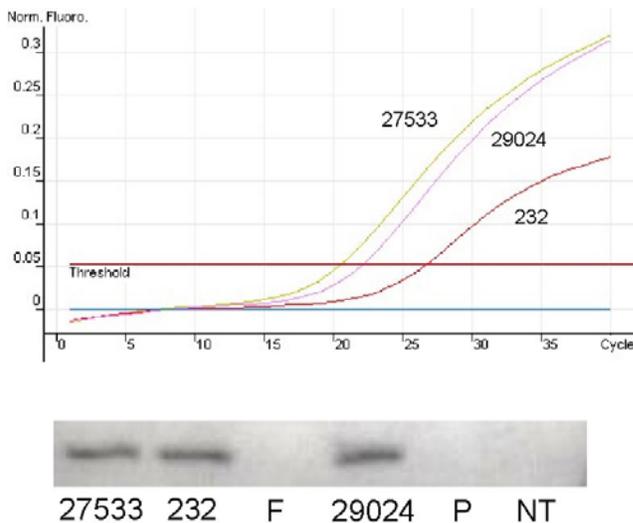


Figure 2. RT-PCR analysis of the five field strains. The upper figure shows the results of the RT-PCR. The lower figure confirms the lack of product by agarose gel.

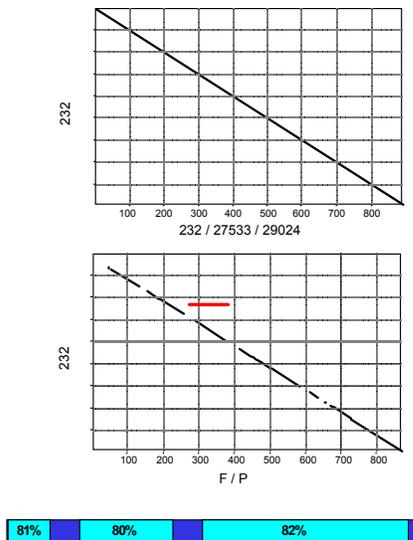


Figure 3. DNA sequence comparisons. The Pustell DNA Matrix graphs are shown in the upper figure. In the lower Pustell plot, the red horizontal bar indicates the location of the RT-PCR target sequence. In the lower bar figure, the three regions (cyan or light gray) indicate less than 100% homology with strain 232. The dark blue or dark gray regions are 100% homologous.

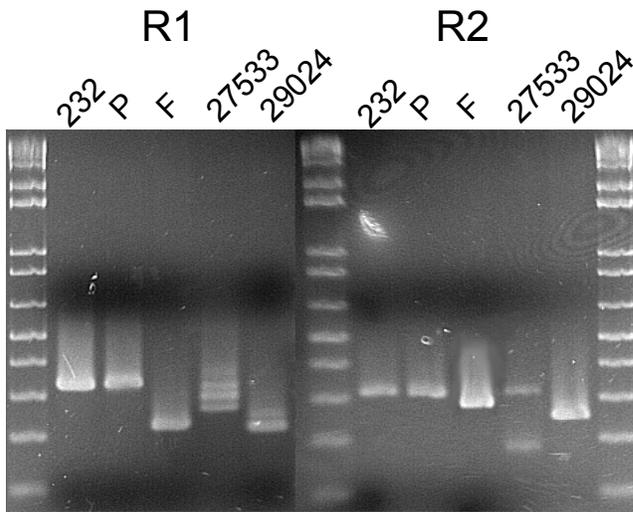


Figure 4. Analysis of the R1 and R2 regions of P97. Shown are PCR products using primers that flank the R1 and R2 regions of P97.

Microarrays. Construction of the arrays involved the quality control of every aspect of the process: PCR reaction optimization, purification of the products, quantification of purified product, optimization of spotting conditions, substrate quality and manufacturer, etc. The final arrays were not completed in time for these studies, but preliminary studies were performed to establish the conditions needed for the genomic analyses.

In initial validation studies with genomic hybridizations, we had difficulty in obtaining standardized fragmentation of genomic DNA by sonication resulting in poor labeling and low signal intensities. Uniform fragmentation of the DNA is necessary to ensure adequate labeling and to ensure maximum hybridization of the labeled probes to the arrays. Nebulization produced more reproducibly sized fragments than could be accomplished by sonication (data not shown).

M. hyopneumoniae is difficult to grow and the low passage field strains even more so. Cell yields are low even with genomic DNA. Since significant amounts of genomic DNA are required for the labeling reactions and subsequent microarray hybridization, we decided to test whether the TempliPhi reaction (Amersham) could uniformly amplify mycoplasma genomic DNA. This reaction is based on a rolling circle model of DNA replication and uses random primers to amplify genomic DNA. To assess the uniformity of amplification, we compared labeled genomic DNA from TempliPhi amplified genomic DNA from strain 232 on the arrays. The results of this study are shown in Figures 5 and 6. Figure 5 shows a portion of the microarray following hybridization with the two labeled probes; only one of the signals is shown, however. Figure 6 shows a plot of the normalized intensity values for each spot at the two wavelengths.

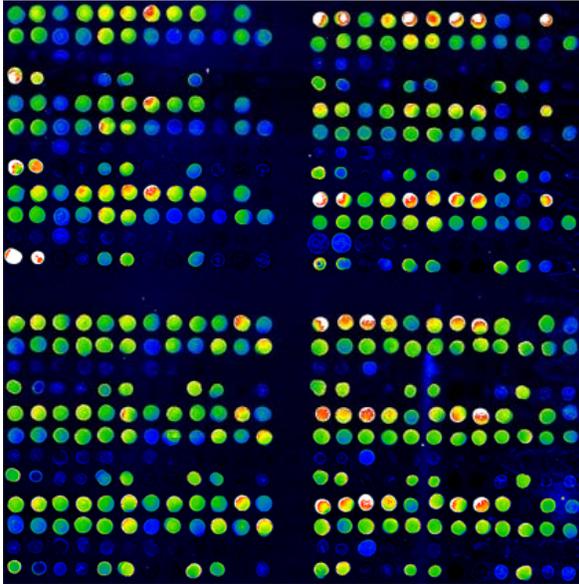


Figure 5. Microarray reacted with genomic and TempliPhi amplified genomic DNA of strain 232. Shown is the fluorescence signal of the labeled genomic 232 DNA. The intensity is represented by false color; blue is low intensity, red, high intensity.

Figure 6. Comparison of genomic and TempliPhi amplified DNA by microarray. The data represents the two normalized intensity values for each spot on the array. The 45° diagonal indicates correlation between spots.

Discussion: The results of this study indicate that *M. hyopneumoniae* field strains vary in their disease potential. We saw significant differences in lung lesion scores between strains F and 29024. In addition, strains 27533 and P gave higher mean lung lesion scores than the challenge strain 232 and strain F although variation in the lesion scores prevented statistically significant differences.

The CCU values at the time of challenge did not correlate with disease severity. Strain P gave almost the same lung lesion score as did strains 27553 and 29024 even though the CCU values of the challenge inoculums were three logs lower. It is also significant that severe disease occurred with organisms grown *in vitro* although the passage number was low. The virulence of strain 232 is maintained by passage through pigs, and lung homogenates are used as the inoculum.

We showed that genomic differences occur in the *M. hyopneumoniae* genome even within a relatively small geographical area (Iowa and surrounding states). These differences can interfere with PCR-based diagnostics although our nested PCR readily identified each strain studied. Real-time PCR is based upon a smaller target size than the nested PCR, and thus may be subject to more variation. Our selected target sequence, which falls within the nested PCR product, failed to detect two of the four field strains used in this challenge study. The assay works well with strain 232 and can be used for research purposes to quantitate numbers of this strain in lung lavage fluids rapidly, but its use as a diagnostic tool for random field studies is questionable. Another target sequence within the homologous regions of the nested PCR product (Figure 3) might be better conserved.

Not surprising were our results of the analysis of the P97 R1 and R2 regions. In previous studies we showed that strain J and 232 differed in the number of repeat units within both regions. This is the first time we have shown differences in local field strains within P97. However, it is not known what impact this variation might have on virulence.

Table 2 summarizes the results of this study by comparing the field strains with strain 232. Each of the strains could not be differentiated from strain 232 by only one of the criteria we studied. Undoubtedly, this indicates only a small portion of the overall genetic differences between strains of *M. hyopneumoniae*. The characterization of these strains could not correlate disease severity to any specific difference. It is likely that virulence is a complex phenotype depending upon a number of variables.

Table 2. Comparison of Field Strains with Strain 232

Strain	CT	LL	PCR	P97
F	Same	Same	Different	Different
P	Same	Increased	Different	Same
27533	Increased	Increased	Same	Different
29024	Increased	Increased significantly	Same	Different

Because of some technical problems, we were unable to complete the microarray studies as originally outlined. We were, however, able to establish the reaction conditions needed to produce the labeled probes for the arrays including TempliPhi amplification and nebulization, as well as optimize printing and hybridization conditions needed for studies of this type.

Lay Interpretation: *Mycoplasma hyopneumoniae* isolated from different pig herds showed variation in disease potential. This is not unexpected since differences in disease symptoms occur widely in the field. There have been few studies of this type, however, because of the difficulty in isolating the organism from clinical samples and growing it in the laboratory. This is also one of the few studies where pure cultures have been used to establish significant disease in clean (pathogen-free) pigs. During the course of these studies, we also demonstrated changes in the genome (chromosome) of the organism in an unexpected region used for diagnostics. This was evident when a newly developed real time polymerase chain reaction test (RT-PCR) was used to study the field strains examined in this study. The advantage of RT-PCR is that quantitative data can be obtained in a short period of time in comparison to the CCU test that requires weeks to complete. This particular RT-PCR assay targets a small segment of the region that has been used previously as a PCR target. That small DNA sequence was found to be in a region that differs from the challenge strain 232, preventing detection by the RT-PCR assay. The nested PCR, however continued to detect all field strains further confirming its utility as a diagnostic tool. These studies demonstrate the need to further test all PCR-based diagnostics with more field strains to ensure accuracy and reproducibility. Variation in a second genome region was both expected and confirmed. This region of the genome has shown variation in the past, but it was clear that a combination of the two tests could enable differentiation of field strains. The sensitivity of PCR diagnostics may even allow differentiation in lieu of culture, a significant advance. Finally, we were able to establish the conditions needed to perform genome-wide comparisons of *M. hyopneumoniae* field strains using microarrays. Although these tests were not completed, our laboratory is well positioned to perform these studies in the near future. These will be the first such studies with an important pig pathogen and promises to enhance our understanding of the mechanisms involved in porcine enzootic pneumonia.