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Analysis of swine antigen-specific antibody responses to *Mycoplasma hyopneumoniae* infection determined by protein microarray

Andrew C. Petersen[a], Jeannett M. Clampitt[a,b], F. Chris Minion[a,*]

[a] Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA 50011-1134, USA
[b] Interdepartmental Microbiology Graduate Program, Iowa State University, Ames, IA 50011-1134, USA

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ABSTRACT

Pigs harbor several different species of mycoplasmas, of which *Mycoplasma hyopneumoniae* presents the most significant economic impact on the swine industry. While ELISAs are the predominant diagnostic assay to measure antibody responses during infection with *M. hyopneumoniae*, the assay itself is only a rough estimate of the total antibody response. It lends little information on pathogen-wide antigen-specific responses. In addition, antibody responses to *M. hyopneumoniae* as measured by ELISA are slow to develop in infected swine. Our goal was to determine if a protein microarray could be more sensitive and informative of the serological responses of pigs to *M. hyopneumoniae* infection. The gene sequences of approximately 50 *M. hyopneumoniae* surface proteins or protein fragments were cloned, mutated to remove UGA codons, expressed in *Escherichia coli* and purified. The arrays were used to interrogate pig sera from various sources. Sera from naturally-infected swine gave some variability in antigen-specific responses, but, unexpectedly, the responses against the C-terminal portion of the major adhesin P97 was weak in all animals, including those that were experimentally infected. In two of four 118-day experimentally-infected caesarian-derived colostrum-deprived pigs, the strongest antibody responses occurred on days 30 and 54 against members of the P97/P102 paralog families. Our Day 0 results in the other two animals indicate that although thought to be mycoplasma free by all known criteria (serology and PCR), they may have harbored an inapparent *Mycoplasma* infection. In summary, the protein microarray has the potential to identify new targets for assay development to enhance sensitivity of antibody-based assays.

1. Introduction

Pigs harbor a number of pathogenic mycoplasma species, including *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis, Mycoplasma hyosynoviae*, *Mycoplasma flocculare,* and *Mycoplasma suis*. Of these, *M. hyopneumoniae* is the most significant, causing the most significant economic impact to producers. Vaccination has provided producers limited relief, but infections continue unabated, often at low levels, in swine herds. Diagnosis of *M. hyopneumoniae* infections can be problematic as well. The serologically-based ELISA test is the assay of choice, but antibody responses have not proven to be a reliable indicator of infection. Serological responses are slow to develop in *M. hyopneumoniae*-infected animals, and strong antibody responses resulting from vaccination or previous infections do not correlate well with protection. Previous studies by this laboratory have detected extensive cross-reactions between *M. hyopneumoniae* and *M. flocculare* (Petersen et al., 2016). Since *M. flocculare* is generally considered a commensal and is rarely tested for, this has the potential for complicating diagnoses by ELISA. Polymerase chain reaction (PCR) assays are also used extensively for diagnosis but also have problems. Although PCR assays are generally very sensitive, the type of samples taken by veterinarians in the field (nasal swabs) are generally not sufficient for use on a per animal basis but can be effective on a herd basis (Kurth et al., 2001). Despite the overall assay sensitivity, nasal swabs do not contain sufficient levels of mycoplasma DNA and thus often yield negative results. Lower tracheobronchial swabs or bronchial lavage fluids are more predictive of infection when tested by PCR (Kurth et al., 2001). However, these types of samples are the most difficult to obtain in the field and are generally not available for diagnostic purposes. Thus, there is a continued need for development of an antibody-based assay that is both sensitive and more indicative of *M. hyopneumoniae* infection.

Recently, this laboratory has generated a series of mycoplasma-specific pig sera by infecting Caesarian-derived colostrum-deprived (CDCD) pigs with four major swine mycoplasma species (Neto et al., 2014). The reason for using CDCD pigs was to prevent previous exposure to mycoplasmas or acquisition of anti-mycoplasma antibodies

* Corresponding author at: 1130 Veterinary Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA 50011-1134, USA.
E-mail address: fcminion@iastate.edu (F.C. Minion).

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through colostrum. Not only were the herds from which these animals originated considered to be mycoplasma free, but these young pigs were extensively tested with different serological tests and by PCR for the presence of mycoplasmas, all of which were negative (Neto et al., 2014). These sera were then used in a series of ELISA assays to assess their antibody cross-reactivity (Neto et al., 2014). It was clear from these studies using a standard ELISA-based assay that the antibody responses to *M. hyopneumoniae* infection was slow to develop, often taking more than 62 days from the day of challenge. This was not true for the *M. hyorhinis* challenged pigs, which developed positive responses by 14 days (Neto et al., 2014). In the same studies, *M. flocculare* challenged pigs were also slow to develop positive antibody responses, taking 62 days or more. In subsequent studies with protein microarrays and hyperimmune rabbit antisera, *M. hyopneumoniae* and *M. flocculare* were highly cross-reactive (Petersen et al., 2016). Clearly, if producers are to better understand the health status of their swine herds, a better, more sensitive and species-specific antibody test is needed for *M. hyopneumoniae*. The study reported here was designed to better understand the development of antibody responses on a protein by protein basis.

2. Materials and methods

2.1. Antisera

The pig sera used in this study came from three sources. The first group of 25 sera was obtained from the Iowa State University Veterinary Diagnostic Laboratory. These sera were positive in the IDEXX or DAKO *M. hyopneumoniae* ELISAs and thus represented the antibody responses of pigs naturally infected in the field. A second group of sera was obtained from pigs infected for a year with *M. hyopneumoniae* strain 232 or *M. flocculare* strain Ms42. These sera thus represented antibody responses in sows with long-term infections. There were two pigs infected with *M. hyopneumoniae* and three pigs infected with *M. flocculare*. A third group of sera was obtained from CDCD pigs following intratracheally challenge with *M. hyopneumoniae* lung homogenate containing strain 232 or *M. flocculare* strain 4843 as described previously (Neto et al., 2014). These pig sera were tested in the IDEXX or DAKO ELISAs and by PCR prior to inclusion in the study (Neto et al., 2014). A Tween 20 ELISA was used for testing all pig sera in this study (Supplemental file 1, Fig. S1). The day -1 value was indicative of the serological responses at the start of the study. These sera were collected on a weekly basis over a 118-day period and stored at −20 °C until use. For the purposes of this study, a subset of the available sera was selected to apply to the protein microarrays covering the entire experimental period to determine the progression of antibody responses. In addition, a pool of twenty 7-day old CDCD pig sera was used as a negative control.

Prior to their use on the array, sera were diluted 1:10 in phosphate buffered saline, mixed with an equal volume of *E. coli* lysate from the expression strain (Petersen et al., 2016), and incubated for 24–48 h at 4 °C yielding a final antibody concentration of 1:20. The antibody-lysate suspension was centrifuged (10,000 × *g*) for 5 min prior to application to the array. For sera from pigs #127 and #143, a final 1:100 dilution was made to more clearly delineate progression of antibody responses over the course of infection.

2.2. Recombinant proteins

Mycoplasma hyopneumoniae genes were cloned by PCR, modified by site-specific mutagenesis reactions, sub-cloned into the expression vector pDEST17 and expressed in *E. coli* BL21-CodonPlus (DE3)–RIPL^{+} as described previously (Petersen et al., 2016). The target genes were selected on the basis of their predicted surface location and lipid modification sequences. Not all targeted genes could be cloned or expressed in a soluble form in our system. The array proteins are described in detail in Supplemental File 2. Purified recombinant proteins were obtained from fully induced *E. coli* using Profinity iMAC Ni-charged resin (Bio-Rad Laboratories) in a batch processing scheme. All protein preparations were screened by immunoblot using an anti-6X His monoclonal antibody (CLONTECH Laboratories, Inc.), and fractions containing expressed proteins were concentrated to a standard concentration of 0.5 mg/mL in printing buffer (150 mM phosphate, 0.01% Tween 20, 4 M urea, pH 8.5).

2.3. Protein microarrays

2.3.1. Array construction

The protein arrays were printed as described previously (Petersen et al., 2016). A BioRobotics MicroGRID II microarray printer (Genomic Solutions, Inc., Ann Arbor, MI) was used to print a serpentine pattern in triplicate on Nexterion H slides (Schott North America, Inc., Louisville, KY). Fourteen arrays were printed to a slide with a pattern of 15 × 15 spots.

2.3.2. Screening of protein arrays

Arrays were blocked (25 mM ethanolamine, 100 mM monosodium phosphate, pH 8.5) for 1 h and washed in PBST (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.5 with 0.1% Tween 20) as described previously (Petersen et al., 2016). A 16 well slide incubation chamber was used to separate the arrays (Nexterion IC-16) and allow individual diluted, adsorbed antibodies to react with individual arrays. Adsorbed pig sera were added to individual arrays, incubated for 1 h at room temperature with shaking followed by washing the slide three times with PBST. Each slide was then incubated with biotinylated anti-swine IgG (H + L) secondary antibody diluted 1:50 in PBST for 1 h at room temperature. In cases where the mouse monoclonal antibody F1B6 was used to detect mhp183.c on the array, a biotinylated anti-mouse IgG (H + L) conjugate was used instead of the biotinylated anti-swine IgG at a 1:100 concentration. After washing an additional three times, Alexa Fluor® 555–labeled streptavidin diluted 1:100 was added for 30 min. The streptavidin was removed by aspiration, the slide was washed twice with PBST, the chamber was removed, and the slide given a final wash in a 50 ml centrifuge tube with PBS for 10 min. Slides were scanned at a laser intensity of 90% and a PMT gain of 70% using a ScanArray 5000 laser scanner (GSI Lumonics, Bedford, MA).

3. Data analysis

Scanned images from the laser scanner were quantified using SoftWorRx Tracker v2.8 (Applied Precision, Inc., Issaquah, WA) (Petersen et al., 2016). As described previously, spot-specific mean signals were corrected for local background by subtracting spot-specific median background intensities using R v3.4.1 (Petersen et al., 2016). The background-corrected mean of the triplicate spots for each protein was then calculated within each array to produce one measure of reactivity with each of the proteins. Heat maps were generated to indicate the degree of reactivity for each protein for each serum.

4. Results

4.1. Printing and quality control of protein arrays

Each of the printed arrays was examined by microscopy. Print confirmation, spot morphology, and relative protein concentrations were determined by probing a slide with anti-6X His monoclonal antibody (Clontech) followed by fluorescein-labeled goat anti-mouse IgG Fab2 antibodies (Cappel) as described above (Supplemental File 1, Fig. S2). Each of the proteins on the array contained a poly-His sequence and could be detected on the array in this way.

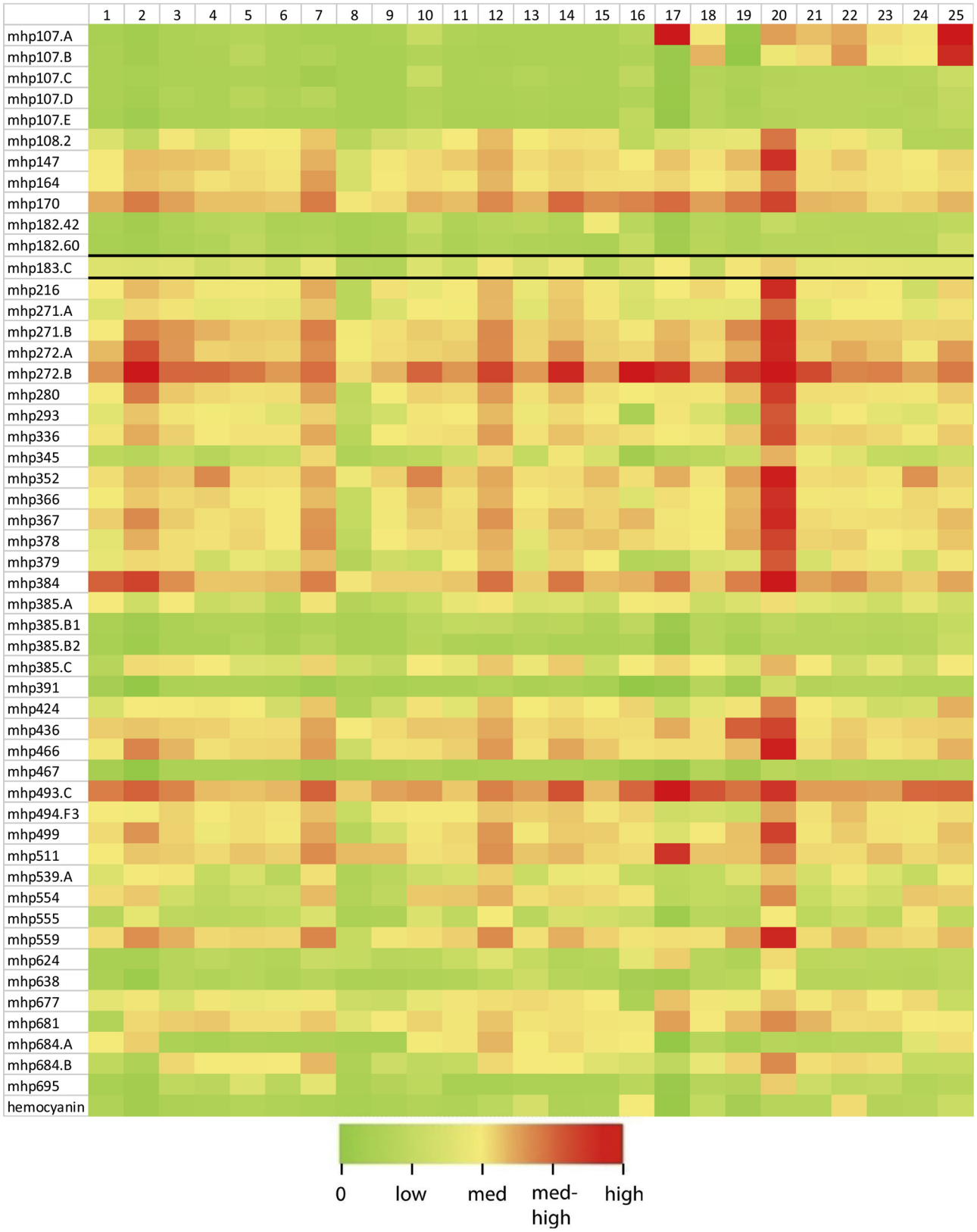

Fig. 1. Heat map of individual sera from naturally-infected pigs that were serologically positive by IDEXX ELISA. The color scale was: green to light green, little to no reaction; yellow, low to moderate reaction; orange to red, high reactivity. Gene products are indicated on the left. Numbers at the top indicate an individual submission of one pig sera to the Veterinary Diagnostic Laboratory. Bracketed is the reactivity toward mhp183.C, the C-terminal fragment of P97.

4.2. Pig sera

Pig sera contained antibodies reactive with minor *E. coli* contaminants in the protein preparations (data not shown). Extensive adsorption of diluted sera with lysates of the *E. coli* expression strain prior to their use on the microarray reduced or eliminated these

Table 1
P97/P102 paralogs of *M. hyopneumoniae* strain 232.

P97 family		P102 family	
mhp183*	P97	mhp182*	P102
mhp107	CH	mhp108	CH
mhp271	CH	mhp272	CH
mhp280	P95	mhp274	CH
mhp385	CH	mhp275	CH
mhp493	CH	mhp384	CH
mhp684	P146	mhp683	CH

CH = conserved hypothetical.
* indicates the prototype gene sequence for each of the families.

reactions (Petersen et al., 2016). Each of the pig sera were adsorbed to eliminate non-specific reactions on the array.

4.3. Heat map analysis

Heat maps were generated to better visualize the results of the study with the Veterinary Diagnostic Laboratory *M. hyopneumoniae* ELISA positive sera (Fig. 1). These sera gave relatively similar antibody responses to the antigens on the array with most animals responding strongly to antigens from genes mhp170, mhp271.A, mhp272.A, mhp272B, mhp384, and mhp493.C (Fig. 1). All of these proteins are members of the P97/P102 gene families except for mhp170 (Table 1). A few of the sera showed reactivity with additional antigens. For instance, one could argue that antigens mhp164, mhp271B, mhp280, mhp336, mhp367, mhp466, mhp499 and mhp511 could also be placed in this group of reactive antigens, but their reactivities are somewhat lower in most of the animals. One animal in particular, sample #20, had reactivities against multiple antigens that were stronger than other sera. A few antigens were uniformly negative on the array as illustrated by the green color. Marked on the figure is mhp183.C, the cilia binding region of the major cilia binding adhesin P97. Surprisingly, the reactivity to this antigen was low in most animals.

Fig. 2 shows the reactivity of sera from animals infected and housed for a year. All of the sera collected pre-infection showed limited reactivity including the control pig #196, which was housed separately throughout the experiment. All of the post-infection sera maintained a high level of reactivity with *M. hyopneumoniae* antigens. Even the *M. flocculare*-infected pigs had a strong response with *M. hyopneumoniae* antigens after a year. This confirmed our previous study with rabbit hyperimmune sera that showed strong cross-reactivity of anti-*M. flocculare* antisera with *M. hyopneumoniae* antigens (Petersen et al., 2016). The highest signals were generated against P97/P102 family members (Table 1), although high reactivity was also detected with several other antigens (mhp147, mhp170, mhp216, mhp436, mhp466, mhp554, mhp559). The reactivity of the *M. hyopneumoniae*- and *M. flocculare* -infected animals was virtually indistinguishable.

Fig. 3 shows the reactivity of the CDCD pigs infected with *M. hyopneumoniae*. These animals were obtained at 12 weeks of age from a herd that was believed to be swine mycoplasma negative. They were negative by ELISA (Supplemental File 1, Fig. S1), PCR, and culture for *M. hyopneumoniae*, *M. hyorhinis*, *M. hyosynoviae* and *M. flocculare* (Neto et al., 2014). Two of the day 0 sera had reactivity with the array (pigs #127 and #143). At necropsy, negative (uninfected) pigs from this herd showed no signs of disease. Pigs from other challenge groups in this study (*M. hyorhinis*, *M. hyosynoviae*, *M. flocculare*) also had no lung pathology at necropsy (Neto et al., 2014). In pigs #199 and #147, the strongest antibody responses were detected on days 30 and 54. The variability in animal responses was extraordinary, with one of the four infected pigs having low antibody levels across all antigens throughout the infection even though lung lesions were apparent at necropsy in this animal. Two of the P102 paralogs, mhp272 and mhp384, were the most reactive, along with one P97 paralogue, mhp493 (Minion et al., 2004). In the animal with a low response, antigens mhp272, mhp367 and mhp493 yielded early (day 8) responses. The raw data upon which the heat maps were developed is included as Supplemental File 3.

5. Discussion

The recombinant proteins used to construct the protein microarray were chosen based on their structure predictions as being membrane proteins (lipoproteins) or members of the P97/P102 gene families (Minion et al., 2004). In some cases, fragments of these genes were spotted to the arrays. In a previous proteomics study, Pendarvis et al. identified all of the proteins on the array except for three, mhp216, mhp554 and mhp559 (Pendarvis et al., 2014). Interestingly, all three of these proteins were recognized by the pig sera (Figs. 1–3). For convenience, we have constructed a comparison image of naturally- and experimentally-infected pig sera (Supplemental File 1, Fig. S5). This image demonstrates how similar the two classes of sera react on the array. Also, a table is included describing the genes and sections of the genes cloned (Supplemental File 2).

The protein microarray is a powerful tool to analyze antigen-specific responses and in this study proved more sensitive than commercial ELISAs. Its sensitivity was unparalleled, capable of detecting and measuring antibody responses when commercial ELISAs had failed to yield positive responses (Neto et al., 2014). Our results indicate that animals thought to be mycoplasma free by all known criteria (serology and PCR) might very well harbor an undetected mycoplasma infection. From previous studies (Petersen et al., 2016), it seemed reasonable that the contaminating infection could be due to *M. flocculare*. It is unlikely that a herd harboring *M. hyopneumoniae* would show no signs of infection over time, as was the case with this herd. There is also anecdotal evidence that this might be true, since the diagnostics for *M. flocculare* are much less developed and are less sensitive than for *M. hyopneumoniae*.

Another species might be the culprit, *Mycoplasma hyopharyngis*. This is a species that is found in swine but is relatively unknown (Erickson et al., 1986). The extent of antigenic cross-reactivity between *M. hyopharyngis* and *M. hyopneumoniae* is not known. We reasoned that it was possible that an inapparent *M. hyopharyngis* infection could be present in the herd from which the CDCD pigs were obtained if *M. hyopharyngis* and *M. hyopneumoniae* cross-react. We examined this by reacting our array with rabbit hyperimmune antisera raised against *M. hyopharyngis*. Fig. S3 in Supplemental File 1 shows the minimal cross-reactivity of the rabbit anti-*M. hyopharyngis* antisera. This suggests that the high antigenic reactivity of pigs 143 and 127 on day 0 was not due to a *M. hyopharyngis* infection, nor was it likely based on previous studies that *M. hyorhinis* or *M. hyosynoviae* might be involved (Petersen et al., 2016). We are unsure about *M. suis*, the causative agent of porcine infectious anemia. Although there are no reports of cross-reactivity between *M. suis* and *M. hyopneumoniae*, this has not been tested. Because of the lack of *M. suis*-specific rabbit hyperimmune antisera, we were unable to perform these studies. We contend that it is most likely that an inapparent *M. flocculare* infection was present in the herd, because of the extensive cross-reactivity (Petersen et al., 2016), the lack of lung lesions in the control pigs at necropsy, and the lack of disease problems in the original herd.

Surprisingly, the pig sera from all groups responded poorly to the mhp183.C and mhp385.C antigens. In comparison with the pig sera, rabbits respond significantly to both these antigens (Petersen et al., 2016). It should be noted that all of the pigs from which sera were obtained for this study were infected with *M. hyopneumoniae* either naturally or experimentally. The rabbits, however, were hyperimmunized with adjuvanted whole cell antigens. This has important ramifications for vaccine trials since many trials have reported using the P97 C-terminal region as a target antigen, the antigen we designated mhp183.C in our arrays. We were concerned that perhaps the protein was not present on the array because it had precipitated out of

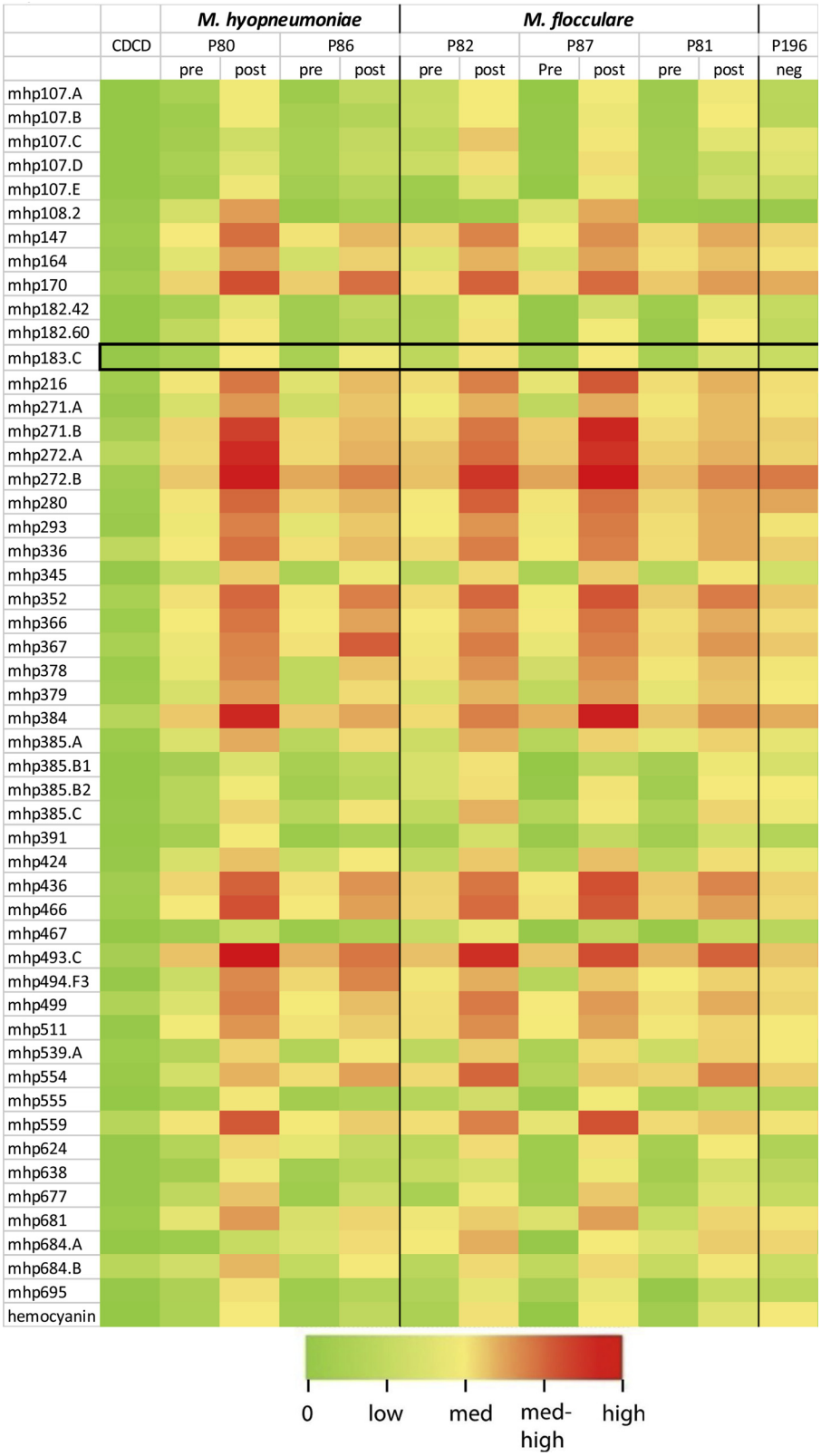

Fig. 2. Heat map of sera from pigs infected with *M. hyopneumoniae* or *M. flocculare* for a year. Pig numbers are indicated at the top; gene products are given on the left. Pre- and post – challenge sera are given. Pig 196 was a control (non-infected) pig held throughout the year in a separate room. CDCD was a combined serum from twenty CDCD 7-day old piglets. Bracketed is the reactivity toward mhp183.C, the C-terminal fragment of P97.

solution or for some other reason. Consequently we reacted one array with the mouse monoclonal antibody F1B6, which specifically recognizes the R1 region of P97 (Hsu and Minion, 1998). Fig. 4 shows that both mhp183.C and a slightly cross-reactive P97 paralog mhp385.C are present on the array. The only explanation is that pigs don't respond strongly to that portion of P97. Another difference between rabbits and pigs is that rabbits respond moderately to the C-terminal portion of P102 (mhp182.42) whereas pigs don't respond to

Fig. 3. Heat map of four CDCD pig sera infected with *M. hyopneumoniae* over a 118-day period. Pig numbers and days post infection are given at the top. Gene products are indicated on the left.

any region of the protein during an infection (Figs. 1–3). Rabbits also respond strongly to mhp494.F3 whereas pigs don't.

Despite the fact that our CDCD pig study revealed an inapparent mycoplasma infection in this herd, there are several important points that can be made. First, the protein microarray is a very sensitive indicator of mycoplasma infection, more so than ELISAs and PCR

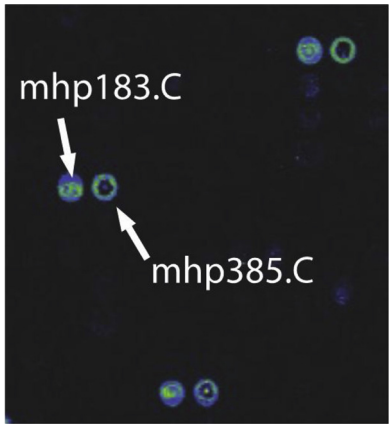

Fig. 4. Microarray reactivity of monoclonal antibody F1B6 (anti-P97R1). The image shows reactivity to the C-terminal fragment of P97 containing the R1 repeat region and a less prominent reactivity to mhp385C, a P97 paralogue. No other protein spots were recognized on the array.

(compare Figs. 3 and S1 of Supplemental File 1). Second, we were able to confirm the extensive cross-reactivity between *M. hyopneumoniae* and *M. flocculare* in pigs (Fig. 2). In fact, we believe this cross-reactivity will confound ELISA development more than any other issue. In our studies, we were unable to differentiate between the two species by antibody reactivity. Any commercial ELISA that has not been tested against sera from *M. flocculare*-infected pigs will potentially generate false positives. This may very well explain the number of weakly positive reactions seen in commercial ELISA assays. The prevalence of *M. flocculare* in pig herds in the field is not known.

By studying field serum samples, we were able to correlate naturally- and experimentally-infected animal antibody responses. The fact that mhp183.C and mhp182.42 were only weakly recognized by any sera from infected animals may indicate the level at which these two proteins are produced by *M. hyopneumoniae* or it may indicate the inability of the pig to recognize and mount a strong antibody response against these antigens. There are several studies that are important for consideration here. First, Adams et al. showed that both of these antigens are transcribed *in vivo,* so we know that they are expressed *in vivo* (Adams et al., 2005). Second, Madsen et al. examined transcriptional differences between *M. hyopneumoniae* grown *in vitro* and those isolated from infected animals (Madsen et al., 2008). In the latter study, there was no indication of any transcriptional differences in these two genes, as one would expect from a pathogen with a niche limited to its specific host that has little need for gene regulation of its adhesins.

Our observations concerning P97 are not unique. Shimoji et al. showed a distinct lack of antibody response against P97 when an *Erysipelothrix rhusiopathiae* strain expressing the C-terminal portion of P97 was used as a vaccine in pigs (Shimoji et al., 2002). Antibody responses against P97 R1 could be generated if a R1-containing subunit peptide was adjuvanted (Conceicao et al., 2006; Chen et al., 2001), but normal whole cell vaccines failed to induce significant antibodies against P97 (Fisch et al., 2016). In contrast, Okamba et al. demonstrated partial protection using an adenovirus expressing the C-terminal portion of P97 (Okamba et al., 2010). However, they cloned a 1676 bp (559 amino acids) fragment from the C-terminus (Okamba et al., 2010, 2007); our fragment was 302 amino acids (906 bp) long. The fragment cloned by Okamba et al. was 256 amino acids longer and included additional antigenic regions 5′ to the R1 region of P97 that may very well have added additional antigenic sites that provided partial protection (Supplemental File 1, Fig. S4).

6. Conclusion

In summary, the protein microarray has the potential to gain new insights into the pathobiology of mycoplasma infections. This approach is also well positioned to identify new targets for assay development to enhance the sensitivity of antibody-based diagnostic assays. Importantly, the protein microarray was able to detect a mycoplasma infection when ELISAs and PCR tests failed. Surprisingly, pig sera from naturally- or experimentally-infected animals responded poorly to the C-terminal fragment of P97 (mhp183.C) and to the C-terminal region of P102 (mhp182.C). Thus, the use of these antigens in vaccine development should be re-evaluated. Several of the P97/P102 paralogues, however, were recognized strongly as expected and might be better targets. The extensive cross-reactivity shown with hyperimmune rabbit sera in previous studies (Petersen et al., 2016) was confirmed with sera from long-term, infected pigs. This also supports the hypothesis that once infected, pigs are colonized long-term, possibly for life.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.02.010.

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