RESEARCH REPORT
SWINE HEALTH

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Title: Development of an Improved *Mycoplasma hyopneumoniae*-Specific ELISA; **NPB #11-057**

Investigator: F. Chris Minion

Institution: Iowa State University

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Industry Summary

Improvement of serological-based diagnostics for *Mycoplasma hyopneumoniae* infections in swine will require identification of mycoplasma-specific antigens and their use in an ELISA-based assay format as well as development of more current technologies. The overall goal of this project was to develop an improved serology-based assay on *M. hyopneumoniae*-specific antigens, follow pig responses to these antigens during infection over an extended period of time, and utilize these antigens in a bead-based assay. This will improve the specificity, reproducibility, and sensitivity of current serological assays. We identified eight *M. hyopneumoniae*-specific proteins that lack cross-reactivity to other swine mycoplasma species. These antigens were cloned and expressed as recombinant proteins for further analysis. Each protein posed unique problems in expression levels, solubility, etc. The proteins are being assessed by a commercial partner for their usefulness in a standard ELISA style assay. The proteins have also been bound to beads, and testing is underway with our defined sera and sera from clinical samples. The defined sera were developed by challenging pigs (4 per group) with one of the following mycoplasmas, *M. hyopneumoniae, M. hyosynoviae, M. hyorhinis,* and *M. flocculare,* and monitoring the infection for a 118 day period. Serum samples were taken throughout the study, oral fluids were collected on a monthly basis, and at necropsy histopathology was performed on the *M. hyopneumoniae* challenged pigs. Serological responses indicated a slow conversion except for the *M. hyorhinis*-challenged pigs. Cross-reactions were noted in the *M. hyopneumoniae* sera with *M. hyorhinis* Tween 20 antigens and in the *M. flocculare* sera with *M. hyorhinis* Tween 20 antigens. Oral fluids from *M. hyopneumoniae* challenged pigs showed a positive IgA response with the Tween 20 ELISA. The ELISA and bead-based assays with the purified antigens are still being tested. (Contact: Chris Minion, VMPM, Iowa State University, fcminion@iastate.edu)

Keywords: Mycoplasma, ELISA, *M. hyopneumoniae*-specific antigens, bead-based assay, protein arrays

Scientific Abstract

The goal of this study was to improve serological assays for *M. hyopneumoniae* infected pigs. Our approach was to focus our assay development on antigens that are *M. hyopneumoniae*-specific and to test these in two different formats, a 96 well ELISA based format, and on beads using Luminex technology. To assist in proper screening, we produced swine sera from mycoplasma-infected pigs. The challenge study used CDCD pigs, four to a group and each group was challenged with *M. hyopneumoniae, M. hyorhinis, M. hyosynoviae,* and *M.*

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For more information contact:
National Pork Board • PO Box 9114 • Des Moines, IA 50306 USA • 800-456-7675 • Fax: 515-223-2646 • pork.org

flocculare. The groups were housed separately throughout the experiment and they were monitored from cross infections by PCR. Sera were obtained throughout the experiment so we could follow antibody responses. In addition, oral fluids were also obtained from each room once a month for analysis. Serological responses indicated a slow conversion except for the *M. hyorhinis*-challenged pigs. Cross-reactions were noted in the *M. hyopneumoniae* sera with *M. hyorhinis* Tween 20 antigens and in the *M. flocculare* sera with *M. hyorhinis* Tween 20 antigens. Oral fluids from *M. hyopneumoniae* challenged pigs showed a positive IgA response. The sera from *M. hyopneumoniae*-challenged animals were also tested on a protein array. The results from that study showed some inconsistencies, possibly due to operator error or blocking issues. Eight *M. hyopneumoniae* proteins were purified, and were bound to beads for testing in a Luminex-based assay. These antigens are also under study in an ELISA assay.

Introduction

Serology due to its low cost and ease of sample collection is the most common tool used to monitor the status of *M. hyopneumoniae* infection within a herd. In addition, testing replacement animals for entry into *M. hyopneumoniae* negative herds to ensure continued negative status is critical for herd health. Two ELISA assay technologies are currently used for *M. hyopneumoniae* diagnostics and consist of either a competitive inhibition assay based on a monoclonal antibody to an internal protein in *M. hyopneumoniae,* or indirect ELISAs that consist of a mixture of membrane-derived proteins as the test antigen. Recent reports from the field increasingly find that the current ELISAs provide conflicting results that are not easily resolved. As a result, the need for an improved assay has become increasingly apparent. The proposed project will develop a new ELISA with improved sensitivity and specificity over currently available tests providing the swine industry with a more effective tool for *M. hyopneumoniae* screening and monitoring on a herd basis.

Objectives

Our long-term objective is to develop an improved diagnostic assay for *Mycoplasma hyopneumoniae* that will optimize our ability to measure exposure and infection. Development of improved diagnostics will increase our ability to successfully eradicate *M. hyopneumoniae* from US swine populations. The specific objective of this proposal is to complete our screening of surface proteins and serological based assay development for *M. hyopneumoniae* detection. This objective will be accomplished by completing the following specific aims:

1. We will test pig oral fluids from animals testing serologically positive for *M. hyopneumoniae* by existing ELISA tests for reactivity to our surface antigen array of 40 proteins to identify antigens recognized by antibodies in these fluids. This will be important in developing new sampling methods for *M. hyopneumoniae* detection.
2. Develop and test swine mycoplasma-specific sera and oral fluids in our pig antigen array. This will involve infecting pathogen-free swine with *M. hyorhinis, M. hyosynoviae,* or *M. flocculare* and collecting sera and oral fluids over time. We will also generate sera from *M. hyopneumoniae*-infected swine and collect sera and oral fluids over a 6-month period to following the waning of the antibody responses and determine to what antigens, again using our protein array.
3. Clones for eight antigens previously been shown specific for *M. hyopneumoniae* and useful as ELISA antigens (see preliminary data) will be constructed with *E. coli* codon usage patterns to facilitate expression in *E. coli* and their downstream purification.

Materials and Methods

<u>Animals and Animal Care</u>

The study was performed at a Biosafety Level 2 facility (Building 29) located at the Veterinary College of Iowa State University. Animal chores were carried out on a daily basis by LAR personnel respecting the facility biosafety protocol. That is, the use of new clean coveralls, rubber and plastic boots, masks, and hair coveralls daily separated for each room since the beginning of the experiment. In addition, during any activities within

rooms (feeding, samples collection, etc.), the personnel involved would go through groups infected with any other mycoplasmas, leaving the one challenged with *M. hyopneumoniae* to be the last visited (this experiment routine was previously established in accordance with Dr. Erin Strait). Rooms consisted of solid flooring, which were hosed down with water daily for cleaning. Water was freely available via a single nipple waterer in each room. Pigs were fed a commercial feed with no antibiotics once daily by caretakers on the floor. Each room has an ante-room where all individuals are required to put on a layer of disposable coveralls, disposable gloves, disposable plastic boot covers, disposable hairnet, and disposable N95 respirator. The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee (#1-12-7289-).

Experimental design and sampling procedures (described separately for Experiments 1 and 2)

Experiment 1- *Mycoplasma hyopneumoniae*, *M. hyorhinis*, and *M. flocculare* challenge

- Twelve 6-week-old female CDCD (Cesarian Derived Colostrum Deprived) pigs were place in 3 challenge groups, four pigs per group (Table 1)
- All pigs were confirmed to be negative for *M. hyopneumoniae*, *M. flocculare*, *M. hyorhinis*, and *M. hyosynoviae* by PCR run in nasal swabs before challenge
- Pigs from the *M. hyopneumoniae* challenge group were tested for the presence of antibodies against *M. hyopneumoniae* before challenge by two ELISA based assays: Tween 20 and DAKO (Oxoid). Animals were confirmed to be negative in both tests
- The procedure used for the challenge was adapted for each pathogen's nature of infection (Table 2)
- Challenge was performed by snaring the pigs and administering half the inoculum into each nostril (Intra-nasal inoculum) or the full amount at once (intra-tracheal) using a disposable syringe
- Blood and oral fluids samples were collected periodically after challenge (Table 3) (Schedule described in the Sample collection section)
- All animals were necropsied at 118 days post-challenge.

Table 1. Scheme for animal placement within rooms at the experimental facility (Experiments 1 and 2).

Rooms #	Pathogen (challenge)	Total # of animals	Animals ID #
2	*M. flocculare*	4	108, 128, 156, 220
3	*M. hyorhinis*	4	110, 134, 179, 213
4	*M. hyopneumoniae*	4	127, 143, 147, 199
19 and 20*	*M. hyosynoviae*	4	104, 105, 106, 107

*Rooms 19 and 20: room 19 had animals # 104 and 107 (challenge with *M. hyosynoviae* strain 3496); and room 20 had animals # 105 and 106 (challenged with *M. hyosynoviae* strain 3491). These four animals were challenge with two different strains of *M. hyosynoviae* as described in parentheses; therefore, were allocated separately in two different rooms (2 animals per room).

Table 2. Description of challenge methods (Experiments 1 and 2).

Pathogen	Strain[1]	Passage (p)/media used for growth[2]	Route of Infection[3]	Inoculum Vol. ml/infectious dose (CCU/ml)
M. flocculare	4843	p6/Friis	Intra-tracheal	10/NA*
M. hyorhinis	3420	p10/BHI	Intra-nasal	$10/10^{2}$
M. hyopneumoniae	232	Lung inoculum/Friis	Intra-tracheal	5/NA*
M. hyosynoviae	3491/3496[4]	P6/Difco+horse serum	IV and IN**	$17^{5}/10^{10}$

[1] Laboratory strain was selected by Dr. Erin Strait.
[2] Strain growth and lung inoculum were prepared by Mat Raymond. A scan copy of his laboratory notebook is included in the email containing the final report.
[3] Route of infection: *M. hyopneumoniae* and *M. flocculare* (7); *M. hyorhinis* (4); *M. hyosynoviae* (2).
[4] *Mycoplasma hyosynoviae* strains 3491 and 3496 were selected based on previous history of each isolate and their ability to grow in the selected media. In addition, there were two animals challenged with each strain (refer to Table 1). Horse serum was used to diminish the risk of anaphylactic shock upon challenge.
[5] A volume of 17 ml (media containing bacteria) was used for the challenge in three consecutive dates (Days post-challenge 0 (2 ml intravenously), 15 (5 ml intranasally), and 39 (10 ml intranasally) to maximize the antibody response. Animals were challenged at with the same strain they received at the first time (DPC 0)
* NA – not applicable – *M. hyopneumoniae* – lung inoculum was used without titration/*M. flocculare* – no titration was done on the media containing it – information verified and confirmed with Matt Raymond
**IV and IN – intravenous and intranasal challenge

Table 3. Calendar of sample collection for groups challenged with *M. hyopneumoniae*, *M. hyorhinis*, and *M. flocculare* (Experiment 1).

Sample type	Day post-challenge
Serum*	-1, 8, 15, 22, 30, 38, 46, 54, 62, 70, 78, 86, 94, 102, 110, 118
Pen-based oral fluids**	-1, 8, 12, 15, 22, 30, 38, 46, 54, 62, 70, 78, 86, 94, 102, 110, 118^

*Serum samples collected and tested individually.
**Pen-based oral fluids samples collected from the group of four animals present in the pen. All four animals were allowed contact with the rope. A volume of 15-20 ml was collected at each time point.
^118 days post-challenge – Necropsy date for all animals of each group.

Experiment 2- *Mycoplasma hyosynoviae* challenge (challenge of this group was carried out before experiment 1, that is why it is described separately)

- Four 14-week-old female CDCD (Cesarean-Derived, Colostrum-Deprived) pigs were place in each of the 2 challenged groups (Table 1)
- All pigs were confirmed to be negative for *M. hyosynoviae* and *M. hyorhinis* by PCR run in nasal swabs and tonsil scraping before challenge and at the end of the study
- The procedure used for the challenge was used in accordance to what has been previously described by Hagedorn-Olsen *et al.* (2)(Table 2)
- Blood samples were collected periodically after challenge (Table 4) (Methodology described in the Sample collection section)
- Animals were necropsied at 66 days post-challenge.

Table 4. Calendar of sample collection for groups challenged with *M. hyosynoviae* (Experiment 2).

Sample type	Day post-challenge
Serum*	-3, 0, 1, 4, 7, 14, 15, 24, 39, 42, 49, 66^

* Serum samples collected and tested individually.
^66 days post-challenge – Necropsy date for all animals.

Animal challenge procedures for Experiment 2

A 1 mL of frozen stock adapted to Difco PPLO Horse Serum (D-HS) was passed into 5 mL of D-HS. This was incubated for 8 days to get really dense growth and turbidity (in case potential contaminant present at very low numbers). A 1 mL of the 5 mL culture was then passed into 25 mL of D-HS. A 100 µL (10^{-1} dilution) sample was then spread plated onto blood (BAP) and chocolate agar for purity verification. Due to their smaller size, 10 µL (10^{-2} dilution) was plated onto brain heart infusion (BHI) agar. All plating was performed in triplicate.

Quantification of inocula was performed by using D-HS media for CFU/mL calculation of the challenge cultures. This was performed via ten-fold serial dilution in triplicate using 2 mL volumes. CCU/mL was also calculated via D-HS containing arginine and phenol red. Inoculation was performed as reported by Hagedorn-Olsen *et al*. (2). Animals were restrained using a humane snare and 2 mL of 10^{10} CCU/mL *M. hyosynoviae* (strain 3491 or 3496) was administered intravenously (IV) using a butterfly catheter in an ear vein. For the negative control group 2 mL of Difco and Horse serum was administered IV using the same procedure.

To further evaluate sero-conversion, 2 pigs each from 3491 and 3496 groups were kept for an additional 52 days (total 66 DPC). These pigs were re-inoculated with 5 mL of 10^{10} CCU/mL *M. hyosynoviae* (using the same strain they were initially challenged with) was administered intra-nasally (IN) on DPC 15 by snaring the pigs and administering half the inoculum into each nostril using a 6 mL disposable syringe. On DPC 39 a third re-inoculation with 10 mL of 10^{10} CCU/mL *M. hyosynoviae* (using the same strain they were initially challenged with) was administered intra-nasally (IN).

Sample collection (applicable for experiments 1 and 2-verify which sample type was used from each experiment as described in exp. design)

Pen-based oral fluids samples were collected using 5/8" cotton rope hung in the center of pen. The rope was cut to length so that the end was shoulder-high to the animals. Ropes were left in place for 20 to 25 minutes or until 15-20 ml of fluid could be collected; however, the floor and animals were always washed before the procedure (aim: diminish environment contaminants load). To recover the oral fluid specimen, the bottom (wet end) of each rope was inserted into a large plastic boot and the oral fluid was manually extracted by squeezing the rope. The sample was decanted into a 50 ml disposable centrifuge tube for storage (Fisher Scientific Co., Pittsburgh, PA, USA). At least 15 ml of oral fluid sample was collected from each pen at each time point (pre and post- challenge). Powder-free nitrile gloves were worn during the collection process and changed between each pen (Microflex Corporation, Drive Reno, NV). Samples were stored at – 20°C until assayed.

Blood, nasal swabs and tonsil scrapings were collected from individual animals. *Blood samples* were collected using a single-use collection system (Vacuum tubes, BD, Franklin Lakes, NJ, USA). Samples were centrifuged at 2000 x *g* for 10 minutes, after which serum was aliquotted into 5 ml tubes (BD Falcon™, BD Biosciences Discovery Labware, Two Oak Park, Bedford, MA, USA). *Nasal swabs* (Collected pre-challenge -1 DPC for confirmation of the animals status) were collected from each naris and placed in 5 ml polystyrene round bottom snap-cap tubes containing 2ml of 1X (PBS InvitrogenTM, Gibco, Grand Island, New York, USA). *Tonsil scrapings* were collected by holding the mouth open with an oral speculum and scraping the surface of the tonsil using a sterilized, blunt stainless steel spoon with an elongated handle (8). Tonsil scraping samples were then collected from the steel spoon using a 15 cm sterile nylon flocked swab (Puritan®) and placed in 5 ml tubes (BD Falcon™) containing 3 ml of sterile 1X PBS (Invitrogen™). All three sample types were stored at – 20°C until assayed.

Lung tissues were collected from all animals necropsied from experiment 1 for histopathology evaluation and PCR testing for all four mycoplasmas (*M. hyopneumoniae*, *M. hyosynoviae*, *M. hyorhinis*, and *M. flocculare*).

Broncheoalveolar lavage was carried out in lungs of all animals from experiment 1, and subsequently, standard culture procedures and PCR were used to test for the presence of either Mycoplasmas or any other pathogen.

All sampling procedures repeatedly carried out throughout the study were executed by the same person and manner.

Necropsy from pigs challenged with *M. hyopneumoniae*, *M. hyorhinis*, and *M. flocculare* (experiment 1)

Description of sampling procedures and summary of results. Animals from all three groups were necropsied at 118 DPC. Lungs were evaluated macroscopically at necropsy by the same person, but no visible lesions characteristic of infection with *Mycoplasma hyopneumoniae* could be seen in any of the 12 animals (3 groups). However, microscopic lesions suggestive of *M. hyopneumoniae* infection were detectable in all pigs challenged with *M. hyopneumoniae* which in combination with positive PCR results defined the presence of active infection in that specific group. Animals from *M. hyorhinis* and *M. flocculare* groups had no microscopic lesion suggestive of *M. hyopneumoniae* infection, and in addition, PCR results from their BALs were negative for the presence of this pathogen (Table 6).

Lung tissue collection at necropsy – multiple pieces (2 cm x 2 cm) of lung tissues were collected from the middle left lobe of all animals. Tissues of each animal were stored separately in formalin (10%) for fixation until processed for histopathology evaluation (H&E).

Bronchoalveolar lavage (BAL) – BAL was collected using 25 ml of PBS 1X solution to wash off the lumen content of the left middle lobe of lungs from all pigs. After collection, samples were kept at -20ºC until real-time PCR was carried out for confirm the presence or absence of *M. hyopneumoniae*, *M. hyorhinis*, *M. hyosynoviae,* and *M. flocculare*. Standard culture procedures using Blood agar and Macconkey (incubated at -37ºC) were carried out in BALs collected from all animals to check for the presence of other bacteria

LABORATORY PROCEDURES

DNA extraction protocols. For all PCR protocols, DNA was extracted using a KingFisher® 96 magnetic particle processor (Thermo Fisher Scientific Inc., Waltham, MA, USA) using the software program AM1836_DW_HV_v3 for high volume (300 µl oral fluids) and 1836_DW_50_v3 (Applied Biosystems, Foster City, CA, USA) for low volume (50 µl of either nasal swabs, BAL, or tonsil scrapings). It is a magnetic bead based protocol. This is the protocol currently used by the Veterinary Diagnostic Laboratory at Iowa State University.

PCR procedures. ***Real-time PCRs for M. hyorhinis and M. hyosynoviae*** For these reactions, amplified products of *M. hyosynoviae* and *M. hyorhinis* were 397 and 161 bp in length, respectively. The 16s rRNA was the target sequence for both pathogens. In brief, 22.5 µl of a solution composed of 12.5 µl of QuantiTect® SYBR® Green master mix (Qiagen® Inc., Valencia, CA, USA), one µl of 10 µM stock solution of reverse and forward primers for either *M. hyosynoviae* or *M. hyorhinis,* and 8 µl of nuclease-free water (Qiagen® Inc., Valencia, CA, USA) was pipetted into each well of a 96 well plate (Fast® PCR plates, Applied Biosystems, Foster City, CA, USA). *Mycoplasma hyosynoviae* forward and reverse primers (Integrated DNA Technologies, Inc., San Diego, California) were: 5'- CAGTTGAGGAAATGCAACTGAAC -3' and 5'- CGTCAG TGATTGGCCACC G -3', respectively. *Mycoplasma hyorhinis* forward and reverse primers were: 5'-TGTTGAACGGGATGTAGCAA -3' and 5'- TGAAGCTGTGAA GCTCCTTTC -3', respectively. DNA-extracted material (2.5 µl) was then added to each well.

RT-PCR was then performed using a 96-well thermal cycler using the following cycling conditions: 1 cycle at 95ºC for 10 minutes, 45 cycles at 95ºC for 15 seconds, 59ºC for 30 sec (*M. hyorhinis*) or 63ºC for 30 sec (*M. hyosynoviae*), followed by a melting curve of 72ºC for 30 sec, 95ºC for 15 sec, 60ºC for 1 min, and 95ºC for 15 seconds (7500 Fast Real-Time PCR System, Applied Biosystems, Foster City, CA, USA). Quality controls

included on each plate consisted of one well of previously DNA-extracted from nuclease-free water (negative control) and pure cultures of either *M. hyorhinis* or *M. hyosynoviae* (positive control). Analysis of amplification curves was performed using the manufacturer's software (7500 Fast Real-Time PCR System, Applied Biosystems, Foster City, CA, USA). Auto baseline was used to determine fluorescence baselines, and the threshold was manually set at 0.04. For either organism, a sample was considered positive if the Ct value was ≤ 44 and the melting temperature (Tm) was 75.7ºC ± 0.5ºC or 81.4ºC ± 0.5ºC, for *M. hyorhinis* and *M. hyosynoviae*, respectively.

Real-time PCR for *M. hyopneumoniae* was performed as described (6). Conventional PCR for *M. flocculare* was performed as described (5).

ELISA Assays. Tween 20 antigens were used in all ELISA serum and oral fluid assays as described (1, 3).

Plate preparation and sample loading. Serum samples were tested in duplicate for the presence of antibodies against all target pathogens using an adaptation of a Tween 20 assay previously described for *M. hyopneumoniae* (1). In brief, the antigens for the ELISAs were extracted from whole-cell cultures of *M. hyorhinis*, *M. hyosynoviae, M. hyopneumoniae,* and *M. flocculare* using a Tween 20 detergent reagent. All mycoplasmas were previously grown in their respective required media (Table 5). Plates were individually prepared for each organism. Wells of 96-well plates (Nunc Immulon 2 HB, Thermo Fisher Scientific Inc., Waltham, MA, USA) were coated with 100 µl of 10 µg per ml of each antigen in carbonate-bicarbonate buffer (pH 9.6). Thereafter, plates were incubated overnight at room temperature and then stored at -80ºC until use. The normal ELISA protocol was followed throughout for serum samples. For oral fluids samples, 150 µl of oral fluid was mixed with 150 µl of Tris buffered saline, and then 200 µl of this final solution was added on the plate (adaptation of Kittawornrat et al., 2012 protocol). One positive (serum from animals of known status) and one negative control (PBS 1X), were run on each plate as quality controls.

Detection of *M. hyopneumoniae* isotype-specific reactions (IgM, IgA, and IgG in serum and oral fluids) - To detect isotype-specific responses, 100 µl of a 1:500 dilution of peroxidase-conjugated goat anti-pig IgA (Bethyl Laboratories, Inc.), goat anti-pig IgM (µ) (KPL, Inc.) and mouse anti-pig IgG (1:500 for oral fluids and 1:10,000 for serum samples) (ICN Biomedicals, Irvine, CA, USA) were dispensed into each well (separate for each conjugate), after which the plates were incubated for 30 min at 37ºC and then washed 3 times (1X ELISA wash solution). The peroxidase substrate system (ABTS® Peroxidase Substrate, KPL, Gaithersburg, MD, USA) was added to each well, plates were incubated for an additional 10 minutes at 37°C, and then 100 µl of stop solution (PRV gI Antibody Test, Idexx Laboratories, Westbrook, ME, USA) was dispensed into each well. Plates were read at 405 nm (Model AL310, Bio-Tek instruments, Inc., Winooski, VT, USA) and the results given as optical density (OD). For each plate, sample results were adjusted by subtracting the OD of the negative control.

Table 5. Mycoplasmas antigens used for plate preparation.

Bacteria	Strain	Media used for growth	Antigen concentration (µg/ml/well)
M. hyopneumoniae	232	Friis	10
M. flocculare	27399	Friis	10
M. hyosynoviae	2861	Dfico+Turkey Serum	10
M. hyorhinis	5-12-97*	BHI	10

* *M. hyorhinis* – there was no specific code assigned for this strain rather than the date of its isolation.

ELISA data analysis. ELISA values more than 3 standard deviation units above background will be considered positive. Sensitivity and specificity were calculated from experimentally produced sera and correlation coefficients were determined comparing field sera to the ISU diagnostic laboratory results.

Protein arrays. *Description*. Genes for the major surface antigens of *M. hyopneumoniae* had been cloned and modified to remove TGA codons (stop codons in most organisms but codes for tryptophan in mycoplasmas). These modified genes have been moved to *E. coli* expression vectors, induced and antigen production analyzed.

Purified proteins from each gene have also been obtained. These proteins express at different levels, some in only quantities and some in higher high amounts. Some genes were cloned in sections for other studies, but purified proteins were available for use in this study. A total of 72 proteins (including a positive control of lysed whole cells) were used in printing protein arrays. A GMS417 Arrayer from Genetic MicroSystems was used to print proteins (concentrations of proteins differed from 125-900 ng/µl) to nitrocellulose. Each protein was printed in duplicate for a total of 144 protein spots. The arrays were blocked with Tris-Saline-Tween 20 (0.1%) solution overnight at room temperature before use.

Array development. For serum, a dilution of 1:50 in TST (Tris-Saline-0.05% Tween 20 was used. A single array was incubated in well of a 24 well plate with 200 µl of diluted sera and incubated for 2.5 h at room temperature. The array was then washed with TST three times and incubated with FITC-labeled goat α-swine IgG (MP Bimedicals, LLC), or rabbit α-IgM and IgA (Bethyl Laboratories, INC) diluted 1:100 in TST. At this stage, All subsequent manipulations occurred in the dark. The arrays were incubated with FITC conjugate for 2.5 h, washed three times and then air dried. Individual arrays were then taped to glass slides and a ScanArray 5000 (GSI Lumonics) used to image each slide. Generally, 8-12 arrays were taped to each slide.

Protein array data analysis. The images were quantified using softWorRx Tracker analysis software (Applied Precision, Inc. Issaquah, Wash.). Spot-specific mean signals were corrected for local background by subtracting spot-specific median background intensities, and duplicate signals were averaged.

Gene cloning and optimization. The presence of TGA codons in mycoplasma reading frames necessitated the mutagenesis of these gene sequences to TGG (TGA codes for tryptophan in mycoplasmas but is a stop codon in other bacteria) to ensure completion of translation in *E. coli*. Previously, the Minion laboratory had cloned and mutated the sequences of all of the proteins studied here. These consisted of all of the major surface antigens of *M. hyopneumoniae*. In addition, mycoplasma genes contain rare codons used by *E. coli* so different cloning and expression hosts had to be studied to maximize the amount of protein product produced. In some cases, the gene sequence was optimized for *E. coli* and synthesized by IDT (Coralville, IA). This required the sub-cloning of the optimized genes into the expression vector pTrcHis and testing of the clone for the appropriate protein produced.

Protein purification. All genes were cloned under control of a *lac* promoter and thus were inducible by IPTG. Induced cells were washed once by centrifugation and then lysed with a solution containing 8 M urea by several freeze-thaw cycles. The insoluble debris was pelleted by centrifugation. Proteins were purified from induced supernatants by metal chelate chromatography using buffers containing 8 M urea. In most instances, the protein-containing solution was dialyzed against Phosphate Buffered Saline to remove the urea following purification. However solubility was an issue with 2-3 proteins and either some urea was added back (1 M) or SDS (0.1%). Expression of proteins was followed by immunoblot using α-polyHis monoclonal antibody (all proteins had a polyhistidine sequence at the N-terminus for purification purposes).

Luminex bead conjugation. The protocol used for the coupling of purified proteins to MagPlax microspheres was the company's protocol (https://www.luminexcorp.com/Downloads/index.htm). Our proteins were in PBS and some included urea and SDS. This enhanced the solubility of the purified proteins but could potentially affect coupling efficiency. The coupling efficiency of each protein was monitored by antisera (either α-polyHis monoclonal antibody or protein-specific antisera).

RESULTS

Objective 1. Animal Studies

Histopathological alterations (described by Dr. Darin Madson – Board Certified Pathologist from the Veterinary Diagnostic Laboratory at Iowa State University). No lesions were found in the lungs of pigs infected with *M. hyorhinis* or *M. flocculare*. Lungs from pigs challenged with *M. hyosynoviae* were not tested. Immunohistochemistry was not performed on lungs from pigs challenged with either *M. hyorhinis* or *M. flocculare* because of the absence of lesions and PCR negative for *M. hyopneumoniae*.

Table 6. Histopathology results from lungs of pigs challenged with *M. hyopneumoniae*.

Pig ID #	Microscopic alterations*
127	Bronchiolar associated lymphoid hyperplasia accentuates greater than 75% of bronchioles in examined sections. Lymphoid nodules are variably sized often singular, but multiple nodules are seen adjacent to few bronchioles. Rare lymphocytes are invading the lamina of few accentuated bronchioles. In addition, few lymphocytes and plasma cells cuff multiple small arterioles. Immunohistochemistry POSITIVE.
143	Bronchiolar associated lymphoid hyperplasia similar to pig A is viewed. Approximately 30% of bronchioles are affected. Lymphoid nodules are frequently small and few arterioles are accentuated by perivascular lymphocytes and plasma cells. In one bronchiole, numerous eosinophils infiltrated the lamina and were seen transmigrating through the epithelium. Immunohistochemistry NEGATIVE.
147	About 10% of bronchioles are accentuated by associated lymphoid hyperplasia. Changes are similar to pig # 143. Immunohistochemistry NEGATIVE.
199	Bronchiolar associated lymphoid hyperplasia is viewed in association with 5-10% of bronchioles within examined sections. No other changes are noted. Immunohistochemistry NEGATIVE.

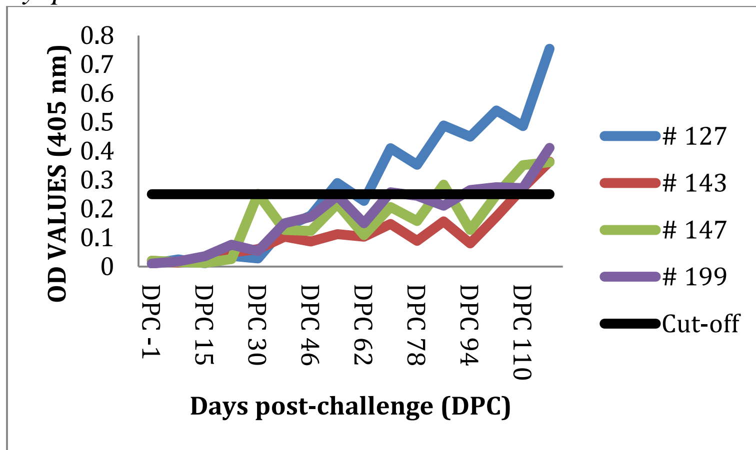
Table 7. Real-time PCR results from bronchioalveolar lavage (BAL) of lungs of pigs challenged with *M. hyopneumoniae*, *M. hyorhinis*, and *M. flocculare*.

Sample ID[1]	Challenge group[2]	*M. hyopneumoniae*	*M. hyorhinis*	*M. hyosynoviae*	*M. flocculare*
BAL pig # 127	*M. hyopneumoniae*	Pos/33.2[3]	negative	negative	negative
BAL pig # 143		Pos/30.4	negative	negative	negative
BAL pig # 147		Pos/35.4	negative	negative	negative
BAL pig # 199		Pos/30.2	negative	negative	negative
BAL pig # 110	*M. hyorhinis*	negative	negative	negative	negative
BAL pig # 134		negative	negative	negative	negative
BAL pig # 179		negative	negative	negative	negative
BAL pig # 213		negative	negative	negative	negative
BAL pig # 108	*M. flocculare*	negative	negative	negative	negative
BAL pig # 128		negative	Pos/35.7	negative	negative
BAL pig # 156		negative	Pos/23.9	negative	negative
BAL pig # 220		negative	negative	negative	negative

[1] Sample ID = BAL (bronchioalveolar lavage) + pig number; [2] Challenge group = pathogen used in the challenge for those animals; [3] Pos/value = Positive/Ct (cycle threshold) value. Negative samples do not have any corresponding Ct value. For *M. hyopneumoniae,* 45 amplification cycles were run; whereas, for *M. hyosynoviae* and *M. hyorhinis*, 44 amplification cycles were run. PCRs were performed in duplicate.

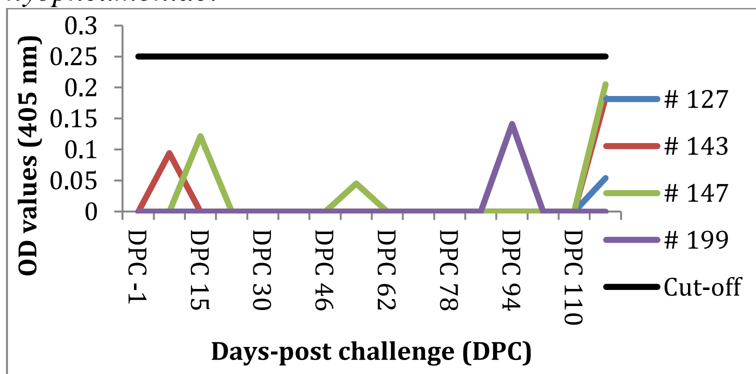
The serum responses to a *M. hyopneumoniae* active infection was low (Figs. 1-3). Possible this was due to the use of CDCD pigs, but it was the only way to ensure mycoplasma-free pigs for the study. Our experience also corresponds with these results; serological responses do not correlate with disease or presence of the organism. Interestingly, most of the pigs did obtain a positive ELISA result on day 94 following the challenge. It is also important to note that these pigs showed significant lung lesions at necropsy (Table 6) and were positive for *M. hyopneumoniae* and negative for other swine mycoplasmas by PCR (Table 7). In summary, our results indicate that it is important to sample older pigs when assessing a herd for the presence of *M. hyopneumoniae* by serology. Cross-reactive ELISA reactions were noted in *M. hyopneumoniae*-challenged pigs on Tween 20 *M. hyorhinis* antigens (Fig. 6) but not on other mycoplasma antigens (Figs. 5 and 7). The oral fluid responses were generally similar to the serum except the major antibody was IgA and not IgG (Fig. 4)

Figure 1. Dynamic of anti-*M. hyopneumoniae* IgG response in serum of pigs experimentally challenge with *M. hyopneumoniae*.



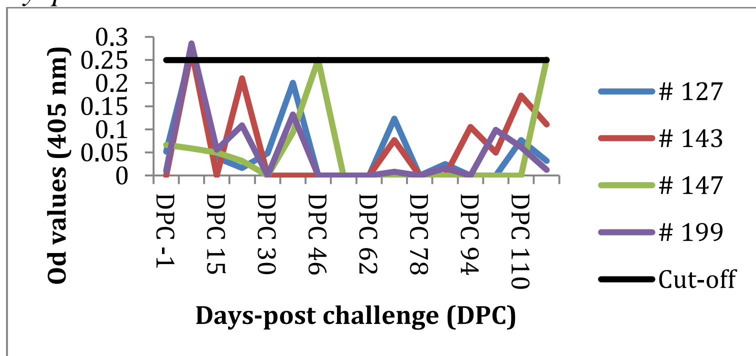
*Mouse anti-pig IgG conjugate was used for this reaction. Cut-off OD value of 0.25 has been previously validated. Thus, OD values above 0.25 are considered positive.

Figure 2. Dynamic of anti-*M. hyopneumoniae* IgA response in serum of pigs experimentally challenge with *M. hyopneumoniae*.



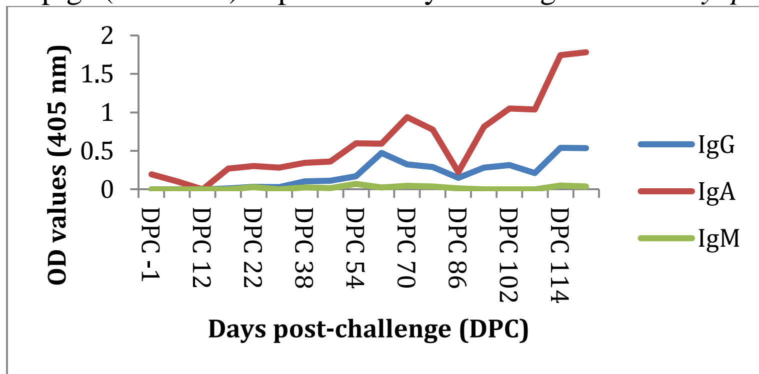
*Goat anti-pig IgA was used for this reaction.

Figure 3. Dynamic of anti-*M. hyopneumoniae* IgM response in serum of pigs experimentally challenged with *M. hyopneumoniae*.



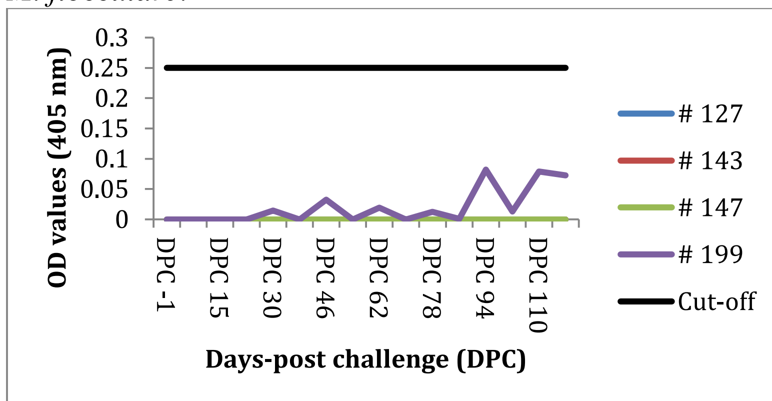
*Goat anti-pig IgM was used for this reaction.

Figure 4. Dynamic of anti-*M. hyopneumoniae* IgG, IgA, and IgM response in pen-based oral fluids of a group of pigs (total of 4) experimentally challenge with *M. hyopneumoniae*.



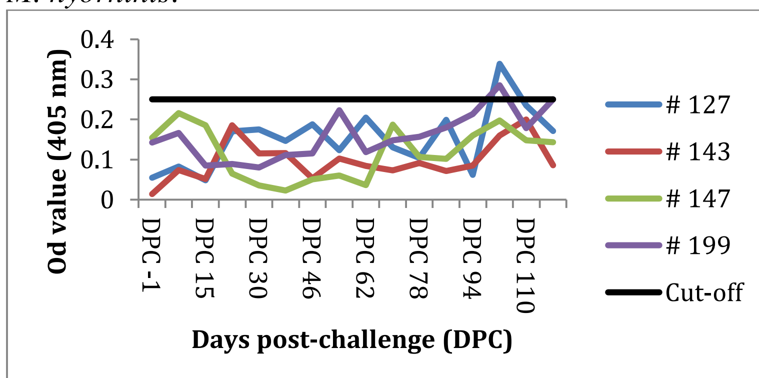
*Mouse anti-pig IgG and goat anti-pig IgA and IgM conjugates were used for this reaction.

Figure 5. Use of a Tween 20 based ELISA to detect antibody cross-reactivity between *M. hyopneumoniae* and *M. flocculare*.



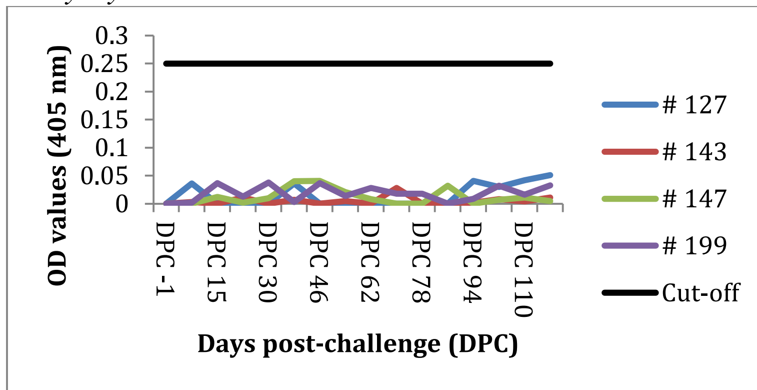
*Mouse anti-pig IgG conjugate was used. Serum samples from pigs experimentally challenged with *M. hyopneumoniae* were tested on *M. flocculare* ELISA plates.

Figure 6. Use of a Tween 20 based ELISA to detect antibody cross-reactivity between *M. hyopneumoniae* and *M. hyorhinis*.



*Mouse anti-pig IgG conjugate was used. Serum samples from pigs experimentally challenged with *M. hyopneumoniae* were tested on *M. hyorhinis* ELISA plates.

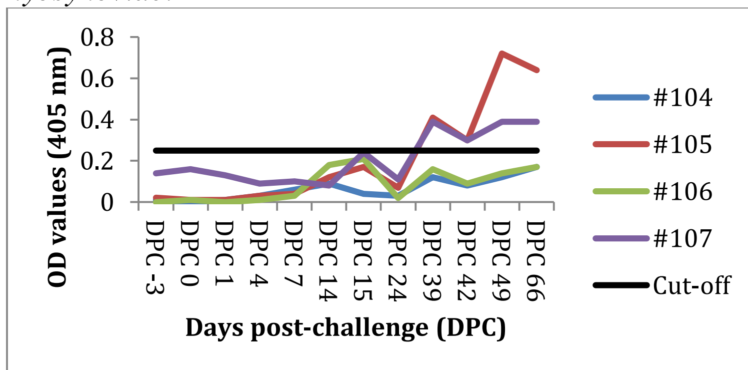
Figure 7. Use of a Tween 20 based ELISA to detect antibody cross-reactivity between *M. hyopneumoniae* and *M. hyosynoviae*.



*Mouse anti-pig IgG conjugate was used. Serum samples from pigs experimentally challenged with *M. hyopneumoniae* were tested on *M. hyosynoviae* ELISA plates.

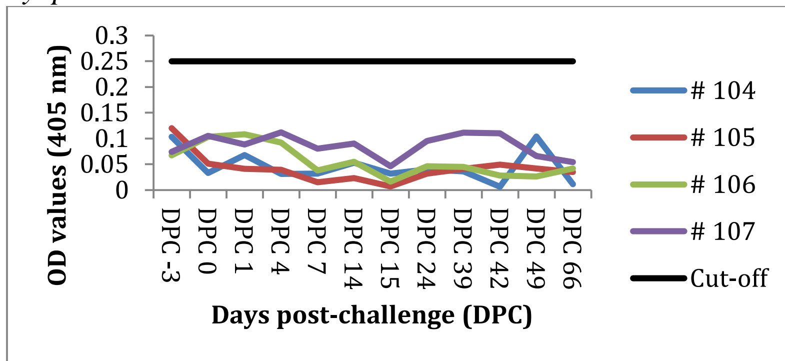
The serological responses to *M. hyosynoviae* showed a positive response in half the animals by day 39 while the other half of the animals never did convert (Fig. 8). These animals seemed to be the most difficult to infect and re-challenged after the first dose. It should be noted that these animals were challenged on a different schedule (necropsy was on day 66) than the other three mycoplasmas (necropsy on day 118). Perhaps if we extended the time frame of the experiment, we would have had conversion in all four animals. We never observed any cross-reactivity between *M. hyosynoviae*-challenged animal sera and the other three mycoplasmas (Figs. 9-11). *M. hyorhinis* challenged animals sero-converted the fastest (Fig 12), at approximately 15 DPC. Sera from those animals did not show cross-reactivity to other mycoplasmal antigens (Figs. 13-15). *M. flocculare*-challenged pigs sero-converted slowly (Fig. 16). Cross-reactivity was observed with the *M. hyorhinis* Tween 20 antigen (Fig. 19) but not the other two antigens (Figs. 17 and 18).

Figure 8. Dynamic of anti-*M. hyosynoviae* IgG response in serum of pigs experimentally challenged with *M. hyosynoviae*.



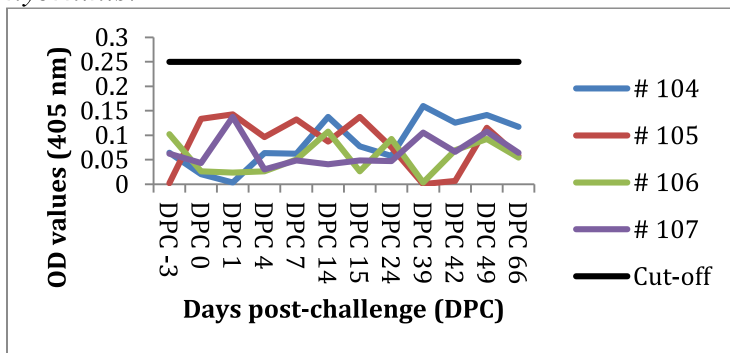
*Results from 4 animals kept up to 66 DPC. Mouse anti-pig IgG conjugate was used. Animals were re-challenged with 5 ml (Intranasally) at DPC 15 and 10 ml (Intranasally) at DPC 39 with the same strain previously used for the IV challenge.

Figure 9. Use of a Tween 20 based ELISA to detect antibody cross-reactivity between *M. hyosynoviae* and *M. hyopneumoniae*.



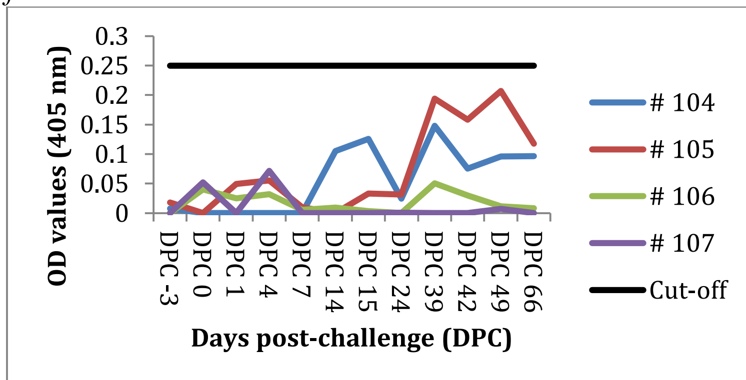
*Mouse anti-pig IgG conjugate was used for this reaction. For this reaction, serum samples from pigs experimentally challenged with *M. hyosynoviae* were tested on *M. hyopneumoniae* ELISA plates.

Figure 10. Use of a Tween 20 based ELISA to detect antibody cross-reactivity between *M. hyosynoviae* and *M. hyorhinis*.



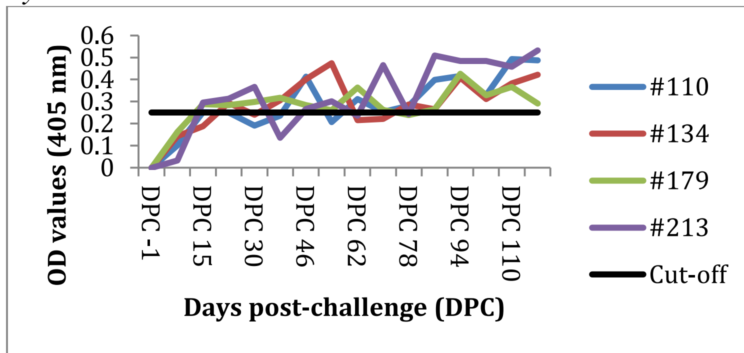
*Mouse anti-pig IgG conjugate was used for this reaction. For this reaction, serum samples from pigs experimentally challenged with *M. hyosynoviae* were tested on *M. hyorhinis* ELISA plates.

Figure 11. Use of a Tween 20 based ELISA to detect antibody cross-reactivity between *M. hyosynoviae* and *M. flocculare*.



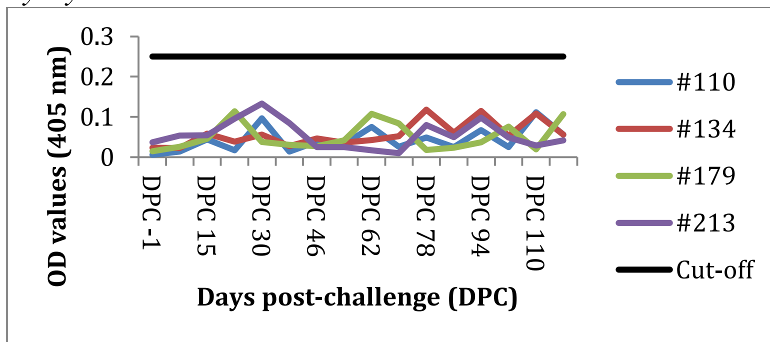
*Mouse anti-pig IgG conjugate was used for this reaction. For this reaction, serum samples from pigs experimentally challenged with *M. hyosynoviae* were tested on *M. flocculare* ELISA plates.

Figure 12. Dynamic of anti-*M. hyorhinis* IgG response in serum of pigs experimentally challenge with *M. hyorhinis*.



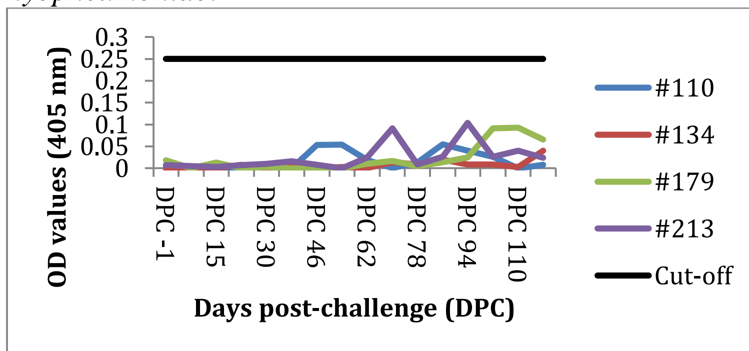
*Mouse anti-pig IgG conjugate was used.

Figure 13. Use of a Tween 20 based ELISA to detect antibody cross-reactivity between *M. hyorhinis* and *M. hyosynoviae*.



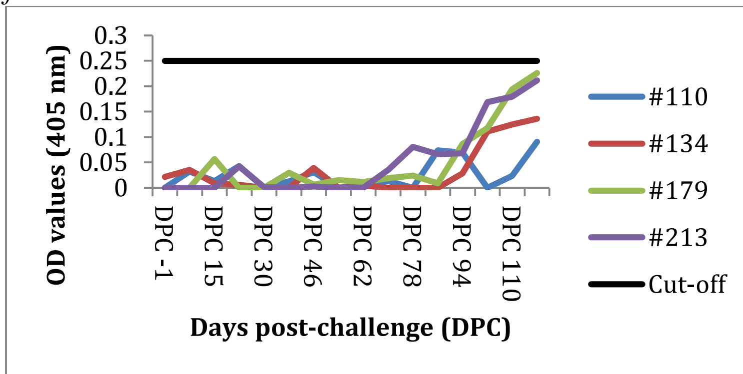
*Mouse anti-pig IgG conjugate was used for this reaction. For this reaction, serum samples from pigs experimentally challenged with *M. hyorhinis* were tested on *M. hyosynoviae* ELISA plates.

Figure 14. Use of a Tween 20 based ELISA to detect antibody cross-reactivity between *M. hyorhinis* and *M. hyopneumoniae*.



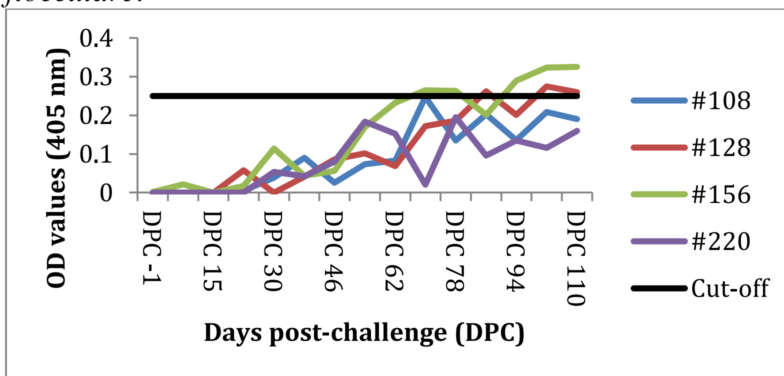
*Mouse anti-pig IgG conjugate was used for this reaction. For this reaction, serum samples from pigs experimentally challenged with *M. hyorhinis* were tested on *M. hyopneumoniae* ELISA plates.

Figure 15. Use of a Tween 20 based ELISA to detect antibody cross-reactivity between *M. hyorhinis* and *M. flocculare*.



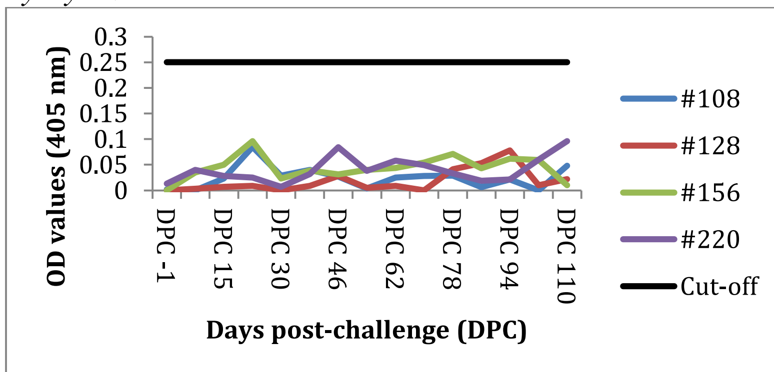
*Mouse anti-pig IgG conjugate was used. Serum samples from pigs experimentally challenged with *M. hyorhinis* were tested on *M. flocculare* ELISA plates.

Figure 16. Dynamic of anti-*M. flocculare* IgG response in serum of pigs experimentally challenged with *M. flocculare*.



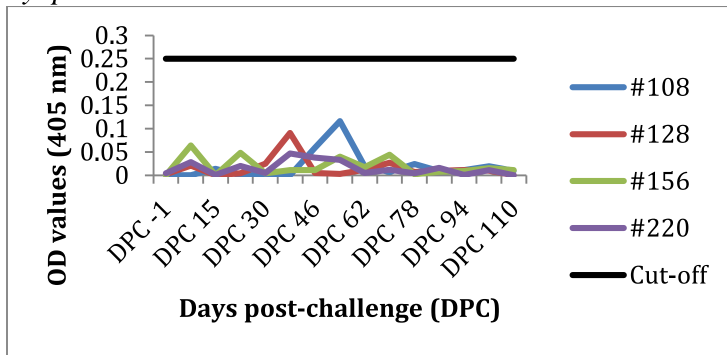
*Mouse anti-pig IgG conjugate was used for this reaction.

Figure 17. Use of a Tween 20 based ELISA to detect antibody cross-reactivity between *M. flocculare* and *M. hyosynoviae*.



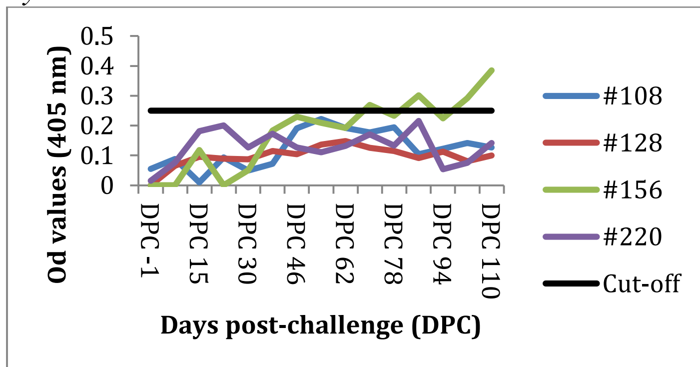
*Mouse anti-pig IgG conjugate was used for this reaction. For this reaction, serum samples from pigs experimentally challenged with *M. flocculare* were tested on *M. hyosynoviae* ELISA plates.

Figure 18. Use of a Tween 20 based ELISA to detect antibody cross-reactivity between *M. flocculare* and *M. hyopneumoniae*.



*Mouse anti-pig IgG conjugate was used for this reaction. For this reaction, serum samples from pigs experimentally challenged with *M. flocculare* were tested on *M. hyopneumoniae* ELISA plates.

Figure 19. Use of a Tween 20 based ELISA to detect antibody cross-reactivity between *M. flocculare* and *M. hyorhinis*.



*Mouse anti-pig IgG conjugate was used. Serum samples from pigs experimentally challenged with *M. flocculare* were tested on *M. hyorhinis* ELISA plates.

Objective 2. Protein array results.

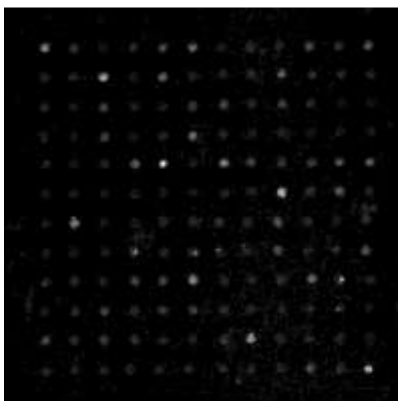


Figure 20. Image of protein array reacted with sera from *M. hyopneumoniae* infected pig.

Sera from the *M. hyopneumoniae* infected pigs were used to develop protein arrays. These sera represented samples taken at various intervals over the 118 day infection period. The results from one animal (pig #199) are shown in Figure 21. The results from the three other infected pigs are not shown. The results generally showed some inconsistency in the reactivity patterns across all animals. The early sera in all pigs were done first (days

1-46) and the latter days (days 54-118) were done later in the study. The early reactivities in all pigs (only pig 199 is shown) were less consistent, probably due to student error and unfamiliarity with the assay (this student was competent with ELISA assays but had no previous experience with microarrays). The yellow highlighted antigens are those that we have shown to be *M. hyopneumoniae*-specific and are being used in the ELISA and bead-based assays (see below).

Oral fluid results with this assay were generally negative. This was probably due to the dilution of the oral fluid used in the assay. The student performing these studies used a 1:100 dilution like he did normally for ELISA assays, but a lower dilution should have been used. Unfortunately we could not go back and redo this experiment because all of the protein arrays had been used up by this time.

[It is important to note that we were unable to repeat these results because we ran out of the protein arrays. To construct more arrays, we would have to purify the antigens again and print the arrays. We do not the financial resources to do that at this time.]

P199	dpc_1	dpc8	dpc15	dpc22	dpc30	dpc38	dpc46	dpc54	dpc62	dpc70	dpc78	dpc86	dpc94	dpc102	dpc118
683-D SD	278.85195	973.9395	405.84295	493.752	435.121	685.0265	910.033	416.489	654.5805	347.2935	421.4255	309.2255	603.408	277.3855	550.0115
683-E SD	272.0506	682.3495	388.3211	583.788	426.474	944.4055	762.222	368.051	572.968	296.1085	448.44	370.1695	447.2555	218.91375	700.3185
683A SD	402.896	1173.734	1203.03645	548.6025	481.0345	1525.705	1188.745	1177.038	1747.846	257.9125	646.305	363.5285	608.5275	207.438	768.472
ADH SD	283.6771	753.445	405.8745	511.2505	417.6825	787.7225	619.547	262.1005	579.9985	349.516	457.495	212.784	845.775	276.416	653.512
F2 SD	274.829	1057.5495	415.0335	728.4655	383.37345	848.391	856.4605	659.1705	578.5755	350.3875	436.923	338.017	236.5165	351.906	911.498
M.hyo WCL	279.41495	463.94	325.5225	416.8075	293.646	587.882	536.7125	376.668	587.7805	299.6945	458.254	438.853	561.56	214.9305	715.728
MH1 1-3 SD	230.83795	982.7465	462.4855	541.371	305.3543	964.2735	867.968	621.048	902.0805	249.333	481.689	266.4215	453.9895	300.508	799.152
mhp 379 E2	302.88525	1184.4805	468.524	716.2435	654.631	1116.7285	756.439	398.3655	448.106	296.881	448.461	460.4795	457.488	371.539	415.131
mhp037 E2	359.222	1210.338	634.0725	748.3745	702.355	1198.5265	1572.909	584.03	670.369	238.554	1057.5545	2174.669	886.454	832.1315	626.922
mhp072 E3	246.9066	1292.642	429.0665	625.481	535.0965	980.9625	995.965	548.6795	640.397	249.29775	662.116	1717.9675	557.6685	548.959	680.5345
mhp107-a SD	360.2995	630.951	442.265	603.8295	440.6885	630.52	670.6465	530.4675	683.317	322.0595	327.267	505.882	362.631	328.3675	619.069
mhp107-b SD	368.49575	1230.787	427.5775	726.2345	518.415	666.5185	1100	490.897	859.1415	290.227	387.84	307.0805	331.0495	343.276	587.704
mhp107-c SD	263.5302	754.0525	401.1715	556.159	456.736	690.7055	635.4365	380.584	480.5635	322.9235	369.1825	415.9095	395.145	373.8355	765.529
mhp107-d SD	321.95405	577.9975	345.459	475.185	286.6599	707.242	734.7825	433.533	542.409	306.578	287.826	344.564	415.3405	383.0755	1753.4065
mhp107-e SD	203.114	552.959	319.718	385.5535	327.848	788.618	530.9505	428.8045	440.2105	366.0225	376.394	546.4235	423.5465	412.117	508.1935
mhp108.2 E1	313.6365	421.6155	257.0963	527.0135	397.3915	686.1675	629.9465	471.449	571.2405	293.6695	715.3	632.828	754.0155	354.159	726.7795
mhp147 E3	259.679	557.1755	295.7421	548.717	322.174	586.773	607.311	305.7795	323.708	374.0205	437.477	400.117	519.0545	404.8355	518.0865
mhp164 E1	234.24135	1521.142	611.796	704.172	485.2615	842.779	984.9225	568.8535	659.0735	259.585	499.1075	479.978	770.573	387.048	733.3045
mhp170 E2	221.4924	648.4935	370.9605	662.6975	336.6879	664.3935	765.4805	501.909	520.6645	262.59625	424.0755	478.838	806.0115	446.9305	559.1875
mhp216 E2	316.72985	1200.9145	494.463	726.428	560.8055	1158.683	1111.49	735.0885	735.517	487.8	439.371	368.2225	471.9255	416.7745	736.8615
mhp271-B SD	278.6648	558.8815	353.2569	497.079	331.996	867.652	426.631	431.3705	428.852	316.8205	455.4275	299.634	346.512	281	634.6855
mhp271-M SD	261.0929	1379.874	562.991	594.681	444.282	923.0055	857.972	606.2645	861.8495	319.682	371.144	442.9165	494.878	369.9375	698.2695
mhp271A E2	295.2147	1447.082	554.0155	759.735	935.273	1286.8055	1035.259	535.8245	469.725	389.738	668.992	347.814	586.27	363.2145	877.7315
mhp271B E1	260.28875	431.515	386.911	531.228	265.84175	711.311	707.225	384.4725	466.6675	295.9955	883.969	394.38	1305.984	453.0235	627.623
mhp272A E1	286.01405	347.242	385.9055	498.17	282.8625	630.8835	680.6665	450.3315	394.9275	343.891	543.7875	464.9945	657.987	440.699	607.0635
mhp272B E1	276.2418	806.4275	344.7522	521.235	451.237	708.411	688.7545	374.7855	555.9295	323.3305	1146.0655	784.233	954.649	460.0695	640.243
mhp280 E1	254.75725	876.5845	428.1075	412.6585	364.4924	698.315	722.8855	440.411	774.9165	366.019	397.426	468.7025	644.359	385.242	750.8695
mhp293 E1	285.1462	1480.0485	481.157	571.7625	538.606	895.1595	961.2925	490.0095	485.865	287.3039	1644.619	1657.8415	860.21	676.4305	483.8525
mhp296 E1	293.84705	832.866	449.5865	550.578	436.923	961.599	695.825	496.8875	456.2015	255.69265	1142.0145	633.7285	772.445	520.146	528.7685
mhp336 E1	257.7973	1186.591	755.6175	934.9585	593.3105	1495.0355	1235.4585	674.317	626.8645	326.015	1897.515	2166.377	977.4145	647.4125	478.414
mhp345 E1	286.43875	911.4575	398.485	529.7155	411.4002	975.408	797.332	648.103	503.303	265.4865	640.039	443.346	621.49	400.365	423.193
mhp352 E1	237.0303	863.1425	376.3015	557.6565	380.5545	764.829	863.564	481.6945	512.0835	338.4605	700.506	445.0025	665.403	479.4425	508.9095
mhp366 E1	229.47945	658.617	335.22025	451.452	345.0335	761.55	831.7035	484.0455	466.471	304.7625	459.1465	493.4685	719.57	355.99	511.5
mhp367 E1	288.556	790.538	632.9005	611.364	470.3695	1044.869	737.0285	628.7275	795.296	548.8975	563.7665	460.916	429.617	383.688	583.648
mhp369 E1	294.01325	1011.5335	401.5575	987.9305	367.8734	773.834	619.8845	441.3725	506.134	345.2875	603.8135	491.645	412.3905	386.97	493.133
mhp378 E1	266.39095	1065.0625	457.891	596.134	431.4255	967.098	795.261	424.583	496.2075	433.0835	1604.5745	394.1295	1139.2855	364.4505	456.8145
mhp384 E1	213.70195	1164.9175	586.8785	554.542	492.8055	829.5235	867.2805	542.111	463.7905	329.323	673.3315	518.835	656.5555	308.41755	572.526
mhp384a SD	348.16455	1464.059	531.953	808.332	468.61125	940.8895	889.6625	531.3105	690.2145	348.971	350.5875	346.01	367.195	353.621	1045.064
mhp384b SD	361.8445	532.697	322.956	551.708	425.541	631.6785	570.466	341.5495	450.129	295.02	364.0905	653.855	384.2925	188.8481	1106.6675
mhp384c SD	402.8115	1128.24	431.58825	752.107	478.4974	619.951	861.0625	486.3115	537.32	318.9825	550.652	348.3725	423.4315	242.7235	651.7925
mhp385-F1 SD	321.82695	615.1925	378.577	400.6305	308.0439	797.958	616.19	397.2495	404.1755	325.687	487.332	282.1015	436.1615	360.1365	914.4505
mhp385-F3	299.9024	1424.331	566.4055	819.70225	438.274	937.5125	856.39175	573.48975	704.2025	312.502	379.8955	280.90075	393.4025	303.28975	735.71175
mhp391 E1	248.3225	1367.61	694.178	798.731	415.1882	885.794	977.361	507.6025	481.833	266.9895	1530.0925	543.1745	1227.0795	449.3285	542.883
mhp424 E1	211.79735	1111.8615	423.7645	689.346	373.92775	693.491	808.846	527.531	473.6775	352.5525	434.4105	332.1395	942.4835	366.142	426.6965
mhp436 E1	269.5765	2119.752	605.1625	692.296	557.324	1421.984	1180.4665	679.7815	748.5875	319.987	534.678	477.347	1319.3255	415.141	536.569
mhp466 E1	252.03985	662.5785	440.114	355.12	467.005	627.1	840.643	377.8215	579.2	295.1865	1067.7015	500.538	573.03	726.9895	435.9395
mhp467 E1	210.14705	515.469	321.81165	502.4055	275.3366	703.0505	560.352	355.601	491.153	351.7	582.0145	368.46	647.4975	374.1495	634.5155
mhp499 E1	268.25935	1003.086	439.1575	569.57	425.5945	616.445	567.174	505.0155	548.432	261.2295	538.0915	376.4955	668.8705	402.9215	676.907
mhp511 E1	248.1005	457.262	385.654	428.937	261.48	608.8015	587.2185	417.0235	575.5715	303.239	542.0275	274.4725	701.6375	532.016	662.2945
mhp536 50ml	229.4074	1233.5735	596.1135	608.194	603.44	829.52	836.1845	459.1725	587.986	293.702	395.1665	253.498	436.1255	368.674	462.3195
mhp554 E1	323.3427	1195.865	573.9765	661.2415	447.329	832.4665	1114.547	536.554	617.2735	291.7585	568.918	323.395	583.742	344.2205	523.236
mhp555 E1	433.7935	1120.4015	568.76235	584.567	355.5916	829.9375	935.092	447.08	735.0685	294.734	493.793	575.173	702.8055	325.161	540.7285
mhp559 E1	292.02465	760.4845	421.3815	666.5825	373.7617	693.973	474.931	373.884	497.4785	323.8775	503.0835	392.7025	624.4815	368.953	680.932
mhp624 E1	286.45985	704.954	456.059	623.177	418.86	593.7605	534.764	467.3915	819.447	341.081	582.503	488.498	1138.0605	382.1845	1049.0365
mhp677 E1	274.57315	509.8465	374.5075	865.9225	353.318	613.018	572.254	401.828	424.492	342.2165	1045.9715	671.387	1161.341	501.885	952.748
mhp681 E1	274.66335	842.702	400.5695	905.4385	463.291	719.6015	714.6195	468.8695	593.3805	312.2395	899.624	402.3895	955.1125	398.0545	755.5915
mhp683 E1	426.5895	1099.887	398.798	571.7465	395.2671	656.1075	779.167	419.9785	430.6775	291.174	921.3255	577.7625	725.7105	390.8625	547.332
mhp683b SD	228.43635	493.6425	377.5936	613.9955	240.72835	741.6715	755.6175	389.675	416.3045	369.76	434.994	438.8415	453.9065	312.99	607.5065
mhp683c SD	305.81665	765.2615	398.0105	600.271	361.46535	753.5075	800.9155	419.2315	432.3905	306.7965	351.121	360.626	441.0695	287.786	536.9465
mhp684A E1	290.80005	1018.3065	474.492	536.2115	526.3745	723.894	978.722	454.769	603.5	245.2214	443.9285	363.9185	560.0865	308.978	417.192
mhp695 E1	290.0455	753.9565	352.6607	598.5875	360.18635	797.0795	631.8675	350.183	475.8305	316.935	473.6795	329.995	543.366	339.33	893.8915
P102 SD	280.59435	1356.1255	656.643	1146.3115	566.455	897.805	1216.5145	565.488	843.029	264.4425	523.2565	447.5225	485.269	467.4455	705.8015
P159.F1 SD	423.232	815.02	437.1481	508.5825	365.92975	843.68	715.699	435.549	335.9255	298.1195	325.383	334.5115	14.2865	965.8265	1107.081
P159.F3 SD	214.63395	1989.8325	1340.8315	910.524	682.411	1257.1105	1422.9095	1040.132	854.375	327.3495	467.1295	411.361	431.5595	269.6365	775.742
P159.F4 SD	262.8097	920.328	459.0515	545.3485	317.98665	731.049	718.1875	531.412	431.175	325.9105	548.216	417.385	667.574	272.2	1026.2965
P216B P SD	233.3553	1015.659	530.293	661.4965	318.29335	933.1155	686.511	511.7175	470.485	352.343	371.0225	234.6555	444.6595	327.1535	735.394
P216C P SD	243.1465	778.817	340.64045	421.2875	368.46625	745.8805	695.622	496.537	413.0825	329.563	529.291	387.375	431.339	365.837	594.7395
P216D P SD	291.3459	430.954	354.145	334.154	282.4497	627.365	679.699	463.0755	416.9725	279.8045	504.481	396.412	522.6715	213.4095	568.6415
P42 P SD	277.7941	733.7135	298.5428	411.631	228.69875	556.0095	566.806	345.394	634.5835	276.578	552.814	292.4525	594.3525	469.277	713.5215
P72 P SD	214.72445	611.7935	296.77365	324.744	282.64185	572.581	589.229	582.6675	732.6715	324.185	369.7805	379.2855	717.662	346.8825	415.0275
r347F1 SD	406.88635	876.969	359.411	898.423	392.647	721.3365	671.5495	424.1265	583.268	333.807	423.53	300.946	417.406	347.113	1116.7015

Figure 21. Protein array results of the sera from pig #199. Highlighted in red are the results that represent values that are 2 times the standard deviation above the control spots. In all pigs, the early days were less consistent and some positive responses may be due simply to background issues.

Objective 3. ELISA and Bead based assay development

All of the eight proteins used in this assay were purified from *E. coli* or *Bacillus megatorium*. For some of the antigens, we had to optimize the coding sequence for *E. coli* to obtain sufficient quantities for these studies; for others we were able to move our constructs into a different *E. coli* background and get reasonable expression levels. In summary, all eight proteins were purified in sufficient quantities to complete the next step, which was to bind the proteins to beads for analysis using the Illumina bead-based platform and to test the antigens in an ELISA based assay.

We monitored protein attachment to the beads using an anti-poly-histidine monoclonal antibody (all of the antigens had a poly-histidine sequence at the amino terminus to facilitate purification). Our results indicated that we were successful with all eight conjugations (data not shown). Our next step is the use of these beads to analyze the sera from our challenged pigs and from clinical samples. All sera are currently available for testing, and as soon as our equipment is repaired and my technician returns from maternity leave after the first of the year, we will complete this phase of the project.

ELISA testing.
During the course of these studies, we were contacted by IDEXX who were interested in developing an improved ELISA. To this end, we sent the eight proteins to the company for testing in their system. Since we had to maximize gene expression in each of these strains, it took some time to find the right *E. coli* strain and/or reclone an optimized sequence. We sent cell pellets with expressed proteins. We have not received their results by this time, but part of the issue is our tardiness in getting the proteins to them.

Discussion
We were successful in completing Objective 1, and have made significant progress in Objectives 2 and 3. We show by ELISA that *M. hyopneumoniae*-challenged pigs are slow to develop antibody responses in comparison to the other three mycoplasmas. This is not entirely unexpected, but the time interval is a bit surprising (94 days before all pigs were serologically positive). *This has implications for pig producers in that only older pigs should be tested by serology for M. hyopneumoniae antibodies*. Some of this delay could be due to the use of CDCD pigs, which are known to be immunologically immature. This, however, was the only way we could ensure that the pigs would be free of mycoplasmas so the sera would contain only antibodies developed in response to a single challenge species.

For Objective 2, we will have to repeat these studies before we are ready for publication because of the variability. This will require purification of all of the proteins on the array in small amounts (~20 µg will be needed for each protein), printing the arrays and redoing the array analysis with all of the antisera. The assay is worth repeating because of its unique nature and potential to ferret out proteins that might prove useful not only for assay development but also vaccine development. The latter would require antigens that are routinely responded to by the host even though they may be cross reactive with other mycoplasma species.

For Objective 3, we are nearing completion of these studies, requiring only that my technician return from maternity leave. He is the only individual trained for this instrument in the lab and he is the one that did all of the conjugations and protein purifications. This should only take a couple of weeks once he returns. We do not know at this time if these antigens will prove to be more sensitive or accurate in detecting *M. hyopneumoniae* infections in pigs. We should be able to answer that once we have tested the series sera from the four *M. hyopneumoniae* challenged pigs and compare our results with the Bead assay with those from the ELISAs (see above).

An updated final report will be submitted once Objective 3 experiments are completed and the results analyzed. Once this is done, we will be sending Dick Hesse at Kansas State some of our beads for further analysis in his laboratory.

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Addendum June 22, 2013

I. Development of an Improved *Mycoplasma hyopneumoniae*-Specific ELISA; NPB #11-057
F. Chris Minion, Principal Investigator
Iowa State University
September 1, 2012; Addendum June 22, 2013

II. Industry Summary

Improvement of serological-based diagnostics for *Mycoplasma hyopneumoniae* infections in swine will require identification of mycoplasma-specific antigens and their use in an ELISA-based assay format as well as development of more current technologies. The overall goal of this project was to develop an improved serology-based assay on *M. hyopneumoniae*-specific antigens, follow pig responses to these antigens during infection over an extended period of time, and utilize these antigens in a bead-based assay. This will improve the specificity, reproducibility, and sensitivity of current serological assays. We identified eight *M. hyopneumoniae*-specific proteins that lack cross-reactivity to other swine mycoplasma species. These antigens were cloned and expressed as recombinant proteins for further analysis. Each protein posed unique problems in expression levels, solubility, etc. The proteins are being assessed by a commercial partner for their usefulness in a standard ELISA style assay. The proteins have also been bound to beads, and testing is underway with our defined sera and sera from clinical samples. The defined sera were developed by challenging pigs (4 per group) with one of the following mycoplasmas, *M. hyopneumoniae, M. hyosynoviae, M. hyorhinis,* and *M. flocculare,* and monitoring the infection for a 118 day period. Serum samples were taken throughout the study, oral fluids were collected on a monthly basis, and at necropsy histopathology was performed on the *M. hyopneumoniae* challenged pigs. Serological responses indicated a slow conversion except for the *M. hyorhinis*-challenged pigs. Cross-reactions were noted in the *M. hyopneumoniae* sera with *M. hyorhinis* Tween 20 antigens and in the *M. flocculare* sera with *M. hyorhinis* Tween 20 antigens. Oral fluids from *M. hyopneumoniae* challenged pigs showed a positive IgA response with the Tween 20 ELISA. The ELISA and bead-based assays with the purified antigens are still being tested. (Contact: Chris Minion, VMPM, Iowa State University, fcminion@iastate.edu)

IV. Scientific Abstract

The goal of this study was to improve serological assays for *M. hyopneumoniae* infected pigs. Our approach was to focus our assay development on antigens that are *M. hyopneumoniae*-specific and to test these in two different formats, a 96 well ELISA based format, and on beads using Luminex technology. To assist in proper screening, we produced swine sera from mycoplasma-infected pigs. The challenge study used CDCD pigs, four to a group and each group was challenged with *M. hyopneumoniae, M. hyorhinis, M. hyosynoviae,* and *M. flocculare.* The groups were housed separately throughout the experiment and they were monitored from cross infections by PCR. Sera were obtained throughout the experiment so we could follow antibody responses. In addition, oral fluids were also obtained from each room once a month for analysis. Serological responses indicated a slow conversion except for the *M. hyorhinis*-challenged pigs. Cross-reactions were noted in the *M. hyopneumoniae* sera with *M. hyorhinis* Tween 20 antigens and in the *M. flocculare* sera with *M. hyorhinis* Tween 20 antigens. Oral fluids from *M. hyopneumoniae* challenged pigs showed a positive IgA response. The sera from *M. hyopneumoniae*-challenged animals were also tested on a protein array. The results from that study showed some inconsistencies, possibly due to operator error or blocking issues. Eight *M. hyopneumoniae* proteins were purified, and were bound to beads for testing in a Luminex-based assay. These antigens are also under study in an ELISA assay.

Objective 1. Additional ELISA results of mycoplasma-infected pigs

All swine sera from the infected groups of pigs were also tested with the DAKO, HIPRA, IDEXX and Synbiotics ELISA assays. The results were scored according to the manufacturer's instructions. Tables 8-11 show the results of these studies. Only the IDEXX ELISA assay showed cross-reactivity in the three groups, and only with the *M. hyosynoviae*- and *M. flocculare*-infected animals (Tables 10-11).

Table 8. Sera from *M. hyopneumoniae* infected pigs.

Pig number	DPC	DAKO	HIPRA	IDEXX	Synbiotics
127	-1	0	0	0	0
143	-1	0	0	0	0
147	-1	0	0	0	0
199	-1	0	0	0	0
127	8	0	0	0	0
143	8	0	0	0	0
147	8	0	0	0	0
199	8	0	0	0	0
127	15	**2**	0	0	0
143	15	1	1	0	0
147	15	1	0	0	0
199	15	1	0	0	0
127	22	1	1	0	0
143	22	1	1	0	0
147	22	1	2	0	0
199	22	1	1	0	0
127	30	1	1	0	0
143	30	1	1	0	0
147	30	1	1	0	0
199	30	1	1	1	0
127	38	1	1	1	0
143	38	1	1	**2**	0
147	38	1	1	1	0
199	38	1	1	1	1
127	46	1	1	1	0
143	46	1	1	1	0
147	46	1	1	1	0
199	46	1	1	1	1
127	54	1	1	1	0
143	54	1	1	1	0
147	54	1	1	1	0
199	54	1	1	1	1
127	62	1	1	1	0
143	62	1	1	1	0
147	62	1	1	1	0
199	62	1	1	1	1
127	70	1	1	1	0
143	70	1	1	1	0
147	70	1	1	1	0
199	70	1	1	1	1
127	78	1	1	1	0
143	78	1	1	1	0
147	78	1	1	1	0
199	78	1	1	1	1
127	86	1	1	1	0
143	86	1	1	1	0
147	86	1	1	1	0
199	86	1	1	1	1
127	94	1	1	1	0
143	94	1	1	1	0
147	94	1	1	1	0
199	94	1	1	1	1
127	102	1	1	1	0
143	102	1	1	1	0
147	102	1	1	1	0
199	102	1	1	1	1
127	110	1	1	1	0
143	110	1	1	1	0
147	110	1	1	1	0
199	110	1	1	1	1
127	118	1	1	1	0
143	118	1	1	1	0
147	118	1	1	1	1
199	118	1	1	1	1

0 = negative, 1 = suspect, 2 = positive

Table 9. Sera from *M. hyorhinis* infected pigs

Pig number	DPC	DAKO	HIPRA	IDEXX	Synbiotics
110	-1	0	0	0	0
134	-1	0	0	0	0
179	-1	0	0	0	0
213	-1	0	0	0	0
110	8	0	0	0	0
134	8	0	0	0	0
179	8	0	0	0	0
213	8	0	0	0	0
110	15	0	0	0	0
134	15	0	0	0	0
179	15	0	0	0	0
213	15	0	0	0	0
110	22	0	0	0	0
134	22	0	0	0	0
179	22	0	0	0	0
213	22	0	0	0	0
110	30	0	0	0	0
134	30	0	0	0	0
179	30	0	0	0	0
213	30	0	0	0	0
110	38	0	0	0	0
134	38	0	0	0	0
179	38	0	0	0	0
213	38	0	0	0	0
110	46	0	0	0	0
134	46	0	0	0	0
179	46	0	0	0	0
213	46	0	0	0	0
110	54	0	0	0	0
134	54	0	0	0	0
179	54	0	0	0	0
213	54	0	0	0	0
110	62	0	0	0	0
134	62	0	0	0	0
179	62	0	0	0	0
213	62	0	0	0	0
110	70	0	0	0	0
134	70	0	0	0	0
179	70	0	0	0	0
213	70	0	0	0	0
110	78	0	0	0	0
134	78	0	0	0	0
179	78	0	0	0	0
213	78	0	0	0	0
110	86	0	0	0	0
134	86	0	0	0	0
179	86	0	0	0	0
213	86	0	0	0	0
110	94	0	0	0	0
134	94	0	0	0	0
179	94	0	0	0	0
213	94	0	0	0	0
110	102	0	0	0	0
134	102	0	0	0	0
179	102	0	0	0	0
213	102	0	0	0	0
110	110	0	0	0	0
134	110	0	0	0	0
179	110	0	0	0	0
213	110	0	0	0	0
110	118	0	0	0	0
134	118	0	0	0	0
179	118	0	0	0	0
213	118	0	0	0	0

0 = negative, 1 = suspect, 2 = positive

Table 10. Sera from *M. hyosynoviae* infected pigs

Pig number	DPC	DAKO	HIPRA	IDEXX	Synbiotics
104	-3	0	0	0	0
105	-3	0	0	0	0
106	-3	0	0	0	0
107	-3	0	0	0	0
104	0	0	0	0	0
105	0	0	0	0	0
106	0	0	0	0	0
107	0	0	0	0	0
104	1	0	0	0	0
105	1	0	0	0	0
106	1	0	0	0	0
107	1	0	0	0	0
104	4	0	0	0	0
105	4	0	0	0	0
106	4	0	0	0	0
107	4	0	0	0	0
104	7	0	0	0	0
105	7	0	0	0	0
106	7	0	0	0	0
107	7	0	0	0	0
104	14	0	0	0	0
105	14	0	0	0	0
106	14	0	0	0	0
107	14	0	0	0	0
104	15	0	0	0	0
105	15	0	0	0	0
106	15	0	0	0	0
107	15	0	0	0	0
104	24	0	0	**0**	0
105	24	0	0	2	0
106	24	0	0	0	0
107	24	0	0	0	0
104	39	0	0	0	0
105	39	0	0	**1**	0
106	39	0	0	0	0
107	39	0	0	0	0
104	42	0	0	0	0
105	42	0	0	**1**	0
106	42	0	0	0	0
107	42	0	0	0	0
104	49	0	0	0	0
105	49	0	0	**1**	0
106	49	0	0	0	0
107	49	0	0	0	0
104	66	0	0	0	0
105	66	0	0	**1**	0
106	66	0	0	0	0
107	66	0	0	0	0

0 = negative, 1 = suspect, 2 = positive

Table 11. Sera from *M. flocculare* infected pigs

Pig number	DPC	DAKO	HIPRA	IDEXX	Synbiotics
108	-1	0	0	0	0
128	-1	0	0	0	0
156	-1	0	0	0	0
220	-1	0	0	0	0
108	8	0	0	0	0
128	8	0	0	0	0
156	8	0	0	0	0
220	8	0	0	0	0
108	15	0	0	0	0
128	15	0	0	0	0
156	15	0	0	0	0
220	15	0	0	0	0
108	22	0	0	0	0
128	22	0	0	0	0
156	22	0	0	0	0
220	22	0	0	0	0
108	30	0	0	0	0
128	30	0	0	0	0
156	30	0	0	0	0
220	30	0	0	0	0
108	38	0	0	0	0
128	38	0	0	0	0
156	38	0	0	0	0
220	38	0	0	0	0
108	46	0	0	0	0
128	46	0	0	0	0
156	46	0	0	0	0
220	46	0	0	0	0
108	54	0	0	0	0
128	54	0	0	0	0
156	54	0	0	0	0
220	54	0	0	0	0
108	62	0	0	0	0
128	62	0	0	0	0
156	62	0	0	**2**	0
220	62	0	0	0	0
108	70	0	0	0	0
128	70	0	0	**2**	0
156	70	0	0	**2**	0
220	70	0	0	0	0
108	78	0	0	0	0
128	78	0	0	0	0
156	78	0	0	**1**	0
220	78	0	0	0	0
108	86	0	0	0	0
128	86	0	0	0	0
156	86	0	0	**1**	0
220	86	0	0	0	0
108	94	0	0	0	0
128	94	0	0	0	0
156	94	0	0	**1**	0
220	94	0	0	0	0
108	102	0	0	0	0
128	102	0	0	0	0
156	102	0	0	0	0
220	102	0	0	0	0
108	110	0	0	0	0
128	110	0	0	0	0
156	110	0	0	0	0
220	110	0	0	0	0

0 = negative, 1 = suspect, 2 = positive

Objective 3. ELISA and Bead based assay development

All of the eight proteins used in this assay were purified from *E. coli* or *Bacillus megatorium*. For some of the antigens, we had to optimize the coding sequence for *E. coli* to obtain sufficient quantities for these studies; for

others we were able to move our constructs into a different *E. coli* background and get reasonable expression levels. In summary, all eight proteins were purified in sufficient quantities to complete the next step, which was to bind the proteins to beads for analysis using the Luminex bead-based platform and to test the antigens in an ELISA based assay.

We monitored protein attachment to the beads using an anti-poly-histidine monoclonal antibody (all of the antigens had a poly-histidine sequence at the amino terminus to facilitate purification and detection in down-stream assays). Our results indicated that we were successful with all eight conjugations (data not shown). Our next step was to use these beads to analyze the sera from our challenged pigs. Our results are below. We do not show the data from Mhp681 because it gave high values with all sera including the day 0 sera prior to challenge. It was also the only protein isolated from *Bacillus*, which may explain the high reactions in some way. In all of the graphs below, the BI 7d pool represents a pool of sera from 30 CDCD pigs at 7 days of age. The CDCD pigs used in this study were challenged at either 6 weeks of age (*M. hyopneumoniae, M. hyorhinis* or *M. flocculare* challenges) or 14 weeks of age (*M. hyosynoviae* challenges). This may explain the higher background at day 0 (or the negative control for each pig as indicated in the figures below).

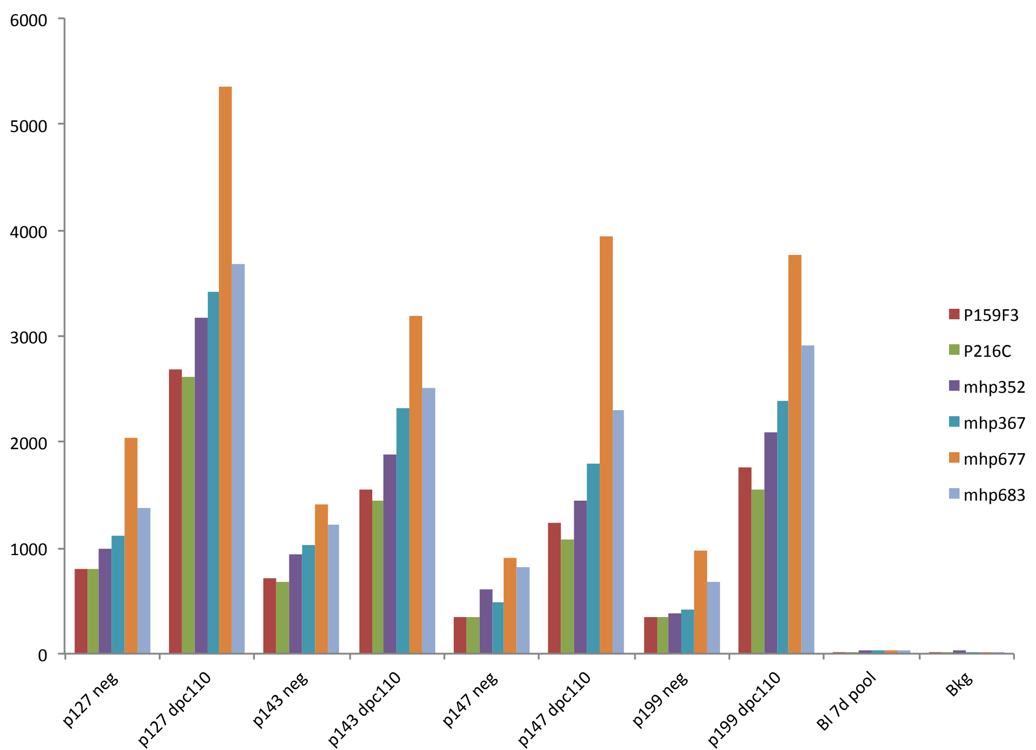

Figure 22. 1:10 Dilution of sera from *M. hyopneumoniae*-infected pigs.

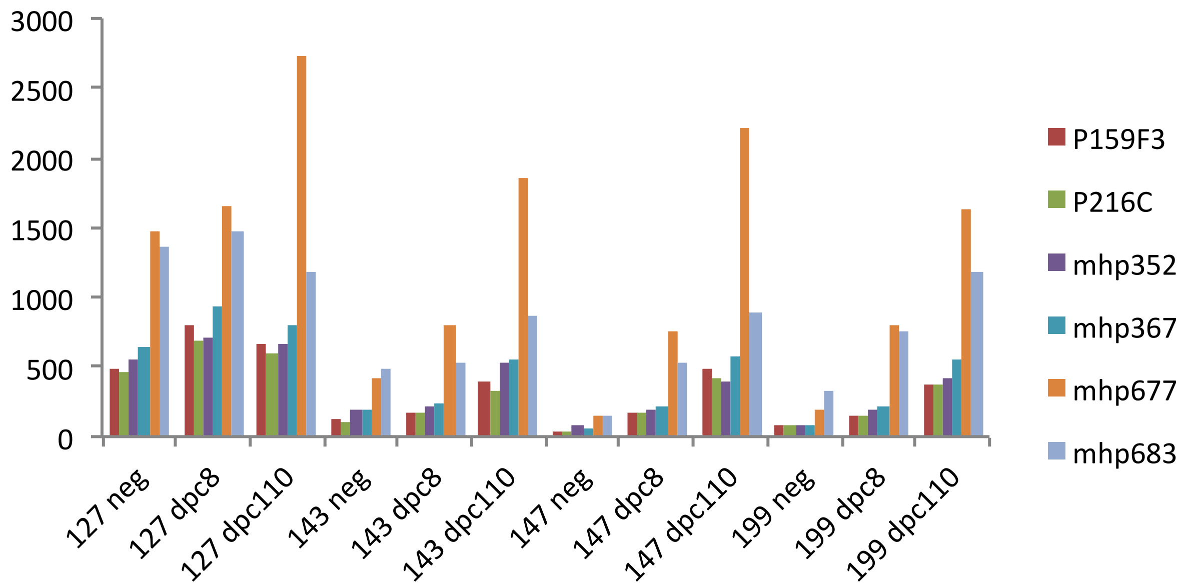

Figure 23. 1:50 Dilution of sera from *M. hyopneumoniae*-infected pigs.

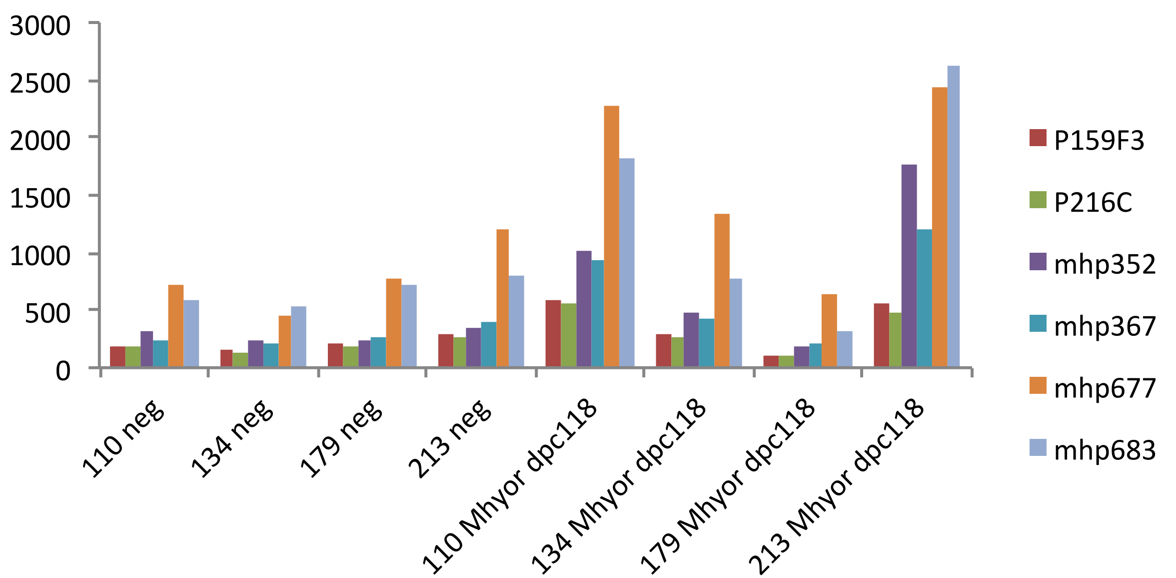

Figure 24. Cross-reactions with sera from *M. hyorhinis*-infected pigs.

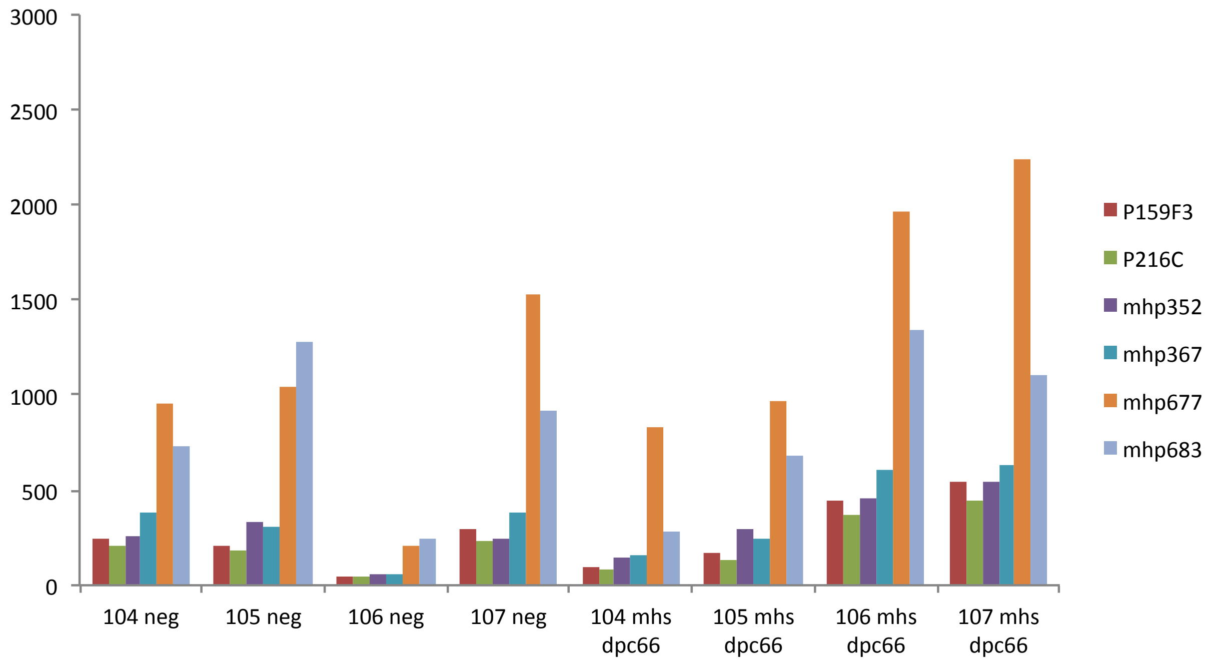

Figure 25. Cross-reactions with sera from *M. hyosynoviae*-infected pigs.

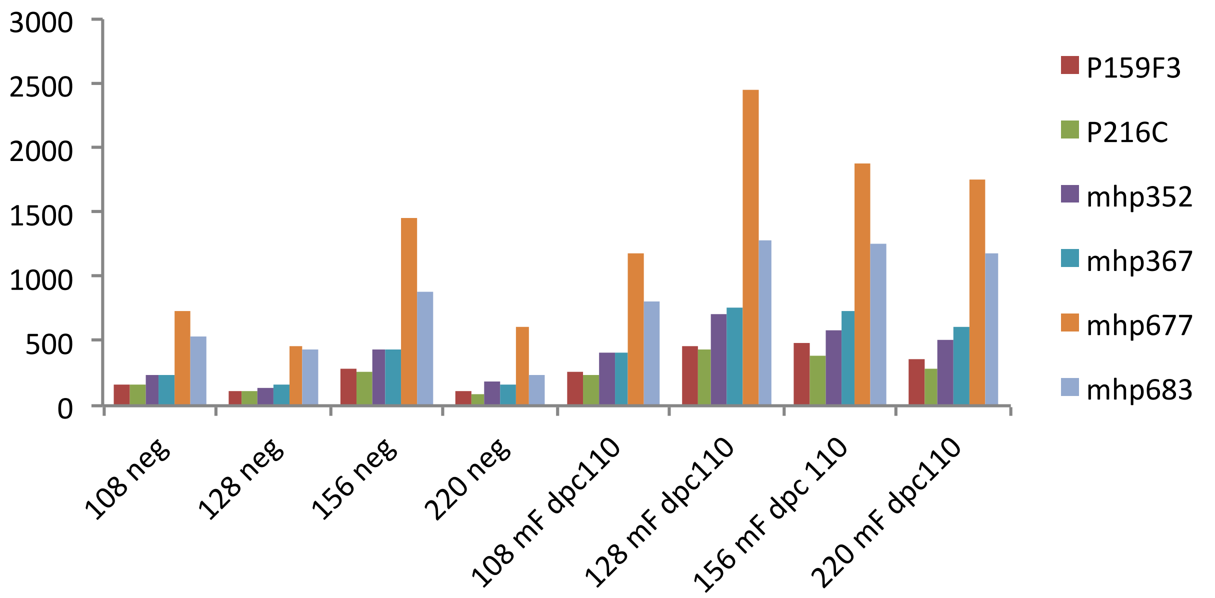

Figure 26. Cross-reactions with sera from *M. flocculare*-infected pigs

ELISA testing-commercial interest
During the course of these studies, we were contacted by IDEXX who were interested in developing an improved ELISA. To this end, we sent the eight proteins to the company for testing in their system. Since we had to maximize gene expression in each of these strains, it took some time to find the right *E. coli* strain and/or reclone an optimized sequence. We sent cell pellets with expressed proteins. IDEXX has indicated an interest in several of the proteins. They are still testing them.

We were also contacted by RnAssays BV (The Netherlands) for access to the proteins as well. We haven't yet supplied them with purified proteins but will once we obtain sufficient quantities.

IX. Discussion
We were successful in completing the three objectives of this proposal. We show by ELISA that *M. hyopneumoniae*-challenged pigs are slow to develop antibody responses in comparison to the other three mycoplasmas. This is not entirely unexpected, but the time interval is a bit surprising (94 days before all pigs were serologically positive by the Tween 20 ELISA). *This has implications for pig producers in that only older pigs should be tested by serology for M. hyopneumoniae antibodies*. Some of this delay could be due to the use of CDCD pigs, which are known to be immunologically immature. This, however, was the only way we could ensure that the pigs would be free of mycoplasmas so the sera would contain only antibodies developed in response to a single challenge species. One interesting observation is the results with the oral fluids from the *M. hyopneumoniae* infected pigs. We are now completing analysis of the oral fluids from other mycoplasma infected groups of pigs.

For Objective 2, we are presently repeating these studies before we send a manuscript off for publication because of the variability we saw in the array data. This will be completed by the end of the summer. The assay is worth repeating because of its unique nature and potential to ferret out proteins that might prove useful not only for assay development but also vaccine development. The latter would require antigens that are routinely responded to by the host even though they may be cross-reactive with other mycoplasma species.

For Objective 3, although we have completed these studies including developing and testing a bead-based assay based on 8 *M. hyopneumoniae* proteins, we may have somewhat different results with second round of array studies. Unexpectedly we had high background with the one protein isolated from *Bacillus* and want to confirm this with the same protein from *E. coli*. Given the results with the Luminex platform, we are not completely convinced these proteins will function as we hoped. Further experiments with the Luminex platform are in progress and hopefully can be completed in a timely manner.