II. Industry Summary

Enzootic pneumonia resulting from Mycoplasma hyopneumoniae infections is an important disease to the US swine industry. Antibodies and immune cells specific to this agent can be detected after vaccination and dams can transfer this immunity to their piglets. It is necessary to understand how cross-fostering can affect passive transfer for Mycoplasma and the protective role of those immune components. Therefore, the objectives of this research were to examine the impact of cross-fostering on the transfer of maternally derived immunity against *M. hyopneumoniae* and influence of the transferred passive immunity on protection from virulent challenge. To examine the first objective, that of examining the effect of cross-fostering on immune transfer, the offspring of vaccinated and unvaccinated dams were cross-fostered at 0, 6, 12 and 20 hrs post-suckling (hps) and humoral and cell mediated immunity in the offspring was assessed. Anti-*M. hyopneumoniae* antibodies were transferred to piglets regardless of source, as long as the piglet was fostered prior to 6 hps. Immune cells were absorbed by piglets that suckled from their own mothers or by a small number of piglets, born from unvaccinated mothers, cross-fostered onto vaccinated sows within the first 6 hps.

To evaluate the second objective, that of protection from challenge in piglets receiving different immune components, one-week old piglets passive administered either *M. hyo* specific immunoglobulin and/were challenged with a virulent strain of *M. hyopneumoniae*. Piglets were divided into groups regarding their status for immune components to Mycoplasma: (1) No immune components, (2) Immunoglobulins + cells, (3) Only immunoglobulins, (4) and only cells. Piglets with or without cells or antibodies were infected with the bacteria, showed coughing, shed the pathogen and had lung lesions associated with Mycoplasma.
Animals born from vaccinated mothers appeared to shed the agent later and in a smaller proportion than the other groups, but differences were not statistically significant. The study indicates that \textit{M. hyopneumoniae}-specific cellular immunity does not transfer into the piglet the same way as humoral immunity. Cross-fostering should be used judiciously and adequate time spent on the sow prior to cross-fostering is critical for successful transfer of immune components.

III. Abstract

\textit{Enzootic pneumonia resulting from Mycoplasma hyopneumoniae} infections is an important disease to the US swine industry. Antibodies and immune cells specific to this agent can be detected after vaccination and dams can transfer this immunity to their piglets. It is necessary to understand how cross-fostering can affect passive transfer for Mycoplasma and the protective role of those immune components. Therefore, the goals of this research were to examine the impact of maternally derived immunity against \textit{M. hyopneumoniae} and potential influence of cross-fostering on immunity and protection from challenge. To examine the first objective, that of examining the effect of cross-fostering on immune transfer, the offspring of vaccinated and unvaccinated dams were cross-fostered at 0, 6, 12 and 20 hrs post suckling (hps) and humoral and cell mediated immunity in the offspring was assessed. Anti-\textit{M. hyopneumoniae} antibodies were transferred to piglets regardless of source, as long as the piglet was fostered before 6 hps. Immune cells were absorbed by piglets that suckled from their own mothers or by a proportion of piglets, born from unvaccinated mothers, cross-fostered onto vaccinated ones within the first 6 hps. To evaluate the second objective, that of protection from challenge in piglets receiving different immune components, one-week old piglets were challenged with a virulent strain of \textit{M. hyopneumoniae}. Piglets were divided into groups regarding their status for immune components to Mycoplasma: (1) No immune components, (2) Immunoglobulins + cells, (3) Only immunoglobulins, (4) and only cells. Piglets with or without cells or antibodies were infected with the bacteria, showed coughing, shed the pathogen and had lung lesions associated with Mycoplasma. Animals born from vaccinated mothers appeared to shed the agent later and in a smaller proportion than the other groups, but differences were not statistically significant.

IV. Introduction

\textit{Mycoplasma hyopneumoniae} (\textit{M. hyopneumoniae}) is a pathogen that affects pigs of all ages and has a worldwide distribution (Ross, 1999). Disease caused by this agent is commonly presented as an uncomplicated chronic pneumonia. \textit{M. hyopneumoniae} is associated with a low mortality rate, however, it becomes endemic (Ross, 1999). One of the most important aspects of \textit{M. hyopneumoniae} is that infection predisposes the colonization of other pathogens, bacterial as well as viral, along the respiratory tract. \textit{M. hyopneumoniae}
infection affects the normal function of the mucociliary apparatus and produces a marked inflammatory response in the respiratory tract (Thacker 2006).

Swine farms become infected with *M. hyopneumoniae* by the acquisition and introduction of carrier animals, infected sows can then colonize their offspring, which after being weaned and mixed with susceptible pigs, will amplify the disease in the herd (Pijoan, 2005). In segregated production systems the most important means of *M. hyopneumoniae* transmission is by sow-to-piglet contact during the lactation period. However, this close relationship also assures the transfer of immunity from the mother to the baby pig via colostrum. It has also been recently demonstrated that *Mycoplasma hyopneumoniae* specific cellular immunity can be transferred from the immunized sow to her offspring (Molitor *et al.*, 2006, Bandrick *et al* 2007).

Immune components such as antibodies (Immunoglobins) and immune cells are transferred to the piglet via colostrum ingestion. Nevertheless, these two types of immunity are not transferred in the same fashion. Immunoglobins are absorbed across the neonatal intestinal mucosa for a selected period of time (approximately 24 hrs) regardless of the immunoglobulin type or even the donor species (e.g. bovine immunoglobulins). Conversely, in order for lymphocytes to cross the neonatal pig’s intestinal barrier, lymphocytes need to be viable and be from the piglets’ mother (Tuboly et al 1988; Williams 1993). Therefore, piglets that are moved to foster sows will be likely deficient in maternal derived cellular immunity, although this has not been tested.

Cross-fostering is a common management practice in which newborn piglets are moved among sows during the first 24 hrs after birth in an attempt to reduce litter variation and supposedly increase the survivability of newborn pigs. The application of cross-fostering assumes that the balance of weight after birth within a litter is more important than normal development and performance individual pigs can have when left with their own mother (Cutler *et al.*, 2006). However, to our knowledge, there are no published studies that have looked at the effect of cross-fostering on the immune status of the newborn pig.

To investigate transfer of antigen specific maternal immune cells to piglets, our research team designed an experiment which was carried out in a commercial farm. In that study, a group of sows was vaccinated with a commercial *M. hyopneumoniae* vaccine while another same sized-group of sows was left unvaccinated. *In vitro* and *in vivo* cellular immune responses specific to *M. hyopneumoniae* were evaluated of offspring from both sow groups. Interestingly, the results showed that newborn pigs from vaccinated sows were able to respond to *M. hyopneumoniae* antigen in a specific manner (Bandrick *et al* 2007). Piglets from unvaccinated mothers did not demonstrate *M. hyopneumoniae* specific cellular immune responses. Since responses were detected in the piglets within 3 days of life we concluded the responses were solely due to transferred immune cells.

Although it is widely accepted that acquired immunity, from natural exposure or via vaccination decreases the detrimental effect of *M. hyopneumoniae* pneumonia,.the mechanism responsible for the effect is unknown. The specific action of antibodies and/or cells involved in the immune response to *M. hyopneumoniae* is not clear.
It is common practice to measure the success or failure of *M. hyopneumoniae* control strategies by measuring seroconversion (antibodies) without knowledge of the relevance of those results. In addition, it has been speculated that the immune factors acquired by the piglet from its mother could be detrimental since passive immunity could interfere with an active immune response i.e post vaccination. Nevertheless, these speculations have not been proven and many studies support the idea that maternal passive interference does not affect protection against *M. hyopneumoniae* (Haesebrouck *et al.*, 2004); however it lowers the antibody response of the piglet (Palzer *et al.*, 2006). Therefore, it is crucial to investigate the role of each immune component, humoral as well as cellular, regarding protection against *M. hyopneumoniae*.

V. Objectives
The overall goal of the proposed research is to examine the impact of maternally (passive) derived immunity against *M. hyopneumoniae* and potential influence of maternal transfer by the management practice of cross fostering. Two specific objectives are proposed to accomplish the overall goal:

1. To evaluate the effect of cross-fostering newborn pigs at different times after birth on the transfer of cellular and/or humoral immunity to *M. hyopneumoniae*.

2. To evaluate the protective role of different immune components passively transferred to piglets against challenge with virulent *M. hyopneumoniae*.

VI. Materials and Methods
The experiment took place primarily in an 800-sow farm, belonging to the University of Minnesota, southwest experimental station, Waseca MN, known to be free of *Mycoplasma hyopneumoniae* and PRRSv infection. For the challenge phase of the study, early weaned piglets were transported to the CVM Isolation Facilities of the University of Minnesota. The experimental challenge with *M. hyopneumoniae* was performed at CVM isolation facilities with pigs from the U of M, southwest experimental station.

Objective 1 Design:
A total of twenty (20) sows were randomly allocated into two groups. Sows were identified and fourteen sows were vaccinated, while six sows remained unvaccinated. The vaccinated sows were immunized twice (5 and 3 weeks pre-farrow) with a commercial *M. hyopneumoniae* killed bacterin (RespiSure®). Proper immunization of sows was confirmed by the detection of antibodies for *Mycoplasma hyopneumoniae* in sow sera, one week prior to farrowing. Four pairs of vaccinated and unvaccinated sows had their offspring cross-fostered within the pair. Ten piglets per sow were included in the study. Piglets were ear tagged in order to identify their mother’s treatment and movement of piglets, from one sow to the other. Two piglets at a time were moved from their mother to the corresponding paired sow, from which 2 piglets were taken and relocated
with the mother from whom the 2 piglets were originally taken. The times at cross-fosterings were: 0, 6, 12 and 20 hrs after birth ± 1 hr. Two piglets/litter remained with their mothers. Only ten piglets per sow were on study, thus, in litter size larger than 10, piglets remained with their mothers but were not tested. Two unvaccinated and two vaccinated sows remained with their own offspring and were evaluated as controls.

Another four pairs of sows, vaccinated and vaccinated, had their offspring cross-fostered, moved across the pair as well. The purpose of this pairing was to test whether immune cells from separate mothers will cross the intestinal barrier and gain access into the newborn pig. Based on previous results from Tuboly et al (1983) we expected the transfer of mycoplasma immune cells to be maternal derived specific.

Farrowing was induced in order to time-match since piglet movement occurred between sows of each treatment group. Farrowing time was recorded at the time of birth of the first piglet.

Twenty four hrs after birth all piglets in the study were subjected to:
- Blood sample collection:
  - For detection of antibodies for *M. hyopneumoniae* by ELISA.

At 3 days of age all piglets were tested for:
- Delayed Type Hypersensitivity (DTH) evaluation: for characterization of in vivo cellular immune response.

A colostral sample was taken from each sow included in the study. Colostrum was processed for cells and fluids by separation on density gradient centrifugation. The fluid portion was assessed for antibody titers to *M. hyopneumoniae*, measured using an ELISA test.

Objective 2 Design:
The second objective was designed to evaluate the effect of cross-fostering on protection afforded by passive transfer of immune components.

A total of 40 piglets were used to accomplish Objective 2 studies. Piglets were divided into 5 groups (eight piglets each) depending on their immune status to *M. hyopneumoniae*. The first four groups of piglets were challenged with virulent *M. hyopneumoniae* and the 5th group was sham inoculated with Phosphate Buffer Saline (PBS).

The five groups were:
1. Piglets with no immune components to *M. hyopneumoniae*.
2. Piglets carrying only specific immune cells to *M. hyopneumoniae*.
3. Piglets carrying only immunoglobulins (antibodies) to *M. hyopneumoniae*.
4. Piglets harboring both humoral and cellular immunity.
5. Piglets with no detectable immunity to *M. hyopneumoniae* (Negative control).
At seven days of age, piglets from groups 1-4 were experimentally infected with a standard infectious dose ($1\times10^5$ ccu/mL) of *M. hyopneumoniae* strain 232. Piglets in group 5 were sham inoculated with PBS. The route of infection was intra-tracheal, in order to assure a homogeneous infection among individuals and groups (Meyns *et al.*, 2006). Piglets were observed for at least 0.5 hr every day. Clinical signs were evaluated by measuring the incubation period and coughing score of each group of pigs. Three and a half weeks after the experimental infection all animals in the study were euthanized at the U of M CVM Diagnostic Laboratory, where lung lesions were scored. Tissue samples from lesions were collected for their histopathological examination. A serum sample was collected from each piglet in order to detect the presence of antibodies to *M. hyopneumoniae*.

The tests and measurements that were used for the evaluation of the samples for both objectives were:

- **DTH testing:** Concentrated and purified *M. hyopneumoniae* (300 µg/ml in 0.1 ml) antigen was injected intradermally into the left inguinal region (Roberts, 1973). Injection sites were clearly marked with livestock paint. Skin fold thickness will be measured 24-36 hrs later with calipers. The mitogen PHA (20 µg/ml in 0.1 ml; Sigma) and biological saline (0.1 ml) were used as controls. Final DTH lesion was determined as orthogonal diameter x skin thickness or D*T.

- **Sample Collection:** Twenty ml colostrum were collected manually from all udders into 50 ml conical tubes following alcohol swabbing of teats. Blood was collected by vena cava puncture in sterile vacutainer tubes.

- **Detection of antibodies for *M. hyopneumoniae* (ELISA):** Serum samples were obtained for evaluation of *M. hyopneumoniae* specific antibodies. Antibodies concentration was measured using a commercial kit for antibody detection (IDEXX® ELISA).

- **Clinical evaluation:** Pigs were observed for *M. hyopneumoniae* specific symptoms, mainly coughing, on a daily basis (30 min). Coughing score was determined by counting the number of coughs per group per day during a period of 15 min.

- **Histopathology:** Samples for histopathological examination were collected from macroscopic lesions and from a consistent location in pigs without visible lesions. Samples were suspended in a 10% formaldehyde solution and processed for H and E staining.

- **Lung lesion score:** The lung lesion score was determined using the protocol described by Pointon *et al.*, 1999. Under this scheme the value of affected lung can reach 100%. The investigators were blinded for the scoring of lung lesions.

- **Data analyses:** Data obtained from both studies was analyzed with commercial statistical software. Results of the *in vivo* and *in vitro* tests were compared among groups for each cross-fostering time; an analysis of variance was be used to compare the results for all time points. Results expressed as proportions were evaluated for statistical significance by Fisher’s exact test. Averages were compared using the Wilcoxon signed-rank test.
VII. Results

Objective I: Effect of cross-fostering on *M. hyopneumoniae* specific Immune Transfer:

From a background of using a *Mycoplasma hyopneumoniae* negative farm, only gilts that were vaccinated against *M. hyopneumoniae* developed humoral and cellular immunity to *M. hyopneumoniae*. No piglets had humoral or cellular *M. hyopneumoniae*-specific immunity prior to colostrum ingestion. All Non-cross(X) fostered piglets from vaccinated gilts had *M. hyopneumoniae*-specific antibodies and the majority had *M. hyopneumoniae*-specific cellular immune responses at 24 hrs. Non-cross fostered piglets from unvaccinated gilts did not have anti-*M. hyopneumoniae* antibodies at 24 hrs and had negative *M. hyopneumoniae*-specific DTH responses.

The proportion of piglets positive to *M. hyopneumoniae* antibodies as determined by ELISA is presented in Table 1 as the number of animal positive by treatment group. In Figure 1 the results of antibodies titers are presented as individual animal based on S/P values:

Table 1. Humoral immunity to *Mycoplasma hyopneumoniae* in x-fostered pigs.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0</th>
<th>6</th>
<th>12</th>
<th>20 hrs</th>
<th>Non X-fostered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinated Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10/10</td>
</tr>
<tr>
<td>Unvaccinated Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0/26</td>
</tr>
<tr>
<td>Vaccinated to Vaccinated</td>
<td>12/12</td>
<td>11/11</td>
<td>11/11</td>
<td>10/10</td>
<td>11/11</td>
</tr>
<tr>
<td>Vaccinated to Unvaccinated</td>
<td>0/10</td>
<td>10/10</td>
<td>10/10</td>
<td>9/9</td>
<td>9/9</td>
</tr>
<tr>
<td>Unvaccinated to Vaccinated</td>
<td>10/10</td>
<td>7/9</td>
<td>1/10</td>
<td>0/8</td>
<td>0/8</td>
</tr>
</tbody>
</table>
The effect of cross-fostering on *M. hyopneumoniae* antibody titer.

Piglets of vaccinated gilts that were cross-fostered before 6 hr had undetectable *M. hyopneumoniae*-specific DTH responses. Two piglets from an unvaccinated gilt that were cross-fostered onto a vaccinated gilt before 6 hr had positive *M. hyopneumoniae* DTH responses. A summary of the responses to the *M. hyopneumoniae* cell-mediated immune assessment from piglets of all groups, at the different cross-fostering times, is presented in figure 2.
Figure 2. Delayed Type Hypersensitivity responses in cross-fostered pigs.

Objective 2: Effect of *M. hyopneumoniae* specific Immune components on Protection from challenge

All piglets were screened prior to challenge as shown to be negative for detection of *M. hyopneumoniae* as determined by *M.hyo* specific PCR.

Piglets in group 5 (sham inoculated) did not show any clinical signs indicative of Enzootic Pneumonia and were negative for detection of *M. hyopneumoniae* DNA throughout the study. Animals from this group did not have gross or microscopic lesions associated with *M. hyopneumoniae* infection.

At 7 days of age, piglets in groups 1 (no immune components) and 2 (immune cells only) did not have detectable antibodies to *M. hyopneumoniae*, while piglets in groups 3 (antibodies only) and 4 (whole colostrum) were positive for antibodies by ELISA.

Clinical signs appeared in the first group of pigs 17 dpi (group 2), while in groups 1 and 4 the onset was at 18 dpi groups and in group 3 at 19 dpi. Pigs in all groups continued to demonstrate coughing until the end of the study. Onset of clinical signs and number of days coughing were compared among groups and no significant differences were found.

The detection of *M. hyopneumoniae* DNA as tested by nested-PCR is presented in Table 2. There were no significant differences detected in the first day of detection in pigs inoculated with *M. hyopneumoniae* or at the end of the study. *M. hyopneumoniae* DNA detection appeared prior to the onset of clinical signs and persisted during the study.

Sample sizes from 7-10 for treatment and non cross-fostered controls of 20.
Table 2. Shedding of *M. hyopneumoniae* DNA in nasal and bronchial swabs, during the acute phase of infection.

<table>
<thead>
<tr>
<th>Treat. group / dpi</th>
<th>0</th>
<th>7</th>
<th>10</th>
<th>14</th>
<th>21</th>
<th>25</th>
<th>Bronchial Swab 25</th>
</tr>
</thead>
<tbody>
<tr>
<td>No cells + No Ig’s</td>
<td>0/5</td>
<td>0/5</td>
<td>2/5</td>
<td>2/5</td>
<td>2/5</td>
<td>3/5</td>
<td>8/9</td>
</tr>
<tr>
<td>Only cells</td>
<td>0/5</td>
<td>0/5</td>
<td>2/5</td>
<td>4/5</td>
<td>4/5</td>
<td>4/5</td>
<td>7/8</td>
</tr>
<tr>
<td>Only Ig’s</td>
<td>0/5</td>
<td>0/5</td>
<td>1/5</td>
<td>1/5</td>
<td>2/5</td>
<td>3/5</td>
<td>9/9</td>
</tr>
<tr>
<td>Cells + Ig’s</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>1/5</td>
<td>1/5</td>
<td>3/5</td>
<td>8/9</td>
</tr>
</tbody>
</table>

Lung lesions associated with *M. hyopneumoniae* were observed in animals from all groups inoculated with the bacteria and are presented in Figure 3. Lung lesion score in sham inoculated animals was significantly different than that in all the groups inoculated with *M. hyopneumoniae*. Lung lesion score in group 4 was statistically different from groups 2 and 3. In order to confirm the association of the lung lesions with *M. hyopneumoniae* infection, samples from all animals were processed for bacterial culture and microorganism able to cause the same type of lesions; no other bacteria were detected. Moreover, blinded histopathologic evaluation of the samples was performed and the lesions were confirmed to be those typical of *M. hyopneumoniae* infection.
Figure 3. Lung lesion score in different groups of inoculated animals.

VIII. Discussion

It has been recently demonstrated that specific immunity to *M. hyopneumoniae* can be transferred from vaccinated mothers to their offspring and that passively acquired immune cells are functional in young animals (Molitor et al., 2006, Bandrick et al 2007). However, it still remains unknown whether those immune components are protective to the neonate and if management practices in the farrowing room, namely cross-fostering, have an effect on the transfer of such maternal components. Therefore, these two studies were designed: (1) to investigate the effect of cross-fostering newborn pigs at different times after birth on the transfer of cellular and/or humoral immunity to *M. hyopneumoniae* and (2) to evaluate the protective role of different immune components passively transferred to piglets against challenge with virulent *M. hyopneumoniae*.

Under the conditions of these studies, anti-*M. hyopneumoniae* antibodies are transferred into piglets regardless of source as long as the piglet is fostered before 6 hr. The studies indicate that *M. hyopneumoniae*-specific cellular immunity does not transfer into the piglet the same way as humoral immunity does. The findings to date have implications on when and if cross-fostering should be undertaken.

To the knowledge of the authors, this is the first time that sows colostrum and piglets were treated in order to obtain groups of animals with none, one or both components of immunity (antibodies and cells) and it is the first time to experimentally challenge young animals to investigate the protective role of such components, alone or in combination.

Although clinical signs in animals from all infected groups were not different, there is a trend in the detection of *M. hyopneumoniae* DNA to have a later onset and to be lower in the first days after infection in
animals with both, antibodies and immune cells specific for Mycoplasma. However, these differences were only numerical. The results in M. Hyo presence represent qualitative assessment. A potential improvement that may lead to more meaningful results is by assessing the quantitative nature of M. hyopneumoniae.

Lung lesions associated with *M. hyopneumoniae* were found in pigs of all infected groups. Nevertheless, the lung lesion score of animals with antibodies and immune cells were higher than the group of animals with each component (only cells or only antibodies), but was not different than the one from animals receiving no M. hyo specific immunity. It is known that mycoplasmal pneumonia is the result of an intense immune reaction in the lung, thus, it can be hypothesized that animals born from vaccinated mothers had more lung lesion due to a higher immune stimulation in the respiratory tract. The fact that these studies were carried out with a small sample size and piglets per treatment were from a single litter suggests further evaluation is necessary.

References

- Le Jan, C; A study by flow cytometry of lymphocytes in sow colostrum; Res Vet Sci, 1994, 57:300-304.