Abstracts concerning *M. paratuberculosis*
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Notes: Original abstract numbers are shown. Those with a "P" suffix were poster presentations the others were oral presentations. These abstracts where retyped by my 18 year-old daughter Katrina and proof read by me. We apologize for any errors that may have been caused in this process. Mike Collins

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#14P

Identification and characterization of a Mn-cofactored superoxide dismutase of *Mycobacterium avium* subsp. *paratuberculosis*.

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Mycobacterium avium subsp. *paratuberculosis* is the etiological agent of paratuberculosis, or Johne's disease. We characterized the Mn-cofactered superoxide dismutase (SOD) (EC1.15.1.1) of *M. paratuberculosis*, which was identified as one of the predominant proteins in the total protein profile of *M. paratuberculosis*. The *sodA* gene was cloned and sequenced, and the transcriptional start site was mapped. This enzyme consists of 207 amino acids and contains a single amino acid difference between the SOD of *M. paratuberculosis* and *Mycobacterium avium* subsp. *avium.* We also showed that the SOD of *M. paratuberculosis* is secreted during growth *in vitro* using a signal peptide independent mechanism.

#15P

Molecular beacons: A new approach for detecting *Mycobacterium paratuberculosis* in bovine fecal samples

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Mycobacterium paratuberculosis is the etiological agent of Johne's disease in cattle, bison and small ruminants. Typically, fecal culture or ELISA-based serology has been used for the diagnosis of infection. This report describes a new approach for diagnosis with fluorgenic reporter molecules called "molecular beacons". After DNA was extracted from bovine fecal material, a molecular beacon which is specific for *Mycobacterium paratuberculosis* IS 900 DNA sequence was utilized. The IS 900 gene was cloned into a pGEM-T Easy Vector and used to generate a standard curve for quantitative measurements. The quantitation of PCR products was performed using a Perkin-Elmer 7700 Sequence Detection System. The results were consistent with that of conventional culture and nested PCR. However, the application of molecular beacons provides a single tube, automated, high through put quantitative PCR system with broad potential in diagnostic testing.

#16P

Genetic analysis of the bovine NRAMP1 Gene: A candidate gene for susceptibility to Mycobacterial infection?

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In mice the NRAMP 1 gene is directly linked to susceptibility to infection by intracellular bacteria, including various mycobacteria. Susceptibility to infection has been linked to a single non-conservative Gly to Asp substitution at amino acid 169 in the murine NRAMP 1 protein. The role of the bovine NRAMP 1 protein in susceptibility/resistance to mycobacterial infection has not been definitively determined. To evaluate potential roles of NRAMP 1 mutations in determining the outcome of mycobacterial infections in the bovine, we have begun a screen for polymorphisms within the bovine NRAMP 1 coding sequence and analyzed the bovine NRAMP 1 gene structure. Comparison of the published cDNA sequences for Bos taurus and bison NRAMP 1 genes reveals fourteen base pair differences. Seven of these genetic differences lead to amino acid substitutions in the Bos taurus NRAMP 1 protein, relative to bison. The presence or absence of these potential differences within various Bos taurus populations (Holstein and Jersey) were assessed by a combination of RFLP on amplification fragments and SSCP using DNA from Bos indicus and Bos taurus as a comparative base. Overall, the bovine NRAMP 1 gene is highly conserved, as are the NRAMP 1 genes of many divergent species. The gene structure of the bovine NRAMP 1 locus also appears to be highly similar to that of the mouse with conservation of intron position as well as length. Identification of bovine NRAMP 1 intron-exon boundaries and intron sequences may lead to new polymorphic markers showing linkage to susceptibility/resistance traits in various cattle populations.

#25

A diagnostic strategy for the early detection of paratuberculosis in young cattle: Design and Preliminary Findings

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Paratuberculosis infection (PTB) seems to be acquired mainly during the first month of age due to contact with infected feces or milk. Infection remains silent in the majority of the animals until the adult age. Sub-clinical infection in adult animals could be associated with absence of clinical signs, shedding, and raise in antibody titers. A strategy for early diagnosis of PTB infection in calves might involve detection of CMI developed at the young age. The objectives of this study are the following: (1) To determine the association between the bacteriological and serological status of the dams (IDEXX ELISA) and the CMI response in the calves up to 7 months of age measured by intradermal johnin and gamma IFN test (2) To evaluate the use of

combined humoral and CMI-based tests to detect PTB infection in calves. Experimental approach: six hundred pregnant dams with positive and suspect ELISA results and 100 pregnant dams with negative results will be selected for this study. It is expected that 50% of them will calf females. These dams and their female calves with be enrolled in the study (n=300 heifers and n=300 dams). Confirmation of the dams' disease status will be performed immediately after calving by fecal culture and/or PCR. Heifers will be followed up until they are 8 months old. Intradermal johnin (PPD) will be injected in the heifers at 30 to 60, 90 to 120, 150 to 180, and 210 to 240 days of age. Simultaneously, blood samples will be collected for ELISA and gamma IFN testing, respectively. Fecal samples from the heifers will be collected between 210-240 days of age for detection of the agent by culture and/or PCR. Expected results: We expect to find a significant association between the serological and/or shedding status of he dams and the presence of a detectable CMI response in the calves during the first seven months of age. The study subjects are being enrolled in August 2000, and results of the first sampling will be presented at the conference. Significance: Early detection of PTB in calves will reduce the costs of raising heifers that will be culled due to the presence of clinical signs or fecal shedding at the adult age.

#26

Comparison of paired fecal cultures and ELISA tests as screening tools for *Mycobacterium avium* subsp. *paratuberculosis* infections in dairy herds

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Successful control programs for *Mycobacterium avium* subsp. *paratuberculosis* will likely incorporate a herd testing program in addition to instituting management practices documented to decrease herd infection levels. The purpose of this study was to report the sensitivity and specificity of a commercially available ELISA diagnostic test relative to fecal culture results when both are applied as screening test in dairy herd with unknown prevalence of *Mycobacterium avium* subsp. *paratuberculosis* infection. Herd testing results were obtained for nine Ohio dairy farms during a six-year period from 1993-1999 from the Ohio Department of Agriculture, Animal Disease Diagnostic Laboratory. Generally, these herds completed paired whole herd fecal cultures and ELISA tests every six months. A total of 4888 paired test results for 1805 cows were compared. The sensitivity of the ELISA test relative to fecal culture was estimated to be 47.0%, and relative specificity was estimated at 97.3%. Kappa values ranged from 0.271 to 0.548 with considerable variability between kit lots. In cows with three or fewer consecutive test dates, 30% of positive ELISA test results lacked confirmation by fecal culture. In cows with four or more consecutive test dates, 51.9% of positive ELISA produces more false negative and false positive results than would be expected based of the sensitivity and specificity information reported by the manufacturer. Kappa values reported here suggest that agreement between fecal culture and ELISA results is moderate. Lack of consistency in ELISA performance relative to fecal culture diminishes the value of this screening test in disease control programs that focus on the identification and removal of infected animals.

#27

Long-term tracking of dairy cattle for infection with *M. paratuberculosis*: Seasonal and production cycle variations in immune status

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Mycobacterium paratuberculosis causes Johne's disease, a chronic granulomatous enteritis that affects approximately 75 to 10% of dairy cattle nationally. The complex interplay of intracellular bacterium and host immune parameters makes diagnosis of Johne's disease highly problematic. Commercial ELISA tests use an absorbed format to block reactivity form environmental Mycobacteria, such as *M. avium*. Although the absorbed ELISA exhibits high specificity (approximately 99%), the sensitivity of this test is low (approximately 20-57%). Contamination problems, sporadic shedding of *M. paratuberculosis* during infection, and long incubation times (3 to 12 weeks), all reduce the utility of direct culture for diagnosis. We have initiated a long-term study to evaluate various assay formats in rapid diagnosis of *M. paratuberculosis* infection and to monitor changes in individual and herd status over time. Over 500 animals have been tracked through bi-monthly sampling using an unabsorbed ELISA format as initial screen, supplemented with an absorbed format on a suspected positive animals. These results have been compared with IgA antigen secreting cell assay, assay of gamma-interferon production from stimulated blood leukocytes, and direct culture. Our findings to date suggest that the unabsorbed ELISA test offers a more sensitive method detecting *M. paratuberculosis* infection. In addition seasonal and time variations in immune response suggest that a single yearly test date is insufficient to effectively screen heard for *M. paratuberculosis* infection.

#64P

Do glucocorticoids shift cytokine profiles from Th1 to Th2? Effects of cortisol and dexamethasone on production of interferon gamma and interleukin 10 in cultured pig splenocytes

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Some experimental evidence suggests that glucocorticoids functions to alter cytokine production from a Th1 to a Th2 pattern. Indeed, under very specific in vitro conditions, cytokine secretion from cultured immune cells can be shifted in this manner. However, most of the evidence for this effect of glucocorticoids has been obtained under rather complex in vitro conditions with dexamethasone, a potent synthetic glucocorticoid. The objective of the current experiment was to revisit the issue of glucocorticoid modulation of Th1/Th2 cytokine production utilizing cultured pig splenocytes. The concentrations of cortisol (CORT) used in the current study were selected to represent physiologically relevant levels observed in pigs undergoing active immune response to disease challenge. Desamethasone (DEX) was included in some treatments for comparison. Splenocytes were obtained from growing pigs and placed in culture in RPMI media containing 10% fetal bovine serum. Cells were exposed to 10 : g/mL concanavalin A (Con A) and Con a plus CORT or DEX. Control dells were not stimulated with Con A or treated with

steroid. Media was obtained after 3,6,16, and 24 h in culture, and later assayed for interferon gamma ((IFN) and interleukin 10 (IL-10) using porcine specific ELISAs. The experiment was repeated two times, with cells from 3 or 4 pigs in the first and second relocated, respectively. Concentrations of both cytokines were increased in Con A treated wells compared to control wells at 12 and 24h (P<. 01). Neither 150 nor 300 nM CORT suppressed IFN, although both concentrations of DEX blunted IFN at 12 (P<. 05) and 24h (P<. 01). In contrast, both 150 and 300 nM CORT and DEX reduced IL-10 after 24 h in culture (P<. 01). Thus, the data suggests that CORT, the major glucocorticoid in the pig, reduced IL-10 but not IFN, even at high physiological concentrations. Under the conditions of he current study, the results do not support the hypothesis that glucocorticoid functions to shift the cytokine milieu from Th1 to a Th2 phenotype.

#82

Development and Evaluation of an Automated System for Isolation of *Mycobacterium avium* subspecies *paratuberculosis* from Bovine Feces.

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Johne's disease, or paratuberculosis, is a chronic infectious disease of cattle and other ruminants with serious economic consequences. *Mycobacterium avium* subspecies *paratuberculosis*, the etiologic agent of this disease, is a slow growing organism that usually infects the host 2_5 years prior to the onset of clinical disease. The organism is shed in the feces in moderate to large numbers during this prepatent period, thus contributing to the exposure of other animals. Culture of the feces remains the most reliable method to detect infected animals. Unfortunately, culture is tedious, costly and lacks sensitivity. Therefore, improved diagnostic methods are needed. The purpose of this work was to evaluate a broth_ based method, adapted from an automated system (MB/BacT system Oganon Technica, Inc.) used for culture of mycobacteria from humans, for the detection of *M. paratuberculosis* in bovine feces. Fecal material from a single, negative animal was autoclaved and frozen in I g aliquots. The fecal material was processed with a double_centrifugation technique that included disinfecting with 0.75% hexadecylpyridinium chloride and incubation with an antibacterial/antifungal cocktail prior to inoculation of broth tubes. Serially diluted organisms (approximately 10^6 to 10^0) were grown in broth alone, in broth with processed feces or with sterile feces spiked with the pathogen prior to processing and inoculation into broth culture. The highest concentration of M. paratuberculosis was detected by the MB/Bact system in as few as 8 d. However, preliminary data indicates that the processing step had a detrimental effect on detection of the organism. These results indicated that this automated system could be used to detect this organism in bovine feces, but that new approaches to processing the feces for culture might have to be explored.

#88P

Lymph node histopathology, bacterial persistence, and cell-mediated immune response to subcutaneous inoculation with *Mycobacterium avium* subsp. *paratuberculosis* in Holstein calves.

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Four-week-old male Holstein calves were given bilateral subcutaneous inoculations in the cervical region with 10^8 CFU/site of *Mycobacterium avium* subsp. *paratuberculosis*. (*Mpt* n=3) or 2 mL of sterile saline (n=3) to evaluate vaccination-induced lymphoid changes, inoculation site alterations, and bacterial persistence. Inoculation sites and superficial cervical lymph nodes were biopsied at 7, 21, and 60 post-inoculation days (PID) and examined histologically. The most consistent lymph node alteration was a sinus histiocytosis in *Mpt*-inoculated calves at PID 7 and PID 21. Skin inoculation sites from *Mpt*-inoculated calves contained scattered acid-fast bacilli (AFB) in the deep dermis at PID 7, large numbers of foamy macrophages and multinucleated giant cells containing massive numbers of AFB at PID 21, with no AFB present at PID 60. *Mpt* was cultured from the lymph node of one *Mpt*-inoculated calf at PID 7. Blood and fecal cultures during the first week PI were negative for *Mpt*. To evaluate cell-mediated immune (CMI) responses of superficial cervical lymph node-derived lymphocytes, cells were isolated at PID 60, PKH67-labeled, and incubated with media alone, conconavalin A, or an experimental PPD preparation for up to 7 days. Lymphocytes were stained for surface markers and for intracellular IFN-gamma, and analyzed using three-color flow cytometry. At PID 60, CD4+, CD8+, and gamma-delta T cells, but not B-cells, from calves inoculated with *Mpt* proliferated in response to PPD stimulation. These T-cell subsets also produced increased amounts of IFN-gamma in response to antigenic stimulation compared to lymphocytes from saline inoculated controls. These results correlated with a moderate intradermal delayed-type hypersensitivity response at PID 90 using USDA standard Johnin and experimental PPD. These results indicate that subcutaneous inoculation of *Mpt* induces a CMI response detected by PID 60 in bovine calves.

#97

Molecular characterization of epitopes from protein p34 of *Mycobacterium paratuberculosis*.

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Johne's disease causes high losses in cattle. The aetiological agent is *Mycobacterium paratuberculosis*. The diagnosis of specific antibodies of he disease is problematic in countries where *Mycobacterium bovis* is endemic. Antibody titers to Mp against the bacteria are low at the beginning of the chronic disease. Later, antibody titers increase and this indicator is considered a bad prognosis; these animals will die with the clinical symptoms of Johne's disease. The immunodominant protein of this microorganism is a proline-rich protein with a MW of 34 Kd (p34). Our goal was to molecularly characterize the epitopes of p34, which are recognized by bovine antibodies. We expressed the carboxyterminal fragment of this protein

(p34-cx) in E. coli. This is the most immunogenic fragment of p34. We used p34cx of affinity purify antibodies form and infected cow that had died of Johne's disease. These antibodies were then used to select clones from a 12-mer phage display library. The predicted amino acid sequence of several of these clones have different levels of identity with the amino acid sequence of p34. Clone 22 has a core of six-amino acids identical to a highly hydrophilic area of p34. Several other 12-mer peptides have between 4 to7 amino acids which could resemble discontinuous epitopes between amino acids 285-295 of p34. The predicted peptides were synthesized and when tested in a direct ELISA they were recognized by serum from Mp infected cattle, but were not recognized by negative nor an anti-*M. bovis* serum, suggesting that these mimotopes resemble Mp specific epitopes.