Abstracts from Oral and Poster presentations at the

Fifth International Colloquium on Paratuberculosis

Madison, Wisconsin

Sept 29 - Oct 4, 1996

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Title The effect of recombinant bovine IFN-gamma and nitric oxide on the interaction of *Mycobacterium avium* ss paratuberculosis with bovine monocytes.

Author(s) Zhao BY, Collins MT, Czuprynski CJ.

Institution Depart Pathobiol Sci, University of Wisconsin, Madison, WI, USA.

Abstract We previously reported that IFN-gamma and LPS can stimulate bovine monocytes to produce nitric oxide, which is blocked by addition of NMMA. The purposes of the present study were to determine the effect of IFN-gamma on the interaction of *M. avium* ss *paratuberculosis* with bovine monocytes, and to find out whether this effect was related to production of nitric oxide by activated bovine monocytes. Freshly isolated peripheral blood monocytes were cultured with different concentrations of IFN-gamma, or NMMA, for 16 to 18 hours, and infected with *M. avium* ss *paratuberculosis*. At the time of infection, LPS was added to some IFN-gamma pretreated monocytes and monocytes cultured with NMMA. Monocytes were then cultured up to 12 days after infection and then lysed with 0.25% SDS at 0, 3, 6, 9 and 12 days after infection. Radiometric counting was used to determine the numbers of viable intracellular *M. avium* ss *paratuberculosis*. Monocytes incubated with IFN-gamma exhibit a trend towards slightly increased phagocytosis of and inhibition of intracellular *M. avium* ss *paratuberculosis* by bovine monocytes. This effect was not altered by addition of LPS and NMMA to IFN-gamma pretreated monocytes. These data suggest that the amount of nitric oxide produced by bovine monocytes may be insufficient to inhibit intracellular growth of *M. avium* ss *paratuberculosis*.

Title Immune responses to the p43 protein encoded by IS900, in naturally and experimentally infected calves.

Author(s) Tizard M, Fifis T[*], Griffiths H, Tennent J[*], Corner L[*], Doran TJ, Hermon-Taylor J.

Institution Depart Surg, St. George's Hosp Med School, London SW17 0RE, UK; [*]Div Anim Health, CSIRO, Parkville, Victoria, Australia.

Abstract Immune response to the p43 protein encoded by IS900 was examined looking at sera IgG and cell mediated response judged by gamma-IFN release in a whole blood assay (CSL). Serological responses were assessed by comparison with a test panel of sera used for validation of the CSL Johne's disease ELISA kit (kindly supplied by Dr. S. Jones, CSL). No correlation was seen indicating that this antigen is poorly recognized at the antibody level. Whole blood, for gamma-IFN release assay, was obtained from calves in a controlled study conducted at the Victoria Institute of Animal Science (kindly supplied by Dr. A. Hope, Dr. W. McDonald and Dr. R. Condron). Groups included 6 calves naturally and 8 calves artificially exposed to *M. paratuberculosis* infection; 4 calves run in isolation (as controls for infection); 9 calves vaccinated with a commercial vaccine; 3 calves run as controls for the vaccinates; one mature cow as an external control. The principle interest was in the gamma-IFN release assay. Responses were seen in 8 of the 14 animals exposed to infection. Responses were seen in only 3 of 9 vaccinates. However 1 of the 4 control calves showed a response to p43 as did 1 of the 3 controls for the vaccinated group. All animals were subjected to postmortem histopathology to establish disease status. The full inference forthese results awaits complete histopathology data. Current interpretation suggests that this antigen may make a useful component of a diagnostic reagent but is unlikely to be sufficient on its own.

Title Seroreactivity of specific *Mycobacterium paratuberculosis* recombinant clone expressing 35KD antigen with clinical and subclinical Johne's disease.

Author(s) El-Zaatari FAK, Naser SA, Graham DY.

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Abstract Paratuberculosis (Johne's disease), is a chronic enteritis caused by *Mycobacterium paratuberculosis* that affects a large proportion of ruminants world-wide and is responsible for

enormous economic losses. Currently available serologic tests have poor sensitivity and specificity. We previously described the construction of an expression library of *M. paratuberculosis* in E. coli. In this study, we used the gene product of a recombinant clone expressing a 35KD (p35) of *M. paratuberculosis* recombinant antigen for serodiagnosis of Johne's disease. The seroreactivity of p35 was evaluated by immunoblotting against sera of 57 reference animals. P35 was recognized by sera from all 12 cattle, 2 goats and 2 sheep (100%) with advanced Johne's disease (Clinical Stage) and by 15 of 20 (75%) cattle with early infection (Subclinical Stage). None, of the 15 *M. paratuberculosis* free cows, 3 BCG-infected cows (Tuberculous cattle) or the 3 cows, artificially inoculated with multiple doses of viable *M. paratuberculosis*, reacted with p35. The overall sensitivity, specificity, positive predictive value and negative predicted value were 86%, 100%, 100%, and 75%, respectively. These data are much higher than those reported using commercially available diagnostic tests. In addition, p35 reacted poorly to sera from tuberculosis, leprosy and Crohn's disease patients. When the DNA fragment encoding p35 gene was used as a probe, it hybridized to DNA only from *M. avium* complex but not to DNA from any other mycobacteria or non-mycobacterial organisms that were tested. Our data clearly suggest that p35 recombinant protein and p35 encoding gene DNA fragment have potential for efficient serodiagnosis of animals with Johne's disease at all stages of the infection and as a probe for identification of *M. avium* complex infection, respectively.

Title Contribution of T cell subsets to the immune response to *Mycobacterium avium* ss paratuberculosis in normal and infected cattle.

Author(s) Bassey EOE, Collins MT, Czuprynski CJ.

Institution Depart Pathobiol Sci, School of Vet Med, University of Wisconsin, Madison, WI, USA.

Abstract The relative contributions and specific functions of T cell subsets in cattle infected with *Mycobacterium avium* ss *paratuberculosis* are not well known. To help elucidate their roles, T-lymphocyte subsets were purified using commercially available monoclonal antibodies to CD3, CD4, CD8, and gamma-delta lymphocyte cell surface markers, and a magnetic bead separation column system. Purity of subsets was verified by flow cytometry. Subsets were assayed for proliferation and interferon-gamma (IFN-gamma) production in response to phytohemagglutinin (PHA) and *M. avium* ss *paratuberculosis* antigens (APPD). IFN-gamma was measured by antibody-capture ELISA, and OD values converted to picograms of IFN-gamma using a standard curve established with recombinant bovine IFN-gamma. The results indicated correlation between proliferation responses and IFN-gamma production in response to specific antigens, but not PHA. CD4+ lymphocytes were the most prolific producers of IFN-gamma. CD8+ lymphocytes produced IFN-gamma to a lesser extent, whereas gamma-delta T lymphocytes produced little or no IFN-gamma. There was no significant difference in the levels of IFN-gamma produced by whole PBMC and CD4+ vs CD8+ cells, and CD4+ vs gamma-delta+ cells in response to PHA were significant at $P<0.01$, but there was no significant difference between CD8+ and gamma-delta + cell IFN-gamma responses. In response to A-PPD, the differences between CD4+ and gamma-delta + cells was significant at $P<0.05$, but levels of IFN-gamma produced to specific stimulation were generally lower. These data for cattle are similar to observations made in other animal species, where CD4+ cells are the major type of T lymphocytes producing IFN-gamma. They further suggest that whatever the role gamma-delta T cells play in paratuberculosis it is not likely to be mediated by IFN-gamma production.

Title Cytokine expression in intestinal tissue of cows infected with *Mycobacterium paratuberculosis*.

Author(s) Sweeney RW, Jones DE, Whitlock RH, Scott P.

Institution Depart Clin Stud-New Bolton Center Depart Pathobiol (Jones, Scott), University of Pennsylvania School of Vet Med.

Abstract The purpose of this study was to determine if cytokine production in intestinal and mesenteric lymph node tissue of cattle naturally infected with *M. paratuberculosis* reflects a Th1-type pattern in early disease and a Th2 pattern in advanced infection. The experimental approach was to measure cytokine mRNA in tissues harvested at slaughter, using a reverse-transcriptase, competitive polymerase chain reaction (RT-cPCR) assay. Eleven adult Holstein cows (from 7 herds) infected with *M. paratuberculosis* were identified by fecal culturing. Four of the cows had clinical signs of paratuberculosis (diarrhea, weight loss) and 7 of the infected cows were asymptomatic. Samples were also obtained from 5 uninfected herd mates of the infected cows. At the time of slaughter, samples of ileum and ileocecal lymph node were frozen in liquid nitrogen immediately after collection, and stored at 70^{o}C until RT-cPCR was performed. For ileum and mesenteric lymph node samples, the IFN-gamma mRNA concentration was highest in the asymptomatic infected group, approximately 3 fold higher than the control and clinically affected cows. The difference between the asymptomatic group and the control and clinically affected groups was significant. No significant difference was detected in IL-4 mRNA concentration in intestinal tissue nor mesenteric lymph node, although there was a trend towards higher IL-4 concentration in the clinically affected cows. These data support the hypothesis that progression of infection to the clinical stage is associated with a reduced Th1 response.

Title Intracellular fate of *Mycobacterium avium* ss paratuberculosis in monocytes from infected and non-infected cows as determined by a radiometric method.

Author(s) Zhao BY, Collins MT, Czuprynski CJ.

Institution Depart Pathobiol Sci, University of Wisconsin, Madison, WI, USA.

Abstract To further understand the pathogenesis of *Mycobacterium avium* ss paratuberculosis infection in the bovine, we investigated the phagocytosis by, and intracellular survival of, *Mycobacterium avium* ss *paratuberculosis* in bovine monocytes from non-infected cows and *Mycobacterium avium* ss *paratuberculosis*-infected cows. The different abilities of these cells to ingest and inhibit intracellular growth of *M. avium* ss *paratuberculosis* were determined either by radiometric (BACTEC) counting of bacilli in monolayer lysates, or by microscopic counting of bacilli in monocytes at 0, 3, 6, 9, 12, and 15 days post-infection in vitro. We observed an early growth phase, during the first 6 days after in vitro infection, followed by mycobacteriostasis or bacterial killing. This is the first report of decreasing numbers of viable intracellular *M. avium* ss *paratuberculosis* in bovine monocytes after 6 days of in vitro infection. In contrast, the numbers of acid-fast bacilli, as detected by microscopic evaluation, continued to increase, suggesting loss of viable bacilli concurrent with bacillary replication. This early growth phase suggests that *M. avium* ss *paratuberculosis* may initially resist antimycobacterial mechanisms of mononuclear phagocytes, or that these antimycobacterial mechanisms are not activated. The later period of mycobacteriostasis, or killing of bacilli, could be related to release of endogenous cytokines that lead to activation of bactericidal mechanisms (ROI, RNI), or nutrient deprivation of intracellular bacilli. Monocytes from infected cows with a strong IFN-gamma response phagocytosed slightly more bacilli than did monocytes of normal cows. There was a trend toward mycobacteriostasis or bacterial killing occurring somewhat earlier in monocytes from *M. avium* ss *paratuberculosis* infected cows than in monocytes from normal cows.

Title Characterization of lymphocyte subpopulations in paratuberculosis vaccinated and non-vaccinated sheep.

Author(s) Mateo A[1], Pinato OC[1], Garrido JJ[2], Juste RA[2], Llanes D[1].

Institution [1]Dpto. de Genetica, Facultad de Veterinaria, Universidad de Cordoba, Cordoba; [2]SIMA, DIAP, Gobierno Vasco, Derio (Bizkaia), Spain

Abstract Cellular immunity plays an important role in the pathogenesis of paratuberculosis. To determine the changes in lymphocyte populations associated with vaccination, a sample of vaccinated and non-vaccinated (positive fecal smear and normal) sheep were submitted to a FACS analysis using a panel of 11 monoclonal antibodies (CD2, CD3, CD4, CD8, CD21, CD31, CD41/61,

CD43, CD45R, and gamma-delta TCR). Results were transformed and submitted to statistical analysis by analysis of variance and principal components multivariate analysis. Significant differences between vaccinated and non-vaccinated sheep were found for CD45R and CD3 markers. Vaccinated sheep showed higher proportions of CD45R and lower of CD3. The same tendencies were observed for normal and for positive sheep when both groups were analyzed separately, although the latter usually showed slightly higher counts than the former. All the markers were positively correlated with the first component of the principal components analysis. However, the second component, although defining a weaker relationship, seemed to define two groups of markers: CD43, CD45R, and CD21 on one side and the rest on the other. When considering the clustering by sample group, the patterns of distribution varied considerably. The most relevant observation was the defined opposition between CD45R and CD2 along the first component in the normal group. In the positive group, a similar situation was observed, but between CD45R and CD3 instead of CD2. These relationships were confirmed by Pearson correlation analysis which showed that CD45R/CD3 correlation was negative and significant ($r=0.996$, $p<0.06$). In the positive group, these relationships became -0.022 (N.S.) and -0.999 ($p<0.02$), respectively. Analyzing the clustering of sheep according to their CD patterns showed a certain segregation of sheep on the second component. Since the low number of sheep made this separation little clear, another set of non-vaccinated sheep (4 positive and 3 normal) with incomplete data was added to the original data. In these conditions, the same pattern of segregation appeared more clearly, with 80% of vaccinated sheep on the left side of the second component, and 100% of normal on the right ($p<0.001$). Positive sheep split between both sides with 33% on the left and 67% on the right. These results show characteristic changes in the cellular immune response to M. a. paratuberculosis sensitization, with CD45R and CD3 appearing as the subpopulation more clearly involved in vaccine protection. The differences between normal and vaccinated CD patterns stresses the relevance of cellular immunity. However, the observation that shedding infected sheep do not specifically conform to any of the two patterns suggests complex cellular relationships in the pathogenesis of this infection.

Title Macrophage Inhibitory Factor - A3 (MIF-A3) a glycolipid compound derived from *Mycobacterium avium* serovar 2 inhibits candidacidal activity of elicited murine peritoneal macrophages.

Author(s) Hines II ME, Cray CC, Elvinger F, Altman NA.

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Abstract Macrophage Inhibitory Factor - A3 (MIF-A3), a glycolipid compound derived from *Mycobacterium avium* serovar 2, has previously been shown to inhibit the candidacidal activity of activated bovine peripheral blood-derived macrophages (MF). This compound appears to be a potent scavenger of reactive oxygen species in zymosan stimulated sheep alveolar macrophages and in a cell-free system. It does not affect phagocytosis of sheep alveolar MF and has no effect on nitric oxide production of g-interferon stimulated murine C57BL/6 thioglycolate-elicited peritoneal-derived MF. In this study, a similar inhibition of candidacidal activity was detected in MIF-A3 treated thioglycolate-elicited murine C57BL/6, C57BL/10, C3H/HeJ and A/J MF. Inhibition of candidacidal activity was demonstrated at MIF-A3 concentrations ranging from 100-400 mg/ml in MF without additional stimulators (exception C3H/HeJ) and in MF additionally stimulated with 200 U/ml g-interferon, 100 ng/ml phorbol myristate acetate (PMA) and 0.4 ng/ml E. coli lipopolysaccharide (LPS) from all mouse strains tested (C57BL/6, C57BL/10, C3H/HeJ and A/J). The decreased candidacidal effect produced by MIF-A3 was dose-dependent and appeared greatest in PMA and LPS treated MF. This effect could be neutralized by the addition of goat anti-MIF-A3 antiserum. Differences in the response to treatment with MIF-A3 of MF from different mouse strains were present and show that MF from the Bcgs mouse strains (C57BL/6 and C57BL/10) are significantly more sensitive to the effect(s) of MIF-A3 than the Bcgr mouse strains (C3H/HeJ and A/J).

Title Modulation of the intracellular growth of *Mycobacterium paratuberculosis* within bovine monocytes by bovine growth hormone and prolactin.

Author(s) Feola RP, Collins MT, Czuprynski CJ.

Institution Depart Pathobiol Sci, School of Vet Med, University of Wisconsin, Madison, WI, USA.

Abstract Our lab is currently interested in the mechanisms responsible for the transition of Johne's disease from a clinically latent infection to clinical disease marked by progressive bacterial multiplication, fecal shedding, chronic diarrhea and wasting. These events have been reported to be associated with parturition and onset of lactation. We developed a hypothesis that the onset of clinical disease may be associated with changes in serum hormone levels due to hormone cycling at parturition. We, therefore, tested several of the major hormones that cycle at this time. Of the hormones tested, we found that two can have a profound effect on intracellular growth of *M. paratuberculosis* in primary bovine monocyte cell cultures. These were bovine growth hormone (GH) and bovine prolactin. In both cases, bacillary multiplication increased to near maximal doubling times when infected monocyte cultures were exposed to hormone for the first 5 days of a 12 day incubation. GH and prolactin treated cultures at day 12 showed a 1.5 to 2 Log10 increase over controls that were not treated with hormone. Furthermore, our data indicated that phagocytosis of *M. paratuberculosis* was decreased in a dose dependent manner at GH concentrations from 1 to 10 ng/ml, and nearly eliminated at a concentration of 50 ng/ml.

Title Clinical biochemical changes in Johne's disease of ruminants.

Author(s) Jones DG, Kay JM.

Institution Moredun Research Institute, Edinburgh, Scotland U.K.

Abstract Plasma samples, obtained at slaughter, from sheep (19), cattle (17), and goats (4) with clinical evidence of *M. paratuberculosis* infection (Johne's disease) were examined for changes in 10 clinical biochemical parameters (total protein, albumin, globulin, calcium, magnesium, phosphorous, creatinine, urea, AST, GGT). Hypocalcaemia and hypoalbumnaemia were a consistent and characteristic finding in all clinical cases compared, at least in the case of sheep and cattle, with animals with no evidence of mycobacterial infection. Goats were obtained from a farm with a history of Johne's disease and were assumed to have been naturally exposed to *M. paratuberculosis*. However, of 21 animals examined, only 4 had evidence of clinical lesions and/or PCR reactivity. Of the biochemical parameters measured, only total protein was consistently affected, presumably reflecting the observed loss of albumin. Plasma antibody titers were significantly higher and lymphocyte reactivity low in clinically affected sheep and goats (no data available for cattle). In both species, decreases in albumin and calcium were correlated with both immune markers. Possible implications of these observations, especially with respect to Vitamin D immunomodulation, will be discussed.

Title Local and systemic cellular and humoral immune responses and cytokine changes in ovine paratuberculosis.

Author(s) Clarke CJ[1], Burrells C[2], Little D[1], Colston A[2], Kay J[2], Sharp JM[2].

Institution [1]Dept Veterinary Pathology, University of Edinburgh, UK; [2]Moredun Research Institute, Edinburgh, UK.

Abstract We have recognized two forms of histological lesions in diseased sheep gut: a multibacillary (lepromatous) form and a paucibacillary (tuberculoid) form. We hypothesize that these correspond to different types of immunological responses. Sheep with clinical paratuberculosis (n=19) and healthy control animals (n=16) were used in the study. The proliferative responses of lymphocytes (isolated from the intestinal lamina propria, mesenteric lymph nodes, and peripheral blood) to *Mycobacterium aviumparatuberculosis* antigens were measured by titrated thymidine uptake. Isolated cells were also cultured with antigen, and the supernatants were assayed for IL-2 activity (IL-2-responsive cell proliferative assay) and IFN-gamma activity (ELISA). Serum samples were assayed for specific antibody. A full necropsy with histological examination of tissues were performed on all animals. A range of immune responses were seen

corresponding to the type of histopathology present. The paucibacillary group was characterized by dominant cell-mediated immune (CMI) responses with high levels of IFN-gamma and IL-2, and lower humoral responses suggesting a TH1 type response. The multibacillary group had significantly weaker CMI responses and IFN-gamma and IL-2 levels, with higher antibody levels, suggesting a TH2 type response. These groups correspond to the recognized tuberculoid and lepromatous types of histopathology, respectively. These results suggest that different mechanisms of pathogenesis, corresponding to the type of pathology seen, may operate in ovine paratuberculosis.

Title Mycobacterial 60 kD and 70 kD heat shock protein specific cellular responses in cattle infected with *Mycobacterium paratuberculosis*.

Author(s) Koets AP[1], Rutten VPMG[1], Hoek A[1], Müller K[2], Wentink GH[3], van Eden W[1].

Institution [1]Dept Immunol, [2]Dept Large Animal Internal Med, Fac Vet Med, Utecht University, The Netherlands, [3]Holland Genetics, The Netherlands.

Abstract It is known that mycobacterial heat shock proteins of pathogenic mycobacteria elicit cellular immune responses in both humans and animals. In the present study, recombinant *M. bovis* HSP 60 kD and 70 kD, and *M. paratuberculosis* PPD antigens were used in blastogenesis assays to test T cell reactivity of cows in various stages of infection with *M. paratuberculosis*. Subsequently overlapping 15-mer peptides from HSP 70 were used in epitope mapping studies. In both infected and vaccinated cows the stage of infection was determined by fecal culture results, clinical signs, and age. Cows from *M. paratuberculosis* free herds, as determined by fecal culture, served as negative control animals. Preliminary results suggest that cell mediated responses against HSP 70 kD and PPD correlate with the stage of infection while responses against HSP 60 kD, which were observed infrequently, did not. Average responses against PPD are higher in non-shedders vs. healthy cows, healthy shedders vs. non-shedders, and clinical cases vs. healthy shedders. Responses against HSP 70 kD parallel the PPD responses, except for clinical cases. In clinical cases the response against HSP 70 kD equals that of healthy negative control animals; while responses against PPD are still above the level observed in healthy shedders. Vaccinated animals have HSP 70 kD responses equal to healthy shedders. HSP 70 kD epitope mapping using PBMC's of a negative control animal, a healthy shedder, and a clinical cow revealed that all three animals recognized conserved aminoterminal epitopes. In addition, the subclinical shedder recognized carboxyterminal epitopes. In contrast, the clinical cow recognized the same epitopes as the negative control but non that were recognized by the healthy shedder. We are in the process of analyzing the nature of the regulatory events apparently causing differences in T cell reactivity in different stages of infection and their relevance for immunopathogenesis.

Title Expression of the IS900 hed gene: implications for diagnosis, treatment and pathogenicity studies of *Mycobacterium paratuberculosis* disease.

Author(s) Doran T.

Institution Department of Surgery, St. George's Hospital Medical School, Cramner Terrace, London, U.K. SW17 0RE

Abstract The *Mycobacterium paratuberculosis* atypical insertion sequence, IS900, encodes a novel gene on the complementary strand to the p43 coding sequence. This gene requires a promoter, ribosome binding site (RBS) and termination codon to be acquired upon insertion into the *M. paratuberculosis* genome, and hence is designated the hed (host expression dependent) gene of IS900. Analysis of IS900 insertion sites suggests that this element targets translation initiation signals in *M. paratuberculosis*, specifically inserting between the RBS and start codon of a putative gene sequence. This would align the hed initiation codon adjacent to a functional RBS and possibly downstream of an active promoter, driving expression of the Hed protein. A termination codon for hed is not generated by the target site and thus will vary in position for each genomic location of the IS900 element. Expression of hed was detected using two specific antibodies to probe Western blots of protein extracts from *M. paratuberculosis*. IS900 is unique to *M. paratuberculosis* and hence any immune response to its derived protein product would be indicative of specific infection. Therefore, recombinant forms of Hed and also p43, expressed and purified from E. coli and baculovirus are being examined as diagnostic antigens in immuno-assays for *M. paratuberculosis* disease. In conjunction, naked DNA constructs for in vivo expression of Hed and p43 have been used to immunize rabbits for future assessment in *M. paratuberculosis* vaccine design. IS900 is the major genetic difference between the closely related *M. paratuberculosis* and *M. avium*. Consequently, this element has been implicated in the increased pathogenicity of *M. paratuberculosis* from a *M. avium* background. The study of IS900 insertion into host gene translation signals and the subsequent post-translational effects of hed expression is beginning to provide a rationale for this element in this evolutionary process. It is hypothesized that the targeting process will result in expression of a number of *M. paratuberculosis* genes being specifically disrupted after IS900 has "hijacked" its translation signals. Such genes may regulate expression of virulence determinants, resulting in constitutive expression in *M. paratuberculosis* and conferring an obligate pathogenic status compared to *M. avium*.

Title Two-dimensional electrophoretic analysis of *Mycobacterium avium* and *M. paratuberculosis* iron-regulated proteins.

Author(s) Hsieh MK, Juste RA, Foley-Thomas EM, Bargar TW, Barletta RG.

Institution Depart Vet and Biomed Sci, University of Nebraska, Lincoln, NE, USA

Abstract Iron, a key nutritional requirement for the in vitro and in vivo multiplication of mycobacteria, regulates the expression of genes involved in its acquisition as well as those encoding cytoplasmic or surface proteins coordinately regulated with components of the iron-sequestration machinery. Mycobactin is an iron chelator produced by most species of mycobacteria with *M. paratuberculosis* being a notable exception. In our studies, we carried out two-dimensional gel electrophoresis to analyze the effect of varying cultural conditions and iron concentrations on *M. avium* and *M. paratuberculosis* protein expression. Approximately 150 polypeptide spots were resolved for cellular and cell-associated proteins. *M. avium* grown on minimal and Middlebrook 7H9 medium revealed unique polypeptides associated with mycobactin synthesis, low and high iron concentration, and conditions inducing ultrastructural changes. *M. paratuberculosis* cells grown on Middlebrook 7H9 medium containing either high iron, low iron or ferric mycobactin revealed fewer differences. Two polypeptides of 15 and 70 kDa were predominantly synthesized in high iron medium, while a 23 kDa polypeptide was overproduced under low iron conditions. In conclusion, our studies indicate a differential response of *M. avium* and *M. paratuberculosis* gene expression to environments with low and high iron.

Title Development of RAPD-PCR for the differentiation of *Mycobacterium paratuberculosis* isolates.

Author(s) Scheibl P, Gerlach GF.

Institution Institut für Mikrobiologie und Tierseuchen, Tierärztliche Hochschule Hannover, Germany.

Abstract The lack of methods for differentiating *M. avium* ssp. *paratuberculosis* isolates has hampered epidemiological studies investigating the spreading of the disease. To contribute to solving this problem we have evaluated a set of 60 decamer primers with a GC-content above 50% alone and in combination in a RAPD-PCR. We succeeded in identifying 10 primers which reproducibly resulted in PCR-products of 200 to 600 bp. Subsequently, DNA was prepared from 12 clinical *M. avium* ssp. *paratuberculosis* isolates. Based on these results it should be possible to investigate the origin of isolates in newly infected herds.

Title PCR variants of *Mycobacterium avium* subsp. paratuberculosis.

Author(s) Nishimori K, Eguchi M, Tanaka K, Nakaoka Y[1].

Institution Hokkaido Branch, National Institute of Animal Health, Hitsujigaoka-4, Toyohira, Sapporo, 062; [1]Tokachi Livestock Hygiene Service Center, Kisen 59-6, Kawanishi, Obihiro, 089-11, Japan.

Abstract Six strains of isolates from 1992 to 1995 possessed multi-PCR products (229bp and the higher molecular products), using primer 1 (139-162) and primer 2 (313-367), common IS900-detecting primer pairs. Multi-PCR products were detected using primer 1 and primer 4 (638-662), but there were only expected products, 454bp and 159pb, using primer 3 (209-233) and primer 4, alternative IS900-detecting primer pairs, and primer 2 and primer 3, respectively. It was suggested that these PCR variants possessed the homologous sequences of primer 1 upstream of the target sequence (139-367).

Title Examination of genetic loci in *Mycobacterium paratuberculosis* disrupted by IS900.

Author(s) Tizard M, Lippett J, El-Zaatari F[1], Doran TJ, Loughlin M, Hermon-Taylor J.

Institution Depart Surg, St. George's Hosp Med School, London SW17 0RE, UK; [1]Gastroenterology Unit, VAMC, Houston, TX, USA.

Abstract Recent observations show that the insertion sequence IS900, from *Mycobacterium paratuberculosis*, has targeted translation initiation signals. This is part of a process which results in expression of a previously cryptic open reading frame now designated the hed (host expression dependent) gene. There are five main RFLP patters observed in *M. paratuberculosis* in which many bands are conserved across strains, even where bands differ significantly the genetic locus may still be the same. We have isolated a 14 of a predicted 18 loci, each of which contain a copy of IS900. Promoter and open reading frame analysis is being performed on each of these genetic loci. One locus, IDL3, contains the PAN promoter. Examination of downstream sequence will reveal the gene regulated by this promoter. Another locus, IDL5, has produced a blue colony, on lacZa complementation screening, indicating an open reading frame fusion from an open reading frame in the IS900 flanking sequence. In vitro transcription-translation of another locus, IDL22 shows expression of a high molecular weight protein. Disruption of gene expression from loci such as IDL3, IDL5 and IDL22 is likely to be responsible for some of the phenotypic differences seen between *M. paratuberculosis* and other members of the *M. avium* complex. One or more loci may be linked to the obligate pathogen phenotype. This could provide a unique opportunity to identify genes involved in regulation of virulence and pathogenicity.

Title HPLC analysis of mycolic acid profiles does not distinguish between *Mycobacterium*

avium and *Mycobacterium paratuberculosis*.

Author(s) Dei R, Tortoli E, Simonetti MT, Bartoloni A, Lillini E.

Institution Instituto Microbiologia and Instituto Malattie Infettive, Università di Firenze, Laboratorio Batteriologia, Ospedale Careggi, Firenze, Instituto Zooprofilattico Sperimentale Regioni Lazio e Toscana, Roma, Italy.

Abstract *Mycobacterium avium* and *Mycobacterium paratuberculosis* are primary pathogens for animals. They belong to the same complex, but are no longer considered as separate species. Differences are based on their growth characteristics, mycobactin requirement, and the natural host-range; although they have different IS sequences, the overall similarity at the genome level justifies the lumping in one species. Among the innovative identification methods, besides genome-based techniques, there is High Performance Liquid Chromatography (HPLC) analysis of cell wall mycolic acids. We have previously shown the usefulness of this new technique for the species identification of mycobacteria, and we have shown the typical profile of *M. avium*. *M. paratuberculosis* strains (the reference ATCC 19698 and animal isolates) were extracted, and the mycolic acid profiles obtained by HPLC were compared to the profile from *M. avium*. The HPLC mycolic acid profile of *M. paratuberculosis* strains were very similar to the *M. avium* profile. Therefore, the technique is not suitable for a rapid species identification of *M. paratuberculosis*. Nevertheless, our data support the view that *M. avium* and *M. paratuberculosis* be considered utmost as subspecies, and confirm HPLC analysis of mycolic acid profiles as the technique most similar to 16S rRNA sequencing or DNA-DNA hybridization.

Title Cloning and sequence analysis of a recA-like gene from *Mycobacterium avium* and *Mycobacterium paratuberculosis*.

Author(s) Ellingson JLE, Stabel JR.

Institution USDA/ARS/NADC, Zoonotic Diseases Research Unit, Ames, IA.

Abstract The RecA protein is widely recognized to have an important role in homologous recombination, DNA rearrangement, repair, and replication. A fragment of *Mycobacterium avium* DNA and *Mycobacterium paratuberculosis* DNA containing RecA-like sequences was identified by hybridization with the Mycobacterium tuberculosis RecA gene. The fragments were isolated by cloning PstI digests of *M. avium* and *M. paratuberculosis* DNA into pBluescript II ±. Southern hybridization and sequence analysis revealed that the 5' terminus of the genes was well conserved across species of mycobacteria. However, Southern hybridization analysis using a specific probe to the 3' terminus of the *M. avium* and *M. paratuberculosis* genes suggests there are two copies of the 3' terminus in each of the respective genomes. This data is important in the understanding of DNA metabolism pathways in pathogenic mycobacteria.

Title The Australian National Johne's Disease Market Assurance Program.

Author(s) Kennedy DJ.

Institution Aus Vet Animal Health Services, Orange, New South Wales 2800, Australia.

Abstract Johne's disease occurs in cattle and goats in south-eastern Australia while northern and western parts are considered to have little or no endemic disease. During the early 1990's, the Australian cattle industries initiated the development of a national program to reduce the spread and impact of Johne's disease. The result is the National Johne's Disease Market Assurance Program, developed and implemented by the industries, in partnership with State veterinary services. It aims to identify and classify herds with a low risk of being infected with *M. paratuberculosis* so that cattle buyers can source cattle with increasing confidence of not buying infected cattle. The program is being launched in 1996 and will be supported by research, training and advisory programs, standardization of diagnostic techniques, control programs in infected herds and rules for control of animal movements. Participation in the market assurance program is voluntary and at the cattle owners' expense. As such, it will be driven by market forces and will appeal to breeders who aim to procure a price premium or retain access to some markets. Herd owners will engage veterinarians, trained and approved for the program, who will be responsible for the herd's compliance with the program's management and testing requirements. Herds and approved veterinarians will be audited. Herds will progress through sequential Tested Negative, or Monitored Negative, status's following negative tests of all animals over 2 years of age, or of a sample of older animals, at intervals of from one to two years. Testing comprises screening by the absorbed ELISA and follow-up of reactors by fecal culture in herds with no previous history or by post-mortem investigation in herds in which Johne's disease had been previously suspected or known to occur. Cattle producers will be encouraged to seek a written and signed vendor declaration of a herd's Johne's disease status before buying cattle for restocking. Johne's disease has been confirmed in a small number of alpaca and is endemic in a small proportion of sheep flocks in areas in south-eastern Australia. Control and assurance programs are being developed.

Title Farm specific approach to paratuberculosis (Johne's disease) control.

Author(s) Rossiter CA, Burhans WS.

Institution NYS Diagnostic Laboratory, College Vet Med, Cornell University, Ithaca, NY, USA.

Abstract Uncertainties in the understanding of Johne's disease make the pursuit of disease control confusing for producers and veterinarians. Generic control recommendations have limited adoption or success because they fail to account for the unique circumstances of individual farms. A systematic and pragmatic approach for developing farm control plans is presented. It targets the development of plans that are adapted to individual farm circumstances, thus more likely to be implemented and effective. While Johne's disease is well recognized to be an insidious disease of economic significance in domestic and wild ruminants, several issues complicate its control on farms. The subclinical chronic nature of the infection makes recognizing the disease difficult and herd level control a lengthy process. Understanding of the pathogenesis and epidemiology of infection is inadequate to definitively predict the cost of the disease, the importance of specific risk factors as control points, and the economic benefit of intervention strategies. Marketing of known infected or exposed animals has regulatory, ethical and economic implications. Availability and commitment of each farm's resources:management, labor, finances, and facilities complicate control proscriptions. As a consequence of these matters, control strategies are not simple or clear; strict control guidelines become difficult to apply, and no single control formula applies in every case. In response to this dilemma, a systematic and pragmatic approach to the control process and how control measures can be uniquely adapted for individual farm circumstances is outlined. An eight point strategy for: 1. collecting information, 2. educating the individuals involved, and 3. designing and implementing a control program customized to the unique farm situation serves as a guide for investigation/discussion on the farm. The eight points include: 1. compiling background information on the farm operation, 2. compiling probable Johne's history and prevalence, 3. identifying risks specific to the farm operation, 4. examining control options for identified risks,

5. considering herd testing strategies, 6. defining control objectives and time frames, 7. defining the intensity of control effort to be pursued, and 8. planning, implementing, and evaluating the control program. The process facilitates a well thought out comprehensive plan that will bear a return on the commitment.

Title Johne's disease on US dairy operations: results from the NAHMS Dairy '96 Study.

Author(s) Wells SJ, Ott SL, Garber LP, Bulaga LL.

Institution US Department of Agriculture-Animal and Plant Health Inspection Service-Veterinary Services, Centers for Epidemiology and Animal Health, Ft. Collins, Colorado and Robbinsville, NJ, USA.

Abstract An assessment of Johne's disease on US dairy operations was part of the National Animal Health Monitoring System (NAHMS) Dairy '96 Study conducted during 1996. Data and biologic samples were collected from randomly-selected dairy producers in 20 states, representing 83% of the national dairy cow population. From February through May of 1996, each respondent dairy producer was visited by a federal or state animal health official and completed a questionnaire assessing producer awareness of Johne's disease and use of management practices reportedly linked to Johne's disease. Blood samples were collected from milk cows on the operations during the visit and sent to the National Veterinary Services Laboratories for *M. paratuberculosis* testing using the IDEXX ELISA. National and regional US seroprevalence of *M. paratuberculosis* at the herd-level will be estimated. In addition, producer familiarity with and recognition of Johne's disease on the operation will be assessed. The frequency of use of certain management practices reportedly linked to the spread of this disease will be assessed. These include: maternity management (calving location, bedding management, use of calving area by sick cows), neonatal heifer management (time of calf separation from dam, washing of teats before colostrum removed), heifer management (physical contact and sharing of feed or water with adult cattle, use of manure handling equipment for heifer feeds, use of Johne's vaccine), and introduction of cattle to the operation.

Title Paratuberculosis in Sweden.

Author(s) Viske D[1], Larsson B[2], Engvall A[2], Bölske G[2].

Institution [1]Swedish Board of Agriculture, Jönköping; [2]National Veterinary Institute, Uppsala, Sweden.

Abstract After having considered the country free from paratuberculosis for 3 decades, one clinical case of the disease in an imported cow was diagnosed in 1993. A stamping-out policy was applied and restrictions were put on the cowshed and pastures for up to three years. This case also initiated several actions from the authorities. Veterinarians in the field and at slaughterhouses were asked to increase the awareness of the disease and to report any suspicion of its presence. All animals that had been imported since 1980 were tested twice (serology and fecal culture) with an interval of at least 3 months. If an imported animal had been slaughtered a serological survey was performed in te herd. A survey in slaughterhouses was also initiated. Samples from the ileum and the ileocecal lymph nodes from adult cattle at emergency slaughter were taken and cultured. Up to now 1900 samples have been analyzed and only one has been positive for paratuberculosis. In all, 24 herds have been declared infected and stamped out. Tracing of the infection backwards and forwards has been carried out. The sources of infection have either been related to imports of animals (8 herds) or to a domestic chain of infection within the limousin breed (15 herds) which is likely to go back to imports of that breed in the 1970's. In one case the source of infection is still unknown.

Title The effect of subclinical Johne's disease on reproductive outcomes in dairy cattle.

Author(s) Johnson-Ifearulundu YJ, Kaneene JB, Sprecher DJ.

Institution Population Medicine Center and Depart Large Animal Clin Sci, Michigan State University.

Abstract A prospective, two group, cohort study was conducted to test the hypotheses that cows that are subclinically infected with *M. paratuberculosis* will have an increased number of days to first service and an increased number of days to conception, than their negative herd-mates. Blood and fecal samples were collected from all cows in their first lactation or 24 months of age and older. The IDEXX Enzyme Linked Immunosorbent Assay (ELISA) test and radiometric fecal culture were used to determine the Johne's status of each subject. Those cows that test positive on either the ELISA or radiometric fecal culture were identified as cases. For each case, three controls were selected from the same herd, matched on lactation number and phase of reproductive cycle. Analysis of the preliminary data (using the Kruskal-Wallis Chi-Square Approximation) has revealed that those cows that tested positive for Johne's disease had a mean of 141.5 days to conception, those cows that were negative had a mean 104.5 days to conception ($p<0.05$).

Title Financial effects of *Mycobacterium paratuberculosis* on mastitis, culling and milk production in clinically normal dairy cattle.

Author(s) Wilson DJ, Rossiter CA, Han HR, Sears PM.

Institution Quality Milk Promotion Services, New York State Paratuberculosis Program, College of Veterinary Medicine, Cornell University, Ithaca, New York.

Abstract Approximately 45 Holstein cows that were *Mycobacterium paratuberculosis*-positive on the basis of fecal culture were maintained at any one time in a 210-cow dairy herd. Paratuberculosis-positive cows were grouped separately from negative cows, but they were otherwise managed identically. During a 1-year study, 180 paratuberculosis-negative cows and 113 clinically normal paratuberculosis-positive cows were identified by fecal culture through the laboratory of the NY State Paratuberculosis Program. Quarter milk samples were asceptically collected for culture of mastitis pathogens: 6,100 milk samples from paratuberculosis-negative cows, and 3,129 quarter samples from paratuberculosis-positive cows. Dairy Herd Improvement Association (DHIA) records were used to monitor milk somatic cell count Linear Score (LS), mature equivalent (ME) milk production, new mastitis infections, and chronic mastitis infections. Beginning in second lactation, and increasing with advancing parity, paratuberculosis-positive cows had lower ME milk production than did negative herdmates. However, rates of new and chronic mastitis infections, as measured by DHIA LS were significantly ($P<0.05$, $P=0.05$, respectively) lower in cows with nonclinical paratuberculosis. Infected cows were culled from the herd at a faster rate than were negative herdmates. Overall, paratuberculosis was associated with economic benefit due to lower rates of mastitis in positive cows, but a net financial loss resulted because of reduced milk production and increased culling rates.

Title Control of ovine Johne's disease in New South Wales.

Author(s) Denholm LJ, Ottaway SJ, Marshall DJ.

Institution New South Wales Agriculture, Agricultural Research and Veterinary Centre, Orange, New South Wales, Australia.

Abstract The accelerating spread of ovine Johne's disease (JD) throughout New South Wales (NSW) sheep flocks has caused considerable concern to the industry. During 1995 a JD Sheep Industry Steering Committee (JDSISC) was formed with specific objectives to take "immediate action to control the spread of JD in sheep in Australia." A Strategic Plan for control of JD in sheep in NSW is being formulated and will be submitted to the NSW Government and industry for endorsement. The Strategic Plan will give priority to procedures for reducing further spread of JD infection and includes detailed proposals for identification of infected flocks, vendor declarations, a market assurance scheme, gazettal of specific JD protected/control/residual zones and associated movement restrictions and special "slaughter only" sales for disposal of infected

sheep. JD-SISC believes financial incentives for disease control will be essential to encourage all affected producers to eradicate JD from their properties, especially as some producers are not severely financially affected by the disease. It is proposed that affected producers will be offered financial assistance under a contractual agreement to implement an approved property disease eradication plan negotiated with the producer. The strategic plan includes a communication strategy. Without a full-scale disease control program, it seems likely that ovine JD will continue to spread throughout the suitable pastoral areas of south-eastern Australia which are currently populated by 50 million sheep. If uncontrolled, Johne's disease has the potential to cause a direct loss to the Australian sheep industry of at least $20 million per annum as well as considerable disruption to sheep trading within Australia and possible disruption to international trade in live sheep and sheep meat products.

Title Distribution of *M. paratuberculosis* in tissues of cattle from herds infected with Johne's disease.

Author(s) Whitlock RH, Rosenberger AE, Sweeney RW, Spencer PA.

Institution Depart Clin Studies, School of Vet Med, University of Pennsylvania, Kennett Square, PA, USA.

Abstract More than 150 adult cattle from seven dairy herds were followed to slaughter to collect tissues from the pulmonary, mandibular, retropharyngeal, hepatic, supramammary, ileocecal and mesenteric lymph nodes and from multiple sites along the jejunum, colon and ileum. Approximately 30% of the cattle that were negative on repeated fecal cultures had culture positive tissues obtained at slaughter. Fifteen (18%) of the 82 cattle with positive fecal cultures had culture negative tissues. Eight animals with negative tissues each had a single positive fecal culture with one colony on one tube. *M. paratuberculosis* was isolated from many tissues throughout the bodies of cattle with heavy bacterial loads (more than 10 colonies per fecal culture tube) and clinical signs of Johne's disease. These results suggests that disseminated infection occurs frequently in animals with Johne's disease.

Title The post-mortem diagnosis of paratuberculosis in farmed deer.

Author(s) de Lisle GW, Wilson CA, Yates GF, Wards BJ, Collins DM.

Institution AgResearch, Wallaceville Animal Research Centre, P.O. Box 40-063, Upper Hutt, New Zealand.

Abstract Deer farming is now a well established industry in New Zealand with over 6,000 farms containing more than a million animals. The first case of paratuberculosis in these deer was recorded in 1985 and during the next six years a further 20 cases were confirmed by bacterial culture. In the 1990s there has been a steady increase in the number of cases of paratuberculosis in farmed deer. In 1995, *Mycobacterium paratuberculosis* was isolated from over 50 deer from more than 30 different farms. A feature of paratuberculosis in deer is lesions in mesenteric lymph nodes which macroscopically and microscopically are very similar to thoe caused by other members of the *Mycobacterium avium* complex and by Mycobacterium bovis. The diagnosis of paratuberculosis in deer is further complicated in some cases by the presence of strains of *M. paratuberculosis* which are extremely difficult to isolate by bacterial culture. To overcome these problems we have evaluated the use of polymerase chain reaction (PCR) tests using primers based on the DNA insertion element IS900. Different DNA extraction procedures have been evaluated in an attempt to develop a robust and sensitive test for routine use in a diagnostic laboratory. PCR tests have proved to be an accurate and reliable test on tissues containing microscopically-visible acid-fast organisms. However, PCR tests are still not able to reliably detect *M. paratuberculosis* in tissues containing very small numbers of bacilli.

Title Mycobacterial infections of free-living deer in Scotland.

Author(s) Reid HW, Stevenson K, Challans JA, Ramage C, Hitchcock D, Sharp JM.

Institution Moredun Research Institute, Edinburgh.

Abstract A survey was conducted of mycobacterial infections in 1235 free-living deer from different regions of Scotland. Selected lymph nodes and spleen were pooled and examined by Ziehl-Neelson, culture and PCR. The overall prevalence of mycobacterial infection was 6.6%. *Mycobacterium paratuberculosis* was found in 2.1% and IS901+*M. avium* in 3.1%. No evidence of infection by *M. bovis* was forthcoming. The PCR procedure was the most sensitive technique, with a greatly reduced delay between sample preparation and result.

Title Johne's disease in a deer herd: accuracy of fecal culture.

Author(s) Stehman SM, Rossiter CA, Shin SJ, Chang YF, Lein DH.

Institution Diagnostic Lab, College Vet Med, Cornell University, Ithaca, NY, USA.

Abstract One to two year old deer in a herd of fallow deer began to show signs of weight loss, patchy alopecia, and occasional intermittent diarrhea. Several severely affected deer from the above age group were killed and samples were submitted for diagnostic testing. Paratuberculosis was confirmed in the index cases. In the remaining herd (70 deer), of 52 deer sampled, 51 were determined to be infected with *M. paratuberculosis* by at least one of the following diagnostic methods; fecal culture, tissue culture, and/or histopathology. As a diagnostic test, fecal culture detected 32 of 51 infected animals with an apparent sensitivity of 62%. Sensitivity of the fecal culture varied from a low of 45% in the most mildly infected deer to 91% in the most severely affected animals. The ileocecal junction was positive in 82% (range 71 to 100%) of cases depending on the age of the animal and severity of infection. In this herd, the palatine tonsil was positive by culture in 88% of 17 deer tested. Cultures were confirmed with an IS900 probe. Paratuberculosis infection and fecal shedding of organism were found in deer as young as 6 months of age. Culture confirmed that the 1 to 2 year old deer were the most severely infected. In this group, *M. paratuberculosis* occasionally caused disseminated infection. Affected tissues included the intestine, liver, multiple lymph nodes, and occasionally lung. Paddocks, on the affected farm, were divided into equal quadrants and the environment was sampled to determine the level of environmental contamination. A total of forty quadrants were sampled including the pond within the enclosure. Of the pasture quadrants sampled, 25 of 36 (69%) were positive for *M. paratuberculosis*. The inflow sediment from the pond was also positive.

Title Paratuberculosis in wild rabbits (Oryctolagus cuniculus).

Author(s) Greig A[1], Stevenson K[2], Pérez V[3], Pirie AA[2], Grant JM[4], Sharp JM[2].

Institution [1]SAC Veterinary Services, Oakbank Road, Perth, Scotland; [2]Moredun Research Institute, Edinburgh; [3]Facultad de Veterinaria, Universidad de Leon, Spain, [4]32 York Place, Perth.

Abstract The hypothesis that the increasing prevalence of cases of paratuberculosis in beef suckler herds in the Tayside area of Scotland was associated with greatly increasing numbers of wild rabbits on some affected farms was tested. In the spring of 1994 rabbits were received from three farms where Johne's disease had been diagnosed and from one farm where the condition had not been recorded. Microbiological and histopathological investigations showed that rabbits on all four sites were infected with and had lesions consistent with paratuberculosis. The preliminary results of a more extensive survey of wild rabbits collected from stock farms across Scotland conducted during 1995/96 will also be presented.

Title Experimental infection of red deer with *Mycobacterium paratuberculosis* and *Mycobacterium avium*.

Author(s) Burrells C, Palmarini G, Porter J, Stevenson K, Sharp JM.

Institution Moredun Research Institute, Edinburgh.

Abstract Experiments were conducted to investigate reports that deer are more susceptible than sheep, cattle or goats to infections with *M. paratuberculosis* and *M. avium* (Nyange, 1990). Groups of 4 red deer calves (Cervus elaphus) were infected orally with either IS901 *M. avium* or *M. paratuberculosis*. Two calves served as non-infected controls. Regular examinations were made of blood cell-mediated immune (CMI) reactions, antibody responses and fecal excretion of mycobacteria throughout the 41 week term of the experiment. Clinical illness was observed only in deer inoculated with *M. avium* and fecal excretion of mycobacteria was demonstrated between weeks 3 and 25 post-inoculation. In contrast, fecal excretion of *M. paratuberculosis* was detected much less frequently between weeks 13 and 21 pi. Differences in immune responses to the two infections were obvious. Essentially, the group of *M. paratuberculosis*-infected deer exhibited low antibody and transient CMI reactivity, whereas both responses were evident early in *M. avium*-infected deer and were sustained throughout the 41-week period of the experiment.

Title Study of epidemiology and pathogenesis of paratuberculosis using RFLP (restriction fragment length polymorphism).

Author(s) Pavlík I, Bartl J, Horvathova A, Rychlik I.

Institution Vet Res Institute, Brno, CR

Abstract RFLP analysis has been used for the study of epidemiology of paratuberculosis. In the present study, DNA from 336 *M. paratuberculosis* strains were examined by RFLP using restriction endonuclease PstI (in selected 306 strains restriction endonuclease BstEII was used in parallel) and a specific probe IS900. After digestion with restriction endonuclease PstI, the strains were differentiated into 9 different RFLP types named A (n=90), B (n=164), C (n=4), D (n=16), E (n=53), F (n=1), g (n=1), H (n=6) and I (n=1). After digestion with the restriction endonuclease BstEII, the strains were differentiated into 14 RFLP types named according to previously presented results by Collins et al (J. Clin Microbiol. 28:1591-1596): C1 (n=173), C2 (n=2), C3 (n=8), C5 (n=1), S1 (n=4) and I1 (n=2). Other strains of 8 RFLP types were provisionally named C8-C15 (n=116). Systematic long term investigation allowed us to perform a regressive epidemiologic study on spreading of the infection in cattle herds of the Czech Republic during the recent 9 years. In 5 farms located in one region, the isolated strains were of the same RFLP type during 3 years. High regional homogeneity can be explained by the same infection source in pastured cattle. Since 1988 RFLP types A-E, I, and C1-2, C8-10, C12-13 and S1 of *M. paratuberculosis* have been isolated from ruminants in the Czech Republic. The variety of isolated RFLP types indicates different infection sources in ruminants kept in the Czech Republic. Identification of different RFLP types allowed us to the monitor both vertical and horizontal spreading of the infection in herds with imported cattle infected with strains of different RFLP types and in infection-free cattle bred in the Czech Republic. It was found, using the same methods, that calves up to the age of 1-2 months after birth being in contact with infected cows are the most susceptible.

Title Associations between subclinical paratuberculosis and milk production, milk components, and somatic cell counts in Wisconsin dairy herds.

Author(s) Nordlund KV, Goodger WJ, Pelletier J, Collins MT.

Institution Depart Med Sci, University of Wisconsin, Madison, WI, USA.

Abstract Associations between subclinical *Mycobacterium paratuberculosis* infection and milk production, milk components, and somatic cell counts were studied in 23 Wisconsin dairy herds with a history of paratuberculosis infection and above-average production. All adult cows in the herds were tested for paratuberculosis by use of an absorbed ELISA, yielding 147 ELISA-positive and 1506 ELISA-negative cows. Milk yield, fat, protein, and somatic cell count data were retrieved electronically from DHI records. Regression analysis showed that ELISA-positive cows had a mature equivalent (ME) milk production of 376 kg (829 pounds) per lactation less than ELISA-negative herdmates based on least squares means (P=0.002). There was no significant difference in lactation average percent fat, protein, or somatic ell count

linear score. When comparing ELISA-positive and negative cow's current ME milk with all previous lactations, there was a significant difference only from the previous lactation (P=0.001). When this difference was examined by parity group, significant difference was confined to cows in the second lactation (P=0.0003). The study showed that subclinical paratuberculosis infections, as determined by ELISA, are associated with a 4% reduction in milk yield.

Title Paratuberculosis vaccination in sheep modifies and limits the development of lesions

Author(s) García Marín FF, Tellechea J, Gutiérrez M, Pérez V, Juste RA.

Institution Dpt. Patología Animal, Medicina Animal, Facultad de Veterinaria, Universidad de León. Campus de Vegazana, s/n. 24071 León, Spain. Servicio de Investigación y Mejora Agraria, Gobierno Vasco. 48016 Derio (Vizcaya), Spain.

Abstract It is well known that vaccination against paratuberculosis significantly reduces the number of clinical cases in field conditions. Different experimental studies have suggested that vaccination does not prevent infection, but modifies and limits the development of lesions. Histopathological methods have been demonstrated to be useful tools to assess the efficiency of paratuberculosis vaccines both in short term experiments and in field conditions. We have used these methods to evaluate vaccine efficacy on a system in which genetic factors have been minimized by the use of twin lamb pairs. One lamb out of each one of eight pairs of twin lambs was vaccinated when it was 15 days old. Fifty days later, all lambs were orally infected with a pathogenic ovine strain of *Mycobacterium avium* subspecies *paratuberculosis*. Three pairs were killed at 150 days post infection and the remaining 5 pairs at 350 d.p.i. On the postmortem, samples were taken for histopathological processing from ileocecal valve, three different segments of ileum, 3 distal, 3 medial, and 3 cranial jejunal Peyer's patches with their adjacent intestinal tissue and from the corresponding mesenteric lymph nodes. Clear differences were observed between vaccinated and non-vaccinated groups at both sampling times after infection. Whereas vaccinated lambs have only regressive-type granulomata limited to intestinal organized lymphoid tissues (Peyer's patches and ileocecal valve), non-vaccinated ones showed spread of granulomatous lesions to other areas of the intestinal wall. In conclusion, this experiment shows that vaccination induces noticeable differences within each pair of experimental animals, and it confirms the efficiency of this tissue sampling protocol since granulomata were located in the organized intestinal lymphoid tissue of all animals.

Title An abbatoir survey of *Mycobacterium paratuberculosis* infection in domestic large and small ruminants.

Author(s) Bazargani TT, Sadegni F, Khanghahi H, Atyabi N.

Institution Depart Clinical Sciences, Large Animal Division, Internal Medicine Section, Vet. Faculty, Univerity of Teheran.

Abstract The infection rates of domestic large and small ruminants with *M. paratuberculosis* were studied at slaughter house. From each animal, smears were taken from terminal ileum (TI), ileocecal valve (IV), ileocecal lymph node (ILN), rectum mucous membrane (RMM), and feces (F). They were stained by Ziehl Neelsen staining method. During this study, 218 cattle, 48 buffaloes, 379 sheep, and 42 goats were examined. The infection rates were 1.48%, 6.25%, 1.8%, and 4.76% for the above mentioned species, respectively. The comparative identification rates of infected animals by each of TI, IV, ILN, RMM, and F samples in large and small ruminants were 100% and 100%, 100% and 100%, 85.7% and 100%, 100% and 58.7%, 57.1% and 55.5%, respectively. The infection rates in Holstein and native breeds were 2.86% and Nil respectively and in cattle no infection was diagnosed in the age less than 2 years. In small ruminants, the infection rates in two different localities were 1.9% and 6.25% and in two different age groups were 0.9% and 2.9%.

Title An attempt to eradicate paratuberculosis from eight infected herds with and without a

vaccination program in a two-year period.

Author(s) Claessens EW, Kalis CHJ.

Institution Animal Health Service, P. O. Box 361, 9200 AJ Drachten, The Netherlands.

Abstract Vaccination experiments showed that vaccination against paratuberculosis is only effective in decreasing the rate of clinical disease, but it is not effective in dereasing the rate of infection itself. Regular feces culturing and culling of positive animals resulted in both vaccinated and non-vaccinated herds having a decrease of the infection rate from 7.2% at the beginning of the two year period till 1.9% at the end. A peak of excretion of *Mycobacterium paratuberculosis* was found in animals at the age of twelve months. Pooling of 5 feces samples showed to be a reliable method to detect positive age groups. The rate of excretion greatly influenced the least necessary time of culturing. Centrifugation of feces with a high speed centrifuge did not influence the sensitivity of the culturing significantly. Pre-cultivation with Dubos medium did not result in higher sensitivity of the culture procedure. Negative cultures in the young stock at the end of the period gives promising expectations for sanitizing these herds.

Title National survey of diagnostic laboratories; the performance of fecal culture and serologic testing for Johne's disease.

Author(s) Whitlock RH, Rosenberger AR, Sweeney RW, Spencer PA.

Institution Depart Clin Studies, School of Vet Med, University of Pennsylvania, Kennett Square, PA, USA.

Abstract Seventy bovine fecal and serum samples were distributed to twenty laboratories for testing. Forty-six samples were obtained fro cattle with at least one previous positive fecal culture. The number of positive cultures in this group of samples from any one laboratory varied from 3 to 24. Thirty-two (70%) of the infected animals were correctly classified by fecal culture in at least one laboratory. Only five of the 1400 fecal samples tested by the twenty laboratories were falsely classified as positive. The DNA probe was positive on six of the 70 fecal samples tested. A commercial test for Johne's disease antibody was positive for three of the 70 sera tested. Several laboratories ran the complement fixation test under different protocols, with variable results. Although culture protocols have been published, many diagnostic laboratories need to update the methods used to detect *M. paratuberculosis* in fecal samples to improve test sensitivity.

Title Application of a rapid lysis method and a nested PCR in a control program of JD.

Author(s) Lillini E, Rovai C, Magrì G, Micarelli F, Dei R.

Institution Instituto Microbiologia, Università di Firenze, Instituto Zooprofilattico Sperimentale Regioni Lazio e Toscana, Roma, Italy.

Abstract Background and Aims. PCR methodology has opened a new field in the diagnosis of Johne's disease (JD). The control of JD relies primarily on good management and early detection of infected animals. We reported a relatively simple extraction procedure and a nested PCR (amplification with primers P90 and P91 followed by amplification with primers P11 and P36, specific for sequences within the IS900 fragment) to detect *M. paratuberculosis* DNA in tissue and fecal samples. The sensitivity of the nested PCR, estimated with a crude extract of the bacterium grown in vitro, was 5fg. Three cows from a herd of 78 animals, had symptoms related to JD. We planned to study the entire herd, and to compare the routine tests with PCR. Materials and Methods. Fecal and serum samples were obtained from the entire herd, and tissue samples from the diseased animals only. An aliquot of the tissue and fecal samples was processed for routine investigations (AFB staining, isolation), and the remaining was stored frozen until PCR processing, to be carried out blindly. The specimens were treated with Proteinase K and SDS, then boiled with Chelex. The diluted supernatant was directly used for the amplification. Results and Conclusions. Of the entire lot of fecal samples, 5 were AFB positive; isolation and serologic results are not yet available. Out of 34 samples so far studied by PCR, 12 were positive. The relatively simple extraction procedure might be exported to routine diagnostic laboratories, and together with the nested PCR might help to speed up the diagnosis of JD.

Title Development of ELISA for the detection of subclinical paratuberculosis infection of cattle.

Author(s) Jark U, Franz B, Gerlach GF.

Institution Institut für Mikrobiologie und Tierseuchen, Tierärztliche Hochschule Hannover, Germany.

Abstract A clinical *M. paratuberculosis* isolate was cultivated in large scale culture. A procedure for efficient preparation of a lipoarabinomannan containing antigen was developed; 25 mg of purified antigen were obtained per gram of bacterial wet weight. An ELISA based on this antigen was developed using a reference-standard-method; it had the best discriminatory efficacy when ELISA Polysorp-plates were coated with 200 ng antigen per well in hypertonic buffer (pH 6.0) at 37°C for 2 hours. Intra- and interassay variation were determined to be 20%

and 27%, respectively. The ELISA was evaluated using the sera of 131 non-randomly selected animals and simultaneously culturing the ileocecal lymph nodes. After determination of the negative and positive cut-off-values positive and negative predictive values of 94% and 93% were calculated with 13 animals giving an intermediate result.

Title Production of g-interferon by peripheral blood mononuclear cells: an important diagnostic tool for detection of subclinical paratuberculosis.

Author(s) Stabel JR.

Institution USDA, ARS, National Animal Disease Center, Ames, IA, USA.

Abstract Peripheral blood mononuclear cells were isolated from noninfected control cows and from cows with either subclinical or clinical paratuberculosis (Johne's disease). Cells were incubated for 6, 12, 24, and 48 hours in complete medium with the following mitogens: concanavalin A (ConA), phytohemagglutininP (PHAP), pokeweed mitogen (PWM), and E. coli lipopolysaccharide (LPS). In addition, cells were incubated for the same time periods with a *Mycobacterium paratuberculosis* sonicate (MpS) and live and heat-killed *M. paratuberculosis* at 10:1 bacteria to cell ratio. After incubation, cell-free supernatants were analyzed for g-interferon (g-IFN) activity. Cells from subclinical cows produced significantly with mitogens, ConA, PHAP, and PWM. Levels of g-IFN were produced by cells isolated from subclinical animals compared to cells from clinical cows and noninfected controls. Stimulation of cells with heat-killed or live *M. paratuberculosis* evoked a similar response. This study indicates that g-IFN production by peripheral blood mononuclear cells in response to *M. paratuberculosis* antigen may be an important diagnostic tool for the detection of paratuberculosis in subclinical animals.

Title Diagnostic tests for detecting *Mycobacterium paratuberculosis* infection in young cattle.

Author(s) McDonald WL, Ridge SE, Hope AF, Condron RJ.

Institution Vic Inst Anim Sci, Agriculture Victoria, Australia.

Abstract Early detection of *M. paratuberculosis* infection, using diagnostic tests suitable for calves, would accelerate progress in control programs for Johne's disease. Limited fecal shedding of *M. paratuberculosis* and seroconversion in cattle less than two years of age has been reported in previous studies. The aim of this study was to evaluate use of current diagnostic tests in detecting infected calves. Twenty-nine calves were obtained by purchase of parturient cows from properties of known Johne's disease status. At birth, the calves were allocated to four groups as follows, (1) control group of four uninfected calves; (2) vaccinated group of eight calves immunized with either the Weybridge vaccine (CVL, Weybridge) or a heat-killed field strain of *M. paratuberculosis* and three unvaccinated calves; (3) artificially infected group of eight calves stomach drenched with either 2g wet weight of *M. paratuberculosis* or 20g of macerated intestinal mucosa from a clinically infected cow; and (4) naturally infected group of five calves reared by dams that were positive for *M. paratuberculosis* by fecal culture. Vaccines were administered to group two calves within twenty days of birth and group three calves were challenged three times, at weekly intervals, wthin one month of birth. Blood and fecal specimens were collected monthly until the calves were slaughtered between the ages of 20 and 30 months. Results from histopathology, tissue and fecal culture, humoral and cell-mediated tests will be presented. In preliminary testing a small number of reactors to the absorbed EIA and fecal culture have been detected. Four calves from groups two, three and four tested positive for serum antibody to *M. paratuberculosis* using the Johne's Absorbed EIA (CSL, Australia). *M. paratuberculosis* was isolated by fecal culture from five calves in group three using modified Roche MB Check system (Becton Dickinson, Australia) and double incubation radiometric culture. Strategies for testing young cattle will be discussed.

Title Long term evaluation of Johne's absorbed ELISA and Gamma interferon (GIF) assays

n an endemically infected beef herd in NSW, Australia.

Author(s) Eamens GJ, Jessep T, Carrigan MJ, Webb R.

Institution NSW Agriculture, Elizabeth Macarthur Agricultural Institute, Camden, Australia.

Abstract Regular 6 monthly testing by absorbed ELISA and g-interferon was undertaken in an *M. paratuberculosis*-infected stud beef cattle herd in NSW over the 9 year period 1987 to 1995. Necropsy examination of 835 cattle was undertaken on test reactors, relatives of infected cattle and routine culls, representing 75% of all animals culled from the herd. 133 infected cattle were identified at slaughter, with 96 of these receiving full necropsy examination. Of these 96, 79% were confirmed histologically and all culturally. On the basis that part examination (histology without culture) had a sensitivity of 79%, and with the exclusion of 19 cattle with equivocal histology, it was assumed that 656 cattle were uninfected and 140 infected. Based on these assumptions, the apparent sensitivity, specificity and positive predictive value (PPV) of a range of criteria for two ELISAs and a GIF assay based on johnin PPD were determined at different time points during the program. ELISA specificity appeared to remain high, but its sensitivity declined to under 40% by year 910. GIF specificity at a fixed criterion (500 johnin-nil units) tended to remain at 80% until year 9 (90%), and the increased specificity was accompanied by a decreased sensitivity equivalent to ELISA. The PPV decreased for both tests throughout the program, being less than 20% for GIF and 30% for ELISA after year 7. For one cut-off parameter for each test, the tandem use of ELISA and GIF together had a combined sensitivity of 74%, a specificity of 79% and a PPV of 37%.

Title Validation of a diagnostic strategy for detection of ovine Johne's disease in New South Wales sheep flocks.

Author(s) Marshall DJ[1], Ottaway SJ[1], Eamens GJ[2], Manchester PE[1].

Institution [1]New South Wales Agriculture, Agricultural Research and Veterinary Centre, Orange, 2800, New South Wales, Australia; [2]New South Wales Agriculture, Elizabeth Macarthur Agricultural Institute, Camden, 2570, New South Wales, Australia.

Abstract The New South Wales sheep industry has developed a strategic plan to progressively eradicate ovine Johne's disease (JD) from the Australian sheep flock. This initiative relies on a suitable diagnostic strategy to identify both infected flocks and those flocks unlikely to be infected to provide a source of clean replacement sheep. Flock diagnosis of JD is limited by the unreliability of fecal culture, the poor sensitivity of serological tests, and the impracticality of sample collection for histology. We propose to base the flock test on ELISA testing a biased sample of the flock. We have determined the prevalence of JD in selected infected sheep flocks by histological examination of terminal ileum collected at slaughter. Prior to slaughter serum was collected from all sheep and body weight and condition score recorded. A significant but not unexpected finding was that sheep infected with JD are more likely to be found amongst sheep of low condition score within the flock. These results demonstrate that the sensitivty of serological diagnosis of JD in sheep flocks can be improved by deliberately biasing the sample of sheep tested to the tail of the flock. The reliability of this strategy is to be assessed in flocks of known JD status.

Title Preliminary methods for growing *Mycobacterium paratuberculosis* using the BBL MGIT™ Mycobacteria Growth Indicator Tube.

Author(s) Stitt DT, Sturm KM, Hagemann PA.

Institution Becton Dickinson, Sparks, MD.

Abstract The ability of the MGITTM system to grow and detect the growth of *Mycobacterium paratuberculosis* was tested under a variety of conditions, including the addition of mycobactin J, egg yolk emulsion and antibiotic mixtures. One-half McFarland suspensions were prepared from ATCC 19851 and ATCC 19698 grown on 7H10 plates supplemented with

mycobactin J. Multiple dilutions were prepared from the suspensions and used to inoculate sets of MGIT tubes at the standard pH of 6.6 or tubes adjusted to 5.7. One-half milliliter of MGITTM OADC was added to each tube, supplements were added, alone or in combination, the tubes were incubated at 37°C and read daily using a 365 nm transilluminator. Tubes containing 1 ml of egg yolk emulsion turned out to be unsuitable. The emulsion apparently was a good enough reducing agent to interfere with the oxygen sensor on the bottom of the tubes and the emulsion also separated out in many cases. Tubes supplemented with mycobactin J grew and fluoresced to indicate that growth had occurred. The time to detection was about the same at pH 6.6 or 5.7. MGIT tubes without mycobactin J or other supplements did not support the growth of either ATCC strains at pH 6.6. However, MGIT tubes adjusted to pH 5.7 supported the growth of both strains without supplements.

Title Modification of a commercial ELISA kit for the diagnosis of paratuberculosis in goats.

Author(s) Velez M[1], Chávez Gris G[1], Collins MT[2], Suárez Güemes F[1].

Institution [1]Vet School, National University of Mexico; [2]Depart Pathobiol, University of Wisconsin, Madison, WI, USA.

Abstract *Mycobacterium paratuberculosis* was first isolated in Mexico in 1975, being prevalent in ruminants from different country areas. The proliferation of this bacterium in infected animals, induces the increase immune humoral response, which is related to the amount of antigen present. It has been mentioned by other researchers, that the presence of lesions is in direct relationship with serum antibody levels. The available serological tests for the diagnosis if this disease in goats are: complement fixation (CF), agar-gel-immunodiffusion (AGID), and ELISA. These tests vary in sensitivity and specificity. The AGID test is the most recommended for the diagnosis of paratuberculosis (Ptb) in goats. The objective of this study was to compare, the AGID test with a modified commercial ELISA kit* in goats. The ELISA kit was originally designed for the diagnosis of Ptb in cattle. The following modifications were considered: higher serum samples dilution (1/350), antigoat-conjugate reagent (1/10,000), when the optical density reached 0.75 nm the reaction was stopped and modification of the time for the absorbance recording. Serum samples from 98 goats were collected in a flock from Topilejo D.F., in which the presence of the disease was already confirmed both bacteriologically and histopathologically. Seven animals had positive results to the ELISA test and 8 to the AGID test. The ELISA positive sera were also positive to AGID. The concordance between these tests was 87.5%. According to these results and the advantages that the ELISA test offers, we considered that it would be a good alternative for the diagnosis of Ptb in goats. Further studies are necessary to determine the specificity and sensitivity of the ELISA test in goats.

Title Detection of *M. paratuberculosis* DNA in blood monocytes or milk macrophages from cattle and sheep affected by Johne's disease by PCR and an oligonucleotide probe.

Author(s) Shin SJ, Chang YF, Kim JB, Stehman SM, Rossiter CA, Lein DH.

Institution Diagnostic Laboratory, College of Vet Med, Cornell University, Ithaca, NY, USA.

Abstract *Mycobacterium paratuberculosis* is the etiologic agent of Johne's disease in cattle, sheep and goats. The diagnosis of Johne's disease in animals is hampered by 1) a lack of a rapid, specific, and sensitive serologic test, 2) slow growth of the organism by fecal culture [4-6 weeks in cattle and goats; usually no growth in culture from sheep], 3) a lack of sensitive PCR direct from fecal samples (not reliable in low and medium shedding cases). In order to improve the diagnosis of Johne's disease in these animals, we are applying the primers described by Vary et al., to set up a PCR and an enhanced chemiluminescence detection system for *M. paratuberculosis*. The DNA was purified from blood monocytes or milk macrophages and was used as a template in the PCR. The PCR products were run on an agarose gel electrophoresis and transferred to nitrocellulose and hybridized with ECL 3'-oligolabelled probe. We have detected PCR positive samples from 3 of 22 samples from cattle milk macrophages, 8 of 31 samples from sheep blood monocytes, and 7 of 21 samples from sheep milk macrophages. The possible application of this technique for diagnosis of *M. paratuberculosis* in animals and for culling the infected animals

are discussed.

Title Cultivation of *Mycobacterium paratuberculosis* in Dubos combined with PCR.

Author(s) Giese SB, Klausen J, Ahrens P.

Institution Danish Veterinary Laboratory, Copenhagen, Denmark.

Abstract A sensitive and fast diagnostic test for paratuberculosis is desirable. Cows from two known positive and three presumed negative herds were examined by cultivation of feces combined with PCR. The feces samples were collected at a field laboratory, decontaminated and cultivated on Löwenstein-Jensen (L-J) with mycobactin at once; then the samples were frozen at -20°C, thawed and recultivated. The feces samples were then sent to the Danish Veterinary Laboratory where they were decontaminated and cultivated in Dubos broth with mycobactin. The broths were analyzed after 1 day, 3 days, 1 week, 2 weeks, and 3 weeks by culture on L-J and by PCR. The L-J's were read once a week for 10 weeks. For PCR, the broths were spun down and the mycobacteria-pellets were lysed by bead beating with zirconium beads. DNA was amplified by PCR using primers specific for IS900. Samples with a low number of bacilli (<100 cfu/gm): Of 22 feces samples positive prior to freezing, only 6 were positive after freezing by primary cultivation on L-J (no pre-cultivation in Dubos), while 18 samples were positive after pre-cultivation in Dubos. Thirteen samples were found positive by PCR. One of these samples were culture negative. Samples with a high number of bacilli (>500 cfu/gm) were highly positive by cultivation prior to freezing. After freezing, these samples were positive in both culture (with and without pre-cultivation in Dubos) and by PCR. These results indicate that freezing of feces samples reduces the sensitivity of cultivation by approx. X10, while pre-cultivation in broth could enhance the sensitivity of cultivation. This combined with the improved PCR technique could considerably reduce the time needed for diagnosing paratuberculosis.

Title Comparison of polyclonal antibodies to three different preparations of *Mycobacterium paratuberculosis* in immunohistochemical diagnosis of Johne's disease in cattle.

Author(s) Stabel JR, Ackermann MR, Goff JP.

Institution USDA/ARS/National Animal Disease Center, Ames, IA, USA.

Abstract Polyclonal antisera were raised in rabbits against preparations of live and heat-killed *Mycobacterium paratuberculosis* as well as cell-wall proteins of *M. paratuberculosis* and evaluated as diagnostic tools in immunohistochemical staining of bovine tissue. Live preparations of *M. paratuberculosis* (LMp) were inoculated at 109/ml either intraperitoneal or intravenous. Heat-killed *M. paratuberculosis* (HKMp) was prepared by treatment of bacteria at 85C for 10 minutes. Cell-wall proteins were isolated from *M. paratuberculosis* and conjugated to keyhole limpet hemocyanin to improve antigenicity (KLH-CWPMp). Heat-killed and KLH-CWPMp preparations were emulsified in incomplete Freund's adjuvant before subcutaneous inoculation of rabbits. At the terminal bleed, antibody titers were higher for HKMp and KLH-CWPMp rabbits compared to the LMp inoculum (1:1024 vs 1:64). The KLH-CWPMp antibody did not cross-react with *M. bovis*-infected tissues. Sensitivity and specificity of immunohistochemical detection of paratuberculosis in bovine tissues was much higher for the KLH-CWPMp polyclonal antibody.

Title A five year history of testing for Johne's disease using the *Mycobacterium paratuberculosis* Antibody Test Kit®.

Author(s) Kramsky JA, Teubert DG, Manning EJB, Collins MT.

Institution Depart Pathobiol Sci, School of Vet Med, University of Wisconsin, Madison, WI, USA.

Abstract Diagnosis of *M. paratuberculosis* infections in cattle by detection of serum antibodies using a commercial absorbed ELISA kit from IDEXX Laboratories, Inc. has been offered by our laboratory as a service since January, 1991. The first year the service was offered, 429 submissions (accessions) constituting 9,812 sera were tested. Each year the number of accessions and sera has increased. In 1995, 3,337 accessions constituting 22,193 cattle sera were tested. In 1995, approximately 65% of samples were from Wisconsin cattle; the remainder were from cattle in neighboring states. Sample submissions have had a seasonal pattern with a greater number of submissions occurring in the fall and spring. Two-thirds of the submissions (accessions) were single samples. Of these, roughly 50% were ELISA-positive for *M. paratuberculosis* antibodies. For accessions with > 50 sera submitted, generally whole herds, 5.90% and 4.95% tested ELISA-positive in 1994 and 1995, respectively. Overall, for past 5 years, while the number of samples submitted has steadily increased, the percentage of positive sera has remained relatively constant at 10%±2%. Clients report that ELISA results correlate well with clinical findings and that the ELISA is a useful tool in a control program for bovine paratuberculosis.

Title A portable, reagentless spectral analyzer for the rapid detection of *Mycobacterium paratuberculosis*.

Author(s) Schlager KJ.

Institution Biotronics Technologies, Waukesha, WI, USA

Abstract All living cells are fluorescent and exhibit "fingerprint" spectral patterns that allow for their detection and identification. The *Mycobacterium paratuberculosis* that causes Johne's disease has a specific fluorescent pattern that may be used for its identification. In addition to its biochemically-based fluorescent spectral pattern, this microbe has a distinctive size and shape pattern that can also be detected through spectral analysis using light scatter measurements. Chemometric analysis techniques such as neural network algorithms allow for the detection of specific bacterial species even in mixtures of multiple bacteria. Some recent experience in the application of fluorometric spectral instrumentation to the identification of bacteria and other microbes in complex matrices will be presented.

Title Application of antibody to recombinant polypeptide a362 and of recombinant DNA probe F57 for diagnosis of Johne's disease.

Author(s) Buergelt CD, Mathelart F, Coetsier C, Vannuffel P, Haveau X, Limbourg B, Denef JF, Cocito C.

Institution Depart Pathobiol, University of Florida, USA; Depart Histology, Catholic University of Louvain, Brussels, Belgium.

Abstract The expressed product (a362) of a *M. ptbc* gene coding for a 34-kDa protein (P34) was used to generate polyclonal antibody in rabbits and monoclonal antibody in rats. The polyclonal antibody was tested for potential to immunohistochemically detect antigen of *M. ptbc* in intestinal and lymph node tissues of infected cattle. Tissues were subjected to different fixation methods, including cryostat sectioning. For comparison, conventional staining methods were included. The recombinant DNA probe F57 was tested on fresh tissues via PCR and DNA hybridization. Preliminary results obtained indicated that the immunohistochemical detection of *M. ptbc* antigen was strong in pluribacillary and sporadic in paucibacillary cases. When compared with conventional stains, the immunohistochemical signal was more sensitive in paucibacillary cases. Choice and time of fixation were critical for detection. The DNA probe F57 was specific and sensitive to detect Johne's disease antigen in fresh and paraffin tissues from infected animals.

Title Evaluation of two ELISAs for Johne's disease in llamas and alpacas.

Author(s) Miller DS[1], Collins MT[2], Smith BB[1], Andersen PR[3], Hope AF[4].

Institution [1]Coll Vet Med Oregon State Univ, Oregon, USA; [2]Depart Pathobiol Sci, UWisc Madison, WI USA; [3]IDEXX Labs, Westbrook, Maine, USA; [4]Vic Inst Anim Sci, Attwood, Victoria, Australia.

Abstract We evaluated two ELISA assays for detecting bovine antibodies to *Mycobacterium paratuberculosis* which were adapted for detecting llama and alpaca (LA) antibodies to *M. paratuberculosis*. A protoplasmic antigen-based assay (Allied Mptb-ELISA) which provides an index score and a whole cell lysate antigen-based assay (IDEXX Mptb-ELISA) which provides an OD value were used. Among a herd of 100 LAs classified as culture negative (CN) for *M. paratuberculosis* and with no history of exposure to the organism, 52 false-positives occurred with the Allied Mptb-ELISA (48% specificity) and 8 false-positives occurred with the IDEXX Mptb-ELISA (92% specificity). For CN LA, the range for Allied Mptb-ELISA scores was 0.1 - 7.1, and the range for IDEXX Mptb-ELISA OD values was 0.034 - 0.384. For the Allied Mptb-ELISA, there was no significant difference (P=0.48) in the distribution of values for CN LA and 9 culture positive (CP) LA, and no CP LA had values greater than 4.7. In contrast, preliminary results indicate that increasing values for the IDEXX Mptb-ELISA are more closely associated with CP LA than are CN LA. For both ELISAs, males and adults (>2 y) had significantly higher values than females and juveniles (<1 y), respectively. This suggests that age and sex specific cutoffs may be needed for interpreting serologic results. Further evaluations of the IDEXX Mptb-ELISA are currently being conducted, and will include determination of the relative performance of a protein G and an anti-llama IgG conjugate. Until additional evaluations can be completed with larger sample sizes, bovine *M. paratuberculosis* ELISAs adapted for use on LA should be interpreted with caution. Mptb-ELISA values classified as positive should be confirmed by culture.

Title An investigation of a pygmy goat herd naturally infected with *Mycobacterium paratuberculosis* via serial fecal culture, Gamma interferon and serum antibody ELISA assays.

Author(s) Manning EJB, Steinberg HS, Collins MT.

Institution School of Vet Med, University of Wisconsin, Madison, WI, USA.

Abstract A longitudinal study was performed on a Minnesota pygmy goat herd with a history of chronic weight loss, abortion and death. A variety of immunological responses over time were ascertained by serial fecal cultures, ELISA and interferon-gamma assays. For instance, five strongly ELISA positive and fecal culture negative goats later became ELISA-negative. Two of 5 culture-positive goats tested positive by the interferon-gamma assay for paratuberculosis using the USDA-licensed kit for use on cattle (IDEXX Laboratories, Inc.). This suggests that the monoclonal antibody against bovine interferon-gamma cross-reacts with goat interferon-gamma as suggested by Woods et al. Gross pathology included serous atrophy of fat, mineralization and caseation of lymph node granulomas. Intestinal wall thickening was not evident grossly. Acid-fast organisms were found in all goats with lesions at gross necropsy (in the ileum, jejunum, cecum and liver). *M. paratuberculosis* was also found in some animals in mesenteric, colic and mediastinal lymph nodes, as well as the colon and lung. Results from the culture of tissues also showed dissemination of the infection: organisms were recovered from mammary, kidney, uterine and splenic tissues as well as those noted via histopathology. Although limited by incomplete records, analysis suggests a strong association between the paratuberculosis test status of each goat and that of its dam. This evidence, coupled with finding a culture-positive fetus and isolation of the organism from the liver suggests the infection disseminates in the latter stages of the disease and may spread to offspring in utero and/or via colostrum or milk.

Title Identification of mycobacteria applying polymerase chain reaction.

Author(s) Valente C[1], DeMas S[2], Scarso S[2], Cuteri V[3], Job L[2], Marangon S[2], Cancellotti FM[2].

Institution [1]Università di Padova, [2]Instituto Zooprofilattico, Padova, 3Università di Perugia, Italy.

Abstract The polymerase chain reaction (PCR) was applied for the identification of field strains of mycobacteria. 28 field strains, cultured on Lowenstein-J medium and identified according to biochemical reactions, were tested in the PCR. The pathological material for culture was collected from different animal species: cow, swine, goat, and poultry. Multiple interspersed negative controls, only reagents without DNA, were included each time a PCR was performed; for the positive control DNA extracted from *M. bovis* and *M. avium* of reference strains (ATCC) were used. The PCR was carried out twice, at different times for all field strains examined. The presence and size of each PCR product was determined by electrophoresis on silver-stained 7% acrylamide gel. Two primers: forward primer, 5'-gggTTTgACATgCACAggAC-3' and reverse primer: 5'-TACggXTACCTTgTTACgAC-3' which codify for 16S subunits, were used for PCR amplification of bacteria included in the Mycobacterium genus. A pair of primers, forward primer: 5'-gAACAATCCggAgTTgACAA-3' and reverse primer, 5'-AgCACgCTgTCAATCATgTA-3', which codify for MPB70 protein, were used for amplification of the *M. tuberculosis* complex. A second method using two further primers codifying for IS986 were then also used to amplify only the *M. tuberculosis* complex. For the amplification of strains within the *M. avium* complex, the following sequence of primers were used: forward primer, 5'-gAACgCCCgTTggCTggCCAT-3' and reverse primer, 5'-gCAACACggTCggACAggCCT-3', obtained from a genomic library. With the primers employed, 26 field strains were included in the genus Mycobacterium (92.85%, 18 of which belonged to the *M. tuberculosis* complex and 2 to the *M. avium* complex. The Mycobacterium genus was well identified as the *M. tuberculosis* complex and the *M. avium* complex, but the PCR was unable to differentiate the species within the complex.

Title Comparison of serological and cellular tests in a paratuberculosis naturally infected herd.

Author(s) Traveria G, Norimine G, Bernardelli A, Cesar N.

Institution CEDIVE, (7130) Chascomus, C.C. 147, Argentina.

Abstract A herd of 350 beef cows aged 2 years and over, have about 3-4 animal deaths per year showing typical diarrhea signs of paratuberculosis. The herd was followed from 1992 to 1995 with simple comparative skin testing, avium PPD and mammalian PPD (SENASA - Argentina). Tests were performed in the neck and the reaction was read 72 hrs post inoculation. Skin fold differences over 3 mm were regarded as positive, with 17% positive reactors. From the herd, 145 animals were chosen at random in 1995 for EIA-IFN-gamma and absorbed ELISA (CSL, Australia) tests were performed according to manufacturer's instructions. Rendering 2% IFN-gamma positive (3 animals), an avium culture OD that was 0.100 above the control culture's OD value was regarding as positive. Responsiveness to *M. avium* PPD was indicated by calculating the ratio less than 0.7 of ELISA OD values for the bovis PPD culture to the avium PPD culture. With the absorbed ELISA, 2.75% of the animals were positive (4 animals) with the positive cutoff value being the negative control plus 0.100. Two cows were IFNgamma , ELISA and skin test positive (skin test was performed 6 months prior to blood sample extraction). Due to contamination, cultures were not available from all animals, but we have recently isolated *M. paratuberculosis* from a symptomatic cow. We have compared these sera with another ELISA-absorbed indirect system using absorbent from M. phlei according to Cok et al method. Sera dilution was 1/20, antigen used was *M. paratuberculosis* PPD (Allied Monitor), conjugate IgG (Denmark) and ABTS as chromogen. Main cross reaction prolem which in Argentina is due to related mycobacteria like *M. bovis*, encouraged us to use sera from a cow with reaction to mammalian PPD as cutoff, prior to absorption with M. phlei soluble antigen for 30 minutes, sera incubation and conjugation for 1 hour. In this ELISA system, 3.4% of animals were positive (5 animals). We also tried to use avium PPD (SENASA-Argentina) as ELISA antigen, but no sera reaction was observed.

Title On the etiology of Crohn's disease.

Author(s) Mishina D, Katsel P, Brown ST, Gilberts ECAM, Greenstein RJ.

Institution VAMC Bronx, NY and Mt. Sinai School of Medicine, NY, USA.

Abstract Crohn's disease is an idiopathic chronic, panenteric, intestinal inflammatory disease. Its etiology may be infectious. Putative organisms implicated are the measles virus and *Mycobacterium paratuberculosis* (*M. ptb*). Empirical anti-mycobacterial treatment is not efficacious in the therapy of Crohn's disease. We suggest that within the spectra of Crohn's disease there are two clinical manifestations, analogous to the tuberculoid and lepromatous manifestations of leprosy. One is aggressive and fistulizing (perforating) the other is contained, indolent and obstructive (non-perforating) (PNAS 91;12721-12724; 1994). Surgical ileal mucosal specimens were from both forms of Crohn's disease as well as controls (n=4 each group). Total RNA was reverse transcribed to cDNA using random primers. RNA was quantified by PCR amplification using published or custom designed primers. Evaluated were; a house keeping gene ß-Actin; a *M. ptb* specific DNA sequence (IS 900); the cytokine IL-1ß (previously shown to differ between the two forms of Crohn's and used to show lack of contamination). Following PAGE and autoradiography, bands were excised, DNA purified, subcloned and sequenced. Controls included *M. ptb* (ATCC). All the cases of Crohn's disease and both samples from the patients with ulcerative colitis showed a band comigrating with the *M. ptb* DNA. There was no signal in the cancer patients. DNA sequence analysis (to date performed on two samples) confirms that this is *M. ptb*. The IL-1ß data show that these esults cannot be ascribed to sample contamination. The finding of *M. ptb* DNA in Crohn's disease has been previously reported. However, a cause and effect relationship has not been established. In part, this is due to the lack of efficacy with empirical anti-mycobacterial therapy. We conclude that clinical trials with anti *M. ptb* therapy are indicated in Crohn's disease. However, patients should be stratified into the aggressive (perforating) or contained forms prior to randomization.

Title Presence of *Mycobacterium paratuberculosis* antibodies in animal health care workers.

Author(s) Chiodini RJ, Thayer WR.

Institution RI Hospital and Brown University, Providence, RI, USA.

Abstract In response to concerns over a disproportionate number of Crohn's disease cases within a animal diagnostic laboratory which employed bench-top handling of *M. paratuberculosis* sera was obtained from all 41 state and federal employees of the laboratory and matched with 168 sera obtained from random screening of human health care workers. Sera were examined by a modified ELISA using a PPD antigen prepared from a human isolate of *M. paratuberculosis*. a significantly higher level of antibodies was detected in the animal health workers as compared to controls (p=0.0009). Sera from the control human health population had a mean OD410 of 0.29±0.10. In contrast, sera from the animal laboratory population had a mean OD410 of 0.44±0.13 (p=0.0009 as compared to controls). Mean OD values of individual animal health employee categories were as follows: bacteriologists engaged in *M. paratuberculosis* culture, 0.56±0.08 (p=0.0026); field, 0.46±0.16 (p=0.0009); general laboratory workers not involved in *M. paratuberculosis* culture, 0.41±0.11 (p=0.0001), and necropsy, 0.40±0.13 (p=ns). Differences in statistical significance of the animal health workers as compared to controls reflect the number of employees in each category; therefore, statistical significance was not suggested with those employees performing necropsy (n=3) even though OD values were similar to those in other groups. There was no significant difference between the individual groups of animal health employees. Of the 3 employees having direct daily contact with *M. paratuberculosis*, 2 had elevated OD reading of 0.58 and 0.67, and the third employee a reading of 0.49 which was equal to 2 SD above the mean of controls. The continued practice of bench-top handling of *M. paratuberculosis* should be discouraged.

Title Adaptation of a bovine ELISA for the detection of *Mycobacterium paratuberculosis* antibody for use with human sera.

Author(s) Cherney TM, Collins MT.

Institution Depart Pathobiol Sci, School of Vet Med, University of Wisconsin, Madison, WI, USA.

Abstract The *Mycobacterium paratuberculosis* Antibody Test Kit® (IDEXX Laboratories, Westbrook, ME) was adapted for use with human sera by checkerboard titration of critical reagents. This bovine ELISA has proven effective in diagnosing paratuberculosis in cattle. Serum from a veterinarian who was twice accidentally inoculated with the vaccine for Johne's disease, was used as a positive control, and serum from a normal, healthy, non-veterinarian was used as a negative control. Optimal discrimination between positive and negative control sera was obtained using a serum dilution of 1:200 in absorbing diluent, an absorption time of 30 minutes, and a polyclonal HRP-conjugated goat anti-human conjugate at a dilution of 1:4000. Using these reagent concentrations, a kinetic ELISA determined the optimum substrate-enzyme reaction time to be 12 minutes. ELISA results were standardized by expression of each optical density value as a percentage of the controls (EV%); negative control=0%, positive control=100%. This prototype assay was used in a preliminary study to evaluate 2 populations of human sera for the presence of *M. paratuberculosis* antibody. One population consisted of 200 serum samples obtained from Red Cross blood donors in Dane Co., WI and the other population consisted of 300 serum samples obtained from Barron Co., WI dairy farm families. The Red Cross samples had EV% values ranging from 41%to 355% with a meanSD of 3.6%±48.8%. The Barron Co. samples had EV% values ranging from -17% to 633% with a mean±SD of 49.2%±99.1%. Statistical analysis (Mann-Whitney test) showed that the 2 populations were significantly different ($P<0.0001$). Results show that this ELISA may be useful in determining the prevalence of antibodies to *M. paratuberculosis* in human sera. This assay will be used to measure exposure rates in selected human populations considered at risk, and in Crohn's disease patients.

Title High-temperature, short-time (HTST) pasteurization of milk containing low levels of *Mycobacterium paratuberculosis*.

Author(s) Grant IR[1], Ball HJ[2], Rowe MT[1,2].

Institution [1]Queen's University of Belfast and [2]Depart Agriculture for N Ireland, Belfast.

Abstract A study was undertaken to determine the efficacy of HTST pasteurization (71.7°C/15 s) when low levels of *Mycobacterium paratuberculosis* are present in milk. The levels of *M. paratuberculosis* employed in this study are considered more likely to be encountered in milk from cattle with Johne's disease than those employed in our previous study (10^8 and 10^4 CFU/ml). Samples (250 ml) of aseptically-drawn cows' milk were inoculated with low numbers (10^3 CFU/ml, 10^2 CFU/ml, 10 CFU/ml or 10 CFU/50 ml) of *M. paratuberculosis* and subjected to HTST pasteurization using Franklin plate heat exchangers. A total of nine bovine strains of *M. paratuberculosis* were tested in triplicate at each of the inoculum levels. Where necessary, low numbers of the organism were concentrated prior to enumeration by centrifuging 10 or 50 ml aliquots of milk (2400 x g, 20 min) and resuspending the pellet of cells in 1 ml of PBS. The most probable number of *M. paratuberculosis* present in the milk before and after pasteurization was estimated by inoculating slopes of Herrold's egg yolk medium containing mycobactin J (HEYM). BACTEC Middlebrook medium supplemented with sterile egg yolk and mycobactin J was also inoculated. Both media were incubated at 37°C for up to 18 weeks and any growth observed was tested for acid-fastness by the Ziehl-Neelsen strain. To date, suspect acid-fast survivors have been observed in a number of the HEYM and BACTEC cultures of HTST pasteurized milk. The acid-fast bacteria were generally present along with other non-acid-fast bacteria arising from the milk. Attempts are being made to decontaminate these cultures prior to IS900-based PCR testing which will confirm whether these suspect survivors are *M. paratuberculosis*.

Title Survival of *Mycobacterium paratuberculosis* and preservation of immunoglobulin G in bovine colostrum under experimental conditions simulating pasteurization.

Author(s) Meylan M, Rings DM, Bech-Nielsen S, Shulaw WP, Kowalski JJ.

Institution The Ohio State University.

Abstract *Mycobacterium paratuberculosis* is the agent of a chronic, fatal, granulomatous enterocolitis in ruminants. Most susceptible to infection are animals in their first month of life, although clinical disease becomes apparent only several years later. The main way of infection is the fecal-oral route, but since excretion of *M. paratuberculosis* has been demonstrated in colostrum as well as in milk of infected cows, concern aroused about feeding mycobacteria loaded colostrum to highly susceptible neonate calves. We investigated the effects of colostrum pasteurization under laboratory conditions, i.e., elimination of the mycobacteria and preservation of immunoglobulins through heat treatment: colostrum samples were spiked with *M. paratuberculosis* (ATCC 19698) in concentrations of 10^4, 10^3 and 10^2 CFU/ml each, and split into two groups, pasteurized and unpasteurized. The pasteurized samples were held for 30 minutes -exclusive of come-up time- at 63°C in a water bath, then cooled on ice. Both groups of samples were subsequently handled similarly: IgG content was measured by single radial immunodiffusion on agarose gel, and the samples were cultured on Herrold's Egg Yolk Medium supplemented with mycobactin J for 16 weeks at 37°C. The mean value for colostral IgG was 44.4 g/l in the unpasteurized samples and 372 g/l in the pasteurized samples, with a mean decrease in IgG concentration of 12.3%, a statistically significant difference (p=0.005). High quality colostrum (>48 g/l IgG) showed a significantly larger mean percental IgG loss than colostrum of lesser quality (20.1% vs 6.7%; p=0.0001), but did not completely eliminate *M. paratuberculosis* from the pasteurized colostrum samples. Although not sufficient by itself to prevent potential infection of neonate calves by feeding colostrum from infected and shedding cows, colostrum pasteurization may be advisable, along with other control measures, to reduce the infection pressure in infected herds.

Title Determination of D-values in studies on the thermal tolerance of *Mycobacterium paratuberculosis*.

Author(s) Sung N[1], Kaspar CW[2], Collins MT[1].

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Abstract *Mycobacterium paratuberculosis* has been cited as the etiologic agent of, or one of the factors associated with, Crohn's disease in humans. Milk has been suggested as a possible source of human infection because *M. paratuberculosis* is both found in raw milk from infected cows and survives pasteurization. The objective of this study was to measure the decimal reduction time (D-value: time to kill one log concentration of bacteria) for *M. paratuberculosis*. D-values are an accepted standard measure of thermal tolerance. D-values were measured for multiple strains of *M. paratuberculosis* suspended in 50 mM lactate buffer (pH 6.8) and milk at 4 temperatures. The D-values were compared with published D-values for other milk-borne bacterial pathogens such as, Listeria monocytogenes, Salmonella typhimurium, and Coxiella burnetii. Viable *M. paratuberculosis* numbers were quantified using a radiometric culture method (BACTEC). After preheating the menstruum for 30 min., 105-106 cells/ml (final concentration) were mixed with the menstruum in 1.5 ml Wheaton vials, and each vial was sealed and immersed in a water bath adjusted to the target temperature. The D-value of *M. paratuberculosis* ATCC 19698 (bovine-origin) in lactate buffer at 71°C (D71°C) was 12.9 sec. The D62°C, D68°C, and D71°C values of strain Dominic (human-origin) in lactate buffer were 139, 46, 21, and 13 sec., respectively. These D-values were similar for all *M. paratuberculosis* strains tested, and higher than those published for Listeria, Salmonella and Coxiella. Coxiella burnetii, the cause of Q fever in humans, is the organism used to set pasteurization time-temperature requirements. These data support the findings of Chiodini et al. that *M. paratuberculosis* may survive in milk which has undergone pasteurization.

Title A novel staining technique for assessing clumping and viability of *Mycobacterium paratuberculosis* cells during pasteurization.

Author(s) Grant IR[1], Ball HJ[2], Rowe MT[1,2].

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Abstract The thermal inactivation curve for *Mycobacterium paratuberculosis* in milk at holder pasteurization temperature (63.5°C) has been shown to exhibit significant "tailing", resulting in survival of low numbers of the organism for extended periods. Results of investigations carried out to determine an explanation for these unusual inactivation kinetics suggested that "tailing" was due to some intrinsic characteristic of *M. paratuberculosis*, and not due to extrinsic factors such as the method of heating or composition of the suspending medium. Clumping of cells was thought to be the most likely explanation for "tailing". Therefore, a novel staining procedure was developed in order to monitor clumping and viability of *M. paratuberculosis* cells in milk of phosphate buffered salne during heating at 63.5°C. This consisted of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) reduction as an indicator of viability followed by auramine O acid-fast staining to visualize all acid-fast cells present. This double staining procedure permitted simultaneous assessment of the extent of cell clumping, and also the proportion and location of viable *M. paratuberculosis* cells in relation to dead cells. It was observed that *M. paratuberculosis* cells that occurred singly were inactivated more readily during heating than cells present in clumps. It appeared that *M. paratuberculosis* cells within clumps were gaining extra protection from the lethal effects of heat from other cells surrounding them. At heating times corresponding to the "tail" region of the thermal inactivation curve, any viable cells observed were always located within clumps. It has been suggested that the "tail" will last until only one viable cell per clump remains. If it is assumed that each clump of cells, rather than each individual cell, produced a colony Herrold's medium then the above observations would explain why no apparent reduction in numbers was observed in the "tail" region of the thermal inactivation curve.

Title Commercial pasteurization of *Mycobacterium paratuberculosis* in whole milk.

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Abstract In order to assess bacterial thermosusceptibility and establish the efficacy of pasteurization, it is preferable to use commercial pasteurizing units because the heating and cooling differentials generated in laboratory heat treatment may not simulate commercial conditions. Seventeen batches of raw milk were loaded with 10^2-10^5 CFU/ml of *Mycobacterium paratuberculosis* and pasteurized in a small scale commercial pasteurizing unit at temperatures ranging from 72-90°C for 15-35 seconds. *M. paratuberculosis* was not isolated from 96% (275/286) of pasteurized milk samples, representing 10^{-4} reductions in mycobacterial concentrations as radiometric culture could detect about one colony forming unit per ml. Viable mycobacteria were not recovered when raw whole milk was loaded with less than 10^4 mycobacteria per ml. Mycobacteria were not cultured in any of 5 batches of milk pasteurized at 72-73°C for 25-35 seconds, which are the minimum conditions applied when this machine is used commercially to correct for laminar flow in the holding tube. Adequate holding time appeared to be more effective in killing *M. paratuberculosis* than higher temperatures in the small number of batches treated. *M. paratuberculosis* was cultured from 5 of the 17 batches of milk, with less than 30 colony forming units of *M. paratuberculosis* surviving per ml. These included 4 of 8 batches heat treated at 72-73°C for 15 seconds and one of 4 batches treated at 82-90°C for 15 seconds. Survival of *M. paratuberculosis* in experimentally inoculated batches of milk in the small-scale commercial unit cannot be directly extrapolated to commercial pasteurization of naturally infected milk in dairy factories because of artificially high mycobacterial loads used in these experiments, possible differences between the thermosusceptibility of laboratory cultured mycobacteria, and features of the small-scale unit. Pasteurization in a small-scale unit used in these experiments was apparently more efficient at killing mycobacteria than laboratory heat treatment systems.

Title Kinetics of nonthermal inactivation of *Mycobacterium paratuberculosis*.

Author(s) Sung N[1], Kaspar CW[2], Collins MT[1].

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Abstract A number of cheeses are made from milk treated by sub-pasteurization temperatures. Low pH and salt are other factors that contribute to the inactivation of bacterial pathogens in cheese. The kinetics of *M. paratuberculosis* inactivation was measured under 12 different pH and salt combinations at 20°C. Culture broths (ca. 10^6-10^8/ml) of bovine origin strain (ATCC19698) and human origin strain (Dominic) were suspended in 50 mM sodium acetate-lactate buffers at pH 4.0, 5.0, and 6.0 with varying NaCl concentrations (0%, 2%, 4%, 6%). Distilled water and lake water were used as controls. Samples were removed after 0, 1, 2, 4, 8, 16, and 28 weeks for enumeration of *M. paratuberculosis*. The radiometric culture method (BACTEC) was used for the enumeration of *M. paratuberculosis*. Viable counts of both strains rapidly declined in solutions with 2%, 4%, and 6% salt (NaCl). Viable *M. paratuberculosis* were not detected after 8 weeks at pH 4 and 16 weeks at pH 5. However, in the absence of salt, *M. paratuberculosis* survived 8 weeks and 16 weeks at pH 4 and 5, respectively. In buffer at pH 6, distilled water, and lake water, both *M. paratuberculosis* strains survived beyond 16 weeks at every salt concentration, but the rate of decline in pH 6 sodium acetate-lactate buffer was higher than in lake water. For each pH and salt combination, decimal reduction time (D-values) were calculated. The D-values in low pH solutions were lower (faster killing) than in high pH solutions, and higher NaCl concentrations also resulted in lower D-values. These data suggest that *M. paratuberculosis* may survive in some cheeses produced with milk heated at sub-pasteurization temperatures.

Title Heat inactivation of *Mycobacterium paratuberculosis* in raw milk using holder-test tube method and lab-scale industrial pasteurization method.

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Abstract Recent evidence has linked Crohn's disease, an inflammatory enteritis, in humans to infection with *M. paratuberculosis*. Although not characterized as a zoonotic agent, *M. paratuberculosis* has been identified in intestinal biopsy tissue from patients with Crohn's One possible source of human exposure to *M. paratuberculosis* is the consumption of dairy products, since presence of *M. paratuberculosis* DNA has been documented in cow's milk obtained from retail markets in Great Britain. Evaluation of optimal time/temperature combinations for effective heat inactivation of *M. paratuberculosis* was determined using either a holder-tube method or a lab-scale industrial pasteurization method. Raw milk was obtained from paratuberculosis - free cows was dispensed into 13 x 100 mm snap-cap polystyrene tubes and placed in a shaking water bath at either 65, 72, 74, or 76°C, with one tube serving as a temperature control. Milk was inoculated with 10^8 CFU *M. paratuberculosis* /ml (strains 19698, Ben and Kay) and aliquots removed at each time point for serial dilution and culture on HEYM. Results indicate that the most effective reduction in viable bacterial numbers was achieved at 72°C, but mean time for optimal killing superseded industrial recommendations of 15 seconds at that temperature. Studies with the lab-scale pasteurizer were conducted in a similar manner. Raw milk (1-2 liters) inoculated with *M. paratuberculosis* (10^4, 10^6, and 10^8 CFU/ml; strains 19698, Ben, and Kay) was poured into the holding vessel and circulated for 15", 72C. Samples were obtained from the output tube at the beginning, middle, and end of the pasteurization run for culture. Results from all experiments conducted with the lab-scale pasteurizer demonstrated that treatment of raw milk inoculated with live *M. paratuberculosis* at 72C, 15", effectively killed all the bacteria. Comparison of these two models for testing heat inactivation of *M. paratuberculosis* indicate that the lab-scale pasteurizer more closely simulates industry conditions and that results from those experiments should be given more careful consideration.