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Title Estimating point prevalence of paratuberculosis in a cattle herd from the results of a screening test by standard methods leads to errors because diagnostic test sensitivity is not constant among herds with differing rates of M. paratuberculosis infection.

Author(s) Sockett DC, Heisey D, Collins MT.

Institution Wisconsin Dept. Agriculture

Abstract Screening tests are often used to estimate the prevalence of disease at a single point in time (point prevalence) in a herd or a given population of subjects. This can be easily done when the sensitivity and specificity of a diagnostic test are known and these two properties of a diagnostic test remain constant for every population of subjects being tested, irrespective of the prevalence of the disease being tested for. Based on study of nine infected dairy herds with paratuberculosis, where all adult cattle in the herds were tested by multiple diagnostic tests for paratuberculosis, we found that the sensitivity of several tests changed depending on the severity of the disease in the population being tested. As a result, the standard equation for estimation of disease prevalence, first described by Marchevsky and cited in most epidemiology textbooks, produces incorrect results. Specifically, use of the Marchevsky equation resulted in under estimation of paratuberculosis prevalence in herds with few test-positive animals, and over estimation of paratuberculosis prevalence in herds with a high number of test-positive animals. This observation has important implications for disease control and eradication programs when screening tests are used to estimate the prevalence of disease in different populations.

Title Prevalence of Mycobacterium paratuberculosis in dairy cattle in South Australia.

Author(s) Vandegraaff R, Barton MD, Barry GD, Van Wijk JGA.

Institution Dept of Primary Industries, South Australia.

Abstract An apparent rise in incidence of clinical Johne's disease and concern within the cattle industry at the lack of accurate prevalence data prompted a survey of cull cattle slaughtered from dairy herds in South Australia from February 1990 to June 1993. A total of 2,551 mature cows culled (for a variety of reasons) from 643 herds in the principal dairy areas of South Australia were sampled for culture, histopathology and serology at slaughter. Ileum, ileocecal valve and associated lymph nodes were cultured and serological tests conducted were the absorbed ELISA and complement fixation tests. *M. paratuberculosis* was detected on culture in 11 (0.43%) of animals in 9 (1.4%) of herds. Infection was found in less than 1% of 2,264 culls from the three major dairy districts of the Central and Southern Hills, the Lower Murray and the South East and in none of 287 culls from the mid-North district. 188 (7.47%) of 2,516 samples were positive at 1:8 serum dilution or above in the complement fixation test, including only two of the culture-positives. This gave a specificity of 92.6%, a sensitivity of 18.2% and a predictive value (of a positive) of only 1.1% when the real prevalence is less than 0.5%. Sixteen samples (0.63% of sera tested) were positive in the absorbed ELISA, including four of the culture-positives, giving a specificity of 99.5%, sensitivity 36.4% and a predictive value of a positive of 25% at a real prevalence of less than 0.5%. The results indicate no significant increase in prevalence over a smaller, localized and less precise estimate made ten years earlier. They also confirm many previous findings that the complement fixation test is of limited value for herd screening or detection of non-clinical cattle in low-prevalence populations and that the absorbed ELISA is more useful in such populations as a herd screen and as a primary indicator of individual high-risk cattle.

Title An epidemiologic study of sheep paratuberculosis in the Basque Country of Spain: serology and some productive data.

Author(s) Adúriz JJ, Juste RA, Saez de Ocariz C.

Institution SIMA, 48016 Derio, Bizkaia, Spain.

Abstract The first objective of this study was to estimate the seroprevalence of ovine paratuberculosis in the Basque Country of Spain. Taking as population all the flocks with more than 100 sheep, about 4504 sheep (2.5% of the total number of heads) in 226 flocks (27.8% of the flocks) were sampled, and sera subjected to Agar Gel Immunodiffusion (AGID) and ELISA. Seroprevalence was found to be 31.4% of the flocks (±5.6) in AGID, and 23.5% (±5.3) in ELISA. 5.8% (±0.7) of the sheep gave positive results in the ELISA and 2.0% (±0.5) in the AGID. The aims of the second part were to investigate the possible association between serology and production factors and to evaluate the effects of vaccination in 10 sheep flocks (about 1400 sheep) with a previous history of clinical cases. About 50% of the adult sheep in each flock, and 50% of the replacer lambs of each year, were vaccinated. The follow-up included a yearly serological study of the whole flock, analysis of the individual productive data, and evaluation of the immunological status of replacers, before and after vaccination. We have found an association between ELISA response and milk production, that, in spite of the influence of other factors, can be estimated in about 20 liters per ELISA index unit. In positive and negative terms, the difference would be of at least a 7.1%. On the other hand, we have been unable to demonstrate a consistent association between seropositivity in the ELISA and fertility. Periodic oscillations of an annual cycle in the reactivities in the ELISA have been observed, which could be related to physiological changes associated with the reproductive cycle, although they cannot be firmly established because of the methodology employed in this study. Moreover, a high proportion (62%) of the sheep tested in the four year follow-up seroconverted at any time in the ELISA, while almost all of the animals with a positive result in the AGID were not present in the following year. A significantly higher proportion of animals vaccinated at birth remained in the flock in the second year compared to the controls (22% vs. 10%; p less than 0.05). This suggests a protective effect of vaccination reaching beyond the traditionally accepted period for the manifestations of paratuberculosis, which could already be present at an early age. The same effect appeared conditioned, in sheep vaccinated in adulthood, by age at vaccination. These results confirm the existence of important losses associated with paratuberculosis but without the typical signs of the disease.

Title Sweden is free from paratuberculosis.

Author(s) Engvall A, Larsson B, Bölske G, Wahlstrom H.

Institution Div of Epiz, Div of Bact, Nat Vet Inst, Uppsala, Sweden.

Abstract Paratuberculosis was introduced into Sweden in the late 19th century through imported cattle (1;2). The disease was again introduced in 1951 by imported beef cattle (3). In 1962 another herd with imported beef cattle was found infected. All outbreaks were controlled by a slaughter policy. Paratuberculosis was made notifiable according to the epizootic diseases act in 1952. Since 1962 no cases of paratuberculosis have been diagnosed except in an imported suckler cow in 1993. Sweden is considered free from paratuberculosis on the following grounds. The disease is notifiable, which means that all suspected cases have to be reported by animal owners and veterinarians. By this way a continuous clinical surveillance is undertaken. All cattle and sheep undergoing sanitary slaughter are examined for signs of notifiable diseases. During 1993, as part of a larger study, relevant parts of the intestines of practically all slaughtered cattle over one year of age were inspected and intestines of all sanitary slaughtered animals were opened and inspected. By this way more than 260,000 normally slaughtered and 13,000 sanitary slaughtered cattle were examined. Two suspect cases were found. Both proved negative by culture. During 1993, a serological survey was performed, involving 4,000 cattle and 3,850 sheep. All cattle sera and all but two sheep sera were negative. The two flocks from which the positive sheep originated are currently subjected to further investigations. 1. Bergman. Scand. Vet. J. 1913; 3, 203-216.; 2. Hoflund. Fors. Forsk. 1954; 11, 67.; 3. Broberg. Swed. Vet. Assoc. 1954; 6, 138-144.

Title Johne's disease in alpacas (Lama pacos) in Australia

Author(s) Hope A, Ridge SE, Condron RJ.

Institution VIAS, Dept of Agriculture, Victoria, Australia.

Abstract Johne's disease was first diagnosed in an alpaca in Australia, from tissues collected at post-mortem, in February 1993. By June 1993, Johne's disease had been confirmed in another 3 alpacas that had shown chronic illthrift and had died. At autopsy, mesenteric lymph nodes were enlarged, fleshy and contained numerous acid fast bacilli. Three of clinical cases were between 12 and 18 months of age, one was 6 years old. All had a common property history. By December 1993, a further 3 subclinically infected alpacas had been identified from 1000 tested using conventional fecal culture. An enzyme-linked immunosorbent assay (ELISA) developed at the Victorian Institute of Animal Science for rapid identification of infected and non-infected alpacas is currently being evaluated. The test is a modification of the absorbed ELISA currently used for the diagnosis of bovine Johne's disease and uses a commercially available anti-llama conjugate. Testing to date suggests that the prevalence of *Mycobacterium paratuberculosis* infection in Australian alpacas is less than 1%. Extensive testing, using both the Elisa and fecal culture, is being used to identify infected herds. Restrictions on stock movements are preventing the spread of Johne's disease from those herds. Disease control on infected properties requires more information on the epidemiology of the disease in alpacas. The source of the infection has not been established.

Title Paratuberculosis in 28 animal species at the San Diego Wild Animal Park

Author(s) Collins MT, Oosterhuis JO.

Institution Dept Pathobiol Sci, Univ Wisconsin, Madison, WI 53706, and San Diego Wild Animal Park, Escondido, CA 92027-9614.

Abstract Prompted by diagnosis of paratuberculosis in 2 blesbok in 1991 and a Chinese Reeve's muntjac in January, 1992, a survey for paratuberculosis of all ungulates at the San Diego Wild Animal Park was initiated. Fecal samples were collected, weighed and 3gm was placed into 30ml of HPC decontaminant/transport medium at the Park, then sent to the laboratory within 3 days collection. Samples were processed to detect growth of mycobacteria in a radiometric culture system (BACTEC) modified to grow *M. paratuberculosis* (*M. ptb*). Most *M. ptb* isolates were detected after 3-5 weeks of incubation and cultures were declared negative if no growth was detected after 7 weeks. The majority of mycobacterial isolates were identified using a commercial PCR amplified DNA probe for *M. ptb* (IDEXX Laboratories, Inc.). When the isolates tested negative with this probe, they were tested using a series of RNA probes for other mycobacterial pathogens, *M. avium*, M. intracellulare, and the TB complex (M. tuberculosis and M. bovis), (AccuProbe, GenProbe, San Diego, CA). Over 80% of the 1500 ungulates at the park were sampled. Over 3,000 fecal and tissue specimens were tested by radiometric culture from January 1, 1992 to December 31, 1993. Seventy-seven isolates of mycobacteria were obtained; 55 were *M. ptb*. No mycobacterial isolates were found to be in the TB complex. Ten saprophytic mycobacterial species that tested negative with the genetic probes were identified by a reference laboratory. *M. ptb* was isolated from 28 different species of animals. The majority of these animals lived in one of three adjacent enclosures and *M. ptb* was isolated from pond water in one of them. As of January 1, 1994, the infection appears limited to roughly one-third of the Park. Infection prevalence in that portion of the park was estimated at 10%. Measures have been instituted to prevent spread of the infection to other parts of the park. In addition, the more heavily infected species of animals, red deer, springbok, muntjac, and blesbok have been removed from the enclosures. Surveillance of animals at the park by fecal culture continues. Movement within and out of the collection is restricted to animals found negative on repeated fecal cultures or from areas/exhibits declared negative.

Title A practice based survey of the frequency of Johne's disease in South West England

Author(s) Cetinkaya B, Egan K, Morgan KL.

Institution Div of Animal Health and Husbandry, Dept of Veterinary Clinical Science, Langford House, Langford, Bristol, BS18 7DU, UK.

Abstract The frequency of Johne's disease in cattle in the South West England was estimated from data collected by telephone interview of veterinarians and farmers. The response rate was 81.6%.

Disease frequency was expressed as the proportion of farms with clinical disease and the cumulative incidence within infected herds. The proportion of farms affected was 1.0% and the cumulative incidence on those farms was 1.9% per year. Similar values were obtained when diagnosis by fecal examination, post-mortem examination and histology were taken into consideration; 0.9% of farms were affected and the cumulative incidence in infected herds was 2.0% per year. The survey was validated against three external reference points. There was good agreement between vaccine use and MAFF records, and the total number of holdings and census data. When responses of veterinarians were compared with those of farmers there was also good agreement on vaccine use (kappa=77.8%), the number of cases reported in the last year of diagnosis (r=0.78) and the total number of cattle within herds (r=0.75). However the results suggest that the total number of cattle holdings was overestimated and consequently the proportion of farms affected may have been underestimated.

Title Seroprevalence of Johne's disease in eleven districts of Buenos Aires, Argentina

Author(s) Moreira AR, Spath EJA, Morsella C.

Institution Instituto Nacional de Technologia Agropecuaria. 7620 Balcarce, Argentina.

Abstract A total of 2530 sera were obtained from 811 beef herds undergoing VIA testing during 1992, belonging to Buenos Aires province (the main cattle breeding province with 20 million heads). The districts sampled were: Ayacucho (n=223); Balcarce (n=96); Alvear (n=247); Bueyrredo'n (n=250); Las Flores (n=235); Maipu (n=258); Magdalena (n=388); Mar Chiquita (n=230); Olavarria (n=243); Rauch (n=138) and Tapalque (n=232). The original design was a district stratified random sample (expected prevalence 50%, error 5%) but the final number of samples was less since only cattle over 24 months of age were studied. The sera were tested for antibodies to *Mycobacterium paratuberculosis* using an indirect ELISA test performed as described by the supplier (Allied Monitor, Lafayette, MO, USA). The adjusted prevalences (using a 58.8% sensitivity and 95% specificity) and their standard errors were as follows: Ayacucho: 17.5 (±2.5); Balcarce: 51.5 (±1.6); Alvear: 7.9 (±1.7); Pueyrredon: 2.5 (±3.1); Las Flores: 23.6 (±2.8); Maipu: 13.6 (±2.1); Magdalena: 23.8 (±2.2); Mar Chiquita: 15.5 (±2.4); Olavarria: 12.9 (±2.1); Rauch: 28.9 (±1.2) and Tapalque': 4.2% (±1.3%). The high prevalences in some districts are similar to the figures found in Canada and USA, there are no similar data from Argentina to compare with. Johne's disease is an emerging and apparently widespread disease in our region which requires the implementation of a control program.

Title Paratuberculosis. Epidemiological study in goats and sheep in the Vouzela area

Author(s) Amado A, Albuquerque T, Afonso AF.

Institution Laboratorio Nacional de Investigaco Veterinaria, Lisboa, and Rosa P, ADS the Vouzela, Portugal.

Abstract A considerable number of goats clinically suspected to suffer from paratuberculosis were reported in the geographic area of Vouzela. Ten suspected animals were studied in our laboratory. Hematological, histopathological and bacteriological tests confirmed the presence of paratuberculosis in these animals. As a high number of animals showed these clinical symptoms we decided to carry out a serological screening in all the small ruminants within the referred area. The total number of animals examined was 4,122 (2,635 goats and 1,487 sheep). Seroreactive animals totaled 10% in goats and 10.2% in sheep. These results are interesting because symptoms of paratuberculosis were only observed in goats, whereas sheep were asymptomatic.

Title Histological confirmation of subclinical infection with M. paratuberculosis in cattle.

Author(s) Condron R, Schroen C, Black C, Ridge SE, Hope A.

Institution Victorian Institute of Animal Science, Department of Agriculture, Victoria, AUSTRALIA.

Abstract Histological examination in culture of tissues is commonly used to assess the accuracy of other diagnostic techniques and to confirm the diagnosis of Johne's disease in cattle slaughtered in control programs. In cattle with clinical disease due to *M. paratuberculosis* infection, granulomatous lesions with plentiful numbers of acid fast bacilli are readily observed in the intestinal mucosa and/or the mesenteric lymph nodes, particularly in the ileocecal region. During the subclinical phase of infection, lesions of Johne's disease are less developed, more localized and organisms may be difficult to detect. In a control program on 25 Victorian dairy herds with Johne's disease, tissues from cattle with a reaction to an absorbed ELISA were examined by culture and histology. Routine histological examination by pathologists experienced with clinical Johne's disease frequently failed to identify subclinical Johne's disease lesions which were detected by further prolonged examination of tissues and confirmed by culture. Very small numbers of acid fast organisms and concurrent parasitic and bacterial enteritis contributed to the failure to identify lesions of Johne's disease. Electron microscopic examination of granulomatous lesions in which acid fast organisms were difficult to detect revealed bacilli with deficient cell walls in both macrophages and giant cells. The significance of these findings with respect to the pathogenesis of the disease and for disease control will be discussed. The findings indicate that in cattle with reactions in the absorbed ELISA or where subclinical infections with *M. paratuberculosis* are suspected stringent histological examination is required. Granulomatous reactions and lesions in which acid fast bacilli are not observed may be due to Johne's disease rather than parasites.

Title Detection of Mycobacterium paratuberculosis in fecal samples, pathological samples and formalin-fixed, paraffin-embedded tissues, by polymerase chain reaction.

Author(s) Guillou JP, Karoui C, Henault S, Thorel MF.

Institution CNEVA/LCRV, 22, rue Pierre Curie, B.P. 67, 94703 Maisons-Alfort Cedex, France.

Abstract The eradication of paratuberculosis is in relation with the performance of diagnostic test used. *Mycobacterium paratuberculosis* grows very slowly. The primary colonies may be expected to appear any time from the sixth to the twelveth week after inoculation. The immunological tests are not sensitive enough. The polymerase chain reaction (PCR) may constitute an alternative to the bacteriological or serological methods. The polymerase chain reaction has been developed, using as target sequence an insertion element of 1,451 base pairs (IS 900), specific for *M. paratuberculosis* (15-20 copies per genome). Two successions of primers has been chosen and optimized by informatic study. The primers are designed to amplify the sequence in double amplification reaction (Nested PCR) (primers 783 and 477 bp respectively). The test was performed in three stages: (1) extraction of bacterial deoxyribonucleic acid (DNA); (2) amplification of the target DNA by means of thermostable DNA polymerase; (3) detection of the amplified DNA by electrophoresis, confirmed by dot blot assay after hybridization with an internal labeled oligonucleotide of digoxygenin. A procedure was performed to allow the detection of *M. paratuberculosis* in preserved fecal samples, kept in ethanol at 70% or at -20°C and an other one from pathological samples disrupted by sonic treatment. A simple procedure was also developed to identify *M. paratuberculosis* from paraffin-embedded tissue sections. This procedure permits the rapid preparation of DNA for PCR from paraffin-embedded samples. Since fewer manipulations are required, it should also reduce the chance of inadvertent contamination of samples by extraneous DNAs. The application of Chelex 100 chelating resin will be helpful for routine treatment of paraffin-embedded samples. The sensitivity and the specificity of methods used, particularly double amplification and hybridization, are discussed by comparing the results obtained by bacterial culture.

Title Pattern of detection of M. paratuberculosis infected cattle in ten dairy herds cultured

every six months for four years.

Author(s) Whitlock RH, Hutchinson LT, Sweeney RW, Spencer PA, Rosenberger AE, Van Buskirk MA.

Institution Univ Pennsylvania, Kennett Square, PA, USA.

Abstract Of the more than 450 cattle over 24 months of age in ten Johne's infected herds cultured at six month intervals, 106 were positive on two or more fecal culture tests. The pattern of detection by fecal culture using a centrifugation technique for each of the ten herds varied enormously. Each of the herd owners were encouraged to remove known infected animals but animals with the higher colony counts were judged the greatest risk to spread the infection and generally culled first. Occasionally animals with only a few colony counts would not be detected again by culture for two years while other animals had a more consistent increase in their colony counts over time. Some animals were detected by only one culture with often less than five colonies total on the four tubes. These animals were not detected again until followed to slaughter and tissues cultured for *M. paratuberculosis*. Nearly always each of these animals with only a few colonies had numerous colonies isolated from a variety of tissues. Approximately 50% of the animals determined to be infected were detected on the first herd culture and the remainder were detected on subsequent herd cultures over the four year period. Thus, sensitive fecal culture techniques were able to identify only half of the infected animals in a herd at one point in time. Unfortunately most of the infected animals not detected by culture were also negative by most serologic tests performed on a subset of the population.

Title Temporal study to evaluate the serum antibody ELISA, gamma interferon test kit, and radiometric fecal culture for diagnosis of paratuberculosis in naturally infected adult dairy cattle.

Author(s) Balzer SE, Teubert DG, Collins MT.

Institution Dept Pathobiological Sciences, Univ Wisconsin, Madison, WI 53706-1102.

Abstract All cattle greater than or equal to 24 months-old in 6 *M. paratuberculosis*-infected Wisconsin dairy herds were sampled every 6 months for a period of 2.5 years (5 times total). Serum antibodies were measured by ELISA using the *M. paratuberculosis* Antibody Test Kit (IDEXX Laboratories, Inc.). The gamma interferon (IFN) response of peripheral blood leukocytes stimulated with *M. bovis* or *M. avium* PPD was determined using the *M. paratuberculosis* Gamma-Interferon Test Kit (IDEXX Laboratories, Inc.). Fecal samples were decontaminated with 1.0% HPC and *M. paratuberculosis* growth was detected using a modified BACTEC radiometric culture system. Whenever possible, ileum and regional lymph node samples from animals that were culled from the herds were collected at slaughter and processed for isolation of *M. paratuberculosis* and for histopathology. A total of 847 animals were tested. Of these, 373 were present at the first herd test and sampled at least twice more. The case definition for paratuberculosis was isolation of *M. paratuberculosis*from a fecal or tissue sample; 106 animals present in herds at the first test fit this case definition. The pattern of conversion to positive test status was variable among cattle. The host response to mycobacteria paradigm is that a cell-mediated immune response, eg. IFN, will precede a humoral immune response. For 34% of cattle, the IFN test became positive before the ELISA. For 34% of cattle, the IFN became positive before fecal culture. For 23% of cattle the ELISA was positive before the animal became fecal culture positive. For 40% of cattle studied, neither the IFN nor the ELISA became positive before the animal became fecal culture positive and was culled from the herd. Other patterns of conversion to test positivity were also examined. The variability observed between animals suggests that the response of cattle to *M. paratuberculosis* infection is rather unpredictable. Some factors that may govern the pattern of responses to infection include age of exposure, dose of *M. paratuberculosis*, and host genetics.

Title The spatial distribution of Mycobacterium paratuberculosis in infected cattle. Implications for pathogenesis and diagnosis

Author(s) van der Giessen J[1], van Dijk L, Bleumink-Plyum N, Eger T, Haagsma J, van der Zeijst B.

Institution School of Vet Med, Utrecht, and CVI, Lelystad. [1]present address: Univ California, Davis.

Abstract The possible spread of *Mycobacterium paratuberculosis* via blood monocytes throughout the body was studied. In addition, the distribution of *M. paratuberculosis* was examined by testing various tissues by PCR. Blood and fecal samples were taken twice a week from five clinically diseased cows. Blood samples were tested directly by PCR and also after first culturing the macrophages. From four cows, blood samples were positive by PCR either directly or after culturing the macrophages. The cows were autopsied and the spread of *M. paratuberculosis* throughout the body was studied by testing various tissues of these animals by PCR. Not only the intestinal tract but several non-intestinal tissues were positive for *M. paratuberculosis* DNA, indicating that paratuberculosis is a generalized infection, spread by infected peripheral blood macrophages. To study the potential to diagnose paratuberculosis directly in blood by PCR, blood samples of an infected dairy herd were tested. Fecal samples of three animals were cultured for confirmation. Blood samples of 60% of the animals were positive by PCR, whereas only 30% of the animals were shedders. The implications of this test for the early detection of infected animals are further studied.

Title Comparison of a commercial serum antibody ELISA, gamma interferon test kit, and radiometric fecal culture for early diagnosis of paratuberculosis in experimentally infected female Holstein calves.

Author(s) Collins MT, Zhao BY.

Institution Dept Pathobiological Sciences, Univ of Wisconsin, Madison, WI 53706.

Abstract Fourteen female Holstein calves were obtained from farms with no serological evidence of *M. paratuberculosis* infection based on serum antibody assays on 100% of adult cattle in the herd. At the age of 4 weeks, 5 calves were orally challenged with 10^8*M. paratuberculosis* by mixing the organism in milk for their evening feeding on 3 consecutive days (high dose group). Another 5 calves were similarly challenged 6 times with 10^6*M. paratuberculosis* (low dose group). Four non-challenged calves served as controls. Every 4 weeks calves were sampled. Serum antibodies were measured using the *M. paratuberculosis* antibody Test Kit (IDEXX Laboratories, Inc.). The gamma interferon response of peripheral blood leukocytes stimulated with *M. bovis* or *M. avium* PPD was determined using the *M. paratuberculosis* Gamma-Interferon Test Kit (IDEXX Laboratories, Inc.). Fecal samples were decontaminated with 1.0% HPC and *M. paratuberculosis* growth was detected using a modified BACTEC radiometric culture system as previously described. At 12-13 months post-challenge, a 1 x 2 cm ileum biopsy and a regional lymph node were surgically obtained from all 14 calves and processed for histopathology and radiometric culture. Four of the 5 high dose calves and 3 of 5 low dose calves have been confirmed infected by isolation of *M. paratuberculosis* from fecal or tissue samples. The study is ongoing and this abstract represents an interim report of results through 18 months post-challenge. No calves have tested positive for serum antibody to *M. paratuberculosis*. No calves have tested positive by the gamma interferon assay, based on evaluation of bovis/avium ratios. Radiometric fecal cultures have been positive sporadically. Specifically, 9 of 100 fecal attempts have been positive for high dose calves and 5 of 100 fecal culture attempts have been positive for low dose calves.

Title A new paradigm for interpretation of paratuberculosis serology: profiling of herds based on multiple thresholds of ELISA.

Author(s) Jacobson RH, Rossiter CA, Chang YF, Shin SJ, Lein DH.

Institution Cornell University, Ithaca, NY, USA.

Abstract ELISA tests for detection of antibody to *M. paratuberculosis* usually lack sensitivity and/or specificity. These deficits are attributable to anergy in recently infected cattle and cross reactivities of antibodies for antigens used in the assays. When specificity is set at about 99%, most ELISAs have a sensitivity ranging from about 40-60%. Of 473 fecal culture-positive

als, only about 40% were detected by an ELISA having a 99% specificity. Three-hundred nineteen of these animals (67%) were low-shedders (less than 10 colonies per culture); only 21% of low-shedders were seropositive. Alternatively, 88 of 94 (94%) high-shedders (greater than 100 colonies per culture) and 58% of moderate shedders (10-100 colonies) were antibody-positive. Because low-shedders account for about two-thirds of infected animals, conventional ELISAs miss many infected cattle. We concluded that if ELISA were to be an effective tool to determine herd seroprevalence, classification of animals as positive or negative based upon a single threshold in ELISA would lead to an unacceptable number of false negative results. Using 1340 samples from farms of known Johne's disease status, we established three arbitrary thresholds for ELISA: the first was 2 times the mean ELISA value for all uninfected animals (n = 867), the second was 3.5 times the mean of all negatives, and the third was equivalent to the 99% specificity level which was 10.2 times the mean ELISA for uninfected cattle. These arbitrary thresholds defined 4 categories of ELISA results. If ELISA values were in the first category, the animals were classified as being at low risk of developing Johne's disease; if in the second, third, or fourth categories, the risk was considered moderate, moderately-high, or high, respectively. A herd profile was then established based on the proportions of animals that fell into the various risk categories. For example, a herd with 95% of the animals in the low- risk and 5% in the moderate-risk categories virtually always depicted a Johne's-free herd, as determined by repeated sequential fecal test. Conversely, if 25% of the animals were in the moderately-high to high-risk categories, such a herd inevitably was infected based on fecal culture confirmation. We conclude that profiling herds via a multi-threshold, rather than a single-threshold ELISA, ia a more accurate means of determining the paratuberculosis herd infection status.

Title Pathologic, bacteriologic and serological findings in 100 ewes and fetuses from paratuberculous sheep flock in South Africa.

Author(s) Bastianello SS, Huchzermeyer HF.

Institution Onderstepoort Veterinary Institute, Onderstepoort, South Africa.

Abstract Specimens were collected at slaughter from 100 ewes and their fetuses. The ewes originated from a paratuberculous flock in South Africa and were in the last 2-3 weeks of gestation with single, twin or triplet lambs. Tissues from all the ewes and fetuses were subjected to histopathological examination (HPE) and cultured for *Mycobacterium paratuberculosis*. Further tests included: examination of ileal smears (ISE) in all the ewes; fecal smears from ewes positive on ISE; and agar gel immunodiffusion (AGID) and Complement fixation (CF) tests in 85 and 80 ewes respectively; AGID on 22 fetuses from 16 ewes; and, CF on 96 fetuses from 67 ewes. True positive cases were taken to be cases positive on HPE or ISE or both. Twenty ewes were positive for paratuberculosis, 19 on HPE and 1 on ISE. Six ewes were positive on ISE, 2 of which were fecal smear-positive. *M. paratuberculosis* could not be cultured from any of the ewes or their fetuses. The AGID test was positive in 27 ewes, 11 of which were positive on HPE, giving a sensitivity of 55%. The CF test was positive in 6 cases, 5 of which were true positive cases, with a consequent sensitivity of 25%. No meaningful conclusions could be made from the results of the AGID and CF tests on the fetuses. Although only limited examinations were performed on the fetuses, the results indicate that in-utero infection is unlikely to occur in field cases of *M. paratuberculosis* in sheep.

Title Evaluation of the response of AGID and Gamma-Interferon tests in lambs infected with Mycobacterium avium subsp. silvaticum and Mycobacterium avium subsp. paratuberculosis and their relation with the diagnosis of ovine paratuberculosis.

Author(s) Pérez V, Chávez Gris G, Gutierrez M, Tellechea J, Badiola JJ, García Marín JF.

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Abstract AGID test is commonly used in the detection of ovine Paratuberculosis. This test detects the humoral immune response of the animals. However, its limitations are well know, due to its low

sensitivity, related to the lesional type that the animal shows. Gamma-Interferon (-IFN) test could eliminate these disadvantages since it is based on the detection of the cellular immune response. In order to clarify these aspects, both tests were evaluated in lambs infected with *Mycobacterium avium* sbsp. *silvaticum* (Group A) and *Mycobacterium avium subsp. paratuberculosis* (Group Z). Samples of serum and blood were taken at different periods post-infection (50, 80, 120, 150, 220, 350 and 500 d.p.i.). Moreover, lambs from both groups were killed at 60, 120, 220 and 500 d.p.i. in order to evaluate the lesions and their correlation with the response in these immunological tests. -IFN test was useful to detect initial phases of the infection, showing positivity from the first day of testing in animals from both groups. This positivity was clearer in group A until the end of the experiment. With respect to AGID test results, animals from group A were positive from the first day of testing, reaching 100% of positivity at 350 d.p.i., while in group Z, positive animals were seen only sporadically and from 120 d.p.i. One of the most important findings was the relationship between the response to AGID and -IFN test and the lesions in group Z. The presence of severe diffuse granulomatous enteritis observed in some animals at the end of the experiment, was related with both positivity to AGID and absence of response to -IFN test. In conclusion, both techniques could be used together because of their complementarity in the diagnosis of ovine paratuberculosis.

Title Efficiency of PCR and culture in the detection of Mycobacterium avium subsp. silvaticum and Mycobacterium avium subsp. paratuberculosis in tissue samples of sheep.

Author(s) Pérez V, Bolea R, Chávez Gris G, Cortabarria N, Juste RA, Badiola JJ, García Marín JF.

Institution Dpto de Patologia Animal. Facultad de Veterinaria. Universidad de Zaragoza. C/. Miguel Servet, 177. 50013-Zaragoza. SPAIN.

Abstract The isolation of *M. avium* subsp. *paratuberculosis* (*Map*) from tissues of infected animals is both difficult and takes a long time. Moreover, the role of *M. avium* subsp. *silvaticum* (*Mas*) must be considered. On the other hand PCR has been proposed as an alternative to culture. This technique permits distinguishing between *Mas* and *Map* and the results can be obtained faster than in the culture. In this study we used tissue samples (ileum and lymph nodes) from experimentally infected lambs with *Map* and *Mas*, and from ewes with natural paratuberculosis infection. Both lambs and ewes had severe or minor lesions. Mycobacteria were extracted using a phase partitioning technique and the mycobacteria lysed by bead beating with zirconium beads. The DNA was extracted using standard techniques and amplified by PCR using primers specific for IS900 and IS901. Culture was made using Lowenstein-Jensen medium with and without mycobactin J and sodium pyruvate. In the experimental animals, those with severe lesions, either infected with *Map* or *Mas* were positive in culture and PCR. Among the animals with minor lesions, the PCR technique was more sensitive than culture in lambs experimentally infected with *Map*. In the group infected with *Mas*, the same animals were detected with both techniques, and in this group, some animals with clear lesions were not detected either with PCR or culture. In the natural infection, all sheep with severe lesion were both culture and PCR positive and among the sheep with minor lesions, the culture detected more animals as positive (60%) than the PCR (30%). All the animals were positive to PCR using primers to detect IS900. Moreover, the primers and the PCR technique used distinguished clearly between the animals experimentally infected with *Mas* or *Map*.

Title Pennsylvania Johne's disease control program (1973 to 1993): a review of the twenty year program.

Author(s) Whitlock RH, Sweeney RW, Hutchinson LT, Van Buskirk M.

Institution Univ Pennsylvania, Kennett Square, PA, USA.

Abstract Pennsylvania implemented a Johne's disease control program because of the early recognition of Johne's disease in the state, the recognized importance of this disease to the agricultural economy and the lack of any effective treatment. The primary objectives of the program were to reduce the prevalence of paratuberculosis in infected herds and to provide farmers with the

means to control the disease through management techniques and diagnostic support. Pennsylvania government believed it had a duty to protect agriculture from Johne's disease and it believed the imposition of restrictions of known infected animals was necessary since the addition of clinically normal Johne's carriers to cattle herds appeared to be the single most important source of infection for herds. Participation in the program required a signed memorandum of understanding between the state and the farmer. The program gradually grew in size with more than 20,000 animals fecal tested annually with additional herds on the waiting list. Indemnity paid to farmers for the slaughter of infected cattle often exceeded $100,000 annually. With continued growth of the program especially in difficult economic times other options for control of Johne's disease in cattle were sought. Implementation of a paratuberculosis test negative program replaced the control program in 1992. The impact of the Johne's control program and the rationale for the change in emphasis will be outlined in detail.

Title Johne's disease control in 25 Victorian dairy herds 1990 - 1993.

Author(s) Ridge SE, Hope A, Condron RJ.

Institution VIAS, Dept of Agriculture, Victoria, Australia.

Abstract A Johne's disease control program based on herd testing with a commercial enzyme-linked immunosobent assay (ELISA) and fecal culture, was implemented in 25 Victorian dairy herds. A whole herd screening test using only the ELISA was conducted at the beginning of the project in 1989-1990 to estimate disease prevalence in each herd, and to allow the progression of disease control in each herd to be modeled using the Collins-Morgan model of Johne's disease epidemiology. Subsequently, while implementing recognized management strategies to control the disease, animals were tested annually with the ELISA and fecal culture. Test positive animals were removed annually prior to the calving period. At the end of the study, the prevalence of disease had decreased from an average herd prevalence of 9.7% in 1990 to 3.2% in June 1993. There were 41 cases of clinical Johne's disease in the study herds in the period July 1990 to June 1991, but only 3 cases of clinical Johne's disease reported from herds culling test positive animals, in the period July 1992 to June 1993. Johne's disease was confirmed by histopathology or culture of post-mortem specimens from 86% of 282 ELISA or fecal culture positive animals examined. *Mycobacterium paratuberculosis* was cultured from 8 (9.2%) of fetuses from 87 subclinically infected ELISA positive cattle, and 6 (50%) of 12 clinically affected ELISA positive cattle. When testing was conducted more than once during each year the greatest number of test positive animals were detected at the mid lactation test. In two herds, no difference was detected between the average production index for ELISA positive cattle and the average production index for the rest of the herd. The results from this study indicate that the Collins-Morgan model can be used to broadly anticipate the results of Johne's disease control in Victorian dairy herds, at least in the early stages of the program. At the end of the project none of the herds could be considered to be free of *M. paratuberculosis* infection. Nevertheless, randomly selected cattle from these herds are considerably less likely to be *M. paratuberculosis* infected than similar cattle selected from the general Victorian dairy cattle population. Recommendations are made regarding the implementation of Johne's disease control programs in endemically infected dairy herds.

Title Horizontal transmission of Johne's disease among young bulls.

Author(s) Rossiter CA, Shin SJ, Jacobson RH, Lein DH.

Institution Cornell University, Ithaca, NY, USA.

Abstract Most US bull studs have herd surveillance programs for Johne's disease (JD) to meet semen export requirements. However, due to lack of sensitivity of tests to detect early infection states and as JD becomes more prevalent, the risk of JD entering bull studs through AI source herds also increases. Because young AI bulls in waiting are typically group housed, the risk of horizontal infection with JD becomes an additional concern. Horizontal spread of JD among young bulls housed in the same pen was demonstrated at a large US Artificial Insemination facility. The primary semen production herd has been paratuberculosis test-negative since the

1970s. In April 1991, a 24 month old bull was detected on annual fecal culture to be shedding *Mycobacterium paratuberculosis*. The bull was group housed with eight other bulls of the same age for the previous 10 months. The infected bull was culture positive (too numerous to count (greater than 100) colony forming units (CFU)) on tissues taken at slaughter in June 1991 including ileum, ileocecal lymph nodes, seminal vesicles, and epididymis. The eight penmates were culture negative on bimonthly fecal samples from March 1991 to September 1992. However, on samples taken November 3, 1992, five of the eight were detected as shedding low (less than 10 CFU per 2 gm) numbers of *M. paratuberculosis*. From March 1993 to present (January 1994), weekly cultures on all eight bulls have remained negative. Serum has been negative ELISA (IDEXX Corp. Portland, ME) and low to moderate risk on KELA ELISA, developed in our lab and used as a herd screening test on the NYS Paratuberculosis Program. Infection in exposed older animals has been shown to occur, but little is known about the progression of infection of shedding or clinical disease, or the influence of health, management or genetic factors on the infection state. This natural experiment provided biologic and epidemiologic evidence that at least five of eight young bulls were horizontally infected with JD. All eight were exposed to the manure of an infected penmate from 16 to 27 months of age, and five were detected shedding *M. paratuberculosis* 19 months later. Persistently negative status on subsequent tests for all eight, however, questions the state or existence of the infection. These bulls are five years of age in 1994 and are being further monitored to answer that question. Horizontal spread of JD infection can occur in group housing conditions. When valuable animals and market are at risk, increased monitoring with multiple tests, heightened management and promoting to the industry the benefits of preventing JD are justified.

Title Effect of Mycobacterium paratuberculosis infection on milk production in dairy cattle.

Author(s) Sweeney RW, Hutchinson LJ, Whitlock RH, Spencer PA, Galligan DT.

Institution Univ Penn School of Vet Med, Kennett Sq. PA, USA, and Penn State Univ, University Park, PA, USA.

Abstract Milk production in 1,706 cows from 14 dairy herds with *M. paratuberculosis* infection was monitored using Dairy Herd Improvement Association (DHIA) records. All cows in each herd were tested on an annual or semiannual basis using the fecal culture test. Milk production and infection status were monitored from July 1, 1987 to January 1, 1992. Lifetime, last lactation, and second-to-last lactation milk production parameters of culture-positive animals were compared with those of culture-negative cows. The majority of infected cows (n=253) were not exhibiting clinical signs of Johne's disease. Mean total lifetime production in the culture negative cows was 41,231 lb (18,741 kg) compared to 36,442 lb (16,564 kg) for the infected cows, but the difference was not statistically significant. Average daily milk production for the infected cows (48.2 lb, 21.9 kg) was significantly less (P less than 0.02) than that of uninfected cows (50.8 lb, 23.1 kg). Daily milk fat and milk protein production were also significantly less for the infected cows. Projected or actual 305 day production (mature equivalent) for the last lactation of infected cows (17,098 lb, 7,772 kg) was significantly (P less than 0.01) less than uninfected cows (18,502 lb, 8,410 kg). In the second to last lactation, the infected cows produced 400 lb (180 kg) less milk than the uninfected cows, but the difference was not significant (P greater than 0.09). These findings demonstrate that cows with *M. paratuberculosis* infection produce significantly less milk than uninfected cows, but the majority of the milk production loss is suffered in the final lactation.

Title Detection of M. paratuberculosis DNA in a goat affected by Johne's disease by an IS900-based PCR and a DIG-labelled probe.

Author(s) Dei R, Zakrzewska K, Vardar T, Rovai C, Fischetti R[1], Lillini E[1].

Institution Ist Microbiologia, Universita di Firenze, [1]Ist Zooprofilattico Sperimentale delle Regioni Lazio e Toscana, Italia.

Abstract *Mycobacterium paratuberculosis*, a slow-growing and mycobactin-dependent mycobacterium, is the etiologic agent of Johne's disease in cattle, and it has been recently

associated with Crohn's disease in humans. The infection is acquired early in life, but it has a long incubation period, during which the animals shed the bacterium with the feces, thus contributing to the spread of the infection. Early diagnosis of infection by isolation or antibody detection is hampered by the lack of rapid, specific, and sensitive techniques. Johne's disease is present in our regions, and we are evaluating the use of biomolecular techniques to detect the bacterium. We used primers P11 and P36, described by Moss et al., to set up a PCR and a DIG-labelled probe specific for *M. paratuberculosis*; the probe was synthesized by a one-step PCR. These primers amplify a 278 bp sequence in the IS900 fragment which is present in multiple copies in the *M. paratuberculosis* genome. We are investigating a herd of goats likely affected by Johne's disease. We received a resected piece of intestine from one of the diseased animals, and fecal samples from the entire herd. The clinical diagnosis of the diseased animal was confirmed histopathologically. We report the results obtained with the intestine. The finely minced tissue was suspended by Lysing solution and digested with Proteinase K; the DNA was extracted by Chelex treatment, and the supernatant used as target in the PCR. After agarose gel electrophoresis a band of the expected size was seen, and the PCR product dot blotted and hybridized with our DIG-labelled probe showed a strong signal, thus confirming the diagnosis. PCR results were obtained in less than two days. Biomolecular techniques seem particularly suitable for the early diagnosis and for the investigation of the role in humans, and the epidemiology of this fastidious bacterium.

Title Economic significance of vaccination against paratuberculosis.

Author(s) Kalis CHJ, Van Schaik G, Dijkhuizen AA, Benedictus G.

Institution Agric Univ Wageningen, Animal Health Service North Netherlands.

Abstract Data of 573 dairy cattle in a field vaccination trial were collected and divided into 269 vaccinated animals and 304 non-vaccinated controls. The economic loss for each culled cow in the non-vaccinated group was Dfl 1.708, and Dfl 1.452 in the vaccinated group. So, vaccinating against *Mycobacterium paratuberculosis* reduced the average economic loss with Dfl 256 per average culled cow and, therefore, turned out to be profitable. For cows with clinical paratuberculosis the decrease in milk production in the vaccinating group was 13%, while 21% in the non-vaccinated group.

Title First results of paratuberculosis therapy in calves experimentally infected by intravenous route.

Author(s) Arrigoni N[1], Belloli A[2], Belletti GL[1], Proverbio D[2], Greppi G[2], Vacirca G[2].

Institution [1]Istituto Zooprofilattico Sperimentale della Lombardia e dell 'Emilia, Italy; [2]Istituto di Patologia Speciale e Clinica Medica Veterinaria, Facolta di Medicina Vet., Universita degli studi di Milano, Italy.

Abstract Two calves, inoculated by intravenous route with *Mycobacterium paratuberculosis* and consequently shedders from the 10th-17th week after the infection, were treated perorally with a combination of rifampicin, streptomycin and pyrazinamide at the doses respectively of 30-25-50 mg/kg/day for a period of seven months from the 33th week after the inoculation. Fecal cultures, performed weekly throughout the duration of therapy and up to six months after, as well as culture examination of tissues, performed after slaughter, were negative.

Title Testing and management strategies for paratuberculosis used in NYS herds.

Author(s) Rossiter CA, Jacobson RH, Chang YF, Shin SJ, Lein DH.

Institution Cornell University, Ithaca, NY, USA.

Abstract The New York State Paratuberculosis (Johne's disease) Program provides NYS farmers with testing, control program, and herd certification services. Responsive to fiscal pressures, focus in

the program generally has shifted from intense fecal culture testing and animal management based on culture status to ELISA testing with fecal confirmation and integration of Johne's disease control into the total farm management system. This testing approach has cut costs while preserving the integrity of our testing service. Herds are "profiled" based on ELISA results that assign animals of the herd to low, moderate, moderately-high, and high risk categories. Fecal cultures are then done on all animals in the latter three categories to confirm specificity of the ELISA findings. Because of the lack of sensitivity of culture, ELISA values sometimes assign animals to moderately-high to high risk categories that are not culture positive until months later. Thus, ELISA and fecal analysis are complementary and can proficiency define herd status at a much reduced cost. If cost is no factor, then full herd fecal analysis and ELISA would be indicated to define both the shedders and potential shedders (high ELISA values). Farm operations in NYS range from tie stall dairies (30-100 cows) to free-stall operations (150-1200 cows) representing both purebred and commercial interests. Testing and Johne's disease management strategies for a given farm depend upon the farm objectives and range from mere awareness of the problem to aggressive Johne's disease eradication. Factors entering into development of a Johne's disease management strategy that conforms to the farms objectives include determination of prevalence, economic and sociologic impact of Johne's disease, epidemiological characteristics, capability to modify management and use of resources, aggressiveness to reach JD management outcomes. A flow chart of alternative approaches is used to demonstrate the management strategies for various prevalence and objectives, their associated requirements, and their expected outcomes. For a herd with endemic Johne's disease, aggressive testing, culling and management is highest cost control and will effectively prevent spread. The initial high costs may well be offset by a long-term economic advantages. Alternatively, a minimal effort is unlikely to slow the progression of Johne's disease. Improvement in control strategies requires proper interpretation of tests, understanding of Johne's disease epidemiology, and consideration of management and economic factors at the farm and global level.

Title Evaluation of antibody responses to immunodominant antigens expressed by Mycobacterium paratuberculosis recombinant clones.

Author(s) El-Zaatari FAK, Naser SA, Whipple DL, Hachem CY, Graham DY.

Institution VAMC and Baylor College of Medicine, Houston, TX., and National Animal Disease Center, Ames IA, USA.

Abstract *M. paratuberculosis* is the causative agent of Johne's disease, a chronic enteritis in ruminants. Simple, accurate, specific and sensitive diagnostic tests to detect clinical and subclinical disease are lacking. The mycobacterial immunodominant antigens are major stimulants of cellular and humoral immunity against pathogenic mycobacteria. The humoral immune responses of animals with Johne's disease were analyzed against *M. paratuberculosis* recombinant clones expressing some immunodominant antigens representing the 65K and 19K heat shock proteins (hsp65 and hsp19), the 32K secreted protein, and the specific IS900-product. The antigenic identity of these proteins was confirmed by immunoblots with specific monoclonal antibodies and/or by hybridization to specific probes. Ten cows, two goats, two sheep with clinical and five cows with subclinical Johne's disease were tested. With the exceptions of hsp65 (and one goat that reacted to recombinant clones representing the IS900), there was no reactivity seen. Three cows (33%) with clinical and 2 of 5 cows with subclinical disease cows reacted with hsp65. In addition, both sheep and neither goat reacted. We conclude that these antigens are not useful for serodiagnostics of Johne's disease.

Title Diagnosis of bovine paratuberculosis using M. phlei-adsorbed sera in a dot-enzyme-linked immunosorbent assay, comparing subjective and objective test evaluation.

Author(s) Bech-Nielsen S, Shulaw WP, Frandsen PL, Jorgensen JB, Ahrens P, Feld NC.

Institution Dept Vet Prev Med, College of Vet Med, The Ohio State Univ, Columbus, OH, USA; Natl Vet Lab, Copenhagen V, Denmark.

Abstract This study describes the response of cattle to use of a dot-ELISA test when using serum absorbed with *Mycobacterium phlei*. In addition, results obtained when using visual observation are compared with those obtained when using a densitometer. Infection status of cattle was determined by fecal culture. Cattle of different level of exposure and disease manifestation were examined. A significant increase of dot-ELISA response was found in animals with heavy *Mycobacterium paratuberculosis* shedding using both unadsorbed and adsorbed serum, as compared to animals that were fecal culture negative or shedding *M. paratuberculosis* at lower levels ($P < 0.05$). Paratuberculosis was diagnosed by visual determination in 29 of 44 (65.9%) of fecal culture positive clinical suspects, and in 85 of 93 (91.4%) of the fecal culture negative cattle when using unadsorbed serum. On adsorbing the serum the sensitivity of the visual determination decreased to 34.1% (15/44) and the specificity increased to 97.9% (91/93). About 75% of the dot-ELISA positive cattle were heavy bacterial shedders at the time of serologic testing with < 1500 colonies/g of feces tested in the fecal culture test. Effects of sensitivity and specificity at various cut-off points were determined by use of video densitometric measurements because sera have not discretely segregated into distinct groups of positive and negative results. Comparing the dot-ELISA results determined by visual vs the objective densitometric measurement showed compatible results for test specificity. Test sensitivity using visual evaluation was 66% and 87.5% using the objective densitometric evaluation for unadsorbed sera at an ELISA cut-off value of 0.2 optical density. This difference was even more pronounced when adsorbed sera were used.

Title Sequential development of cell-mediated immune response and humoral immune response in cattle experimentally infected with M. paratuberculosis.

Author(s) Yokomizo Y, Mori Y, Shimoji Y.

Institution National Institute of Animal Health, Tsukubashi, Ibarakiken, Japan.

Abstract The success of efforts to control bovine paratuberculosis depends on the early detection of infected animals, hereby allowing for removal of carriers. Recently, Wood et al. described an enzyme immunoassay for interferon- (IFN- EIA) as an in vitro assay for cell-mediate immune (CMI) response, which endowed excellent sensitivity in detecting subclinically infected animals. The object of this investigation was to evaluate the potential usefulness of INF- EIA as an early diagnosis of bovine paratuberculosis. At 4 to 5 days of age, 6 calf were inoculated orally with 500 mg of *M. paratuberculosis* and blood samples collected periodically for 12 months were subjected to the INF- EIA, the lymphocyte-transformation (LT) test. These animals began fecal shedding at 3 to 4 months after the inoculation and remained so throughout the study. Positive results in INF- EIA an LT test were obtained 2 to 4 months after inoculation. The magnitude of the CMI response to johnin PPD was higher than that to bovine PPD. The INF- EIA titer (ng/ml) tended to remain at highly positive level more consistently than LT titer (stimulation index) in the animals. Positive conversion of ELISA antibody titer was detected 8 to 10 months after the inoculation. The results have substantiated the profile of immunological response of paratuberculosis that CMI response precede humoral immune response, and also indicate that INF- EIA may become a useful tool for the early diagnosis of subclinical paratuberculous cattle.

Title The need for employing standardized susceptibility testing methods and interpretive criteria which provide optimal information for evaluating the response of intestinal mycobacterial isolates to anti-infective agents.

Author(s) Lambrecht RS, Schroederus SK, Yang H, Collins MT.

Institution The University of Wisconsin-Milwaukee, The University of Wisconsin, Madison, USA.

Abstract Performing antimicrobial susceptibility tests on intestinal isolates of *M. paratuberculosis* and *M. avium* has been somewhat problematic due to a number of factors including, relatively slow growth, intracellular survival, cidal versus static activity and resistance to many anti-infective agents. While the commercial radiometric broth technique has proved to be a valuable approach

to determining susceptibility of human mycobacterial isolates, current endpoint determination does not take into account a number of important kinetic parameters and does not necessarily provide information on cidal activity. We examined the effects of selected antimycobacterial agents including monensin (ionophore), ethambutol, rifabutin and ciprofloxacin, alone and in combination with one of several cytokines, interferon, tumor necrosis factor and GM-CSF on growth of enteric mycobacterial isolates. Extracellular and intracellular susceptibility were performed in microwells containing Middlebrook broth only or macrophage cells in culture respectively. At selected time points following incubation, aliquots from the wells were inoculated into radiometric vials for determination of static or cidal activity. A lysing agent was added to wells of infected macrophages prior to inoculation. Susceptibility was determined using established procedures which compare treated groups to a 1% survival population of the inoculum. We conclude that additional and valuable information including kinetics, combinatorial effects and cidal or static activity can be obtained and is essential in evaluating the antimycobacterial capabilities of potential therapeutic regimens. While certain antimicrobial agents proved effective either alone or in combination in inhibiting growth of extracellular mycobacteria, these same treatments were not able to inhibit intracellular mycobacteria in a similar manner and the effects were mostly static resulting in regrowth when drugs were removed. Addition of cytokine treatment in select cases resulted in cidal activity.

Title Development of a species-specific enzyme-linked immunosorbent assay for diagnosis of Johne's disease in cattle.

Author(s) Machtelinckx L, Vannuffel P, Gilot P, Dieterich C, Naerhuyzen B, Coene M, Cocito C.

Institution Univ of Louvain Medical School, Brussels, Belgium. Limbourg B, Centre de Depistage des Maladies du Betail, Erpent, Belgium.

Abstract A 23 kDa protein of the *M. paratuberculosis* A36 complex is immunodominant in Johne's disease. The DNA fragment corresponding to the carboxylic end of the protein coded for a polypeptide containing B-cell epitopes present in all tested *M. paratuberculosis* strains1. This a362 polypeptide, which represents the first specific recombinant antigen of *M. paratuberculosis*, was used to develop an ELISA test for paratuberculosis serology. The a362-ELISA was applied to the sera from 300 randomly chosen bovine representative of a large Belgian region. The distribution of the ELISA values frequency classes was then analyzed according to a mixture population model2. A bimodal distribution was found to be the most appropriate to accommodate our results: it included 87.7% of low titer sera and 12.3% of high level sera. This 12% prevalence of a362-seropositivity in the Belgian population was close to those estimated by others for paratuberculosis in different countries. The optimum cut-off value separating these two populations was estimated by the method of Anderson3, assuming that the prevalence of antibodies directed to peptide a362 is equal to the proportion of the high lg titer population (p=0.12) and that the cost of a false negative diagnosis is twice as great as that of a false positive one. Accordingly, five sera with ELISA-absorbance values corresponding to the determined cut-off were chosen as reference points in each ELISA test. The serological test was then applied to some 30 reference sera from paratuberculous-certified cattle belonging to the U.S. National Repository (MT Collins, Univ. Wisconsin) and to five Belgian herds: two herds of healthy animals (H1=110, H2=65), two herds in which paratuberculous cattle were found (P1-144, P2=64) and one herd with tuberculosis (T=38). Sensitivity of the a362-assay was 70% according to the reference sera. For the healthy and tuberculous herds, the distribution of anti-a362 lg followed a monomodal model, whereas a bimodal distribution was found for the herds with paratuberculous animals, indicating that some 40% were found positive to the assay. This figure is probably an underestimation of the a362-ELISA sensitivity because not all animals were infected by *M. paratuberculosis*. The sensitivity of the assay is estimated to be in the range of 40.8 to 70%. From the percentage of false positive results in herds H and T, we can estimate that the specificity of the a362-ELISA with respect to healthy and tuberculous animals is 95.0%. In conclusion, the sensitivity of the a362-ELISA, based on a recombinant product, is practically similar to that of other ELISA tests, based on natural antigens or on mixtures of mycobacterial components. [1] J Clin Microbiol, 1993, 31:947-954. [2] J Virol Meth, 1990, 27:135-14; [3] An introduction to multivariate analysis. 1958. Wiley NY, 126-131.

Title Importance of differential diagnosis of paratuberculosis in sheep.

Author(s) Afonso A, Lage M, Amado A, Albuquerque T.

Institution Laboratorio Nacional de Investigaco Veterinaria, Estrada de Benfica, 701, 1500 Lisboa.

Abstract The authors present a clinical case in a herd of one thousand sheep, whose presumptive diagnosis made by the farmer's assistant veterinarian, was paratuberculosis. Biochemical, pathological, serological, bacteriological, virological and parasitological tests, were done on some animals that presented with the most extensive clinical symptoms. The obtained results led us to conclude that the first presumptive diagnosis wasn't confirmed. All symptoms and lesions were due to a strong parasitic infection caused by Moniezia expansa and Dictyocaulus filaria.

Title Isolation of Mycobacterium paratuberculosis using Dubos medium combined with ELISA and PCR.

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

Institution Nat Vet Lab, Copenhagen, Denmark.

Abstract The control of paratuberculosis depends on early identification of animals shedding bacteria, especially those animals not showing any clinical signs of disease. Clinically healthy cows older than two years were examined by cultivation of feces combined with ELISA and PCR. The feces samples were decontaminated and cultivated on Lowenstein-Jensen (L-J) with mycobactin as well as in liquid Dubos with mycobactin. The samples cultivated in Dubos were analyzed biweekly by culture on L-J, ELISA and PCR. The L-J were read once a week for 14 weeks. For ELISA and PCR and Dubos samples were spun down and the mycobacteria-pellet were lysed by bead beating with zirconium beads. DNA was amplified by PCR using primers specific for IS900. By ELISA antigen was detected with a monoclonal antibody raised against *M. paratuberculosis*. Of 14 animals shedding bacteria 6 were detected by primary cultivation on L-J, while a further 8 animals were detected following cultivation in Dubos. Of the 14 animals shedding M. paratuberculosis only 3 (shedding an innumerable amount of bacilli) were detected by PCR and ELISA. These results indicates that a prior cultivation in liquid medium could enhance the sensitivity of cultivation. This combined with an improved PCR technique will be a powerful diagnostic tool.

Title Hybridization capture of mycobacterial DNA from sample extracts prior to IS900 and IS902 PCR.

Author(s) Millar DS, Tizard MLV, Ford JG, Withey S, Hermon-Taylor J.

Institution Dept Surgery, St. George's Hospital Medical School, London SW17 ORE, UK.

Abstract Polymerase chain reaction (PCR) has been widely used in the detection of mycobacteria. PCR is particularly applicable where the relevant pathogens may be difficult to culture or are in low abundance. The overall sensitivity of PCR assay may however be substantially reduced due to a vast excess of non-target DNA and inhibitory substances in the sample. pPN14 plasmids containing IS900 were digested with SmaI/SacI to remove the optimal *M. ptb* specific PCR 413 target site from the 5' region and religated. Primers p8 5'-TbGTGGCGTTTTCCTTCGGTG-3' and p21 5'-GbCGCTCGAGTAGCCGCGTTC-3' were then used to amplify 5'-biotinylated 513bp capture probes from the 3' end of IS900 where homology with IS902 is greatest. In the test, 50mcl of buffer containing an excess of 5'-b capture probe was added to 450ml as sample DNA extract, boiled for 5 min, incubated overnight 65°C, cooled 25°C and 15mcl of a suspension of streptavidin-coated M280 Dynabeads added. After 2h the beads were captured magnetically and the supernatant discarded. PCR IS900 and IS902 was carried out directly on the beads. Titration experiments demonstrate the ability of this system to detect 2-20 *M. ptb* in a ml of buffer. 100mg fecal samples from 10 bovines suspected of Johne's disease and of shedding *M. ptb* in low abundance were boiled in 1ml NaI Geneclean buffer for 20 min and the DNA extracted on glass beads. Direct IS900 and IS902 PCR was negative on all samples.

Mycobacterial DNA was then captured as described and PCR repeated. 6 animals tested strongly positive for *M. ptb* and one for *M. avium* subsp *silvaticum* (*M. avs*). Hybridization capture may contribute to the simple automatable detection of *M. ptb* and *M. avs* in clinical and environmental samples.

Title Phagocytic and killing capacity of bovine bone marrow-derived macrophages infected with Mycobacterium paratuberculosis.

Author(s) Buergelt CD, Pontzer CH, Williams E.

Institution Univ of Florida, Gainesville, FL USA.

Abstract We developed a culture system from bone marrow-derived neonatal calf macrophages for the assessment of phagocytosis and killing of *Mycobacterium paratuberculosis*. In the presence of fibroblast-derived macrophage colony-stimulating factor the bone marrow cells were cultured for 8 to 10 days in HMEM medium supplemented with 10% FBS and 5% HS. Conditioned macrophages were infected with *M.ptb* and subjected to luminol-dependent chemiluminescence. Controls included uninfected macrophages and macrophages infected with *Listeria monocytogenes*. Oxidative respiratory burst activity was measured in a luminometer and results were recorded via a phagocytosis software program. Maximal intensity of respiratory burst was expressed in mV and obtained in the first 10-12 minutes of the recording cycle irrespective of cycle length and time of cell incubation after infection. Simultaneous treatment with rBoIFN-gamma resulted in a noticeable increase of respiratory burst peak activity during the same time period. Our results suggest that *M.ptb*. stimulate oxidative bacteriocidal mechanisms during cellular ingestion by bone marrow-derived macrophages which can be augmented by cytokines. A second assay system involved S-methionine labeled *M.ptb* and labeled *Listeria monocytogenes* subjected to RboIFN-gamma activated bovine bone marrow-derived macrophages. Short term assays with interferon treated macrophages showed no significant mycobacteriocidal effect by macrophages infected with such labeled *M.ptb* bacilli. Killing of *L. monocytogenes* by activated macrophages was noticed. We conclude that infected macrophages primed with interferon need a second signal or longer time intervals for potential mycobacterial killing. The applied isotope incorporation into metabolically active bacilli, however, provides a rapid and sensitive quantitative tool for the assessment of antimycobacterial effects of bovine macrophages.

Title A role for the 65-kDa stress protein in the cellular immune response to M. paratuberculosis infection in sheep.

Author(s) Colston A, McConnell I, Bujdoso R.

Institution Dept of Veterinary Pathology, Univ of Edinburgh, Scotland.

Abstract The mycobacterial 65-kDa stress protein has been shown to be a major target for the cellular immune response to pathogenic mycobacteria in humans and animals. T cells reactive with the mycobacterial 65-kDa stress protein have been isolated from infected individuals which may imply a role for T cells in the immunopathogenesis of mycobacterial infections. For these reasons the 65-kDa stress protein of *M. paratuberculosis* was chosen for the investigation of the immune response to *M. paratuberculosis* infection in sheep. We have generated recombinant *M. paratuberculosis* 65-kDa stress protein (rhsp65) and have investigated the response of T cells to this antigen. Peripheral blood mononuclear cells (PBMC) were used to assess T cell responses to rhsp65 in an in vitro proliferation assay. PBMC purified from M. paratuberculosis-infected, rhsp65-primed, CFA-M.75 primed or non-primed animals were cultured in vitro with various concentrations of rhsp65. Flow cytometry was used to characterize the T cell subsets involved in proliferative responses to rhsp65. PBMC from infected and rhsp65-primed animals showed significant proliferative responses to rhsp65 in vitro. No significant proliferative responses were seen by PBMC from CFA-primed or non-primed animals. FACS analysis suggested that CD8+ or gamma/delta T cells may be involved in the in vitro proliferative response to rhsp65. Immunohistochemical analysis was used to investigate the presence of *M. paratuberculosis* 65-kDa stress protein in infected ileum. An anti-rhsp65 mab reacted with lamina propria cells in infected ileum but not in non-infected ileum. This investigation has demonstrated that paratuberculosis-infected animals have T cells that recognize, and are capable of responding by proliferation to, *M. paratuberculosis* 65-kDa stress protein in vitro. Furthermore, this protein was present in infected tissue. These preliminary findings support a role for *M. paratuberculosis* 65-kDa stress protein in the immunopathology associated with ovine paratuberculosis.

Title Phenotypic characterization of intestinal lymphocytes in ovine paratuberculosis.

Author(s) Little D, Clarke CJ, Alzuherri HM.

Institution Dept Veterinary Pathology, Univ Edinburgh, Veterinary Field Station, Roslin Midlothian, UK.

Abstract Characterization of the T-cell subsets in intestinal lesions in sheep with paratuberculosis may provide important information on understanding the pathogenesis of this disease. To determine the phenotype and distribution of lymphocytes in the normal sheep intestinal mucosa and in *Mycobacterium paratuberculosis* infected sheep, immunohistochemistry was performed on 12 normal and 12 naturally infected, clinically diseased sheep. Immunoperoxidase staining was carried out on frozen sections of ileum using monoclonal antibodies against ovine CD4, CD8, gamma T-cell receptor (TCR). In both sample groups, cells appeared to be non-randomly distributed throughout the lamina propria. Higher densities of lymphocytes were present in villus than in crypt areas. CD8+ cells were located principally around the epithelial basement membrane, whereas CD4+ cells were localized towards the central villus area of the lamina propria. Very few CD4+ cells were present within the epithelium. Lymphocytes bearing the gamma T-cell receptor were more widely distributed, both in epithelial and lamina propria compartments. The mean relative percentages of total positively staining cells by monoclonal antibody were CD4+ 37.1/37.9, CD8+ 52.0/46.3, gamma TCR 11.0/15.8 percent in normal and diseased ileum respectively. This indicates a significant relative increase in the percentage of gamma TCR bearing cells in paratuberculosis infection (P less than 0.05).

Title Comparative investigations of the resistance and T cell response of C57BL/6 and C3H/He mice to infection with Mycobacterium paratuberculosis.

Author(s) Veazey RS, Horohov DW, Krahenbuhl JL, Taylor HW, Oliver JL, Snider III TG.

Institution USA

Abstract The susceptibility and T cell responses of Bcg resistant (C3H/He) and susceptible (C57BL/6) strains of mice were compared at various intervals after intraperitoneal infection with *Mycobacterium paratuberculosis*. Both mouse strains developed non-specific macrophage activation by 10-15 days after infection. Mycobacterial counts progressively decreased in the C3H/He mice while remaining relatively constant in the C57BL/6 mice. Lymphocytes were harvested from peritoneal exudate cells (PEC) spleens, intestinal epithelial lymphocytes (IEL), lamina propria lymphocytes (LPL), Peyer's patches (PP) and mesenteric lymph nodes (MLN) and labeled with monoclonal antibodies to CD3, CD4, CD8, gamma/delta TCR, CD25, and CD44 for flow cytometric analysis. Innate strain differences in the proportion of T cell subsets were detected in both control and infected mice. Uninfected C3H/He mice had higher proportions of CD3+ cell sin the spleen, MLN, PP, LPL, ands IEL while uninfected C57BL/6 mice had higher proportions of gamma/delta+ and CD8+ cells. Marked increases in gamma/delta+ and CD8+ cells were detected in the PEC's and other tissues of both strains in response to infection. Higher CD4/CD8 T cell ratios were observed in most lymphoid tissues of C3H/He mice, while increased proportions of CD8 and/or gamma/delta T cells were present in C57BL/6 mice. Expression of CD25 by PEC's decreased in C57BL/6 mice and increased in C3H/He mice in response to infection. Expression of CD44 was higher in C57BL/6 mice throughout the study and increased in both strains in response to infection. These results suggest that decreased proportions of CD4+ cells and a downregulation of IL-2 production or function may play a role in the persistence of *Mycobacterium paratuberculosis* infections.

Title Adoptive transfer of congenic spleen cells reduces the severity of clinical disease and pathology in SCID Bg mice infected with Mycobacterium paratuberculosis (M. PTB) of bovine origin.

Author(s) Mutwiri G, Rosendal S, Kosecka U, Yager J, Perdue M, Butler DG.

Institution Univ Guelph, McMaster Univ, Ontario, Canada.

Abstract We have previously reported on the use of the SCID bg mouse infected with *M. PTB* as a model to study paratuberculosis. Here, we investigated the influence of immune reconstitution (by adoptive transfer of congenic spleen cells) on clinical disease and pathology in SCID bg mice previously infected with *M. PTB*. Thirty six SCID bg and 12 BALB/c mice were used in eight groups of six mice each. Test mice were injected intraperitoneally (ip) with 10^6 cfu of *M. PTB*. Immune reconstitution was achieved by injecting SCID bg mice with 3.0 X 10^7 spleen cells obtained from either naive or BALB/c mice previously injected ip with 106 cfu of live *M. PTB*. The mice were necropsied after being observed for 12 wks (10 wks after immune reconstitution). All mice injected with spleen cells (n=24) were successfully reconstituted as evidenced by the production of plasma Ig's and the presence of CD4+, CD8+ and B220+ lymphocytes in the spleen. While three (3/6) of the infected (but not reconstituted) SCID bg mice developed clinical disease, none of the infected reconstituted mice did (n=12). The mean body weight of the former group did not change during the experimental period: The mean body weights of other 7 groups (n=42) however increased. Reconstitution with either naive spleen cells (n=6) or spleen cells from infected BALB/c mice (n=6) reduced the severity of clinical disease, macroscopic and microscopic lesions, and bacterial load within liver lesions (p=0.05). Spleen indices (spleen wt/body wt X 100) were significantly increased (p=0.05) in all infected mice (n=24). We conclude that immune reconstitution of *M. PTB* infected SCID bg mice with spleen cells from BALB/c mice (naive or *M. PTB* sensitized) reduces the severity of the experimental disease. Given this abrogation in disease and the much larger number of CD4+ compared to CD8+ cells identified after reconstitution, we attribute this largely to the CD4+ population of lymphocytes. This model should permit further evaluation of independent and cooperative interactions of cells and mediators of the immune system responding to *M. PTB* infection.

Title Histopathological and morphometric changes in the intestine in ovine paratuberculosis.

Author(s) Clarke CJ, Little D.

Institution Dept of Veterinary Pathology, Univ of Edinburgh, UK.

Abstract Ovine clinical paratuberculosis is characterized by a chronic granulomatous enteritis. Various forms of intestinal pathology have been reported possibly corresponding to the stage or level of the host immune response. As part of a wider program of investigation, diseased (24) and control (24) sheep have been necropsied and gross and histopathological assessments of intestinal and other tissue lesions have been made. In addition morphometric measurements of gut tissues have been performed using image analysis. Results demonstrate marked changes in the diseased group concerning mucosal thickness, villus atrophy, cellular infiltration, etc. Within the diseased group various subgroups are recognizable and relate particularly to infiltrating cell type and mycobacterial load. There is significant correlation between mycobacterial load and the type of cellular infiltration seen in the gut. However serological analysis is less clear cut with AGID positive results detected in many animals with various advanced lesions. Histopathological and morphometric analysis of intestinal lesions proves a data base for assessing possible correlations with further immunohistochemical and molecular investigations.

Title Pathological findings in lambs experimentally infected with Mycobacterium avium subsp. silvaticum and Mycobacterium avium subsp. paratuberculosis.

Author(s) García Marín JF, Chávez Gris G, Pérez V, Badiola JJ.

Institution Dpto de Patologia Animal, Facultad de Veterinaria, Universidad de Zaragoza, C/. Miguel Servet, 177, 50013-Zaragoza, Spain.

Abstract *Mycobacterium avium* subsp. *silvaticum* (*Mas*) has been observed to produce intestinal lesions in ruminants, such as calves or deer, resembling those of Paratuberculosis, and suggesting that *Mas* could be involved in this disease of ruminants. In order to study the lesional aspects of the infection with *Mas* and *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) and to establish possible differences or similarities between both

infections, an experimental infection with *Mas* and *Map* in two lamb groups was carried out. The lambs were killed at different times: 60, 120, 220, 500 and 650 d.p.i. being the digestive system, especially the gut associated lymphoid tissue, studied in detail. Gross and microscopic lesions were clearly different in both infections, the animals infected with *Mas* showing typical lesions of intestinal tuberculosis, located only in the lymphoid tissue (jejunal and ileal Peyer's patches and lymph nodes); these lesions showed a clear tendency to regression with time. However, the lambs infected with *Map* showed focal lesions (microscopically different from those produced by *Mas*), only located in Peyer's patches, more in jejunal than in ileal, that progressively involved the mucosa and the mesenteric lymph nodes. In some lambs, a diffuse granulomatous enteritis was seen at the last slaughter. In conclusion, the type of lesions and the evolution in the time were different in both infections.

Title Histopathological and morphometrical comparison of granulomatous lesions in BALB/c and C3H/HeJ mice inoculated with Mycobacterium paratuberculosis.

Author(s) Tanaka S, Sato M, Taniguch T, Yokomizo Y.

Institution National Institute of Animal Health, Tsukuba, Ibaraki, Japan.

Abstract Susceptibility to *Mycobacterium paratuberculosis*, strain ATCC 19698, infection was compared histologically and morphometrically between BALB/c (BcgS) and C3H/HeJ (Bcgr) mice. The granulomatous lesions, containing *M. paratuberculosis*, in the liver and spleen of BALB/c mice increased progressively until 32 days after inoculation. In contrast, both the granulomatous lesions and number of bacteria decreased drastically in C3H.HeJ mice beginning at 6 weeks post inoculation. There were histological differences in granulomatous lesions between BALB/c and C3H/HeJ mice, indicating that the two strains differ in the functional activity of cellular immunity. Intestinal lesions similar to those seen consistently in bovine paratuberculosis were observed first at 6 weeks after infection and later disseminated widely, especially in BALB/c mice. Our results suggest that the innate susceptible BALB/c mice and the innate resistant C3H/HeJ mice may be a useful and convenient model for further studies of immunoregulation and antimicrobial therapy of paratuberculosis.

Title Cytokine profile changes in the intestine of ovine paratuberculosis.

Author(s) Alzuherri HM, Clarke CJ, Woodall CJ, Little D.

Institution Dept of Veterinary Pathology, Univ of Edinburgh, UK.

Abstract *Mycobacterium paratuberculosis* causes a chronic granulomatous enteritis in adult sheep. The lamina propria intestinal macrophage appears to be pivotal in the pathogenesis of this disease. The granulomatous pathology and wasting seen in clinical Johne's disease is consistent with certain biological activities of the monokines TNF-alpha and IL-1. The purpose of this study was firstly to compare the phenotype of intestinal lamina propria macrophages from healthy control sheep and diseased animals. Secondly to compare the levels of cytokines provided by lamina propria cells from control and disease sheep. Lamina propria cells were isolated from gut tissue and phenotyped using FACS and immunohistochemistry. Preliminary results show increased numbers of VPM 65 (ovine macrophage monoclonal antibody) - reactive cells in diseased gut. However cell surface expression of this and some other markers examined appears not to be significantly altered. Cytokine message levels were determined by PCR on intestinal mucosa using oligonucleotide primers specific for ovine TNF-alpha and LI-1. Preliminary results from agarose gel analysis show increased signals for both cytokines in diseased compared with control tissue. Increased levels of TNF-alpha and IL-1 NRNA in the lamina propria suggest that these cytokines may be important mediators in the pathogenesis of paratuberculosis. Further studies to localize cytokine production are in progress.

Title Attenuation of tumor necrosis factor and interleukin-6 activities in cattle infected with Mycobacterium paratuberculosis.

Author(s) Stabel JR.

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

Abstract Little is known about regulatory mechanisms of immunity in cattle infected with *M. paratuberculosis*. Although *M. paratuberculosis* activates both humoral and cell-mediated immunity, chronic states of infection are associated with this disease. This appears to be due to the ability of this intracellular pathogen to survive and replicate within resident macrophages of infected hosts. Acquired resistance to mycobacterial infection is thought to be mediated by activation of macrophage microbicidal activity through sensitized T cells. Since T cells can stimulate bacteriocidal and/or bacteriostatic activity in macrophages via secretion of various cytokines, it is likely that this activation pathway is arrested in some manner during mycobacterial infection. We have evaluated the effects of subclinical and clinical Johne's disease in cattle on transcription of TNF in vitro. Peripheral blood mononuclear cells from noninfected control cows subclinically (low/intermittent shedders) and clinically (high/chronic shedders) infected animals were incubated in RPMI-1640 medium with 10 mcg concanavalin A/ml and 10 ng phorbol myristate acetate (stimulated) or medium alone (nonstimulated) for 18 hours. Total RNA was extracted from cultured cells by guanidium isothiocyanate lysis, slot-blotted onto nitrocellulose membrane and probed with a digoxigenin labeled-mouse TNF cDNA insert followed by a digoxigenin labeled-chicken beta-actin cDNA insert. Tumor necrosis factor mRNA expression was significantly reduced in both nonstimulated (28%) and stimulated 48-57% cell cultures from clinically infected cows compared to subclinical and noninfected control animals. In addition, expression of TNF mRNA in cells isolated from clinical cows was significantly lower for cells stimulated in vitro with ConA/PMA compared to nonstimulated cell cultures. Serum TNF measured in samples taken at the same time cell isolations were performed was undetectable regardless of infection status. In contrast, serum IL-6 was negatively correlated with level of infection with lower serum IL-6 observed in Johne's infected cows compared to noninfected controls. These results indicate that TNF transcription is reduced during clinical disease suggesting that a corresponding decrease in translation of TNF could be causative factor in the loss of protective immunity at this stage of infection. Alternatively, a reduction in the level of TNF transcription could be protective mechanism invoked by cells to safeguard against the toxic effects of TNF during periods of chronic inflammation.

Title Temporal changes in the number of alpha/beta (CD4+ and CD8+) and gamma peripheral blood lymphocytes in normal and experimentally M. paratuberculosis-infected Holstein calves

Author(s) Zhao BY, Collins MT.

Institution Dept Pathobiological Sciences, Univ Wisconsin, Madison WI 53706.

Abstract The relative percentage and absolute numbers of peripheral blood mononuclear cells (PBMC) bearing CD4, CD8, or gamma T lymphocyte receptors were investigated in calves with experimentally induced paratuberculosis. Five female Holstein calves were orally challenged with 10^8 *M. paratuberculosis* at the age of 4 weeks. Four breed, age, and sex-matched calves served as controls. Every 4 weeks to 20 months post-infection, PBMCs were isolated, labeled with commercial monoclonal antibodies against the CD3, CD4, CD8, or gamma T cell receptors, stained using a second fluorescein-labeled antibody, and analyzed by flow cytometry. For each calf at each sampling, a total white blood cell count and differential were performed in order to calculate absolute numbers of each T cell subset from the flow cytometry data. The 5 calves orally challenged with M. paratuberculosis were confirmed infected by culture or surgically obtained ileum and regional lymph nodes. A linear increase in number of CD4+ T cells from 627/mcl to 2828/mcl occurred in both control and infected calves from ages 4 to 14 months. CD8+ T cell numbers varied considerably between sample dates but were relatively constant over the study period (576 ± 100/mcl). The number of gamma T cells in control calves increased from 1420 ± 395/mcl to 3020 ± 747/mcl from month 4 to month 10 of the study, and then declined to levels comparable to the controls. The number of gamma T cells in the infected calves remained constant and significantly lower than in control calves (1208 ± 386/mcl). Peripheral blood neutrophil counts were significantly lower in the *M.*

ected calves over approximately the same time period. The *M. paratuberculosis* infection appears to have caused a decrease in number of peripheral blood neutrophils and gamma T cells. Mechanisms for this observation are not known, but could include recruitment of the affected cells to the site of infection, direct cytotoxic effect of *M. paratuberculosis* products on these cell populations, or indirect effects of cytokines induced by the infection on bovine neutrophils and gamma T cells.

Title A simple, cost-effective radiometric method to quantify Mycobacterium paratuberculosis from in vitro infected bovine monocytes and macrophages.

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

Institution Dept Pathobiological Sciences, Univ Wisconsin, Madison, WI 53706-1102.

Abstract Mechanisms of intracellular survival of *M. paratuberculosis* (*M. ptb*) in macrophages are poorly understood and have been difficult to study for lack of precise methods to quantify viable *M. ptb*. Conventional plate counting gives low viable counts due to clumping of the organism and plates often become contaminated or dehydrated during the prolonged incubation required. Microscopic counts of infected macrophages on coverslips are tedious, time consuming, affected by clumping and can not distinguish viable from nonviable *M. ptb*. We previously described a radiometric (BACTEC) method for quantification of slow growing mycobacteria (Appl. Env. Microbiol. 54:910,1988). In the present study, we demonstrate application of this technique to the study of ingestion and intracellular killing of *M. ptb* by bovine monocytes and monocyte-derived macrophages (Mo). Freshly isolated monocytes were infected with *M. ptb* (10:1 organism to cell ratio). After 2 h, and 4, 8, 12, and 16 days of infection, the monolayers were lysed with SDS. The lysates were inoculated into BACTEC 12B vials supplemented with mycobactin. Growth was monitored every two days on a BACTEC 460 and cumulative growth index (CGI) readings were calculated. CGI values were translated into viable *M. ptb* counts on the basis of a standard curve. Results were obtained in 20 days and at less cost in labor and materials than for other counting methods. *M. ptb* counts by microscopic examination of stained coverslips of infected Mo monolayers and by BACTEC quantification of *M. ptb* from Mo lysates were compared. Over 90% of maximal *M. ptb* uptake by Mo occurred within 20 min. The effects of cytokines and several other factors affecting the number of viable *M. ptb* numbers of Mo will be reported.

Title Iron-containing compounds alter expression of cell wall proteins in enteric mycobacteria and offer potential alternative strategies for inhibiting intracellular growth and replication.

Author(s) Lambrecht RS, Collins MT, Mjanger AK.

Institution University of Wisconsin-Milwaukee, University of Wisconsin, Madison, USA.

Abstract Like most bacteria, the enteric mycobacterial pathogens, *M. paratuberculosis* and *M. avium*, require iron for growth and replication. Virtually no iron is available in mammalian tissues as it is complexed by host iron-binding proteins (IBP's) such as transferrin, lactoferrin and ferritin. Most enteric bacteria overcome iron deprivation in the host environment either by synthesizing high affinity iron-chelating/uptake systems or by directly binding specific IPB's. We sought to examine the influence of iron-containing compounds on mycobacterial growth. In a chemically defined medium, growth and replication of clinical isolates of mycobacteria derived from intestinal tissues were shown to be regulated by controlling the amount of iron either in the form of iron salts or bound to specific host IBP's at various levels of saturation. All mycobacterial strains ceased growth under conditions devoid completely of iron. Intracellular growth of mycobacteria, using the J774 murine peritoneal macrophage-like host cell line, was also influenced by iron availability. Mycobacteria-infected macrophages demonstrated increased uptake of ferric-transferrin by immunohistochemical staining which was accompanied by increased burdens of mycobacteria observed visually and confirmed quantitatively. Selected strains were cultivated under conditions of iron-deprivation and iron-repleteness, and cell envelope fraction examined for variations in expression of proteins. Up to 8 different iron-regulated envelope proteins (IREP's) were demonstrated in SDS-PAGE preparations and are being examined further for iron acquisition/transport roles. We conclude that iron plays an important role in intestinal mycobacteriosis and suggest several alternative strategies for inhibiting mycobacterial growth including: 1) iron-witholding or deprivation approaches, 2) use of cytokines to down-regulate transferrin receptor expression on host cells, and 3) use of antibiotics which enter and kill mycobacteria through iron-uptake systems.

Title Identification of an immunogenic 40kDa protein that may be specific for some strains of M. avium.

Author(s) Inglis NF, Davies RC, Stevenson K, Nyange J, Burrells C, Sharp JM.

Institution Moredun Research Institute, 408 Gilmerton Road, Edinburgh EH17 7JH, Scotland.

Abstract Members of the *M. avium* complex are over 95% homologous and differentiation between them is notoriously slow and difficult. Particular difficulties are encountered in discriminating *M. paratuberculosis* from other mycobactin-dependent members of the group eg wood pigeon bacillus (syn. M. a. silvaticum). We have identified a 40kDa protein that appears to be expressed in IS901/902+ strains of *Mycobacterium avium* but not in *Mycobacterium paratuberculosis*. The amino terminal twenty amino acids of the protein share no significant homology with any proteins currently available in the OWL database. The protein stimulates strong cell-mediated and humoral immune responses in sheep and deer. Such responses are observed only in animals infected with IS901/902+ strains of *M. avium*. The gene encoding the 40kDa protein has been isolated from a gtll expression library of *M. avium* and is currently being characterized.

Title Isolation and characterization of a serine protease from Mycobacterium paratuberculosis.

Author(s) Cameron RN, Inglis NF, Klausen J, Sharp JM.

Institution Moredun Research Institute, Edinburgh, NVL, Copenhagen, Denmark.

Abstract A lambda gtll genomic library of a deer isolate of *M. paratuberculosis* was screened with serum from a sheep with Johne's disease. A number of clones were identified, two of which

were selected for further study after being found to contain overlapping fragments of the same gene. Antibody elusion studies showed these clones to correspond to a 34kDa protein in *M. paratuberculosis*. Both clones have been fully sequenced and were found to contain an open reading frame of 1083bp, translating to a protein of 361 amino acids. The sequence also contained a possible Shine Dalgarno sequence, start codon, and appeared to code for a signal peptide of 39 amino acids, and a pro-peptide of 15 amino acids. Although the amino acid sequence showed no great overall homology to any previously described protein in the EMBL database, it did contain a motif (GDSGG) corresponding to the active serine residue in a chymotrypsin-like serine protease, in which His57 and Ser195 are brought together to form the three dimensional active site of the protease. A similar organization can be found in the *M. paratuberculosis* 34kDa protein (His102 asp133 Ser215). These three sites, however, show a greater homology (over 70%) to those detected in the HtrA proteins of *Escherichia coli* (His131 Asp161 Ser136). *Salmonella typhimurium* (His132 Asp162 Ser137), and *Brucella abortus* (His 152 Asp182 Ser257), than to the sites in chymotrypsin. These HtrA proteins are thought to be serine proteases, and these three homologous sites may form the three dimensional active site of the enzyme. The native 34kDa protein has been affinity-purified using monoclonal antibodies, and the N terminal sequence of the protein matched the deduced amino acid sequence of the two genomic clones. Experiments are underway to determine any proteolytic activity of the native protein.

Title Monoclonal antibody against a Mycobacterium paratuberculosis antigen cross-reactive with membrane leukocyte antigen in various species.

Author(s) Moreno A, Pintado CO, Perez de la Lastra J, Molina A, Friend M, Llanes D.

Institution Departamento de Genetica. Facultad de Veterinaria. Universidad de Cordoba. Spain.

Abstract Polyclonal and monoclonal antibodies against mycobacteria are frequently reactive with heat shock protein (hs60, Ubiquitin,..). These proteins present in all organism, have been implicated in autoimmune disease. Here we describe a monoclonal antibody (MF.D4B6) that recognizes an epitope on a 25/45 kDa antigen on *Mycobacterium paratuberculosis*. The reactivity of MF.D4B6 antibody was studied by Western-blot on different bacterial species such as Mycobacterium phlei, *Escherichia coli*, *Bacillus subtilis* and PPA-3, a commercial antigen from *Mycobacterium avium*. In all of them, the epitope on the 25/45 kDa was detected as well as additional antigens. Furthermore membrane leukocyte antigens from goats and sheep (45 kDa), cattle (65 kDa), swine (52 and 65 kDa) and humans (30 and 60 KDa), were detected by MF.D4B6. Cross-reactive monoclonal antibodies such as MF.D4D6 are useful for studying cross-reactive epitope in the autoimmune process related to mycobacterial disease.

Title A glycolipid compound derived from Mycobacterium avium serovar 2 that inhibits the candidacidal activity of macrophages scavenges reactive oxygen species, but has no effect on nitric oxide production.

Author(s) Hines II ME, Scherer T, Silwany O, Lauredo LT, Wanner A, Stein-Streilein J, Altman NH, Abraham WM.

Institution Univ of Miami, Miami FL, USA.

Abstract A glycolipid compound designated Macrophage Inhibitory Factor A3 (MIF-A3), isolated from *M. avium* serovar 2 (formerly *M. paratuberculosis* strain 18), has been shown to prevent the killing ofCandida albicans by activated bovine peripheral blood macrophages. Because both superoxide (O2-) and nitric oxide (NO) have been implicated in macrophage killing, it is possible that MIF-A3 could inhibit one or both of these systems. The present study tests this hypothesis. In a cell-free system, MIF-A3 significantly inhibited O2- production by xanthine-xanthine oxidase at 200, 400, and 800 mcg/ml concentrations [16.4±2.3%, 15.6±2.2%, and 17.0±3.1% respectively (mean ± SE, n=6-12,$p < 0.05$)], while the 100 mcg/ml concentration (7.5±3.0%) was not significantly different from a control glycolipid (5.7±2.7%). Similar results were obtained when hydrogen peroxide (H2O2) production was measured colorimetrically in sheep alveolar macrophages (2.0 x 106) stimulated with opsonized zymosan

with or without pretreatment with various concentrations of MIF-A3 (100-400 mcg/ml) Zymosan stimulated alveolar macrophages produced 10.3±1.6 mcM H2O2, which was reduced to 5.5 mcM by the addition of 12,500 U/ml catalase. Stimulated alveolar macrophages pretreated with 200 and 400 mcg/ml MIF-A3 produced significantly less H2O2 [6.8±0.6 and 5.7±0.7 M, respectively (mean ± SE, n = 4-8, P < 0.050], while h2O2 production in alveolar macrophages pretreated with 100 mcg/ml was not significantly different (8.6±0.4 mcM). Phagocytosis as determined by counting zymosan particles within alveolar macrophages was unaffected. In addition, MIF-A3 caused a concentration dependent reduction of measurable H2O2 when incubated directly with exogenous H2O2. In contrast, NO production in murine C57BL/6 thioglycolate-elicited peritoneal macrophages (measured colorimetrically) after pretreatment with various concentrations of MIF-A3 (100-400 mcg/ml) and stimulation with lipopolysaccharide and -interferon was not significantly altered (n=3). These findings suggest that MIF-A3 acts as an oxygen radical scavenger which may be important in intracellular survival of mycobacteria.

Title Isolation of DNA from Mycobacterium paratuberculosis by Matrix Solid Phase Dispersion (MSPD).

Author(s) Hines II ME, Barker SA, Snider TG III.

Institution Univ of Miami, Miami, FL, USA.

Abstract A new method for the lysis and subsequent isolation of nucleic acids from *Mycobacterium paratuberculosis* is described using the technique of matrix solid-phage dispersion (MSPD). An *E. coli* strain containing a plasmid pUC19 was similarly treated for comparison and to show plasmid DNA could also be obtained. Bacteria were blended with octadecylsilyl (C18) derivatized silica to obtain complete cellular lysis. The blended material was used to prepare a column, which was eluted with various solvents and Tris-EDTA buffer (TE buffer). After subsequent purification by phenol-chloroform extraction and polyethylene glycol (PEG) precipitation, isolation of cellular nucleic acids was confirmed by spectrophotometric analysis and ethidium-bromide agarose gel electrophoresis. The highest yield of DNA was obtained when the nucleic acids were eluted with TE buffer alone, which was equally effective in recovering plasmid DNA from *E. coli* as the commonly used alkaline-lysis technique. Since the nucleic acids were effectively recovered with one solvent, the use of a column became unnecessary. Restriction endonuclease digestion of the DNA obtained was performed to show the applicability of the MSPD technique as a method for the lysis and subsequent isolation of nucleic acids from mycobacteria.

Title A Polymerase chain reaction designed for the detection of both M. paratuberculosis and M.avium subsp silvaticum.

Author(s) Dumonceau JM, DeBeenhouwer H, VanGossum A, Adler M, DeRijk P, Drowart A, Portaels F.

Institution Erasme Hospital, Brussels, Belgium and Institute of Tropical Medicine, Antwerpen, Belgium.

Abstract BACKGROUND: Johne's disease (JD) is due to *M. paratuberculosis*. Nevertheless, 10% of cases could be due to *M. avium* subsp *silvaticum*. Polymerase Chain Reaction (PCR) assays developed for a rapid diagnosis of JD should also detect that mycobacteria. METHOD: Three Insertion sequences (IS) which were showed to be multi-copy elements specific for *M. paratuberculosis* (IS900), *M. avium* (IS901) and *M. avium* subsp *silvaticum* (IS902) were compared. As 4 highly conserved areas were identified, we designed 2 sets of primers for use in a nested PCR. The area spanned by the inner primers contains recognition sites for restriction enzymes which are different if the PCR product is derived from *M. paratuberculosis* or *M. avium* and *M. avium* subsp *silvaticum*. RESULTS: We tested 36 strains, including 12 *M. paratuberculosis* and *M. avium* subsp *silvaticum*, 8 *M. avium* and 16 other unrelated mycobacterial species. All of the 12 *M. paratuberculosis* and *M. avium* subsp *silvaticum* strains were detected and correctly differentiated by their restriction pattern. Two of the 8 *M. avium* strains and none of the 16 unrelated mycobacterial strains were also found to be positive.

CONCLUSION: We report a single PCR assay for the detection of both *M. paratuberculosis* and *M. avium* subsp *silvaticum* which are then distinguished by restriction of the PCR products obtained.

Title IS900 encodes a second ORF, hed 900 on the -ve strand.

Author(s) Doran TJ, Tizard MLV, Hermon-Taylor J.

Institution Dept Surgery, St. George's Hospital Medical School, London SW17 ORE, UK.

Abstract *M. paratuberculosis* (*M.ptb*) possess the species specific, atypical insertion sequence, IS900 is 1,451bp and was reported to have a single open reading frame (ORF) possessing the translation signals necessary for expression of a protein of 399 amino acids, termed p43. Recently, Murray et al. isolated a promoter sequence, PAN, from *M.ptb*, located proximal to the 3' end of an IS900 element. PAN could drive expression of a second previously unreported ORF from IS900, encoded on the complementary strand to the p43 gene. We have designated this ORF as hed 900. Intriguingly hed 900 also requires sequences adjacent to, and outside IS900 to enable translation of a protein product. In fact, by virtue of site specific insertion into the sequence 5'AAGGNG*(4-6)NCATG3' (where the asterisk denotes the site of insertion), IS900 appears to target putative translation initiation sequences of host genes, thereby providing the necessary regulatory sequences for hed 900 expression. The termination codon for hed 900 is not generated by the target insertion site, and thus varies in position for each IS900 element. The gene products of IS900 represent specific antigens from *M.ptb*. As such, we are undertaking a number of approaches to express and purify Hed 900 in order to establish the potential of this protein as an antigen recognized during *M.ptb* infection in ruminant Johne's disease and in humans during Crohn's disease. Secondly, we are interested in the post-translational effects of hed 900 expression on *M.ptb*. IS900 has been implicated in the increased virulence of *M.ptb* from *Mycobacterium avium* background. A study of IS900 insertion and hed 900 expression may establish a role for this element in this evolutionary process.

Title Purification and characterization of the p43 antigen of M. paratuberculosis encoded on the positive strand of IS900.

Author(s) Tizard M, Sumar N, Griffiths H, Doran T, Hermon-Taylor J.

Institution Dept Surgery, St. George's Hospital Medical School, Tooting, London SW17 ORE, UK.

Abstract The p43 antigen encoded by the positive strand of IS900 has been shown to be expressed in a processed 29kDa form in *M. paratuberculosis*. Since the initial identification of P43 a number of recombinant forms of the protein have been produced in *E. coli*. p43 is a highly species specific antigen and the aim of this work was to produce sufficient purified material to evaluate its potential as an immunodiagnostic reagent for *M. paratuberculosis* in Johne's disease and human Crohn's disease. Initially a good purification of p43 was achieved by DNA-cellulose affinity chromatography. Though the yield was 100-200 *g and purity 90% the method was inapplicable due to degradation of the DNA matrix by residual nuclease activity. Both hydrophobic interaction and ion exchange chromatography were employed as upstream processes to remove this nuclease activity. However p43 was found to bind very tightly to all of these matrices and could only be eluted in denaturing buffers incompatible with DNA-cellulose chromatography. Successful purification has been achieved by engineering the coding sequence and adding 10 histidine residues to the amino, or carboxy, terminus of the protein. The decahistidine tailed forms of p43 have been recovered at 90% purity, with yields of approximately 10 mg/l of bacterial culture, in a single step using immobilized Ni2+ affinity chromatography in the presence of 8 M urea. Downstream processing of this material by preparative SDS PAGE chromatography, using the Bio Rad Prep Cell system, has yielded material >99% pure (by SDS PAGE analysis). We have generated mg quantities of essentially pure p43 which is currently being evaluated for application in immunodiagnosis of ruminant and human *M. paratuberculosis* infection. This material should be suitable for biological assay including ELISA applications, lymphocyte transformation assays, skin testing in animals

and possibly vaccination.

Title Epitope mapping of IS900 +ve strand p43 using sera from humans with chronic enteritis Crohn's disease (CD).

Author(s) Sumar N, Tizard MLV, Austen BM, Hermon-Taylor J.

Institution Dept of Surgery, St. George's Hospital Medical School, London SW17 ORE, UK.

Abstract Eighty overlapping alpha-N biotinylated 15 residue synthetic peptides were obtained spanning the entire p43 sequence. Each peptide was bound 7.5 mcg/ml to duplicate streptavidin-coated wells of microtiter plates with streptavidin-only and conjugate-only controls. 100 mcl of human serum diluted 1:40 with PBS containing protease inhibitors was added to the wells and incubated for 1h. After washing, plates were developed using rabbit anti-human Ig-HRP conjugate followed by ABTS substrate. Plates were read at 405nm on an Anthos automated plate reader connected to a computer. A program was written which expressed the duplicate mean optical density (OD) for immunorecognition of each peptide by CD sera, against mean OD + 2SD for that peptide using normal sera. The resulting profile immediately demonstrates significant antibody binding by CD sera to specific sequence determinants within p43. Sera from 8 of 12 (75%) normal subjects showed weak antibody binding to one or more of 4 domains, peptides 22-24, 34-37, 40-43 and 64-67. Sera from all 9 (100%) CD patients so far tested demonstrated significant antibody binding (>mean normal + 2SD) to one or more of 4 different domains, peptides 28-32, 54-58, 71-73 and 74-80. Mean recognition of the major carboxyterminal epitope on p43 by CD sera was particularly strong exceeding mean normal +2 SD >twofold. IS900 has proved uniquely specific for *M. paratuberculosis* (*M.ptb*). The present findings are consistent with the concept that most of the human population has been exposed to *M.ptb*, and further implicate this organism as a chronic enteric pathogen in humans. A change in specific immune recognition of *M.ptb* may accompany the switch from benign intestinal cohabitation to disease state.

Title DNA fingerprinting as a tool for epidemiological studies of paratuberculosis in ruminants and Crohn's disease.

Author(s) Pavlík I, Bejckova L, Fixa B[1].

Institution Vet Res Inst, Brno, Czech Republic, [1]2nd Dept Med, Charles Univ, Hradec Kralove, Czech Republic.

Abstract DNA, isolated from more than 100 mycobactin-dependent strains of *Mycobacterium paratuberculosis*, was digested with restriction endonuclease Pst I and hybridized with the fragment IS900. The strains were isolated from more than 80 domestic ruminants (cattle, sheep and goats) in 30 herds and 9 countries, and from patients suffering from Crohn's disease (strain Linda, U.S.A., and Fryba, Czech Republic). Three types of strains, tentatively designated A (bovine, ovine and Linda), B (bovine, ovine and Fryba) and C (ovine and caprine) were identified by RFLP. Differences in RFLP patterns were found between the strains isolated from mesenteric lymph nodes and from intestinal mucosa of one cow and one sheep. A single type was identified in cattle and sheep kept in one farm. All the three RFLP types were found in a mixed sheep-and-goat farm in the Czech Republic with frequent purchases and sales of animals. A within-region dominance of a single type was observed in strains isolated in the Czech and the Slovak Republics. The strain Fryba belonged to the RFLP type identified in strains isolated in the same region from more than 10 herds of cattle and sheep. Type B strains were isolated in 1993 from a herd imported in 1990 and kept in a strict separation, while only the type A was identified in the remaining cattle and sheep herds in the same region during the past 6 years.

Title Complete nucleotide sequence of a gene encoding the bacterioferritin subunit of Mycobacterium avium subspecies silvaticum.

Author(s) Inglis NF, Stevenson K, Hosie AHF, Sharp JM.

Institution United Kingdom

Abstract A gene encoding the bacterioferritin of *M. avium* ss. *silvaticum* has been cloned, sequenced and expressed. Translation of a 477 bp open reading frame revealed a deduced sequence of 159 amino acid residues which was shown to share up to 92% homology with the bacterioferritins of five bacterial genera. The recombinant bacterioferritin exhibits serological cross-reactivity with *M. avium* ss. paratuberculosis and *M. avium* ss. *silvaticum* and a protein of 20-22 kDa in sonicates of M. leprae.

Title Mycobacterium avium complex isolated from swine doesn't possess IS901.

Author(s) Nishimori K, Eguchi M, Nakaoka Y, Onodera Y, Tanaka K.

Institution Hokkaido Branch, Nat Inst of Anim Hlth, Hitsujigaoka-4, Sapporo, Japan, :Tokachi Livestock Hyg Serv Cent, Kawanishi, Obihiro, Japan, :Kamikawa Livestock Hyg Serv Cent, Higashitakasu, Asahikawa, Japan.

Abstract The presence of mycobacterial insertion sequence IS901 in strains of mycobacteria, mainly *Mycobacterium avium* complex, isolated from swine was examined with PCR using IS901 specific primers (1776bp products) and primers specific for IS901 insertion sites (300bp or 1776bp products), that were descried by Kunze et. al. Four kinds of DNA extraction methods for the PCR were compared. InstaGene matrix (Bio-Rad) allowed faster and easier preparation of PCR amplifiable DNA than the NaOH-heating extraction and the SDS extraction of pretreated mycobacteria, and it provided more effective protection against the destruction of DNA than the boiling extraction. Four in 5 reference strains of serovar 1 to 3 possessed IS901. Reference strains of serovar 4, 5, 6, 8, 9, 10, 11, and 21 possessed only IS901 insertion sites. Other reference strains of serovar 7, 12, 13, 14, 15, 16, 17, 19, and 20 possessed the novel sequence (about 1070bp). All of 110 Strains of *M. avium* complex isolated from swine didn't possess IS901. It was suggested that no possession of IS901 was characteristic of swine-derived strains of *M. avium* complex.

Title Detection of specific T-cell reactivity in sheep infected with Mycobacterium avium subspecies silvaticum and paratuberculosis using two defined mycobacterial antigens.

Author(s) Burrells C, Inglis NF, Davies RC, Sharp JM.

Institution Moredun Research Institute, 408 Gilmerton Road, Edinburgh.

Abstract Two antigens from *Mycobacterium avium* subsp. paratuberculosis (M. a.paratuberculosis) and *Mycobacterium avium* subsp. *silvaticum* (M. a.*silvaticum*) were employed in two different assays to measure the cell-mediated immune reactivity of ovine peripheral blood lymphocytes. Using a standard lymphocyte stimulation assay, proliferative responses to purified protein derivative (PPD) of *M. a.paratuberculosis* were detected but did not discriminate a) between immunized and infected animals or b) between animals infected with *M. a.paratuberculosis* and those infected with M. a.*silvaticum*. In contrast, with defined antigens, responses to a purified 30 kDA alpha antigen from M. a.paratuberculosis were observed only with lymphocytes from sheep inoculated with live organisms whereas a P40 protein from *M. a.silvaticum* elicited in vitro responses only with lymphocytes derived from sheep inoculated with live *M. a.silvaticum*. When a sandwich enzyme-linked immunosorbent assay was used to detect the release of interferon-gamma, responses were observed when lymphocytes from animals given live mycobacteria were cultured in the presence of a 30 kDa antigen. With lymphocytes from immunized animals responses were only slight. P40-induced release of interferon-gamma was observed only with lymphocytes from animals given live *M. a.silvaticum*. Lymphocytes from 2 sheep serving as negative controls did not respond to any of the antigens in either of the assays.

Title Patterns of gene expression in Mycobacterium paratuberculosis (M.ptb) and other mycobacterial species in response to stress and intracellular survival.

Author(s) Richardson NGB, Tizard MLV, Ford J, Butcher PD, Hermon-Taylor J.

Institution Dept Surgery and Medical Microbiology. St. George's Hospital Medical School, London SW17 ORE, UK.

Abstract In vivo labeling of proteins using 35S-methionine followed by one or two dimensional analysis is a convenient method for studying the pattern of gene expression in *M.ptb* and other mycobacteria under a variety of controlled environmental conditions in the laboratory. *M.ptb* strain Dominic, a human disease isolate of *M. avium* and M. bovis BCG, were cultured in Dubos medium, supplemented with mycobactin in the case of *M.ptb*. Organisms in the mid log phase of growth were transferred to RPMI 1640 methionine deficient medium in the presence of 35S-met and incubated for 2h at 37°C or 45°C heat shock. Cells were harvested, washed in Tris buffer and lysed by beating with < 100 mcm glass beads for 5 min. After boiling, proteins were separated on 10% SDS-PAGE, the gels dried and autoradiographed. In other studies the mycobacteria were grown intracellularly in THP-1 human macrophages for 24h prior to 35S-met labeling. After lysis of the macrophages, labeled mycobacteria were harvested by centrifugation and the pattern of 35S-met mycobacterial proteins obtained as described. The profile of labeled proteins from all three mycobacteria in conventional culture and their responses to heat shock were remarkably similar. Substantial differences were however observed in the pattern of labeled proteins when the organisms were cultured in THP-1 macrophages. These methods are applicable to the study of adaptation in mycobacteria in response to the intracellular environment.

Title Identification of Mycobacterium paratuberculosis by high pressure liquid chromatography (HPLC) analysis of mycolic acid extracts.

Author(s) Collins MT, Glickman SE, Kilburn JO.

Institution Univ Wisc, Madison, WI and Div Bacterial and Mycotic Dis, Centers for Disease Control Prevention, Atlanta, GA.

Abstract *Mycobacterium paratuberculosis* is indistinguishable from *M. avium* by 16s rRNA sequence analysis. Consequently, the commercial rRNA probe (AccuProbe for *M. avium* complex, GenProbe, San Diego, CA) can not distinguish between the two organisms. DNA homology with *M. avium* is roughly 99%. Phenotypically, *M. paratuberculosis* is much slower growing, dependent on mycobactin for in vitro cultivation and capable of causing a chronic inflammatory bowel disease in ruminants known as Johne's disease, while *M. avium* is essentially avirulent for nonimmunocompromised mammals. *Mycobacterium paratuberculosis* has also been found in human patients with Crohn's disease. Genetically, the only way to distinguish *M. paratuberculosis* from *M. avium* is to test for the presence of the insertion element IS900. A PCR-amplified DNA probe for IS900 is commercially available (IDEXX Laboratories, Inc. Westbrook, ME). To determine if HPLC analysis of mycolic acid extracts could identify *M. paratuberculosis*, over 15 isolates of the organism were subjected to HPLC as previously described for identification of mycobacteria. Initially the computer program could not distinguish the chromatogram of *M. paratuberculosis* from that of *M. intracellulare*. However, visual inspection of the HPLC pattern revealed subtle differences. Once the HPLC pattern of *M. paratuberculosis* was entered into the pattern recognition library, the system successfully distinguished *M. paratuberculosis* from *M. intracellulare* and *M. avium* grown on the same culture medium. HPLC analysis may provide a more rapid and cost-effective means of identifying *M. paratuberculosis* than use of genetic probes.

Title Utilization of iron by pathogenic mycobacteria.

Author(s) Kemsell KE, Tizard MLV, Hermon-Taylor J.

Institution Dept Surgery, St. George's Hospital Medical School, London, SW17 ORE, UK.

Abstract Mycobacterial pathogens are in the main, intracellular and proliferate within immune mononuclear cells such as monocytes. In addition to surviving killing by the bactericidal

mechanisms of these cells, intracellular parasites must compete for essential nutrients. One of the most important of these is ferric iron, the availability of which is severely restricted by the host. Pathogens have evolved a variety of mechanisms for obtaining iron from host proteins such as the use of siderophores. Mechanisms of iron utilization by pathogenic mycobacteria, including M. paratuberculosis, from a variety of eucaryotic iron sources were investigated by growth in supplemented Dubos medium. Laboratory M. paratuberculosis strains were capable of growth in the absence of mycobactin. Growth was not stimulated by addition of various iron sources except microperoxidase (heme) to a small extent. Other factors such as fatty acids are thought to be more essential for growth stimulation, even under conditions of iron limitation. Ovotransferrin and inorganic ferric iron were found to be strongly stimulatory for growth of avian derived *M. avium*.

Title Comparison of cellular and extracellular proteins expressed by various isolates of Mycobacterium paratuberculosis and related mycobacteria.

Author(s) White WB, Whipple DL, Stabel JR, Bolin CA.

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

Abstract A search was initiated for *M. paratuberculosis*-specific proteins which may be useful for the diagnosis of Johne's disease. Protein expression profiles of 10 isolates of *M. paratuberculosis*, *M. avium* 18 (formerly *M. paratuberculosis* 18), and one isolate each of *M. avium* serotype 2, *M. avium* serotype 8, and M. bovis BCG were compared. SDS-PAGE analysis of [35S] methionine-labeled extracellular proteins revealed variability among the *M. paratuberculosis* isolates. SDS-PAGE analysis of [35S] methionine-labeled cellular proteins divided the *M. paratuberculosis* isolates into 2 groups based on a difference in the level of expression of a 28,000-dalton protein. Protein expression profiles of the *M. paratuberculosis* and *M. avium* isolates were similar. However, 2-dimensional gel analysis of [35S] methionine-labeled cellular proteins resolved 4 proteins, with molecular *Mas*ses of 28,000, 32,000-, 32,000-, and 42,000-daltons, which were expressed at a higher level in *M. paratuberculosis* than in *M. avium*. Two proteins, with molecular *Mas*ses of 43,000- and 60,000-daltons, were expressed at a higher level in *M. avium* than in *M. paratuberculosis*. Western (Immuno) blot analysis, using antiserum from a cow clinically infected with *M. paratuberculosis* as the primary antibody, suggested that the 42,000-dalton protein may be specific for *M. paratuberculosis*. A lambda gt11 expression library and a lambda DASH genomic library of *M. paratuberculosis* DNA were constructed to facilitate cloning of the gene encoding the 42,000-dalton protein and other genes encoding proteins with diagnostic potential.

Title Distribution of insertion sequence IS901 and a 40 kD protein in Mycobacterium avium strains.

Author(s) Ahrens P, Giese SB, Klausen J, Inglis N, Fuursted K.

Institution Nat Vet Lab, Copenhagen, Denmark, Moredun Res Inst, Edinburgh, UK, Statens Serum Institut, Copenhagen, Denmark.

Abstract *Mycobacterium avium* infects a number of animal species and have become the most common disseminated bacterial infection in AIDS patients. The bacillus is also found in the environment. Using PCR specific to IS901, and ELISA based on monoclonal antibodies specific for p40 as well as serotyping, strains from different sources were examined for the distribution of these markers. IS901 and p40 were found concomitantly in all of the examined strains. These two markers were found in "wood pigeon" isolates as well as in *M. avium* strains isolated from animals (found in most serotype 2 isolates and in some serotype 1 and serotype 3 isolates). The two markers were not found in *M. paratuberculosis* isolates. Approximately 50% of the isolates from animals contained the two markers. The markers were not identified in strains isolated from the environment. The distribution of serotypes and markers differed in human and animal isolates, suggesting that animals are not the source of human infection. Marker positive and marker negative strains were isolated from animals with approximately equal frequency and the strains could not be distinguished by cultivation or by the pathological changes caused by

the bacteria. This could indicate that the two markers are not directly related to virulence. Though the two markers seems to have substantial potential to differentiate between strains of the *M. avium* complex, the presence of the markers was found inconsistent with the present grouping of mycobacteria.

Title Recombinant clone expressing Mycobacteria paratuberculosis antigen recognized by antibodies from Crohn's disease patients.

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

Institution VAMC and Baylor College of Medicine, Houston, TX, USA.

Abstract Crohn's disease is an inflammatory bowel disease of unknown etiology that shares clinical and histological features with mycobacterial infections. Recent data using improved culture techniques and specific PCR-based assays have strengthened the association of *M. paratuberculosis* with Crohn's disease. To provide more direct evidence of an etiological association, we studied the relationship between *M. paratuberculosis* and the immune system at the humoral immune response level. We used sera from patients with Crohn's disease, tuberculosis, leprosy, ulcerative colitis, sarcoidosis, and non-inflammatory bowel disease controls (patients with gastric and duodenal ulcer) in SDS-immunoblots of M. para sonicate and recombinant clones selected from an *M. paratuberculosis* expression library to study the humoral immune response to M. para antigens. Both Crohn's disease and control sera reacted with *M. paratuberculosis* sonicate without an identifiable specific pattern. However, sera from 8 of 10 Crohn's disease, 3 of 4 tuberculosis, all 6 leprosy and 5 sarcoidosis patients reacted with a recombinant clone expresses a 38K protein (p38). Only 4 of 10 ulcerative colitis and 4 of the 15 controls reacted with p38. The difference between reactivity between Crohn's disease and ulcerative colitis, controls or both was significant ($p < 0.05$). The fact that sera from Crohn's disease patients reacted with the same high frequency as the sera with known mycobacterial infection is further evidence to support the hypothesis of the mycobacterial etiology in Crohn's disease and perhaps sarcoidosis.

Title Occurrence in Crohn's disease of antibodies directed against a species-specific recombinant polypeptide of Mycobacterium paratuberculosis.

Author(s) Vannuffel P, Dieterich C, Naerhuyzen B, Gilot P, Coene M, Fiasse R, Cocito C.

Institution Univ of Louvain Medical School, Brussels, Belgium. Machtelinckx L. Innogenetics, Ghent, Belgium.

Abstract Clinical symptoms of Crohn's disease, a chronic human enteritis of undefined etiology, mimic those of Johne's disease of ruminants, which is induced by *Mycobacterium paratuberculosis*, hence the hypothesis of a common mycobacterial etiology. Recently, a species-specific polypeptide (a362) of *M. paratuberculosis* was obtained by genetic engineering technology from an immunodominant protein of the *M. paratuberculosis* A36 complex. Sensitivity and specificity of a362 prompted its use in an enzyme-linked immunosorbent assay (ELISA) for paratuberculosis. This assay correctly diagnosed the tested blood samples from infected cattle[1]. In the present study, such an a362-based ELISA was used to compare the serological response of Crohn's patients with healthy controls. The a362 polypeptide was used for serological analysis of 50 healthy subjects and 73 Crohn's patients. Healthy subjects were blood donors from the Belgian Red Cross Organization and Crohn's patients were diagnosed at the Saint Luc University Clinics by conventional clinical, radiological, endoscopic and histological criteria. Statistical comparison of ELISA-data from the two populations was made using the nonparametric Mann-Whitney U test. Crohn's patients had significantly higher IgG ($P < 0.05$) and IgA ($P < 0.001$) titers than controls. Anti-a362 IgA titers were then analyzed using a mixture population modeling[2]. A monomodal Gaussian distribution of control values, compatible with a single population, was observed; whereas the bimodal distribution of Crohn's patients sera supported the occurrence of two populations. In 64% of Crohn's patients, the anti-a362 IgA level was comparable to that of controls whereas titers were significantly higher in 36% of the patients. Several hypothesis can be proposed to explain the occurrence of two Crohn's subpopulations. In the case of patients with high anti-a362 IgA titers, *M. paratuberculosis* may be the etiological agent. In other cases, mycobacteria, such as *M. kansasii* or *M. tuberculosis*, might be responsible for some of the 64% Crohn's cases diagnosed negative by the a362 test, which is species-specific. On the other hand, the level of anti-*M. paratuberculosis* is possibly low in some steps of the disease, as it is the case for tuberculoid leprosy or primary tuberculosis. Finally, some Crohn's disease patients may perhaps

recognize mycobacterial B-cell epitopes different from those present in the a362 polypeptide, on which our assay is based. The use of the species-specific a362 assay would lower the high background level of antibody directed against mycobacteria in Crohn's patients (due to the presence in the intestinal tract of different mycobacteria) and restrict the response to specific target. Moreover, IgA should be preferentially involved in such enteropathy as Crohn's disease, and differences with respect to controls are expected to be more evident, whenever a study is restricted to a specific immunoglobulin. [1] J. Clin, Microbiol. 1993, 31:947-954. [2] J. Virol. Meth. 1990, 27:135-144.

Title Mycobacterium paratuberculosis in intestinal tissue from patients with Crohn's disease demonstrated by a nested primer polymerase chain reaction.

Author(s) Lisby G, Andersen J, Engbæk K, Binder V.

Institution Univ of Copenhagen, Herlev Hospital, DK-2730 Herlev, Denmark.

Abstract Background: The etiology of Crohn's disease has so far remained unknown, but the current research has concentrated upon autoimmunity and/or mycobacterial infection. With the appearance of the highly sensitive polymerase chain reaction (PCR), the possibility of detecting nucleic acids from even a few present microorganisms has emerged. Methods: We have used a sensitive variant of the polymerase chain reaction (nested primer PCR) for detection of a multi-copy insertional element (IS900) specific for *Mycobacterium paratuberculosis*. This technique was applied to DNA extracted from fresh as well as paraffin-embedded intestinal tissue obtained from patients undergoing resection of inflamed intestinal tissue due to Crohn's disease. Results: In fresh intestinal tissue from 11 of 24 patients with Crohn's disease, 2 of 10 patients with ulcerative colitis and 3 of 28 patients with other colonic disorders specific *M. paratuberculosis* DNA was found. In paraffin embedded tissue from 4 of 58 patients with Crohn's disease and from none of 72 control patients (55 patients with ulcerative colitis and 17 patients with colonic cancer), specific *M. paratuberculosis* DNA was found. Conclusions: Whether these microorganisms were connected to the inflammatory bowel disease, or their presence were mere coincidence, cannot be stated. We find this presence interesting and encouraging for further investigations.

Title Heat sensitivity of Mycobacterium paratuberculosis in cows' milk at pasteurization temperatures.

Author(s) Grant IR, Ball HJ, Rowe MT.

Institution Queen's Univ of Belfast and Dept of Agriculture for N Ireland, Belfast, Northern Ireland, UK.

Abstract A study was undertaken to determine if *Mycobacterium paratuberculosis* survives pasteurization of milk. Twelve strains of *M. paratuberculosis* of bovine, ovine and caprine origin, were inoculated into aseptically-drawn cow's milk of low initial bacterial count at levels of 10^8 cells ml-1 or 10^4 cells ml-1 and pasteurized in the laboratory by two methods: (1) the standard holder method in which inoculated milk (5 ml) in stoppered tubes was heated to and held at 63.5°C for 30 minutes in a water-bath, exclusive of come-up time; and (2) a High Temperature-Short Time (HTST) method in which inoculated milk (250 ml) was transferred to a Franklin Plate Heat Exchanger situated in a water-bath and heated to and held at 71.7°C for 15 seconds, exclusive of come-up time (equivalent to commercial HTST pasteurization). Additional heating times of 5, 10, 15 and 20 min at 63.5°C were also included to enable the construction of a thermal death curve for the organism at this temperature. Viability after pasteurization was determined by growth on Herrold's egg yolk agar slopes containing mycobactin and in BACTEC Middlebrook 12B radiometric medium supplemented with mycobactin and egg yolk suspension. Both media were incubated at 37°C for up to 18 weeks. Any growth observed was tested for acid-fastness by the Ziehl-Nielsen stain. The thermal death curve obtained for *M. paratuberculosis* was concave in shape exhibiting a rapid initial death rate followed by significant "tailing" which would tend to indicate survival at low levels after pasteurization. In general growth was detected earlier by radiometry than by conventional

lture on Herrold's slopes, and heat-treated *M. paratuberculosis* required a longer incubation period than unheated controls before growth was detected by either method which suggests the existence of sublethally-injured *M. paratuberculosis* following pasteurization. Preliminary results suggest that the organism may not have been completely inactivated by pasteurization at 63.5 and 71.7°C under laboratory conditions. However, the levels of *M. paratuberculosis* employed in this study are unlikely to be encountered in raw milk under normal circumstances and were selected to represent a "worst case" scenario.

Title IS900 PCR testing for Mycobacterium paratuberculosis (M. ptb) in units of whole pasteurized cows milk widely obtained from retail outlets in England and Wales.

Author(s) Millar DS, Ford J, Sanderson JD, Tizard MLV, Kempsell K, Lake RJ, Hermon-Taylor J.

Institution Dept of Surgery, St. George's Hospital Medical School, London SW17 ORE, UK.

Abstract Cartons and bottles of whole pasteurized cows milk were purchased from retail outlets throughout central and Southern England and south Wales month-by-month from 1st September 1991 to 31st March 1993. These were brought into a dedicated unit in the department and processed under experimental conditions which rigorously excluded contamination artifact, monitored by simultaneous process controls. Each container was cleaned with 100% ethanol, a 15 ml aliquot of milk aspirated and centrifuged 41,000xg for 1 hr yielding cream, almost clear whey and pellet fractions. These were separated and made up to the volume of the whey. 500 mcl aliquotes of each fraction were then boiled for 20 min, centrifuged and 5 mcl of supernatant assayed in triplicate by IS900 PCR, with individual PCR negative and positive controls. The lower limit of detection of this system, previously established in spiked samples, lay in the range 500-1000 *M.ptb* organisms per ml. The results showed that coming in surges particularly in Feb/Mar and Sept/Oct in which up to 1/4 of samples were affected, an overall 6.25% (21 of 336) of cartons or bottles tested positive by IS900 PCR. In 18 of 21 positives (86%) the PCR signal was present in cream and/or centrifugal pellet which is where the PCR signal segregates in milk spiked with intact *M.ptb*. 575 liquid culture flasks derived from 19 PCR positive milks, 34 PCR negative milks and multiple buffer and medium-only blanks currently incubated for between 8 and 26 months, await meticulous testing for viable *M.ptb*. The results to date are consistent with an intermittent contamination of pasteurized cows milk supplies by intact *M.ptb*.

Title Seroreactivity of Crohn's patients against paratuberculosis antigens.

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Abstract A role for mycobacteria in Crohn's disease has been suggested. Isolation of *M. paratuberculosis* from Crohn's lesions added further support to this relationship. Several studies have investigated the presence of antibodies against *M. paratuberculosis* in Crohn's patients. While most of these have observed no real differences in the level of antibodies between Crohn's patients and controls, others have reported significant differences. This work was carried out to test that relationship using samples available to us. A total of 197 human sera from two hospitals in the Basque Country, including 66 Crohn's cases, 52 ulcerative colitis cases, 31 active pulmonary tuberculosis cases and 48 healthy controls from blood donors were tested. The sera were absorbed with a suspension of *M. phlei* (1/1), incubated overnight at room temperature and further diluted up to 1/100 with PBS-TG. The sera were subjected to a conventional ELISA against PPA-3 antigen in duplicate wells, using as conjugate rabbit anti-human IgG labeled with horseradish peroxidase. Statistical analysis showed significant differences in mean IgG reactivity between Crohn's and tuberculosis patients and controls and ulcerative colitis patients. Comparison of the frequency of positive results in Crohn's patients (13.6%) and in controls (0.0%) showed significant differences ($p < 0.01$), although the highest proportion of positives was found among the tuberculosis group (32.3%). In comparing our results with previous published studies, we submit that the failure to find differences in some of those studies could potentially be explained by the way the results were interpreted.

tivity among mycobacterial antigens, differences in antigen preparation and testing methodology, changing patterns of sensitization between different populations, and individual variability in the type of disease are known factors influencing the association between humoral immune response and mycobacterial infections that could also account for some contradictory results in studies on serologic responses against mycobacterial antigens in Crohn's disease. Analysis of our results together with all the other published data allowing a positive/negative classification shows a definitely increased reactivity of Crohn's patients against mycobacterial antigens of the *M. avium*-paratuberculosis group ($p < 0.001$). This conclusion lends further support to the hypothesis of a role of mycobacteria in the etiology of this disease.