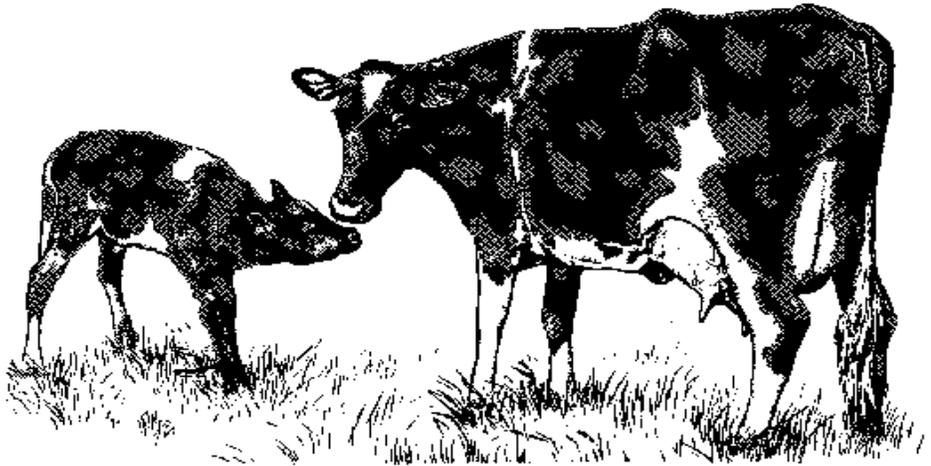


The Paratuberculosis Newsletter - Online



**An Official Publication of the
INTERNATIONAL ASSOCIATION for
PARATUBERCULOSIS
and Other Intestinal Mycobacterioses**

The Paratuberculosis Newsletter – the online edition

The development and expansion of the internet has led to a revolution in how we obtain information. Access to databases such as Pubmed has resulted in scientists obtaining most of their information sitting in front of a computer screen rather than spending time in libraries searching among the dusty rows of journals. The revolution has also resulted in the majority of written communications with scientific colleagues being sent by all but instantaneous email rather than by post. In the bottom of the Southern hemisphere the time has long past where we had to wait for a couple of months for the mail to arrive by boat from Europe or North America. In step with these changing times, the International Association for Paratuberculosis has decided that the Paratuberculosis Newsletter should become an online publication. The reasons for this change include;

- More efficient and rapid distribution of information.
- Greater opportunities for including coloured graphics

The change to an online edition of the Paratuberculosis Newsletter is an opportune time to reassess the format and content of this publication. There is a need to reassess the purpose of the Newsletter and how best it can meet the needs of the members. This can only come about by members making their views known to our Association.

We are only an email away!

The issues concerning Paratuberculosis include -----

- The difficulties of detecting infected animals, especially those with subclinical infections.
- The lack of cost effective control programmes, especially where there are no government subsidies to support disease control schemes.
- The uncertainties concerning the role *M. paratuberculosis* may play as a human pathogen.
- The technical difficulties of carrying out research on this slow growing pathogen.
- The shortage of research funding for Paratuberculosis.

Many of these issues have not changed since the Association was established many years ago. The greatest change has been the increasing concerns relating to what role *M. paratuberculosis* has as a human pathogen. This concern is reflected in the steady increase in the numbers attending the Paratuberculosis Colloquia, with a record attendance at the 7th International Meeting at Bilbao.

Proposed content of the online Newsletter

Information concerning the activities of the International Association for Paratuberculosis, including publications and forthcoming conferences.

Links to existing online information.

Regional initiatives that are not readily available to the wider Paratuberculosis community.

Conference abstracts

Commentary on new landmark research publications on Paratuberculosis.

Geoffrey de Lisle

Editor in Chief

Geoffrey.delisle@agresearch.co.nz

AgResearch

Wallaceville Animal Research Centre

P.O. Box 40-063

Upper Hutt

New Zealand

Message from the President:

January 20, 2004

The pace of scientific work on the slowest growing of pathogens, *M. paratuberculosis* (YES, that is the name I prefer I'm not ashamed to say so), continues to quicken. Genome sequencing is paying dividends in creation of new genetic and antigenic targets for diagnosis and vaccine development (see the recent papers from John Bannantine (7ICP Merkal Award winner) et al. Funding agencies are finally recognizing the importance of *M. paratuberculosis* to animal agriculture and providing more appropriate levels of funding. Even human health agencies are coming forward with research funding.

Our colleague John Hermon-Taylor continues to provide strong evidence indicating that we are working with a zoonotic agent. Another IAP colleague provided a nice review of this issue in *Lancet* last August. Very soon we should learn of results of anti-mycobacterial drug therapy for Crohn's disease from Australian gastroenterologists.

National programs continue to grow and *even get funding*. In 2003 the U.S. Dept. of Agriculture pumped over \$21M into state and national programs for education, expansion of the infrastructure to sustain a national control program, and subsidies to help producers use existing resources for paratuberculosis control. It was the U.S. dairy industry who largely made this level of funding from the U.S. congress possible.

Veterinary practitioners continue to put Johne's disease on the program of their meetings as they strive to keep up with advances on a disease which, for those who graduated from veterinary school before 1980, was considered rather rare and has now become unfortunately all too common. This month will see the birth of an online veterinary continuing education program that provides a series of six training modules on paratuberculosis followed by an on-farm experience with risk assessment and herd management plan design. This helps to bring practitioners up to speed as the pace of discovery about paratuberculosis quickens and will certify practitioners allowing them to get paid for on-farm risk assessments in U.S. cattle herds. Check out this website: <http://vetmedce.org>, get a free user account and look under the courses tab.

The International Association for Paratuberculosis is responding by enhancing its website with the primary goal of enhancing communication among its members. Our hope is to make distribution of our Newsletter easier and faster, to centralize and simplify submission of abstracts for the Colloquia, and to centralize and make membership management easier and more member-friendly.

The 8th International Colloquium on Paratuberculosis is not so very far off. I look forward to seeing you all in Copenhagen, August 2005. Start thinking about abstracts which will be due later in 2004. And, watch the IAP website: <http://paratuberculosis.org> and the charming website site created by our Danish hosts specifically for the 8ICP for more information: <http://www.8icp.dk/>.

Best wishes for a prosperous and productive new year.

Mike Collins, President, IAP
mcollin5@wisc.edu



Visit the website for further details, <http://www.8icp.dk/>



The First International Colloquium on Research in Paratuberculosis, June 16-18, 1983, Ames, Iowa, USA. (27 in photo)



The Seventh International Colloquium on Paratuberculosis, June 12-14, 2002, Bilbao, Spain.

Claus Buergelt, Geoff de Lisle, Finn Saxegaard, Marie-Francois Thorel, Yuichi Yokomizo and Robert Whitlock attended both conferences.

The First ICP, Programme

Welcome to the Paratuberculosis Colloquium and NADC P.A. Berry

Moderator R.S. Merkal

The current status of clinical diagnosis of Johne's disease G.F. Hoffsis

The incidence of paratuberculosis in New England, R.J. Chiodini, H.J. Van Kruiningen

Johne's disease in Wisconsin cattle – a survey in cull cows. J.M. Arnoldi, S. Hurley

An analysis of case reports of *Mycobacterium paratuberculosis* in cattle, sheep and goats at Iowa State University teaching hospital, January 1, 1974 – December 30, 1982, W.J. Owen

The isolation of *Mycobacterium paratuberculosis* from animals in the United States – A 16 – year summary, W.D. Richards, S.K. Harris, J.J. Jarnagin

Observations on the economic impact of Johne's disease (paratuberculosis) on cattle herds in Illinois, T.A. McPherron, C.L. Coglianese, E.H. Jaster, J.W. Urbance, R.E. Mock, C.R. Oetzel, D.F. Parrett

Moderator S. Hurley

The incidence of Johne's disease in cattle and goats in Victoria, Australia, P.J. Coloe, M. Harrison

Diagnosis and control of paratuberculosis, H. Rieman, B. Abbas, Lonnerdal

Comparison of the use of mycobactin J and mycobactin P for the detection of Johne's disease in Australian dairy herds, P.J. Coloe, D. Lightfoot, W. Fromm

Experimental infection of North American wild ruminants with *Mycobacterium paratuberculosis*: Clinical signs and pathology, E.S. Williams, S.P. Synder, J.C. de Martini, K.L. Martin

Moderator A.M. Hintz

Modifications in the techniques for cultivation of *Mycobacterium paratuberculosis*, D.L. Whipple, R.S. Merkal

Comparison between three diagnostic tests for detection of paratuberculosis in goats, C. Ramirez, J. de Lucas

A method for avoid false-positive reactions in an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of bovine paratuberculosis, Y. Yokomizo, H. Yugi, R.S. Merkal.

Comparison of ELISA and gel diffusion precipitin tests for paratuberculosis in cattle, sheep and goats, P.A.S. Lyle, R.S. Merkal

Serological tests for detecting Johne's disease in sheep, G.W. de Lisle, E.P. Wall

Experimental exposure of cattle to *Mycobacterium paratuberculosis*, C.O. Thoen, J.J. Owen, E.M. Himes, M.R. Hall

Some observations on naturally occurring paratuberculosis (Johne's disease) in cattle herds in Nebraska, Colorado, and Minnesota, C.O. Thoen, C.D. Stumpff, D. Tupper, E.M. Himes, T.A. Petersburg

Diagnosis of clinical and non-clinical paratuberculosis by immunological and bacteriological methods, J.B Jorgensen

Moderator **W.D. Richards**

Mycobacterial carbohydrate antigens: a review and some observations on their analysis and preparation in serological tests for paratuberculosis, E.A. Sugden, B. Brooks, A.H. Corner, J.R. Duncan, M.M. Garcia, B. McGuire, R. Michaud, B.S. Samagh, J. Stevens, C. Turcotte.

Crossed immunoelectrophoresis and immunoelectrophoretic blotting – application to antigen and analysis of *M. paratuberculosis* and the development of improved serodiagnostic tests, B. Brooks, B. Brooks, A.H. Corner, J.R. Duncan, M.M. Garcia, B. McGuire, R. Michaud, B.S. Samagh, E.A. Sudgen, J. Stevens, C. Turcotte.

Observations on the application of currently available diagnostic methods (serology, lymphocyte stimulation and culture) to naturally infected domestic ruminants, J.R. Duncan, B. Brooks, A.H. Corner, J.R. Duncan, M.M. Garcia, B. McGuire, R. Michaud, B.S. Samagh, E.A. Sugden, J. Stevens, C. Turcotte.

Diagnosis of clinical and subclinical Johne's disease in goats using the agar gel immunodiffusion (AGID) test, D.M. Sherman, R.J.F. Markham, F. Bates

Diagnosis of clinical Johne's disease in cattle using the agar gel immunodiffusion (AGID) test, D.M. Sherman, R.J.F. Markham, F. Bates

The use of the lymphocyte transformation test in the diagnosis of bovine paratuberculosis, C.D. Buergelt, R.S. Merkal, J.R. Duncan

An evaluation of lymphocyte transformation, enzyme-linked immunoassay and complement-fixation tests for the detection of infected animals in a dairy herd with endemic Johne's disease, C.R. Wilks, A.W.D. Lepper, C.J. McCaughan, M. Kotiw

Indirect immunoperoxidase test for the diagnosis of paratuberculosis, C.D. Buergelt, H.T. Hguyen

Moderator **P.A.S. Lyle**

The possible influence of intracellular iron storage on the pathogenesis of Johne's disease, A.W.D. Lepper, C.R. Wilks

The effect of prolonged elevation of dietary iron on the pathogenesis of Johne's disease in dairy cattle with special reference to changes in copper, phosphorus, and zinc metabolism, and the immune response to antigens of *M. paratuberculosis*, A.W.D. Lepper, C.R. Wilks, C.H. Embury, M. Kotiw

Fatty acids as a means of identifying *Mycobacterium paratuberculosis*, P.J. Coloe, H.F. Slattery, D. Lightfoot

Separation by high performance liquid chromatography of fatty acids from *M. paratuberculosis*, A.M. Craig, R.C. Burdett

In vivo studies of antimicrobial agents against *Mycobacterium paratuberculosis*, A.M. Hinz, R.S. Merkal, D.L. Whipple, P.A.S. Lyle

Johne's disease: a case study with clofazimine therapy in a dairy cow, R.H. Whitlock, T. Divers, J. Palmer, H.M Acland, J. Bruce, E. Tulleners

Paratuberculosis in sheep, cattle, goats and reindeer in Iceland: a result of an import of a flock of sheep from Germany in 1933. The control of the disease. S. Siguroarson, E. Gunnarsson

Moderator **S.K. Harris**

Results of a field evaluation of a whole cell bacterin, S. Hurley, E. Ewing

The effect of vaccination on the excretion of *Mycobacterium paratuberculosis*, J.B. Jorgensen

An unclassified *Mycobacterium* species isolated from patients with Crohn's disease, R.J. Chiodini, H.J. van Kruiningen, W.R. Thayer, R.S. Merkal, J.A. Coutu

Mycobacterium paratuberculosis antibodies in Crohn's disease patients, W.R. Thayer, J.A. Coutu, R.J. Chiodini, R.J. van Kruiningen, R.S. Merkal

Comparative study of mycobactin-dependent strains – pathogenicity for the calf, M.F. Thorel, P. Pardon, J. Marly, K. Irgens, P. Lechopier

Open forum for livestock producers

Moderator **P.A. O’Berry**

Johne’s disease and the producer, T.A. McPherron

Efficacy of bacterins, S.S. Hurley

Diagnostic methods, R.S. Merkal

Related mycobacterial infections, M.F. Thorel, R.J. Chiodini

Moderator **H.W. Moon**

Potential liability of persons selling cattle infected with Johne’s disease, J.K. Matson

Points of interest from the first International Colloquium

- Yuichi Yokomizo presented his pioneering work on an absorbed ELISA. His work was the forerunner to a large number of subsequent studies and the development of commercially available absorbed ELISA kits.
- Rod Chiodini described the isolation of mycobactin dependent *Mycobacterium sp.* from Crohn’s disease patients culture using medium especially developed for culturing *M. avium* subsp. *paratuberculosis*. This ground breaking study was in large part responsible for identifying the possibility that *M. avium* subsp. *paratuberculosis* is also a human pathogen.
- Major concerns were raised about the deficiencies of the diagnostic tests and many of the tests are still being widely used today. It can be argued that the improvement in diagnostic tests over the last 20 years has only been incremental. Many of the concerns raised in 1983 are still relevant in 2003.
- The first Colloquium was notable for the complete absence of any papers on the use of molecular biological techniques for investigating paratuberculosis. The first evidence of the DNA revolution was seen in the second Colloquium.

Genome Sequencing

http://pathogenomics.umn.edu/map_index.htm#publication

The genome sequencing of *Mycobacterium avium* subsp. *paratuberculosis* strain K-10 is now complete and annotated (2nd February, 2004, <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=genome>). The genome of K-10 is a single circular chromosome ~ 4.83 base pairs in length, with 69.30% GC content, and contains over 4000 open reading frames. The manuscript is under preparation.

Papers using information from the sequencing of the *Mycobacterium paratuberculosis* genome

Genome scale comparison of *Mycobacterium avium* subsp. *paratuberculosis* with *Mycobacterium avium* subsp. *avium* reveals potential diagnostic sequences.

Bannantine JP, Baechler E, Zhang Q, Li L, Kapur V.

J Clin Microbiol. 2002 Apr;40(4):1303-10.

National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa 50010, USA. jbannant@nadc.ars.usda.gov

The genetic similarity between *Mycobacterium avium* subsp. *paratuberculosis* and other mycobacterial species has confounded the development of *M. avium* subsp. *paratuberculosis*-specific diagnostic reagents. Random shotgun sequencing of the *M. avium* subsp. *paratuberculosis* genome in our laboratories has shown >98% sequence identity with *Mycobacterium avium* subsp. *avium* in some regions. However, an in silico comparison of the largest annotated *M. avium* subsp. *paratuberculosis* contigs, totalling 2,658,271 bp, with the unfinished *M. avium* subsp. *avium* genome has revealed 27 predicted *M. avium* subsp. *paratuberculosis* coding sequences that do not align with *M. avium* subsp. *avium* sequences. BLASTP analysis of the 27 predicted coding sequences (genes) shows that 24 do not match sequences in public sequence databases, such as GenBank. These novel sequences were examined by PCR amplification with genomic DNA from eight mycobacterial species and ten independent isolates of *M. avium* subsp. *paratuberculosis*. From these analyses, 21 genes were found to be present in all *M. avium* subsp. *paratuberculosis* isolates and absent from all other mycobacterial species tested. One region of the *M. avium* subsp. *paratuberculosis* genome contains a cluster of eight genes, arranged in tandem, that is absent in other mycobacterial species. This region spans 4.4 kb and is separated from other predicted coding regions by 1,408 bp upstream

and 1,092 bp downstream. The gene upstream of this eight-gene cluster has strong similarity to mycobacteriophage integrase sequences. The GC content of this 4.4-kb region is 66%, which is similar to the rest of the genome, indicating that this region was not horizontally acquired recently. Southern hybridization analysis confirmed that this gene cluster is present only in *M. avium* subsp. *paratuberculosis*. Collectively, these studies suggest that a genomics approach will help in identifying novel *M. avium* subsp. *paratuberculosis* genes as candidate diagnostic sequences.

Characterisation of IS901 integration sites in the *Mycobacterium avium* genome.

Inglis NF, Stevenson K, Heaslip DG, Sharp JM.

Division of Bacteriology, Moredun Research Institute, International Research Centre, Pentlands Science Park, Bush Loan, Penicuik, Midlothian EH26 0PZ, UK.
neil.inglis@mri.sari.ac.uk

Data are presented on the identification and characterisation of 17 chromosomal integration loci of the insertion element IS901 in the *Mycobacterium avium* (cervine strain JD88/118) genome. Thirteen of these integration loci have been mapped to their corresponding positions on the *M. avium* strain 104 (an IS901(-) strain) genome (The Institute for Genome Research (TIGR) unfinished genome-sequencing project). Sequence data for both upstream and downstream sequence flanking regions were obtained for 12 insertion loci, while upstream sequence was obtained for five others. A consensus IS901 insertion target sequence compiled from all 17 integration sites was in broad agreement with earlier reports that were based on only two such loci. Analysis of IS901 integration site flanking sequences revealed that, like IS900 in *M. avium* subspecies *paratuberculosis*, IS901 inserts preferentially between a putative ribosome-binding sequence (RBS) and the translational start codon of an open reading frame (ORF). In BLAST X and BLAST P searches of the GenBank database, these ORFs were shown to share significant homologies with a number of other prokaryotic genes.

Mycobacterial interspersed repetitive units (MIRU) differentiate *Mycobacterium avium* subspecies *paratuberculosis* from other species of the *Mycobacterium avium* complex.

Bull TJ, Sidi-Boumedine K, McMinn EJ, Stevenson K, Pickup R, Hermon-Taylor J.

Mol Cell Probes. 2003 Aug;17(4):157-64.

Department of Surgery, St George's Hospital Medical School, Cranmer Terrace, SW17 0RE, London, UK. tim.bull@sghms.ac.uk

Mycobacterial interspersed repetitive units (MIRU) comprise short tandem repeat structures found at multiple loci throughout the *Mycobacterium tuberculosis* genome and

have been used for typing these pathogens. We have identified MIRU at 18 conserved loci throughout the common portions of the *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and *M. avium* subspecies *avium* (MAA) genomes. Six of these loci were found to differ between MAA and MAP in the number of tandem repeat motifs occurring at each MIRU locus. Locus specific PCR at 4 of these loci segregated MAP into two major groups, which could be differentiated from ovine-pigmented strains of MAP and the MAP vaccine strain 316F. The same PCR differentiated MAA into five MIRU profiles. PCR at either MIRU locus 1 or MIRU locus 4 distinguished between MAP and all other *M. avium* complex (MAC) tested. PCR at both loci 1 and 4 also distinguished MAP from *Mycobacterium intracellulare*. MIRU typing may provide an additional simple and rapid procedure for the differentiation between MAP and other MAC.

Genomic homogeneity between *Mycobacterium avium* subsp. *avium* and *Mycobacterium avium* subsp. *paratuberculosis* belies their divergent growth rates.

Bannantine JP, Zhang Q, Li LL, Kapur V.

National Animal Disease Center, USDA-ARS, 2300 N, Dayton Ave, Ames, IA 50010, USA. jbannant@nadc.ars.usda.gov

BACKGROUND: *Mycobacterium avium* subspecies *avium* (*M. avium*) is frequently encountered in the environment, but also causes infections in animals and immunocompromised patients. In contrast, *Mycobacterium avium* subspecies *paratuberculosis* (*M. paratuberculosis*) is a slow-growing organism that is the causative agent of Johne's disease in cattle and chronic granulomatous infections in a variety of other ruminant hosts. Yet we show that despite their divergent phenotypes and the diseases they present, the genomes of *M. avium* and *M. paratuberculosis* share greater than 97% nucleotide identity over large (25 kb) genomic regions analyzed in this study. **RESULTS:** To characterize genome similarity between these two subspecies as well as attempt to understand their different growth rates, we designed oligonucleotide primers from *M. avium* sequence to amplify 15 minimally overlapping fragments of *M. paratuberculosis* genomic DNA encompassing the chromosomal origin of replication. These strategies resulted in the successful amplification and sequencing of a contiguous 11-kb fragment containing the putative *Mycobacterium paratuberculosis* origin of replication (*oriC*). This fragment contained 11 predicted open reading frames that showed a conserved gene order in the *oriC* locus when compared with several other Gram-positive bacteria. In addition, a GC skew analysis identified the origin of chromosomal replication which lies between the genes *dnaA* and *dnaN*. The presence of multiple *DnaA* boxes and the ATP-binding site in *dnaA* were also found in *M. paratuberculosis*. The strong nucleotide identity of *M. avium* and *M. paratuberculosis* in the region surrounding the origin of chromosomal replication led us to compare other areas of these genomes. A DNA homology matrix of 2 million nucleotides from each genome revealed strong

synteny with only a few sequences present in one genome but absent in the other. Finally, the 16s rRNA gene from these two subspecies is 100% identical. CONCLUSIONS: We present for the first time, a description of the oriC region in *M. paratuberculosis*. In addition, genomic comparisons between these two mycobacterial subspecies suggest that differences in the oriC region may not be significant enough to account for the diverse bacterial replication rates. Finally, the few genetic differences present outside the origin of chromosomal replication in each genome may be responsible for the diverse growth rates or phenotypes observed between the avium and paratuberculosis

Characterization of genetic differences between *Mycobacterium avium* subsp. *paratuberculosis* type I and type II isolates.

Dohmann K, Strommenger B, Stevenson K, de Juan L, Stratmann J, Kapur V, Bull TJ, Gerlach GF.

J Clin Microbiol. 2003 Nov;41(11):5215-23

Department of Infectious Diseases, Institute for Microbiology, School of Veterinary Medicine Hannover, D-30173 Hannover, Germany.

A combination of representational difference analysis and comparative DNA sequencing revealed that four type I (sheep) isolates of *Mycobacterium avium* subsp. *paratuberculosis* were differentiated from nine type II (bovine) isolates by the presence of an 11-bp insertion in a novel *M. avium* subsp. *paratuberculosis*-specific region of genomic DNA. Further, our studies show that *M. avium* subsp. *paratuberculosis* type I isolates contain three type-specific loci that are missing in *M. avium* subsp. *paratuberculosis* type II but are present in *M. avium* subsp. *avium*. Taken together, the results are consistent with the hypothesis that *M. avium* subsp. *paratuberculosis* type I strains are an evolutionary intermediate between *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis* type II isolates or share a subset of *M. avium* subsp. *avium* type-specific loci through horizontal transfer.

Expression and Immunogenicity of Proteins Encoded by Sequences Specific to *Mycobacterium avium* subsp. *paratuberculosis*.

Bannantine JP, Hansen JK, Paustian ML, Amonsin A, Li LL, Stabel JR, Kapur V.

J Clin Microbiol. 2004 Jan; 42(1): 106-14.

National Animal Disease Center, USDA Agricultural Research Service, Ames, Iowa 50010. Biomedical Genomics Center and Departments of Microbiology and Veterinary Pathobiology, University of Minnesota, Minneapolis, Minnesota 55488.

The development of immunoassays specific for the diagnosis of Johne's disease in cattle

requires antigens specific to *Mycobacterium avium* subsp. *paratuberculosis*. However, because of genetic similarity to other mycobacteria comprising the *M. avium* complex, no such antigens have been found. Through a comparative genomics approach, 21 potential coding sequences of *M. avium* subsp. *paratuberculosis* that are not represented in any other mycobacterial species tested ($n = 9$) were previously identified (J. P. Bannantine, E. Baechler, Q. Zhang, L. Li, and V. Kapur, *J. Clin. Microbiol.* 40:1303-1310, 2002). Here we describe the cloning, heterologous expression, and antigenic analysis of these *M. avium* subsp. *paratuberculosis*-specific sequences in *Escherichia coli*. Nucleotide sequences representing each unique predicted coding region were amplified and cloned into two different *E. coli* expression vectors encoding polyhistidine or maltose binding protein (MBP) affinity purification tags. All 21 of the MBP fusion proteins were successfully purified under denaturing conditions and were evaluated in immunoblotting studies with sera from rabbits and mice immunized with *M. avium* subsp. *paratuberculosis*. These studies showed that 5 of the 21 gene products are produced by *M. avium* subsp. *paratuberculosis* and are antigenic. Immunoblot analysis with a panel of sera from 9 healthy cattle and 10 cattle with clinical disease shows that the same five *M. avium* subsp. *paratuberculosis* proteins are also detected within the context of infection. Collectively, these studies have used a genomic approach to identify novel *M. avium* subsp. *paratuberculosis* antigens that are not present in any other mycobacteria. These findings may have a major impact on improved diagnostics for Johne's disease.

U.S. expert recruited to Johne's disease fight

A leading American livestock disease expert, Dr John Bannantine, has been awarded a McMaster Fellowship to support CSIRO Livestock Industries' efforts to reduce the impact of Johne's disease.

A molecular biologist with the US Department of Agriculture's National Animal Disease Center in Ames, Iowa, Dr Bannantine will work for six months at CSIRO Livestock Industries' Australian Animal Health Laboratory (AAHL) in Geelong, Victoria.

<http://www.csiro.au/index.asp?type=mediaRelease&id=Prbannantine>



NATIONAL JOHNE'S DISEASE CONTROL PROGRAM

Quarterly National Coordinators' Report

July – Sept 2003

Prepared
by
David Kennedy and Evan Sergeant

For more information and lists of assessed MAP herds and flocks
See the Johne's Information Centre website

<http://www.aahc.com.au/jd/index.htm>

THE NATIONAL JOHNE'S DISEASE PROGRAM

Highlights of this quarter included:

- The September meeting of stakeholders agreed that the future management of OJD would be based more on vendor declaration, voluntary flock assurance and vaccination.
- A national scheme of crediting sheep flocks for assurance was developed.
- Under the National BJD Strategic Plan, the beef and dairy industries developed means of assuring herd BJD status.
- The draft DeerMAP was written but implementation will be delayed while new tests are formally approved.

People

Lorna Citer has been appointed Program Manager, John's disease and Training at Animal Health Australia. Lorna replaces Ralph Hood who is now CEO of Animal Health Australia.

Lorna brings to the position a breadth of experience including veterinary practice, on-farm QA programs and tertiary teaching.

Cattle

National BJD Strategic Plan

The Management Committee for the program met for the first time in July and endorsed a broad range of projects.

Beef Industry Initiatives

Under the Strategic Plan, the beef industry is investigating options for financial assistance to infected herds and developing a herd assurance system to help prevent the spread of BJD. One of the main features is a proposed new assurance category called *Beef Only* based on a history of no BJD in the herd and limited contact with dairy cattle. The *Beef Only* proposal recognises that pure beef herds in Australia have a low risk of being infected.

Pilot sales will be held in early 2004 to assess the demand, practicality and veracity of the *Beef Only* classification.

Dairy Industry Initiatives

About half of the samples have been collected in the survey of raw and pasteurised milks at factories in eastern Australia. Results are expected in mid 2004.

The National Dairy BJD Work Group has developed a means of scoring dairy herds for their BJD assurance. Herds will be allocated scores, based on their history,

disease control activities and testing. Scores will initially range from 1 for known infected and suspect herds that have not herd tested, through a range of scores for infected herds progressing in control programs through herds with negative herd tests, to 10 for herds in the Free Zone or at the top level of the CattleMAP.

It is proposed that the score 0 will be held in reserve for two to three years, after which time, herds that have not tested will be demoted to this score. The assurance scoring scheme also allocates 1 or 3 additional credit points for calves reared under calf rearing QA programs.

The major dairy companies have agreed that a standard calf management program, designed to reduce the exposure of calves to adult faeces will become a "desirable" element of all on-farm QA programs in 2004.

The industry is funding research on a bulk herd test (for instance, on milk) to enable routine inexpensive assessment of all dairy herds for the risk of infection and potential contamination of the farm and product.

Disease Situation

The numbers of known infected herds reported by the States are presented in Table 1.

Table 1. Number of known infected cattle herds Sept 2003 (NAHIS).

	Sept 03	June 03	June 02
NSW	143	136	146
VIC	1129	1124	1154
TAS	21	20	16
SA	55	50	44
NT	0	0	2
Qld	0	0	1
	1348	1330	1363

No infected herds were reported in WA.

Market Assurance

The total number of herds in the CattleMAP fell by 1% during the quarter.

Fifty-two new herds joined CattleMAP and 75 herds progressed their status to MN2 or MN3. Seventy-four herds (4%) were reported as leaving the program or as being disbanded, with most of these being in NSW.

Table 2. Numbers of assessed herds in CattleMAP, Sept 2003.

	MN1	MN2	MN3	
NSW	210	311	314	835
VIC	219	140	17	376
SA	124	136	18	278
TAS	51	60	0	111
QLD	0	0	0	0
	604	647	349	1600

No MAP herds were found to be infected this quarter.

Sheep

Future Direction

The September meeting of stakeholders considered the outcomes of the national consultation on the discussion paper, *National Framework for the Future Management of OJD in Australia*. The meeting strongly endorsed the seven principles identified in the discussion paper.

In summary these are:

1. Control (limit) the spread of OJD between farms and regions.
2. Minimise the impact of OJD regulation on affected producers.
3. Minimise the effect of OJD on animal welfare, farm productivity and trade.
4. Maintain complementary and mutually recognised approaches between jurisdictions to enhance the effectiveness of regional control and minimise its impact on / across regional trade.
5. Engage producer support.
6. Encourage responsible management of OJD with enhanced trading opportunities available to farmers as

an incentive to undertake disease control.

7. Improve understanding of OJD through continued research and development.

The stakeholders also supported the second of the three options that were proposed for control. Option 2, *Flock risk-based trading program*, proposed:

- trading based on flock risk management;
- removal of zones for the regulatory control of stock movements;
- use of vendor declarations; and
- increased availability of vaccine

In NSW, the Bull Review, commissioned by the new Minister for Agriculture, recommends a similar approach to managing OJD in that State. It includes allowing Rural Lands Protection Boards in low prevalence *exclusion areas* to protect themselves by self-funded programs if there is broad producer support in those areas.

Disease Situation

Table 3. Number of known infected sheep flocks Sept 2003 (NAHIS).

	Sept 03	June 03	June 02
NSW	1213	1150	840
ACT	-	-	1
VIC	118	98	45
TAS	41	40	30
SA	57	55	15
	1429	1343	931

No infected flocks were reported in WA or Queensland.

Market Assurance

Fifteen new flocks entered the SheepMAP and 61 progressed to MN2 or MN3 status. Twelve flocks withdrew from the program.

Table 4. Numbers of assessed flocks in SheepMAP, June 2003.

	MN1	MN2	MN3	
NSW	76	125	114	315
VIC	58	55	39	152
SA	74	79	94	247
TAS	11	14	8	33
QLD	3	3	1	7
	222	276	256	754

Two MN2 and one MN1 flock were found to be infected, all in heavily infected regions of NSW and South Australia.

A SA MN2 flock was found to be infected by surveillance testing under NOJDP destocking Trial 1.1. The flock had restocked with sheep from another MN2 flock. NSW MN1 and MN2 flocks were found infected by Sample Test and traceforward investigation respectively.

Forty SheepMAP flocks have been found infected with OJD in the past six years. All but four were MN1 status at the time of infection being detected. The others were MN2 flocks.

Goats

Future JD management

Animal Health Australia has established a working group with the Goat Industry Council of Australia to develop a new approach to JD management in goats.

Disease Situation

Table 5. Number of known infected goat herds Sept 2003 (NAHIS).

	Sept 03	June 03	June 02
NSW	9	10	12
VIC	8	6	8
TAS	6	6	5
SA	7	6	0
WA	0	0	0
	30	28	25

Update on OJD and BJD in goats

Factors associated with JD in 27 infected goat herds in Australia was reviewed and the main points are that:

- Twenty-one herds were infected with Cattle type *M paratuberculosis*, with three quarters of these herds located in the BJD Residual and Control Zones. Three herds are infected with S type, two of which are in the NSW OJD Management Area and the type is unknown in 3 Victorian herds.
- In 18 of the herds infected with C type, the infection had been detected since 2000. A cluster of SA cases detected in 2003 is thought to have resulted from a breakdown in a control program dating back to 1993. The three S-type infections had been detected since 1998.
- Many of the infected herds are quite small; only one third of those with C-type infections had 50 to 400 goats. The three S-type infected herds were larger and ranged from approximately 100 to 300 goats.
- Most of the cluster of herds in SA detected in 2003 have destocked, or intend to do so, and one Victorian herd has achieved RD2 status.
- Two large goat herds are vaccinating to control C-type infection under approved disease management

programs. The Gudair vaccine was developed from a cattle strain of *M paratuberculosis* and is registered as an aid in the control of JD in goats. All goats in the herds were vaccinated initially, and from now on the kids will be vaccinated. Vaccination site reactions have not been a major problem.

Table 6. Numbers of assessed herds in GoatMAP, Sept 2003.

	MN1	MN2	MN3	
NSW	18	14	0	32
VIC	5	1	0	6
SA	15	4	0	19
TAS	1	0	0	1
QLD	0	0	0	0
	39	19	0	58

One new herd entered the MAP and five progressed. One herd was disbanded.

Camelids

Disease Sitrep

Careful management of JD in the alpaca industry since the initial outbreak 10 years ago continues to deliver results.

Testing in NSW and Victoria this quarter cleared another three herds that had been under restrictions.

Table 7. Number of known infected alpaca herds, Sept 2003 (NAHIS).

	Sept 03	June 03	June 02
NSW	0	0	1
VIC	2	2	4
TAS	0	0	0
SA	0	0	0
	2	2	5

The number of herds in the AlpacaMAP was reported to have slumped from 202 to 128 during the quarter. This was largely due to a drop in NSW from 127 to 59, as a result of herds being suspended as their renewals had not been effected in time.

In the other States, 8 new herds entered the AlpacaMAP and 29 progressed to MN2 or MN3.

Table 8. Numbers of assessed herds in AlpacaMAP, Sept 2003.

	MN1	MN2	MN3	
NSW	3	24	32	59
*				
VIC	6	15	13	34
SA	5	6	23	34
TAS	0	0	1	1
QLD	0	0	0	0
	14	45	69	128

Deer

Disease Sitrep

Last quarter's report stated that there were 15 infected herds in Victoria. This was an administrative error. The correct number was 8.

Table 5. Number of known infected deer herds, Sept 2003 (NAHIS).

	Sept 03	June 03	June 02
NSW	1	1	0
VIC	8	8	7
TAS	0	0	0
SA	2	2	1
Qld	0	0	1
	11	11	9

DeerMAP

The Deer Industry Association approved the draft DeerMAP but its national endorsement and implementation may be delayed a few months.

The pooled faecal culture and ELISA test developed under a RIRDC research project have to comply with another couple of steps in the approval process required by the Sub-Committee on Animal Health Laboratory Standards (SCAHLs).

Research and Development

Update of OJD Research

by Peter Rolfe, Meat & Livestock Australia

Harvest year for OJD research, 2004.

Two key events will be held in 2004 to capture and summarise the outcomes of the OJD research conducted in recent years. These are an update of R&D in March 2004 and a wider conference later in 2004. An independent review of the value of the R&D program will be conducted as part of this process.

Destocking study (OJD.001 or Trial 1.1).

Infection had been detected on 25 of 35 farms that have restocked sheep after the 15 month period of decontamination. An intensive investigation into the reasons for the failures has been conducted on 8 farms. The inability to detect infection in restocked sheep and failure to control lateral spread across farm boundaries are the major factors limiting the success of destocking.

After considerable consultation it has been agreed that little additional useful information was likely to come with continuing the study without modifying it. The trial will continue however so as to allow:

- two year post-destock testing on all farms;
- release of those farms that are clear of infection but do not wish to continue,
- three year testing on the uninfected farms that wish to continue.

Current evidence suggests that destocking *per se* is adequate to kill the organism in most but not all circumstances. Simple precautions such as fencing high-risk areas will further enhance the decontamination. This knowledge has had a major impact on current decisions about how OJD should be controlled in Australia in the future.

Vaccine evaluation.

The results continue to indicate that the efficacy of the vaccine in terms of reduction in mortalities and the reduction in shedding of organism is approximately 90% in vaccinated sheep. This is consistent with the assumptions used to model the effect of vaccination. On all farms, unvaccinated sheep started to shed bacteria in faeces 12-18 months before vaccinated sheep.

A study has commenced to monitor the long-term effect of vaccination on farms with varying prevalences of infection. It will also examine the effect of vaccinating uninfected adult sheep onto an infected farm.

A study to assess the effect of vaccine lesions on carcass has shown, at least initially, that there are no significant losses due to carcass damage.

Modelling the spread of OJD.

A computer model of the spread of OJD on farm and between farms has been completed. Two publications have been produced and these have been combined with a copy of the model and power point presentations for use by advisors for presentations to producers and will be distributed early in 2004.

Basic research into the pathogenesis of OJD.

Staff have been recruited to this project and research will commence in earnest in early 2004 to develop much better understanding of the early stages of disease development. This project is designed to lead to improved early diagnosis and possibly more effective vaccines.

A parallel program of work in the cattle disease will be complementary and utilise the critical mass of research capability in Australia.

David Kennedy
Evan Sergeant
AHA's National Coordinators

9 December 2003

Ovine paratuberculosis : field diagnosis

Bernardelli, A.¹; Cicuta, M.E.²; Nicola, A.⁵; Roibón, W.R.²; Boehringer, S.I.²; Benítez, M.C.²; Barceló, M.C.²; Alonso, B.^{5,6}; Schneider, M.³; Zumárraga, M.⁴; Estéves Madero, J.⁷.

¹ Reference Laboratory of Paratuberculosis OIE, DILACOT- SENASA-República Argentina

¹ Reference Laboratory of Tuberculosis bovine OIE ,DILACOT-SENASA

² Microbiology Department- FCV/UNNE .

³ Animal Pathology Department- FCV/UNRC

⁴ Biotechnology Institute ,CICV y A INTA Castelar.

⁵ Brucellosis Department DILACOT-SENASA

⁶ Mycobacteria Department DILACOT-SENASA

⁷ Virology Department DILACOT-SENASA

Corresponding author : e-mail : ameb@fibertel.com.ar

ABSTRACT

The aim of this study was to use PARACHEK™ Johne's Absorbed EIA-CSL, gamma interferon test IFN BOVIGAM™-CSL , Mycobacterial Growth Indicator Tube MGIT, BACTEC 960 BECTON-DICKINSON, PCR with IS900 probe, to diagnose paratuberculosis in sheep from the Argentine North East (ANE) and compare the results with those of the comparative skin testing using bovine and avian PPD (B-PPD and A-PPD), agar gel immunodiffusion (AGID), Ziehl-Neelsen stain, and culturing using Löwenstein-Jensen and Herrold's (with/without mycobactin). Plasma, serum and faeces were collected from 66 Romney Marsh and Corriedale cross-bred sheep from three farms from Corrientes, Argentina **Results:** Skin tests with B-PPD versus PARACHEK™-CSL) had a specificity of 89%, A-PPD versus PARACHEK™-CSL had a specificity of 88% , Comparative B-PPD and A-PPD skin test *versus* IFN BOVIGAM™-CSL had a specificity of 98% and 88% respectively. The former *versus* culture had a specificity of 83%, IFN (BOVIGAM™-CSL *versus* culture : 100% and 94% respectively , PARACHEK™-CSL *versus* culture had a specificity of 88% and a sensitivity of 33%. All AGID tests were negative.

Nine mycobacterial isolates were obtained from fecal cultures: 3 from Herrold's with mycobactin, 2 from Herrold's without mycobactin and 4 from MGIT-BACTEC 960, plus mycobactin. **IS900 was shown by PCR to be present in 6 of the isolates.**

Conclusion : The joint use of tests will tend to clarify the infection status of animals and may result in lower costs and improved testing efficiency. Future research will be necessary to further define the sensitivity of the tests and determine how they can be optimally used as part of a disease control programme.

INTRODUCTION

Paratuberculosis (Johne's disease) is a chronic granulomatous enteropathy of the ruminants, caused by *Mycobacterium avium* subsp. *paratuberculosis*. The infection is commonly transmitted by the fecal-oral route through contaminated pastures, water or milk. Intra-uterine infections have also been recorded (Whittington et al., 2000). In Argentina it has been diagnosed in cattle, sheep, goats, deer, alpacas and llamas (Bernardelli et al., 1981/7/99; López et al., 1990; Moreira et al., 1993/9; Cicuta et al., 1995) and elsewhere also in rabbits, bison, rhinoceros, other herbivores, and primates (Meylan et al., 1995; Cousins et al., 2000; Whittington et al., 2000). Crohn's disease in humans has similar histopathology, bacteriology and clinical symptoms to paratuberculosis in domestic animals (Engstrand's, 1995; Whittington et al., 2000).

Paratuberculosis is a significant economic problem causing a 6 to 25% reduction in

milk production, weight loss and loss of commercial value. The chronic diarrhoea that is a classical clinical sign in affected cattle, is only observed in 10 to 20% of cases in sheep, goats and deer where the most common clinical symptom is loss of weight, with hypoproteinemia and maxillary oedema and muscular atrophy (Stehman, 1996). Young animals in the first 30 days of life are the most susceptible to infection and clinical disease is not commonly observed until the animal is 2 to 5 years old. Experimental studies (García Marín et al., 1992) have demonstrated that doses so low as 10^3 *M. avium* subsp. *paratuberculosis* have produced microscopic lesions in intestine of lambs.

Infected animals will contaminate pastures and provide a source of infection for other animals. The bacteria, if protected from sun light and drying can remain infective in soil for at least one year (Collins, 1994). The complex cell wall of the Mycobacteria is rich in lipids (mycolic acids) which confers the acid-resistant characteristic and are immune modulation properties (Clarke et al., 1997).

After the ingestion, the mycobacteria penetrates by endocytosis the cells of the ileal Peyer's patches and are transported in vacuoles inside the subepithelial macrophage target cell. The mycolic acids play an important role in the intracellular resistance mechanisms (Pérez et al., 1999). *M. avium* subsp. *paratuberculosis* resists the intracellular degradation and multiplies inside the macrophage causing the production of a cascade of cytokines like tumour necrosis factor (NTF), interleukines (IL) and derived IFN- γ that cause immunopathologic effects and maintains a chronic inflammatory response (Clarke et al., 1997). The lesions are characterized by variable thickness of the small intestine mucosa, with granular appearance, and edema of the lymph nodes. Most of the tissues have an extensive infiltration of epithelioid cells in the mucosa with abundant acid fast bacilli (AFB). Occasionally a yellow-orange colour is observed due to pigmented ovine strains (Clarke and Little, 1996).

Conclusive evidence of host specificity for different strains of *M. paratuberculosis* does not exist although restriction fragment length polymorphism (RFLP) through IS900 and IS1311 probes have detected different patterns and segregation of strains among different species and geographical regions. A high degree of genetic homogeneity has been observed among ovine strains. The nominated type C (=cattle) infect predominantly cattle and the type S (=sheep) sheep, indicating a certain degree of host specificity, preference or adaptation (Cousins et al., 2000; Whittington et al., 2000).

Serology methods have been improved; complement fixation tests (CD) have been used in cattle and agar gel immunodiffusion test (AGID) is the most efficient test in sheep. Yokomizo et al. (1985) introduced a variant to the enzyme-immune assay (ELISA), modified by Cox et al., 1991 where sera is absorbed with soluble antigen of *M. phlei* to increase the specificity by eliminating cross reactions with other mycobacteria and related organisms (Hilbink et al., 1994). It measures only IgG 1 antibodies, against protoplasmatic *M. avium* subsp. *paratuberculosis* antigens. It has a sensitivity of 40 to 75% and a specificity of greater than 97%, depending on the different assays and prevalences (Hietala, 1992). Sweeney et al. (1994) demonstrated that ELISA was able to detect 75 % of shedding animals. Due to its high specificity, the ELISA is a good test for screening herds at the beginning of a control program.

Cellular immunity and fecal shedding often precede humoral immunity, consequently, serologic tests like AGID, complement fixation (FC) and ELISA predominantly detect animals in advanced periods of illness. Clinical cases, with visible lesions usually have high levels of IgG and IgM antibodies in serum (Merkal, 1973; Clarke et al., 1996). ELISA, CF and AGID have sensitivities ranging from 87 to 100% in clinically affected sheep, but much lower sensitivities (20%) in sheep with subclinical infections (Stehman, 1996). The CF is not considered useful in small ruminants due mainly to cross reactions with corynebacteria and

other mycobacteria; AGID detects antibodies 3 to 9 months after fecal shedding; the sensitivity in clinical cases is 50% and 27-29% in subclinical cases. The specificity is about 100% (Hietala, 1992).

An indirect method to measure lymphocyte T stimulation is IFN test that measures the production of the cytokine *in vitro* through stimulation of lymphocytes with avian-PPD or Johnin. Although this test has been originally developed to detect bovine tuberculosis, it is also used to diagnose paratuberculosis. The sensitivity of the IFN test is between 70 to 94% and the specificity from 97 to 99%, when it is used as screening test (Hietala, 1992). When it is compared with pre-absorbed ELISA, IFN was able to detect more cows with subclinical infections while ELISA detected a greater percentage of clinically affected animals and was relatively insensitive for identifying subclinical infections (Stabel, 1996; Travería et al., 1999).

The current study was carried out in a region of 1.2 million sheep, where avian PPD reactors are present (Cicuta et al., 1995; Cicuta, 1999) and, where sheep mix with cattle that also have paratuberculosis (16,7% of dairy cattle positive to ELISA and 1% of beef cattle, Moreira et al., 1993 and 13,3% positive to avian PPD over 202 bovines, Cicuta et al., 1995). The study has provided additional information on paratuberculosis in this region and will be a useful basis for formulating better control strategies.

The analysis of the PCR restriction fragments products, offers a quick and a relatively simple method for the identification of mycobacterial species (Devallois et al., 1997). This method was developed based on the polymerase chain reaction (PCR) and the amplification of the gene corresponding to the heat shock protein hsp65 present in all mycobacteria, and the subsequent digestion with the restriction enzymes BstEII and HaeIII (Telenti et al., 1993). This methodology has special value in the differentiation of atypical mycobacteria.

MATERIALS AND METHODS

The gamma interferon test kit BOVIGAM™-CSL, PARACHEK™ John's Absorbed EIA - CSL, MGIT (Mycobacterial Growth Indicator Tube) and BACTEC 960, BECTON-DICKINSON, PCR IS900, were used to examine samples from 66 sheep from the Argentine North East (NEA) and the results compared with the tuberculin skin test with Bovine and Avian-PPD, AGID (Agar Gel Immunodiffusion Test), Ziehl-Neelsen stain and culturing using Löwestein-Jensen and Herrold's, with and without mycobactin, medium. The animals were Romney Marsh and cross-bred Corriedale, coming from three farms of Corrientes, Argentina.. Blood and feces were collected from each animal.

1- Comparative Skin Test

The comparative skin test was carried out with B-PPD prepared with AN5 strain of *Mycobacterium bovis* (1 mg/ml protein concentration) and 32.5000 IU/mg/ml. Serial #60 and A-PPD produced with D4 ER strain of *Mycobacterium avium*, with a concentration of 0.5 mg/ml and 25.000 IU/ml. The tuberculins were prepared and provided by the Mycobacteria Department under the Technical Control and Laboratory Bureau of DILACOT from the National Agri-food Health and Quality (Servicio Nacional de Sanidad y Calidad Agroalimentaria, SENASA). Each tuberculin (0.1ml) was inoculated in the skin of the axilla with automatic precision syringe (B-PPD on the right and A-PPD on the left). The thickness of the skin was palpated and measured with a calliper immediately prior to injection and 72 hours after inoculation. A positive reaction to B and A-PPD when the diameter of the reactions were 3mm or greater and negative when less than 3 mm. Results analysis were evaluated through a Scattergram classifying the herd in positive reactors, suspicious or negative to A-PPD or to B-PPD.

2. Gamma Interferon (BOVIGAM–CSL) : It was carried out according the manufacturer's instructions.

3.Serology

a-The sera were tested with an absorbed PARACHEK™ Johne's absorbed EIA, (CSL Ltd., Melbourne, Australia) according to the manufacturer's instructions. The cut-off value was calculated as recommended by the manufacturer as the absorbance of the negative control plus 1.The difference between the sample absorbance and the cut-off was multiplied by 100 to obtain the ELISA "titre".

b- Serological testing with the agar gel immunodiffusion test (AGID) was done as recommended by Thorel 1996 and Merkal 1978 . The PPA (Paratuberculosis Protoplasmatic Antigen) used was made by Allied Monitor Inc.USA.

4.Bacteriology

Faeces were cultured according to Merkal (1973). In short, they were treated with hexadecylpyridinium chloride (0.7%) and streaked on solid Löwenstein–Jensen and Herrold's medium with and without mycobactin and incubated at 37°C. Sediment of decontaminated faeces was also been cultured in the MGIT system (Mycobacteria Growth Indicator Tube) adding mycobactin in each tube (30 microlitres) and placed in the BACTEC™ MGIT™ System for Mycobacteria Testing for five months. Ziehl-Neelsen staining was carried out on all samples at the end of the incubation period immediately before the media were discarded. Isolates were confirmed as *M. paratuberculosis* by PCR using IS900 probe.

5.Genetic Analysis (PCR IS900)

To corroborate the biochemical identification of the isolates, the insertion element IS900 of *Mycobacterium avium* subsp. *paratuberculosis* was amplified. The PCR was carried out according to Collins et al. (1993) producing a DNA fragment of 217 bp.

6.Correlation among skin test, Gamma interferon, serology and culture results.

The records from all positive cultures, all negative and seropositive animals, Gamma Interferon test and comparative skin test were used to study the correlations among them. The study was carried out by Medcal ROC curves, tables 2x2 and histogram.

RESULTS

1- Comparative skin tests

Assay for cell-mediated Immunity (CMI)

The Scatterdiagram (Figure 1) provides a summary of the results of the 66 animals studied:

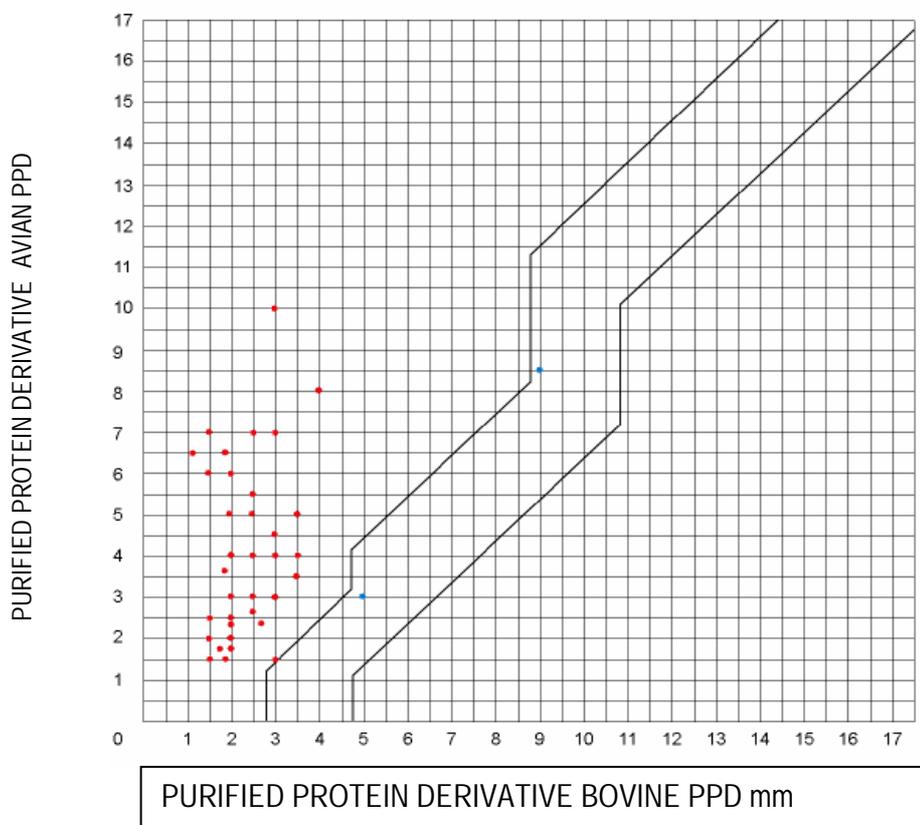
Positive reactors to Avian PPD : 15

Suspected: 2

Positive Reactors to Bovine PPD: 0

These results are consistent with a flock with paratuberculosis.

Figure 1 . Scatterdiagram of the avian and bovine skin test results



2. Gamma interferon (BOVIGAM™-CSL)

The comparative results are summarised in Tables I and II..

The results were ordered, as follows

a) in 2 x 2 tables, in such a way that in each cell the resulting quantity of the combination of the coincident and dissident diagnoses is consigned, giving as marginal results the Sensitivity, Specificity, positive Probability of a true one and Probability of a true negative (Pr.T - and Pr N). See Table III.

1.- A-PPD and Avian-BOVIGAM™

The comparison between A-PPD and Avian-BOVIGAM™ of 62 animals, shows an 88% specificity, however the sensitivity is null so any value is consigned .

2.- B-PPD bovine and Bovine-BOVIGAM™

In the case of B-PPD and bovine BOVIGAM™ 98% specificity was found but the sensitivity is meaningless because only a single case of positive B-PPD was found.

Table 1: Comparison between tests.

Methods	SENSITIVITY	SPECIFICITY	AGREEMENT	SENSITIVITY		SPECIFICITY	
				Low Lim.95%	Upper Lim.95%	Low Lim.95%	Upper Lim.95%
Skin Test Avian PPD vs. BOVIGAM	0%	88%	73%	0%	0%	76%	96%
Skin Test Bovine PPD vs. BOVIGAM	0%	98%	96%	0%	0%	88%	99%
Skin Test Avian PPD vs. PARACHEK	0%	88%	70%	0%	0%	76%	96%
Skin Test Bovine PPD vs. PARACHEK	0%	89%	85%	0%	0%	78%	96%
Skin Test Bovine-Avian PPD vs. CULTURE	0%	83%	65%	0%	0%	70%	92%
Avian PPD BOVIGAM vs. CULTURE	0%	94%	84%	0%	0%	84%	98%
Bovine PPD BOVIGAM vs. CULTURE	0%	100%	100%	0%	0%	100%	100%
PARACHEK vs. CULTURE	33%	88%	83%	4%	77%	77%	95%

Table II. Avian PPD skin test vs. BOVIGAM™ Avian

	PPD +	PPD -	Total	Pr.V.- (*)	Pr.V.+ (**)
Bovigam.av +ve	0	6	6	0%	
Bovigam.av -ve	11	45	56		80%
Total	11	51	62		

				Low.Lim.95%	Upper. Lim. 95%
Sensitivity.	0%			0%	0%
Specificity.		88%		76%	96%

3.Serology

a-ELISA PARACHEK™ –CSL

Skin test with bovine and avian PPD versus PARACHEK™-CSL ,sensitivity : 0% ,specificity 89% agreement : 85% and sensitivity : 0% ,specificity : 88% , agreement : 70%.

3.- A-PPD and PARACHEK™

On 51 samples it was observed 88% specificity and 0 sensitivity, that is to say that on 13 negatives A-PPD none was detected by PARACHEK™, on the other hand of 51 negatives A-PPD, 45 were detected.

4.- B-PPD and PARACHEK™

In this comparison as was the case for B-PPD vs. bovine-BOVIGAM™ the sensitivity was null since there was only one value, however the specificity was 98% (it detected 46 cases of a total of 47).

b-AGID Yielded negative results throughout the samples.

Table II. Comparison of tests.

**SHEEP THAT REACT TO SKIN TEST, BOVIGAM, PARACHECK™,
vs. CULTURES**

A- BOVINE and AVIAN PPD vs CULTURES

	PPD +	PPD -	Total	Prob.T. N	Prob.T. P
Cult.+	0	9	9	0%	
Cult.-	14	43	57		75%
Total	14	52	66		

Bovine PPD + = 1

		Low. Lim.95%	Upper Lim.95%
Sens	0%	0%	0%
Spec.	83%	70%	92%

B- AVIAN BOVIGAM vs CULTURES

	BOVG+	BOVIG-	Total	Prob.T. N	Prob.T. P
Cult.+	0	3	3	0%	
Cult.-	6	48	54		89%
Total	6	51	57		

		Low. Lim.95%	Upper Lim.95%
Sens	0%	0%	0%
Spec.	94%	84%	98%

C- BOVINE BOVIGAM vs CULTURES

	BOVG+	BOVIG-	Total	Prob.T. N	Prob.T. P
Cult.+	0	0	0	Sin datos	
Cult.-	0	48	48		100%
Total	0	48	48		

		Low. Lim.95%	Upper Lim.95%
Sens.	0%	0%	0%
Spec.	100%	100%	100%

D- PARACHECK vs CULTURES

	PARACH+	PARACH-	Total	Prob.T. N	Prob.T. P
Cult.+	2	7	9	22%	
Cult.-	4	53	57		93%
Total	6	60	66		

		Low. Lim.95%	Upper Lim.95%
Sens	33%	4%	77%
Spec.	88%	77%	95%

References: Prob.T.N.: Probability true negative – Prob.T.P.: Probability true positive

4. Bacteriology

Nine isolates from faeces were obtained from the 66 samples cultured: 3 in Herrold's with mycobactin, 2 in Herrold's without mycobactin and 4 in MGIT –BACTEC 960, plus mycobactin (Table III).

5. Genetic Analysis

PCR IS900: Six of them was positively amplified.

See Table III.

6. Correlation among skin test , gamma interferon BOVIGAM™ -CSL ,ELISA PARACHEK™-CSL, and cultures (Table III).

Table III Results from the faecal cultures of 66 sheep

ISOLATION

Animal No	Herrold's without Mycobactin	Herrold's with Mycobactin	MGIT BACTEC 960 with Mycobactin	PCR IS 900
7	Posit	Negat	Negat	Posit
8	Negat	Posit	Negat	Posit
11	Negat	Posit	Negat	Posit
14	Negat	Posit	Negat	Negat
23	Negat	Negat	Posit	Posit
26	Posit	Negat	Negat	Posit
28	Negat	Negat	Posit	Negat
36	Negat	Negat	Posit	Posit
51	Negat	Negat	Posit	Not done

DISCUSSION

During the first stage of the infection in paratuberculosis only small numbers of bacilli are shed in the faeces and faecal cultures are usually negative; in the second phase faecal shedding begins in apparently healthy animals and skin test reactions with A-PPD, Ziehl Neelsen stain, faecal culture (Herrold's with mycobactin and MGIT-BACTEC960), PCR and γ -IFN (BOVIGAM™-CSL) are often positive. Some infected animals may recover spontaneously, while in others the infection may progress. The development of the clinical disease is accompanied by a decrease in skin test reactivity and an increase of humoral immunity with positive serology to ELISA and AGID. The animals that shed bacilli can be detected by faecal culture with conventional media (Herrold's with mycobactin) and MGIT-BACTEC960 and also by PCR IS900. Symptoms may be absent for years in animals with subclinical infection who may spread infection through faecal shedding.

The use of comparative tuberculin test as screening procedure is an economical method as a herd screening procedure.. The pre and post inoculation difference reading for each tuberculin, expressed in millimeters and a scatterdiagram gives a quick vision of the flock situation.

Interpretation of diagnostic tests.

Fecal and tissue culture: Detection of the causative organism by culture should be definitive for paratuberculosis (test specificity of 100%). Some strains, for example those of sheep and bison, are difficult to isolate and require longer incubation times of up to 150 days.

Agarose gel immunodiffusion (AGID): It has been successfully used in control programs and in sheep it has been reported to have a similar sensitivity to an absorbed ELISA. Neither test can reliably detect subclinically infected sheep.

Absorbed Enzyme-linked Immunosorbent assay (a-ELISA): In cattle a-ELISA is more sensitive than AGID and CF. The sensitivity of the technique is comparable to CF in clinical cases: 65% for positive true animals, 36% for false negatives, 55% for false positives and 45% for true negatives (Ridge & Sockett), these values increase in subclinically infected animals.

Skin testing: It has a low specificity but is useful in detecting animals in the first stages of infection. As the PPDs possess common antigens with other mycobacteria, a way to reduce non specific reactions is to carry out the comparative test using avian and bovine PPD.

Gamma Interferon (γ -IFN): It measures the secretion of this cytokine by sensitised lymphocytes as part of cellular response to *Mycobacterium avium* or *M. paratuberculosis* antigen stimulation. The gamma interferon test is relatively expensive.

PCR IS900: The sensitivity of the assay is less than culture but the specificity is high (approx 100%). The test is expensive, requires specialised equipment and is technically difficult, especially as a routine diagnostic procedure.

MGIT-BACTEC960 Its use allows for a quicker diagnosis than that obtained with conventional cultures using solid media. Contamination is reduced by the addition of antibiotics and positive bacterial growth is detected by fluorescence. The identity of the cultures can then be confirmed by rapid DNA tests.

The test selection depends on the level of sensitivity required at individual level or that of the flock. The joint development of diagnostic techniques will tend to clarify the situation which implies less costs of sampling and major efficiency of the results. Additional research will be necessary to show the certainty of diagnosis in order to determine how best the tests can be used for the systematic control of the disease.

CONCLUSION

The combined use of the different tests will tend to better clarify the infection and disease status of animals and may result in a more cost effective diagnosis of paratuberculosis. Future research on the tests will be necessary to determine how best they can be used for the control of the disease. The chronic nature of paratuberculosis highlights the need to define the optimal times for using each diagnostic technique. This is required if the different tests are to be used efficiently as part of a disease control programme.

ACKNOWLEDGEMENTS

We are grateful to Julio Tinao for her excellent technical assistance. A. Bernardelli is fellows of the RELACTB (Red Latinoamericana y del Caribe de Tuberculosis)

REFERENCES

- Bernardelli, A. and Torres, P. Bovine paratuberculosis and tuberculosis in a field study. Abstracts of the Sixth International Colloquium on Paratuberculosis, Melbourne, Australia, pp.32, 1999.
- Bernardelli, A.; Nader, A.J. Diagnóstico de paratuberculosis bovina con tuberculina aviar o johnina. Veterinaria Argentina, IX (83): 164 – 169, 1992.

Bernardelli, A.; Nader, A.J.; Moreira, A.R.; Debenedetti, R. y Esteves Madero, J. Evaluación de distintas técnicas para eliminar la paratuberculosis de un rodeo de cría. *Revista de Medicina Veterinaria* 72 (3): 223 – 230, 1991.

Bernardelli, A.; Sanguinetti, R. Diagnóstico de la paratuberculosis bovina. *Veterinaria Argentina* IV (34): 332 – 340, 1987.

Cicuta, M.E. Validez de la prueba tuberculínica en el diagnóstico de paratuberculosis bovina en el nordeste argentino. *Revista de Medicina Veterinaria*, 80 (2): 72 – 74, 1999.

Cicuta, M.E.; Boehringer, S.I.; Roibón, W.R.; Bernardelli, A.; Bakos, E.; Benitez, M.C. ; Kunert, J.A. y Aragón, L.R. Paratuberculosis in cattle and sheep of the North East of Argentine. *The Paratuberculosis Newsletter*, 7 (1): 18 – 23, 1995.

Clarke, C.J. The pathology and pathogenesis of paratuberculosis in ruminants and other species. *J.Comp.Path.* 116: 217 – 261, 1997.

Clarke, C.J. and Little, D. The pathology of ovine paratuberculosis: gross and histological changes in the intestine and other tissues. *J. Comp. Pathol.* 114 (4): 419-37, 1996.

Collins, M.T. Clinical approach to control of bovine paratuberculosis. *JAVMA* 204 (2): 208 – 210, 1994.

Collins D., Stephens D., de Lisle G., Comparison of polimerase chain reaction tests and fecal culture for detecting *Mycobacterium paratuberculosis* in bovine faeces. *Vet. Microbiol.* 36: 289-299, 1993.

Cousins, D.V.; Williams, S.N.; Hope, A. and Eamens, G.J. DNA fingerprinting of Australian isolates of *Mycobacterium avium* subsp. *paratuberculosis* using IS900 RFLP. *Aust. Vet. J.* 78 (3): 184-90, 2000.

Da Silva Rocha A., da Costa Leite C., Torres H.M., de Miranda A. B., Pires Lopes M.Q., Degrave W.M., Suffys P.N. Use of PCR-restriction fragment polymorphism analysis of the hsp65 gene for rapid identification of mycobacteria in Brazil. *J.Microbiol. Methods*, 1999.

Devallois A., Seng Goh K., and Rastogi N.. Rapid identification of Mycobacteria to species level by PCR- restriction fragment length polymorfism analysis of the hsp65 gene and proposition of an algorithm to differentiate 34 Mycobacterial species. *J. Clin. Microbiol.* 35:2969-2973, 1997

Engstrand, L. *Mycobacterium paratuberculosis* and Crohn's disease. *Scand. J. Infect. Dis. Suppl.*, 98: 27 – 29, 1995.

Green, E.P.; Tizard, M.L.; Moss, M.T.; Thompson, J.; Winterbourne, D.J.; Mc Fadden, J.J. and Hermon-Taylor, J. Sequence and characteristics of IS 900, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. *Nucl. Acids. Res.* 17 (22): 9063-73, 1989.

Hietala, S. The options in diagnosing ruminant paratuberculosis. *Veterinary Medicine*: 1122 – 1132, 1992.

Hilbink, F.; West, D.M.; de Lisle, G.W.; Kittelberger, R.; Hosie, B.D.; Hutton, J.; Cooke, M.M. and Penrose, M. Comparison of a complement fixation test, a gel diffusion test and two absorbed and unabsorbed ELISA's for the diagnosis of paratuberculosis in sheep. *Vet.Microbiol.*, 41: 107 – 116, 1994.

Jorge, M.C.; Schettino, D.M.; Torres, P. y Bernardelli, A. Primera descripción de infección concomitante tuberculosis y paratuberculosis en ovinos lecheros en Argentina. *Rev.Sci.Tech.Off.Int.Epiz.*, 19 (3): 800 – 809, 2000.

Juste, R.A.; Saez de Ocariz, C.; Marco, J.C. and Aduriz, J.J. Comparison of different media for the isolation of small ruminan strains of *Mycobacterium paratuberculosis* *Vet. Microbiol.* 28: 385 – 390, 1991.

López, B.; Ritacco, V.; Kantor, I.N.de; Debenedetti, R.; Nader A.J. and Bernardelli, A. Evaluation of an ELISA for the serodiagnosis of bovine paratuberculosis. *The Paratuberculosis Newsletter*, pp 5-6 1990.

Merkal, R.S. Laboratory Diagnosis of Bovine Paratuberculosis. *JAVMA*, 163 (9): 1100 – 1102, 1973.

Meylan, M.; Nicolet, J.; Busato, A.; Bunens, A. et Martig, J. Étude de prévalence de la paratuberculose dans la région du Plateau de Diesse. *Schweizer Archiv für Tierheikunde*, 137 (1): 22 – 25, 1995.

Moreira, A.R.; Paolicchi, F.; Morsella, C.; Zumarraga, M.; Cataldi, A.; Bigi, F.; Alito, A.; Piet, O.; van Soolingen, D.; and Romano, M.I. Distribution IS900 of restriction fragment length polymorphism types among animal *Mycobacterium avium* subsp. *paratuberculosis* isolates from Argentina and Europe. *Vet. Microbiol.* 70 (3-4): 251-9, 1999.

Moreira, A.R.; Späth, E.J.A. y Morsella, C. Seroprevalencia de paratuberculosis bovina en siete partidos de la cuenca del Salado. *Rev.Arg.Prod..Anim.* 13, Sup. 1, 67, 1993.

Nader, A. y Bernardelli, A. Utilización del test tuberculínico intradérmico comparativo en ovinos como prueba de screening en paratuberculosis. *Rev.Med.Vet.* 67 (6) 335-8, 1986.

Pérez, V.; Tellechea, J.; corpa, J.M.; Gutiérrez, M. and García Martín, J.F. Relation between pathologic findings and cellular immune responses in sheep with naturally acquired paratuberculosis. *Am.J.Vet.Res.* 60 (1): 123/7, 1999.

Ridge, S.E.; Morgan, I.R.; Sockett, D.C. *et al.* Comparison of the Johne`s absorbed EIA and the complement-fixation test for the diagnosis of Johne`s disease in cattle. *Aust. Vet J.* 68, 253-257, 1991

Stabel, J.R. Production of γ -interferon by peripheral blood mononuclear cells: an important diagnostic tool for detection of subclinical paratuberculosis. *J.Vet.Diagn.Invest.* 8: 345-350, 1996.

Stehman, S.M. Paratuberculosis in small ruminants, deer and South American camelids. *Veterinary Clinics of North America: Food Animal Practice*, 12: 441-455, 1996.

Travería, G.E.; Baldo, A.; Silvestrini, M.P.; Bernardelli, A.; Romero, J.R. y César, N. The effects of skin testing with avian tuberculin on antibody responses to paratuberculosis. *The Paratuberculosis Newsletter*, 11 (2), 1999.

Telenti,A.; Marchesi, F.; Balz, M. et al. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J. of Clin. Microbiology*, 31 (2): 175-178, 1993.

Whittington, R.J.; Fell, S.; Walker, D.; McAllister, S.; Marsh I.; Sergeant, E.; Taragel, A.; Marshall, D.J. and Links, I.J. Use of pooled fecal culture for sensitive and economic detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in flocks of sheep. *J. Clin. Microbiol.* 38 (7): 2550-6, 2000.

Whittington, R.J.; Hope, A.F.; Marshall, D.J.; Taragel, C.A. and Marsh I. Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis*: IS900 restriction fragment length polymorphism and IS 1311 polymorphism analyses of isolates from animals and a human in Australia. *J. Clin. Microbiol.* 38 (9): 3240 – 3248, 2000.

Yokomizo, Y.; Yugi, H. And Merkal, R.S. A method for avoiding false-positive reactions in an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of bovine paratuberculosis. *Jpn.J. Vet. Sci.* 47 (1): 111 – 119, 1985.