The Paratuberculosis Newsletter

September 2008

An official publication of the
International Association for Paratuberculosis

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Notes from the Editor

This issue of the newsletter has the highest number of contributors in 2008, a trend that I hope will continue. The issue contains a section with the header "Debate" and I hope that future newsletters will contain more scientific opinions, so that we can continuously stimulate each other, not only with data, but also with ideas. Opinions given in the newsletter are not those of the International Association on Paratuberculosis. However, I will invite members to share their opinions. The primary parameter for inclusion is that the opinion should be meaningful and understandable.
No contributions are peer-reviewed, and conclusions drawn rest solely on the authors. Some of the work presented is work-in-progress, which should be considered if citing the studies. Hopefully you will enjoy all this issue. And hopefully, you will contribute to the next one.

Søren Saxmose Nielsen
Editor

DEADLINE FOR NEXT ISSUE: November 15, 2008.

All contributions should be sent to ssn@life.ku.dk

1. IAP Business

International Association for Paratuberculosis

Financial Report -- Second Quarter 2008 (4/1/08 – 6/30/08)

	Checking	Savings	CD	Total
Opening balance (1/1/08)	$10,705.70	$35,772.26	$52,599.91	$99,077.87
Q1 Closing balance (3/31/08)	$14,621.31	$35,818.16	$53,191.74	$103,631.21
Q2 Closing balance (6/30/08)	$15,332.76	$35,860.27	$53,751.45	$104,944.48

Receipts

	Dues	Book Sales	Interest	Total Receipts
Q1	$4100.00	$50.00	$677.33	$4827.73
Q2	$1600.00		$601.82	$2201.82
Year to date	$5700.00	$50.00	$1279.15	$7029.55

Expenses

	Credit card processing fees	Open Journal System Fee	Total Expenses
Q1	$274.39		$274.39
Q2	$101.05	$787.50	$888.55
Year to date	$375.44	$787.50	$1162.94

Net income

Q1	$4553.34
Q2	$1313.27
Year to date	$5866.61

-submitted 8/4/08, Raymond W. Sweeney, VMD; Secretary-Treasurer

10th International Colloquium on Paratuberculosis

The 10th International Colloquium of the International Association for Paratuberculosis will take place on the University of Minnesota campus in Minneapolis, Minnesota, Sunday, August 9, through Friday, August 14, 2009. More details, including a tentative schedule, will be available from a link on the web site http://www.cvm.umn.edu/outreach by mid-September.

Dr. Scott Wells is the Colloquium chairman. Please contact him regarding general meeting questions or suggestions at wells023@umn.edu.
Drs. John Bannantine and Srinand Sreevatsan serve as the Scientific Program co-chairs, and are forming a Scientific Program Committee to establish the 10 ICP scientific program. Logistical questions or concerns should be sent to the Veterinary Continuing Education Office at vop@umn.edu or + 1 612 624 2268 / + 1 800 380 8636.

2. Communications from members

Claim for a basic level in paratuberculosis control programs

Walter Baumgartner, Johannes Lorenz Khol

Clinic for Ruminants, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria

Many different countries have established voluntary programs to control paratuberculosis and prevent further spreading of the disease. In other countries compulsory registration for paratuberculosis is performed, or strict control and stamping out programs are in action. Due to its long incubation period, difficulties in laboratory diagnostic and other specific attributes of *MAP*, paratuberculosis is a problem that can not be solved by one country alone. Beside the ongoing discussions about the improvement of laboratory procedures, the genome of the bacterium and other important scientific topics a supraregional discussion about a basically on farm program against paratuberculosis should be started. First steps in such supraregional control program could include:

1. on farm hygienic and management measures
2. elimination of animals suffering from clinical paratuberculosis
3. agreement on livestock trading

These three cost effective and easy to perform steps could be the beginning of a transnational basic program in the fight against paratuberculosis and the protection of free herds and areas and could be implemented immediately. Beside all differences in actions and opinions this could be considered as a “minimum level” which could be achieved in reasonable time and would be accepted by many countries.
To gain improvement concerning this important topic we emphatically support and demand for a panel discussion or workshop at the next ICP in Minneapolis in August 2009. We hope that this initiative is supported by the members of the IAP and that the organisers of the coming ICP will include our suggestion in the congress program.

Response from the colloquium organisers

We as organizers of the 10 ICP Meeting in Minneapolis in August 2009 recognize the need by countries to develop best management practices for control of JD. Our planning process currently includes availability of time for elective workshops on various topics of interest by meeting participants, one of which could include this topic.

Milk quality assurance for paratuberculosis in the national Dutch dairy herd

M.F. Weber

GD Animal Health Service, PO box 9, 7400 AA Deventer, The Netherlands

In January 2006, a bulk milk quality assurance programme (BMQAP) for paratuberculosis in Dutch dairy herds was initiated. The aim of the BMQAP is to reduce the concentration of *Mycobacterium avium* subsp. *paratuberculosis* (Map) in milk delivered to the milk factories. The BMQAP is run alongside the pre-existing Intensive Paratuberculosis Programma (IPP), which aims at low-risk trade of cattle between herds.

The BMQAP starts with an initial assessment consisting of a single herd examination. Test-negative herds enter a surveillance procedure consisting of biennial herd examinations. Test-positive herds enter a control procedure consisting of annual herd examinations and culling of test-positives. All herd examinations are done by milk- or serum-ELISA. Positive ELISA results can be confirmed by faecal culture.

Based on the results achieved in 2006 and 2007, the Dutch dairy industries have recently taken the lead to increase the uptake of the BMQAP. In 2008, the majority of costs for participating farmers are covered by the dairy industries.

The joint efforts of the dairy industries have resulted in a major increase of the uptake of the BMQAP. By July 1st, 2008, approximately 15.000 (75%) of the 20.000 Dutch dairy herds had entered the BMQAP, alongside approximately 500 (2.5%) herds in the IPP (Fig 1).

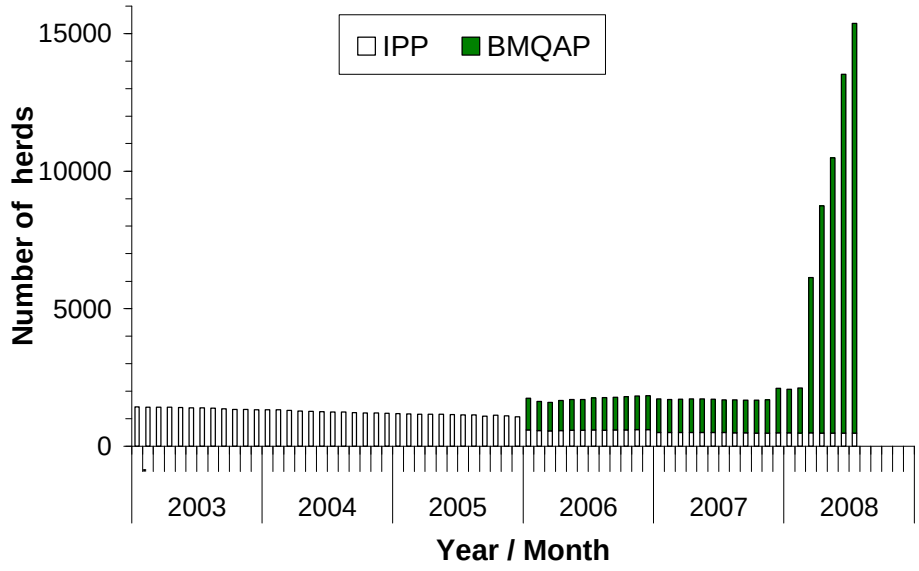

Figure 1. Number of Dutch herds participating in the Bulk Milk Quality Assurance Programme (BMQAP) and Intensive Paratuberculosis Programme (IPP).

3. Short scientific reports

Use of expression of Foxp3 in bovine peripheral blood mononuclear cells in studies of pathogenesis of paratuberculosis

William C. Davis

Dept. Veterinary Microbiology/Pathology, CVM, Washington State University, Pullman, WA USA 99164-7040. e-mail: davisw@vetmed.wsu.edu.

The mechanisms accounting for the loss of protective immunity to paratuberculosis remain to be elucidated. Disease progression appears to be consistently associated with a loss of cell mediated immunity and a concomitant increase in humoral immunity (Rideout et al., 2003). The trigger for initiation of this transition could be that *Mycobacterium avium* subsp. *paratuberculosis* (*Map)*, over time, erodes the capacity of antigen presenting cells (APC, dendritic cells (DC), and macrophages (MΦ)) to sustain a Type I immune response mediated in part by CD4 memory T cells producing IFN-γ. Failure to completely eliminate *Map* infected DC and MΦs would allow for the gradual increase in infected cells and dysregulation of antigen processing and presentation.

The cytokine microenvironment in lesional areas of ileum and lymphoid tissue containing large numbers of APC could change from an environment dominated by the presence of IFN-γ and IL-12 to one dominated by IL-10 and TGF-β, cytokines known to modulate the immune response to intracellular pathogens. Exposure of monocytes entering the lesional areas to bacteria before differentiation to immature DC (iDC) could lead to the differentiation of regulatory DC (DCr) that promote differentiation of regulatory T cells (Tr) producing IL-10 and TGF-β that down-regulate Type 1 $CD4^+$ and $CD8^+$ T cell responses (Mariotti et al., 2002; McGuirk and Mills, 2002; McGuirk et al., 2002; Niedergang et al., 2004). Comparison of cytokine profiles in tissues and peripheral blood mononuclear cells (PBMC) obtained from cows at the pre-clinical and clinical stages of disease support this possibility (Buza et al., 2004; Khalifeb and Stable, 2004a; 2004b; Stabel, 2000; Sweeney et al., 1998). Preliminary studies currently in progress in our laboratory now provide evidence that Tr cells may indeed be involved in modulation of the immune response to *Map* and pathogenesis. A transcription factor forkhead box P3, Foxp3 (Vignali et al., 2008) has been used as a marker to distinguish $CD4^+/CD25^+$ effector T cells from $CD4^+/CD25^+$/Foxp3+ Tr that secrete IL-10 and TGF-β (Mahic et al., 2008). We recently developed monoclonal antibodies to bovine Foxp3 to study the potential role of Tr in regulation of immune responses to infectious agents (Seo et al., 2008) Flow cytometric analysis of the immune response to *Map* in experimentally infected calves has shown that there are only a few $CD4^+/25^+$ T cells in freshly isolated peripheral blood mononuclear cells (PBMC) and PBMC cultured in medium alone. Stimulation with PPD, soluble *Map* antigens (SAg) or live *Map* elicits a strong proliferative response dominated by $CD4^+$ T cells expressing CD25 and 11 other activation molecules (Koo et al., 2004). $CD8^+$ T cells also proliferate, but usually comprise a smaller proportion of cells responding to Ags. Current studies with experimentally infected calves have revealed little or no expression of Foxp3 in freshly isolated cells or cells cultured with or without Ag (unpublished data). In contrast, examination of PBMC from naturally infected cows at a pre-clinical stage of disease, with a strong reaction in a commercial *Map* ELISA, has shown that Foxp3 is highly expressed in activated $CD4^+/CD25^+$ memory cells present in PBMC cultured in RPMI alone and PBMC cultured in the presence of PPD, SAg, or live *Map* (see Figure 1). Foxp3 was not detected in $CD4^+/CD25^+$ T cells present in freshly isolated PBMC. It was only detected in activated proliferating PBMC. Quantitative RT-PCR is in progress to determine the cytokine profile present in these cells.

The points of interest are that culture alone can lead to activation and expression of Foxp3 in some PBMC present in blood from animals that have a strong antibody response in a *Map* ELISA. Further studies are needed to determine if the $CD4^+/CD25^+$ cells present in the cell preparations are the ones that become activated and express Foxp3. Second is the

observation that all activated Ag stimulated cells express Foxp3 at this stage of disease. Further studies are now needed to show that activated Foxp3 positive cells possess immunosuppressive activity and that a strong reaction in the *Map* ELISA is a consistent indicator of the appearance of $CD4^+/CD25^+$ cells with potential immunosuppressive activity (Mahic et al., 2008). The development of mAbs to bovine Foxp3 affords new opportunities to study the mechanisms of pathogenesis of paratuberculosis at the cellular level.

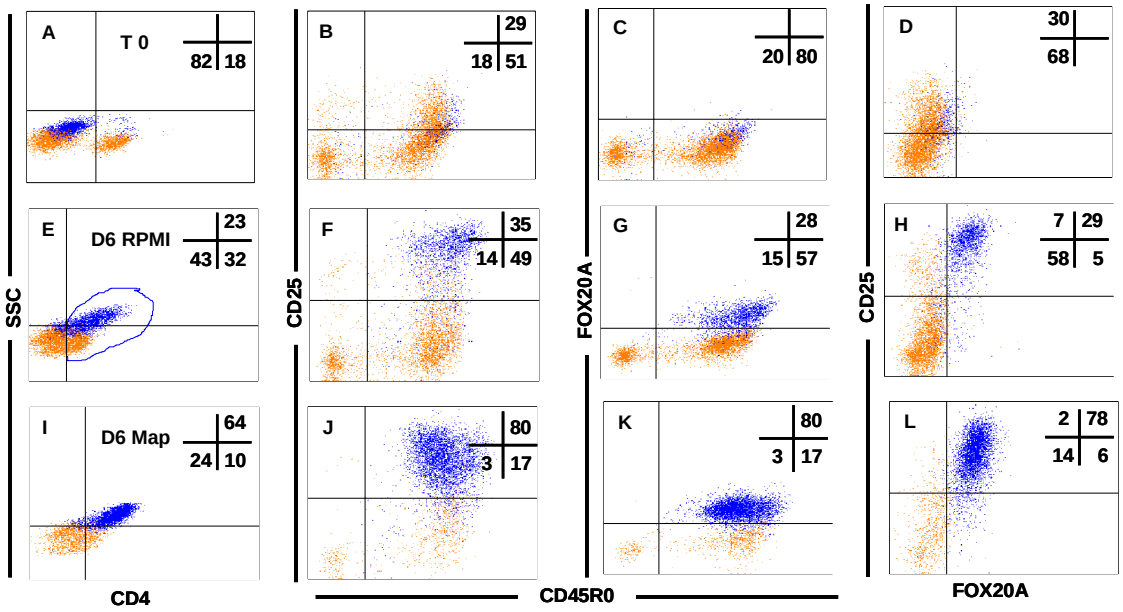

Figure 1. Representative dot plot flow cytometric profiles of PBMC obtained from *Map* ELISA positive Holstein cows (N = 3) labeled with mAbs specific for CD4 (IL-A11A, IgG2a), CD45R0 (IL-A116A, IgG3), CD25 (CACT116A, IgG1), and Foxp3 (FOX20A, IgG2b). The cells (2 X 10^7) were labeled at the initiation of culture (T0, A - D) and after 6 days of culture in RPMI culture medium alone (E – H) or with live Map (K10, 10^7 in 20 ml of culture medium, I - L). Two electronic gates were placed on the cells using side (SSC) and forward light scatter (FSC) to distinguish small un-activated (orange) cells from large activated proliferating cells (blue). CD4 positive T cells were isolated with a third electronic gate to analyze expression of CD25 and Foxp3 on naïve (CD45R0 negative) and memory (CD45R0 positive) CD4 T cells. The figures in the upper right quadrant in the dot plots show the percent of cells negative or positive for the respective molecules expressed on CD4 T cells. As shown, a small proportion of CD4 memory T cells expressed CD25 at T0 (plot B) but not Foxp3 (plot C) in freshly isolated PBMC. Expression was less than one percent on naïve cells. Following culture in RPMI alone, expression of CD25 and Foxp3 was upregulated on activated memory T cells (F – H). Essentially all activated cells co-expressed both molecules (H). Proliferation of CD4 T cells was more extensive following culture with *Map*. Activated but not resting (un-activated) CD4 memory T cells co-expressed CD25 and Foxp3 (J – L).

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Use of blotted tissue impressions for rapid PCR identification of *Mycobacterium avium* subspecies *paratuberculosis*

J. Elliot Williams[1*], Claus D. Buergelt[1], Pablo Pinedo[1], Gilles R.G. Monif [1,2]

[1]*College of Veterinary Medicine, University of Florida, Florida, USA.*
[2]*College of Medicine, University of Oklahoma-Tulsa, Oklahoma, USA.*
**Corresponding Author: J. Elliot Williams; E-mail: WilliamsE@vetmed.ufl.edu*

Abstract
Simple polymerase chain reaction (PCR) and nested PCR both identified *Mycobacterium avium* subspecies *paratuberculosis* DNA in blotted tissue impressions done on bovine tissue of cows with documented Johne's disease. Simple PCR based upon IS*900*, identified 64% of ileocecal lymph nodes, 69% of mesenteric lymph nodes and 71% of ileal tissue samples from cows with documented paratuberculosis. The addition of nested PCR identified 100% of ileocecal lymph nodes, 100% of mesenteric lymph nodes and 100% of the ileal tissue. This PCR based test allows for rapid identification of and is a definitive diagnosis for MAP.

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the pathogenic causation of a chronic granulomatous intestinal disease (Johne's disease) in domestic and wild ruminants (Chiodini and others 1984). Necropsy studies have traditionally involved histopathological examination in which sections stained with acid-fast stain constituted the principal tool for analysis (Whitlock and Buergelt, 1996; Clarke, 1997). The detection of acid-fast bacilli infers, but does not document, that infection is due to MAP. The introduction of diagnostic probes based on specific bacterial DNA sequences has allowed the rapid identification of pathogens, and at the present a series of PCR tests based on MAP DNA insertion sequence (IS*900*) are available (Harris and Barletta 2001). The use of a nested PCR aiming at IS*900* with applicability for the demonstration of MAP DNA in tissues, blood and milk has been reported (Gwozdz et al., 1997; Buergelt and Williams 2004). It is hypothesized that this technique applied to blotted tissue impressions could be a rapid and effective method for MAP identification in bovine tissue samples.

The purpose of this short communication is to describe a rapid and simple technique that permits definitive determination of the presence of MAP in bovine tissues by the application of direct and nested PCR techniques.

The study population consisted of 14 adult cows subjected to necropsy and primarily derived from a Holstein herd located in Alachua County, Florida, USA. Each animal had been documented to have clinical Johne's disease confirmed by agar gel immunodiffusion (AGID), ELISA titer, and/or nested PRC performed on blood and milk. At the time of necropsy a pre-absorbed ELISA (Allied laboratories Inc., Ames, Iowa, USA) test was performed using a crude soluble protoplasmic antigen (Allied Monitor Missouri, Fayette, MO, USA), following a previously reported protocol (Braun et al., 1990). Test sera were pre-absorbed with *Mycobacterium phlei* and ELISA results were calculated from wave length readings at OD 405 nm. Results were recorded as negative (<1.5), inconclusive (1.5 to 1.9), low positive (2.0 to 2.5), and high positive (>2.5) as reported previously (Buergelt and Williams, 2003; 2004). Simultaneously, an AGID test was performed by use of the same crude, protoplasmic antigen that was used for the ELISA. Petri dishes were poured with sterile 1% agarose prepared in 0.1 M Tris-HCL buffer at pH 10. Well distances were 8 mm and well sizes were 4 mm for the six peripheral wells and 3 mm for the central well. Peripheral wells were inoculated with 45 µL of test sera. The central well contained 35 µL of antigen. A positive control serum from a cow proven to have paratuberculosis was used. Final analytical readings were done after 48 hours.

Pertinent tissue samples (Table 1) were aseptically collected during the necropsy procedure. Tissues were cut in such a way that "touch-press" blots could be made on a standard glass slide. A 200 µl aliquot of 0.2 N NaOH was applied to the slide and a sterile razor blade was used to scrape the tissue from the slide immediately after application. The

mixture was removed with a pipette (Fig. 1) and placed into a 1.5 Eppendorf screw-cap vial and boiled for 20 minutes @110 degrees Centigrade, spun for 1 minute at 500 g and then tested by PCR technology. A second slide was stained for acid-fast bacilli using acid-fast stain.

Table 1. Comparison of J1-2 Nested PCR vs., P90-91 based PCR in the detection of *Mycobacterium avium* subspecies p*aratuberculosis* in bovine selected necropsy tissues.

			P90-P91[†]	J1-J2[‡]		P90-P91[†]	J1-J2[‡]		P90-P91[†]	J1-J2[‡]	
Cow Id	AGID	ELISA (ER)[¥]	PCR ICLN	PCR ICLN	ICLN Stain[§]	PCR MLN	PCR MLN	MLN Stain[§]	PCR Ileum	PCR Ileum	Illeum Stain[§]
NO3-541	+	4.7	-	+	P	+	+	P	-	+	P
NO3-583	+	3.3	-	+	P	-	+	M	+	+	M
NO3-648	+	1.6	+	+	P	+	+	P	+	+	M
NO3-718	+	5.6	+	+	P	+	+	M	+	+	M
NO3-216	+	4.5	-	+	P	-	+	M	n.t.	n.t.	n.t
NO4-267	+	4.9	+	+	P	-	+	P	-	+	P
NO4-369	+	3.8	-	+	M	n.t.	n.t.	n.t	n.t.	n.t.	n.t
NO4-423	+	3.8	+	+	P	+	+	M	n.t.	n.t.	n.t
NO4-752	-	3.9	+	+	M	+	+	M	+	+	M
NO4-754	+	3	+	+	M	+	+	M	n.t.	n.t.	n.t
NO4-757	-	3	+	+	P	+	+	M	n.t.	n.t.	n.t
NO4-286	-	2.2	+	+	M	+	+	M	n.t.	n.t.	n.t
NO4-422	-	3	+	+	M	+	+	M	+	+	M
NO4-420	+	2.3	-	+	P	-	+	P	n.t.	n.t.	n.t

[§] Stain=acid-fast stain; P= paucibacillary; M= multi-bacillary n.t.= not tested
[†]=simple PCR; [‡]=nested PCR
[¥]ER=ELISA ratio: <1.5:negative; 1.5-1.9: inconclusive; 2.0-2.5: low positive; >2.5: high positive

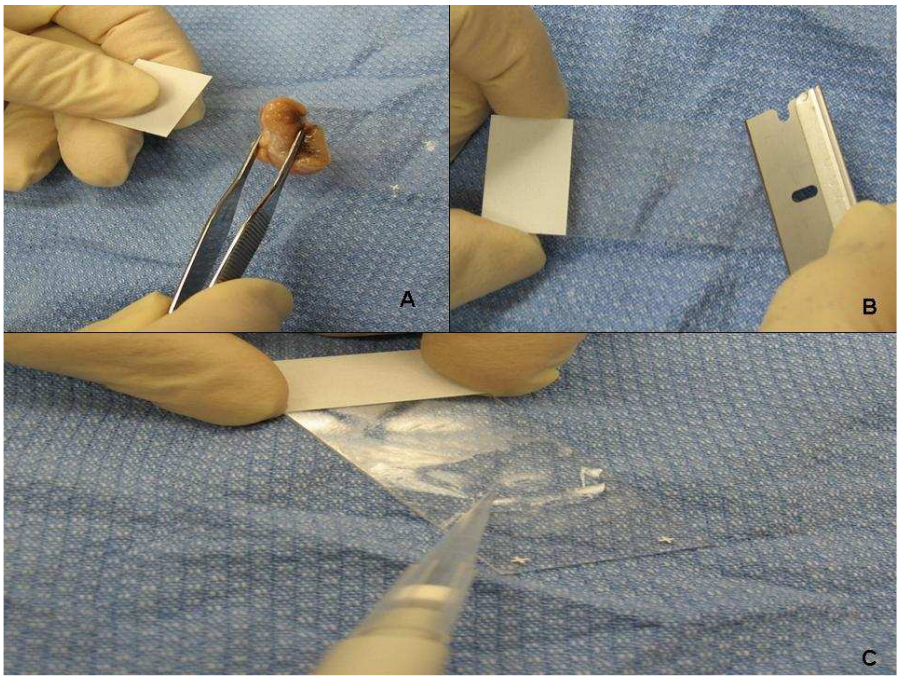

Fig. 1. Blotted tissue impressions: (A) Tissue is pressed onto slide several times; (B)Using a razor blade, tissue is scraped on NaOH solution several times with a back and forth motion; (C) A filtered pipette tip is used to remove homogenate for collocation in tube.

Samples were subjected to a nested PCR. The first reaction was based on forward primer, P90 5'-GAAGGGTGTTCGGGGCCGTCGCTTAGG-3', reverse primer, P91, 5'-GGCGTTGAGGTCGATCGCCCACGTGAC-3' which target a 413 bp sequence of IS*900* in

MAP. The second reaction used 1 µl of amplified product and forward primer, J1, 5'-TGATGGCCGAAGGAGATTGGCCG-3' and reverse primer, J2, 5'-GTTGAGGTCGATCGCCCACGTGAC-3' which overlap and span a 333 bp region within the insertion sequence (Vary et al., 1990; Gwozdz et al., 1997; Buergelt and Williams, 2004). The protocol for the first stage PCR was 35 cycles at 94^{o}C for 30 s at 58^{o}C for 15 s and at 72^{o}C for 60 s. The protocol for the second stage (nested reaction) consisted of 36 cycles of 30 s at 94^{o}C, 15 s at 63^{o}C and 60 s at 72^{o}C. A volume of 10 µl of the PCR product was run on 1.5% agarose gel by electrophoresis in TAE running buffer (Continental Lab Products, CA, USA). Extracted DNA from an isolate previously obtained from a clinically affected cow confirmed at necropsy by histopathology and culture was used as positive control and sterile water was used as negative control for the PCR assay in each of the reactions. Gel inspection was done using ultraviolet light and recorded with a computerized digital camera (UVP Transilluminator System, BIO-RAD Laboratories, Segrate, Milan, Italy).

Sections of at least one of the following three tissues were examined by light microscopy; small intestine, mesenteric and ileocecal lymph nodes. Specimens were stained with hematoxylin-eosin stain to determine the density of bacilli present.

The test results are summarized in Table 1. All tissues studied that exhibited advanced histopathology characteristics for disease in that organ system were confirmed positive upon acid-fast staining.

As previously reported (Gwozdz et al., 1997; Buergelt and Williams, 2004), the use of nested primers over a previously amplified segment of IS*900* considerably increased the sensitivity of the process. Simple PCR based upon the IS*900* and using primers P90-91 identified 64% of the ileocecal lymph nodes, 69% of the mesenteric lymph nodes and 71% of the ileal tissue samples from cows with documented MAP enteritis. The addition of the J1-2 set of primers identified 100% of ileocecal lymph nodes, 100% of the mesenteric lymph nodes and 100% of the ileal tissue. This PCR based test allows for rapid identification of and is a definitive diagnosis for MAP.

The authors gratefully acknowledge the financial support provided by the Florida Dairy Farmers Association and Infectious Diseases Incorporated.

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Equine Johne's Disease: Are Equine *Mycobacterium avium* and *M. avium* subsp. *paratuberculosis* truly *M. avium* and *M. avium* subspecies *paratuberculosis*?

Gilles R.G. Monif, J. Elliot Williams, Barbara J. Sheppard

Sheppard et al. (2008) reported a case of necrohemorrhagic enterocolitis with underlying granulomatous enteritis and lymphadenitis. The mycobacterium was identified by the USDA diagnostic laboratory at Ames Iowa as *Mycobacterium avium* using 16s rRNA. The investigators extracted from formalin fixed tissue the organism's DNA. The extracted DNA was tested using base and nested primers based upon the IS*900* and IS*1311* insertion sequences. The nested IS*900* and IS*1311* primers identified the isolate as being closer to *Mycobacterium avium* subspecies *paratuberculosis* (Map) than *M. avium*. A second equine case due to similar etiology is now reported.

An 8 year old gelding quarter horse presented with anterior enteritis. He was treated for 5 days with reflux, lidocaine and Banamine. He did well until he became febrile (T=102.9 – 103.3). Fluid obtained by abdominocentesis revealed the presence of 202,100 white blood cells per cc. At surgery, multiple transmural jejunal and ileal masses were identified. Microscopic examination revealed a necrohemorrhagic enteritis imposed upon a granulomatous ulcerative enteritis. Intralesional acid fast bacilli were present. Multi-focal areas of fibrosis were present. The animal's owner elected to have the horse euthanized.

The mycobacterium's DNA was extracted from formalin fixed tissue and tested using base and nested primers based upon the IS*900* and IS*1311* insertion sequences. Both the nested IS*900* and IS*1311* sets of primers identified the organism as being *Mycobacterium avium* subspecies *paratuberculosis*. The critical observations were not what primers tested positive for Map, but those base primers which did not test positive. Neither the base IS*900* nor the IS*1311* base sets of primers were positive. Comparable data were present in the original Sheppard case cited. The combined data from these two horses suggests mycobacterium causing equine Johne's disease appears to be genomic polymorphic variant between the two mycobacterium species.

Equine Johne's disease may be a more common event than currently recognized. Necropsy analysis of horses is the exception and not the rule. The presence of multiple areas of fibrosis and the presence of a moderate number of plasma cells indicate the chronicity of the disease process. The overlying acute inflammatory and necrotizing hemorrhagic histopathology suggests that, in both cases, an intervening acute disease appears to have unmasked an underlying chronic process.

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Overview of a milk real-time PCR assay for *Mycobacterium paratuberculosis* detection

Warren Foshaug[1], Heather Donahue[1], Jason Lombard[2], Todd Byrem[1], Preetha Biswas[1]

Antel BioSystems, Inc. Lansing, MI 48910[1] and National Animal Health Monitoring System (NAHMS) USDA:APHIS:VS:CEAH, Fort Collins, CO 80526[2]

This report describes the development of an *M. paratuberculosis* (Map) PCR test for use in milk, and a brief overview of results from a large scale field trial of the method. The test is intended to complement a Johne's disease screening and management program.

Fifty mL of bulk tank milk sample is processed by centrifugation and bead-beating, followed by DNA extraction using DNeasy spin columns (Qiagen, Inc., Valencia, CA). Real-time PCR is performed with IS*900* as the target sequence using an Applied BioSystems 7500 Real-Time PCR System and TaqMan PCR reagents (Applied BioSystems, Foster City, CA). Milk samples with a Ct value of less than 41 in each of two replicates are considered positive. Preliminary assays indicate the test may be able to detect as little as 1 cfu/mL of milk.

A large-scale trial of the procedure was as a component of a USDA study, NAHMS Dairy 2007 (National Animal Health Monitoring System, http://nahms.aphis.usda.gov/dairy/index.htm). Bulk tank PCR test results were compared to environmental fecal culture data, where a given dairy farm was considered positive if one culture tube or flask (HEYM) from any of six environmental samples had a confirmed Map colony. Of 515 dairy farms, 384 were culture positive for Map, while 131 were negative. The bulk milk PCR detected Map in 153 of the culture positive samples, and 8 of the culture negative samples (Table 1). Therefore, the relative sensitivity of the test is 39.8% and the relative specificity is 93.9%. In addition, the PCR signal intensity increased (lower Ct value) in the farms that had a higher number of culture positive environmental sites, suggesting a higher level of Map contamination in these farms (data not shown). As determined by PCR, 31.2% of participating dairy farms had bulk tank milk positive for Map.

The rapid turnover of milk-based PCR assay over traditional culture method offers a quick means to screen dairy herds for the presence of Map infection. The sensitivity of the assay suggests it should not be used as a stand-alone test, but it will prove a valuable addition to an industry wide Johne's disease testing and control program. The relatively high percentage of dairy farms with Map positive bulk milk, whether through shedding or contamination of the milk, demonstrates the need for additional efforts to evaluate the quality of the milk supply.

Table 1. Comparison of bulk milk real-time PCR to environmental sample HEYM culture

		HEYM Culture		
		Positive	Negative	Total
Milk PCR	Positive	153	8	161
	Negative	231	123	354
	Total	384	131	515

Abstracts of MAP posters presented by Dr Irene Grant's research group at the recent Society for Applied Microbiology Summer Conference held in Belfast, 7-10 July 2008

Antibacterial activities of naturally-occurring compounds against *Mycobacterium avium* subsp. *paratuberculosis*

Stella Wong[1], Irene Grant[1], Mendel Friedman[2], Chris Elliott[1] and Chen Situ[1]

[1]Institute of Agri-Food and Land Use, Queen's University Belfast, Belfast, UK
[2]Western Regional Research Centre, USDA, Albany, California, USA

Aims: To investigate the antibacterial activities of 19 naturally-occurring compounds (including essential oils and some of their isolated constituents, apple and green tea polyphenols and other plant extracts) against the animal pathogen *Mycobacterium avium* subsp. *paratuberculosis* (*Map*).
Methods and Results: The naturally-occurring compounds were evaluated against three strains of *Map*, a bovine isolate, a raw milk isolate and a human isolate, using a macrobroth susceptibility testing method. *Map* was grown in 4 ml Middlebrook 7H9 broth containing 10% OADC, 0.05% Tween 80 (or 0.2% glycerol) and 2 µg/ml mycobactin J supplemented with five concentrations of each test compound. The optical density of the cultures at 600 nm was recorded at intervals over an incubation period of 42 day at 37^{o}C. The lowest concentration of test compound in test tubes with no visible or detectable bacterial growth was considered to represent the minimum inhibitory concentration (MIC). Six of the 19 compounds were found to inhibit the growth of *Map*. The most effective compound was trans-cinnamaldehyde with an MIC of 25.9 µg/ml, then cinnamon oil (26.2 µg/ml), oregano (68.2 µg/ml), carvacrol (72.2 µg/ml), 2,5-dihydroxybenzaldehyde (74 µg/ml) and 2-hydroxy-5-methoxybenzaldehyde (90.4 µg/ml).
Conclusions: Six naturally-occurring compounds exhibited anti-*Map* activity. Three of the six inhibitory compounds were aldehydes (*trans*-cinnamaldehyde, 2-hydroxy-5-methoxybenzaldehyde and 2,5-dihydroxybenzaldehyde). Trans-cinnamaldehyde is the main active ingredient (81%) in cinnamon cassia oil. The phenolic compound carvacrol is the major component in oregano oil. These results suggest that the aldehyde group of the three compounds and the hydropobic-hydrophilic nature of carvacrol may be important for antimicrobial activity. No difference in compound activity was observed between the three *Map* strains studied.
Significance of Study: Currently there is no drug approved to treat Johne's disease. The anti-*Map* naturally-occurring compounds identified in this study may have the potential to be included in therapeutic drugs for treatment of Johne's disease in farm animals, and possibly also in human medicine against Crohn's disease, or as food additives for dairy products.

Optimisation of a phage amplification assay for enumeration of viable *Mycobacterium avium* subsp. *paratuberculosis*

Antonio Foddai, Irene Grant and Chris Elliott

Institute of Agri-Food and Land Use, Queen's University Belfast, Belfast, UK

Aims: To optimise phage amplification assay conditions to permit accurate quantification of viable *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) by investigating burst time, burst size and how correlation between plaque counts and colony counts is affected by incubation time.
Methods and Results: Four week old cultures of four different *Map* strains in Middlebrook 7H9 broth containing 10% OADC supplement and 2 µg/ml mycobactin J were inoculated with D29 mycobacteriophage (10^8 pfu/ml) and incubated at 37°C before viricide treatment and plating in Middlebrook 7H9 agar along with *Myco. smegmatis* mc^2155 cells to yield plaques. Burst time was determined in three ways: Method 1 – OD_{600nm} was measured at 15 minute intervals post-D29 infection until a reduction in OD due to lysis of cells was observed; Method 2 – viricide treatment was carried out after different incubation times post-D29 infection up to 240 min and pfu were monitored until a reduction in pfu was observed; Method 3 – D29 infected *Map* cultures were treated with viricide 15 min post-D29 infection and then incubation was extended for periods up to 280 min before plating; pfu were monitored until pfu counts plateaued. Colony counts (cfu) for comparison with plaque counts were obtained by plating the original *Map* cultures on Herrold's egg yolk medium containing 2 µg/ml mycobactin J and incubating plates at 37°C for 4-6 weeks. The burst time for *Map* indicated by the three methods was between 180-220 min at 37°C.
Conclusions: The burst time obtained for *Map* is much longer than that of fast-growing *Myco. smegmatis* (60 min). The difference between pfu and cfu counts decreased as incubation time increased (1.5-2 $\log_{10}$ at 60 min, 1-1.5 $\log_{10}$ at 210 min) but 100% correlation between pfu and cfu counts was never achieved, which suggests that aspects of the test other than incubation time still need to be optimised.
Significance of Study: This study has provided new information about burst time of *Map* following D29 mycobacteriophage infection which has led to some improvement in the phage amplification assay. However, further optimisation is needed before the assay could be used to accurately quantify viable *Map* in a sample.

Abstracts of presentations from a workshop on paratuberculosis risk and control held at Adelphi Hotel, in conjunction with at Society of Veterinary Epidemiology and Preventive Medicine, Liverpool, March 26, 2008. The workshop was chaired by George Gunn, SAC Epidemiology Research Unit, Inverness, Scotland, United Kingdom

Dissecting Paratuberculosis Tools

Objective: To seek informed feedback and advice on all aspects of paratuberculosis risk and control through a series of interactive presentations reviewing ongoing and linked European Community (EC) Work Package projects in ParaTBTools.

Workshop description: The EC remain concerned about the control of paratuberculosis (*Mycobacterium avium* subspecies paratuberculosis; Map) in livestock populations and the putative link to Crohn's disease in humans. Control is often seen to be restricted, in part, by the limited abilities of the current diagnostic tests and vaccines. In 2006, following a competitive tender process within Framework 6, a large International consortium led by the Netherlands was funded to "Develop improved tools for the detection of paratuberculosis in livestock, *M. paratuberculosis* in food & the assessment of risk for human exposure". Work in progress was presented during the workshop:

- G Gunn provided context through an overview of the full project (5 themes) and how it relates to Theme 4 – Risk & Control.

Current updates were provided for the four Work Packages within that theme:

- Søren Nielsen & Nils Toft lead a discussion on diagnostic accuracy, prevalences and prevalence study design.
- Ross Davidson described the use of existing epidemiological modeling frameworks of Map dynamics in dairy herds to quantify the benefits in disease eradication and reduction in prevalence per unit improvement in the diagnostic test sensitivity and vaccine efficacy.
- Alistair Stott explored the hierarchy of interdependent economic models in the toolbox: decision analysis methods for farm level; economic welfare analysis for regional level and partial equilibrium modelling for national level research.

Overview of Full EU Paratuberculosis Tools Project

George Gunn

SAC Epidemiology Research Unit, Stratherrick Rd., Inverness, Scotland, UK

In 2006 the EU commissioned a consortium project led by Dr. Douwe Bakker from Lelystad in The Netherlands: "Development of improved tools for the detection of paratuberculosis in livestock, *M. paratuberculosis* in food & the assessment of risk for human exposure". This is a 4.5 Million-Euro project involving 28 partners over a 3-year period. The presentation will try to provide an outline for the full project before focussing on Theme 4 "Risk & Control" that encompasses the Work Packages to be discussed in more detail within the rest of this workshop. The five thematic areas are:

TA 1: Diagnostic Tools – Leader Dr. Douwe Bakker (Lelystad)
TA 2: Host Pathogen Interaction - Leader Dr. Jim McNair (Belfast)
TA 3: Food Safety - Leader Dr. Michael Rowe (Belfast)
TA 4: Risk & Control - Leader Prof. George Gunn (Inverness)
TA 5: Crohn's Disease - Leader Dr. Ingrid Olsen (Oslo)

The 16 Work Packages are:

Work Package 1: Standardisation of protocols for diagnosis of PTB and detection of MAP. WP Leader - Bakker; Lelystad (Netherlands)
Work Package 2: Novel antigens to detect MAP. WP Leader - Willemsen; Lelystad (Netherlands)
Work Package 3: Develop improved methods for the diagnosis of PTB and detection of MAP. WP Leader - Garrido; Berreaga (Spain).
Work Package 4: Cattle infection model for MAP. WP Leader - McNair; Belfast (UK).
Work Package 5: Association between ruminant immune responses and pathology for MAP. WP Leader - Guilloteau; Nouzilly (France).
Work Package 6: Optimisation and validation of blood based tests. WP Leader - Jungersen; Copenhagen (Denmark).
Work Package 7: improved culture and molecular methods to detect MAP in milk products. WP Leader - Rowe; Belfast (UK).
Work Package 8: Standardisation methods to isolate MAP in milk products. WP Leader - Donachy; Belfast (UK).
Work Package 9: Assessment of the degree of inactivation of MAP in dairy product. WP Leader - Hammer; Kiel (Germany).
Work Package 10: Evaluation of existing test strategies and prevalence estimates. WP Leader - Nielsen; Copenhagen (Denmark).
Work Package 11: Paratuberculosis control: how good the tools? WP Leader - Hutchings; Edinburgh (UK).
Work Package 12: Assessment of the farm business risk and economically optimum control strategies. WP Leader - Stott; Aberdeen (UK).
Work Package 13: Qualitative & Quantitative risk assessment for presence MAP in food products. WP Leader - Guitan; London (UK).
Work Package 14: Characterisation of immune responses to MAP in humans. WP Leader - Olson; Oslo (Norway).
Work Package 15: Isolation and characterisation of human MAP isolates. WP Leader - Bull; London (UK).
Work Package 16: Dissemination of the results. WP Leader - Aranaz; Madrid (Spain)

Assessing the Prevalence of Paratuberculosis in Europe

Søren Saxmose Nielsen[1], Nils Toft[2]

[1]University of Copenhagen, Copenhagen, Denmark, and
[2]Danish Meat Association, Kjellerup, Denmark

Reliable and unbiased prevalence estimates of a disease are essential for both choosing a particular control strategy and for a risk assessor modelling the disease and its consequences. To facilitate these tasks, our aim was to assess the animal and herd level prevalence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in farmed animals in Europe. Since no perfect diagnostic tests exist for detection of MAP, a number of different tests are used. Thus, it was necessary to review the accuracy of currently available diagnostic tests, prior to assessing the prevalences in order to compare populations.

A literature review of the reported diagnostic test evaluation studies was conducted, and summarised based on animal species (cattle, sheep, goat and deer), test-type (faecal culture (FC), ELISA and interferon-γ), target condition (MAP infected, infectious and affected), target population, and other relevant information (Nielsen and Toft, 2008a).

The quality of the test evaluation studies was generally poor and the main reasons for exclusion from further comparison were variations in study design, selection bias and poor reporting of the studies. Given that very few studies were of reasonable quality, no formal statistical comparisons were made. We concluded that sensitivities and specificities varied considerably and stratification by different target conditions was deemed relevant. Although, many test evaluation studies have been published, there is a great need for well designed test evaluation studies following the guidelines outlined in e.g. Greiner and Gardner (2000).

In the second step of the literature review, we collected information from available prevalence studies. However, the design of many studies was either poor or a test with unknown accuracy had been used. The most reliable prevalence studies in cattle suggested between-herd prevalences of >50% and within-herd prevalences around 20% (Nielsen and Toft, 2008b), but these estimates could only be supported by data from a few countries.

A general challenge in estimation of test-accuracies and prevalences for MAP is the lack of a reference test. Many test-evaluations are carried out on a study population that is not representative of the target population, ultimately resulting in biased estimates. However, different types of latent-class methodologies exist and could be further used. We have demonstrated that Bayesian mixture-models can produce more precise prevalence estimates than the classical cut-off based models (Nielsen et al., 2007). In addition, if selection bias is avoided, more reliable prevalence estimates should be achievable. These methods are reasonably new in veterinary medicine and have yet to gain a wider acceptance.

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Rates of return in paratuberculosis control per unit improvement in diagnostics and vaccines

Ross S. Davidson[1], Glenn Marion[2], George Gunn[1], Michael R. Hutchings[1].

[1]SAC, West Mains Road, Edinburgh EH26 0PH
[2]Biomathematics, Statistics Scotland, Kings Buildings, Edinburgh EH26 3HH

The control of paratuberculosis (*Mycobacterium avium* subspecies *paratuberculosis*; *Map*) in livestock populations is often seen to be limited, in part by the limited abilities of the current diagnostic tests and vaccines. In 2006 a large European consortium was funded to improve on these existing epidemiological tools. Here we use an existing epidemiological modeling framework of *Map* dynamics in dairy herds to quantify the benefits in disease eradication and reduction in prevalence per unit improvement in the diagnostic test sensitivity and vaccine efficacy. The simulation model was able to present the abilities of the current tests and vaccines within a continuum. We explored two aspects of the diagnostic test sensitivity; the proportion of infected animals that test positive prior to the onset of clinical disease and the mean infected time that the diagnostic detects an infected animal. We discuss the results in relation to the likely gains in disease control per unit improvement of the epidemiological tools. We conclude that significant improvements in paratuberculosis control can be achieved with improved tools, however, the results suggest the need for a long term approach to paratuberculosis control.

Dissecting economic tools for decision support in Paratuberculosis control

Alistair W. Stott[1], Habtu T. Weldegebriel[1], Luiza Toma[1] and George J. Gunn[1].

[1]SAC, West Mains Road, Edinburgh EH26 0PH

The EU FP6 project 'Paratbtools' aims to develop better tools to detect and diagnose *Mycobacterium avium* subspecies *paratuberculosis* (*Map)* in animals and foodstuffs. Such tools provide the basis for improved control of Paratuberculosis (PTB) in animals and hence reduced exposure to *Map* in man. Economic tools are required to ensure that the improved detection and diagnostic tools and the information they generate provide efficient decision support and so yield the greatest benefit to stakeholders. Decision makers concerned range from farmers through to national and international policy developers. Economic tools are required at all these levels. Starting with tools that aid dairy cow replacement in response to PTB at farm level (Stott et al., 2005), we developed a Markov chain model to generate the data needed for further economic analysis. This may be used to aid investment in disease prevention and control at farm level (Stott and Gunn, 2008). We have also used it to assess the benefits to stakeholders (consumers and farmers) of a hypothetical eradication of PTB from the Scottish dairy herd (Weldegebriel et al., 2008). Our results suggest a £14.3m gain in discounted economic surplus to consumers, £13.4m gain to farmers currently infected with PTB but a £10.7m loss to PTB-free producers (due to lower milk prices without compensating milk yield increases). The net gain to society was £17m. These results can be compared with the costs of eradication, with or without improved detection/diagnosis tools and give policy makers an indication of how the costs of eradication might be fairly shared between stakeholders according to benefit. To account for the impact of reduced prevalence of PTB on international trade and on the environment we are also developing a partial equilibrium model of trade in milk/beef between Scotland and the EU (see McCalla, A.F., and Revoredo, C.L., 2001, for a review of such models and Rich et al., 2005 for an overview of possible application to animal disease).

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4. Debate

What If? A Contrarian's Questioning of the Natural History of Bovine Infection Due to *Mycobacterium avium* subspecies *paratuberculosis*

Gilles R.G. Monif, M.D.

Johne's disease is a progressive chronic granulomatous disease of the bovine gastrointestinal tract caused by *Mycobacterium avium* subspecies *paratuberculosis* (Map) (Stabel, 1998). The adverse economical impact of disease globally is estimated at 1.5 billion dollars annually (Chi et al., 2002). In 1999, the economical loss to the United States dairy industry was estimated at $220 million dollars (Ott et al., 1999).

The adverse economical impact of Map on the dairy industry has been greatly reduced by either systemically administered, killed Map vaccines or commitment to a herd management policy of testing and removing all animals with a significant level of shedding as demonstrated on fecal culturing and/or with a diagnostic titer as determined by a commercial Map ELISA test. In such herd, the net reduction has been a marked reduction in the number of animals developing clinical diseases and impact on milk and fat production. The basic problem with both disease prevention schemes is that neither precludes the prevalence of Map within the herd (Kormendy, 1994; Kalis et al., 2001).

The focus on disease has obscured the natural history of Map bovine infection. What is best delineated from an epidemiological perspective is the progression of Map infection to its end-stage consequence, Johne's disease. The pathogenesis of disease is not the natural history of Map bovine infection.

The pathogenesis of disease is considered to involve three stages. In stage I, organism presence is primarily within macrophage-like cells within the gastrointestinal tract. While some evidence of Th-1 cellular immunity can be demonstrated, the organism is rarely found within the feces. No demonstrable B-cell stimulation indicated by the production of specific antibodies is evident. In stage II, the organism can now be intermittently found within feces. Varying degrees of Map antibody production is demonstrable. In stage I and II, the animal is clinically normal or near normal. Stage III is characterized by a chronic progressive diarrhea and weight loss. Once diarrhea is well established, the process ultimately results in death of the animal; although, a few animal have reverted to relative good health status.

A second source of scientific data applicable to understanding the pathogenesis of Map induced disease comes from the experimental induction of Map infection/disease in host animals. Lymph node involvement has been demonstrated when an infecting dose of live mycobacteria is introduced into the gastrointestinal tract (Wu et al., 2007). Demonstration of mycobacteria within a lymph node is consistent with site specific availability for antigen processing. Disease induction reflects an imbalance of a Th2 over a TH1 immune response.

The primary focus of serological tests has been the identification of animals with or at high risk of developing Johne's diseases. Other herd management programs have focused upon removal of significant Map fecal shedders. The underlying presumption inherent in both programs is that animals within the herd constitute the reservoirs of infection.

What if infected and diseased cows are the disseminators of infection, but not the ultimate reservoir of infection?

Map strains are global in their distribution. Map produces disease in a wide range of wild and domesticated herbivores. Epidemiological data coupled with the relative inability to eradicate Map shedding from tightly screened dairy herds appear to infer that Map is embedded in the food chain of herbivores as well as possibly their water supply.

What If: Map bovine infection parallels epidemiologically mycobacteria infection as documented in the human model system; more specifically that induction of disease is the exception and not the rule?

In the human model system due to *M. tuberculosis*, disease is a rarity; however, evidence of *M. tuberculosis* cell-mediated immunity can be demonstrated years after the initial infection in a large number of exposed individuals.

Preliminary analysis of two year serial analysis of a large dairy herd within the Florida Johne's Disease Dairy Herd Demonstration Program has revealed that approximately 10-11% of dairy cows tested, will annually demonstrate the presence of non-diagnostic and diagnostic Map specific ELISA antibodies; however, of the cows not culled, approximately 75% of the previous sero-positive sera will be negative on subsequent annual examination (Harrel and Gauthier, unpublished data). Of the remaining 25% of sero-positive cows with persisting Map antibodies, only 15-20% exhibited a titer-increase indicative of disease status disease while others exhibit diminishing Map antibody levels.

What if: Map is a zoonotic disease as has been suggested by the recovery of Map from the blood and breast milk of women with Crohn's disease and the frequency of identification in gastrointestinal tract tissue sample derived from patients with Johne's disease? (Anon., 2000; Hermon-Taylor, 2000; Naser et al., 2000; 2004; Sechi et al., 2005)

If the reservoir of infection is embedded in the food chain of herbivores and Map can jump species lies and produce disease in unrelated species (Fridriksdottir et al., 1999; Whittington et al., 2001), will brucellosis-like eradication programs work?

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5. Paratuberculosis Calendar

Please report to Søren Nielsen (ssn@life.ku.dk) should you have knowledge of any events that you find relevant to include in the calendar.

2009

August 9-13, 2009. 10th International Colloquium on Paratuberculosis, St. Paul/ Minneapolis, Minnesota, USA.

August 10-14, 2009. 12th International Symposium on Veterinary Epidemiology and Economics. Durban, South Africa (http://www.isvee12.co.za).

August 25-28, 2009. *M. bovis* V Conference, Wellington, New Zealand (http://www.mbovisconference.org/)

6. List of Recent Publications

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