

The Paratuberculosis Newsletter

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Table of Contents

TABLE OF CONTENTS	86
1. SHORT SCIENTIFIC REPORTS	87
Identification of <i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i> (Map) in a herd of dairy water buffaloes (<i>Bubalus bubalis</i>) in southern Brazil	87
The Canadian Johne's Disease Initiative	92
Comparative evaluation among 36 combinations of decontamination and isolation protocols for <i>Mycobacterium avium</i> subspecies <i>paratuberculosis</i> (MAP) from bovine raw milk	93
Serological survey of <i>Mycobacterium avium</i> subspecies <i>paratuberculosis</i> in dairy cattle in Espírito Santo state, Brazil	94
Improved diagnosis of paratuberculosis using the lipopentapeptide L5P, a specific antigen of <i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i>	95
2. COMMENTS & OPINIONS	97
Insanity	97
3. EVENTS.....	100
11 th International Colloquium on Paratuberculosis 2012	100
4. LIST OF RECENT PUBLICATIONS.....	101

DEADLINE FOR NEXT ISSUE: February 15, 2011.

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Søren Saxmose Nielsen
Editor

1. Short Scientific Reports

Identification of *Mycobacterium avium* subsp. *paratuberculosis* (Map) in a herd of dairy water buffaloes (*Bubalus bubalis*) in southern Brazil

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ABSTRACT

Paratuberculosis (Johne's disease) is a chronic disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map) characterized by granulomatous enteritis and lymph node enlargement. Amongst large ruminants, it affects domestic dairy cattle and water buffaloes, in addition to other species. The purpose of this study was to isolate Map from clinical cases of paratuberculosis among water buffaloes. Tissue, milk and fecal samples were collected from a herd of dairy water buffaloes (*Bubalus bubalis*) with clinical signs of paratuberculosis. Additionally, bulk tank raw milk samples were analyzed for the presence of Map using HEYM, and the diagnosis was confirmed by polymerase chain reaction (PCR). Five Map strains grown on HEYM were isolated from five animals with suspected with paratuberculosis, which was confirmed by PCR using IS900 primers. Map was isolated from cooling tanks in this herd. Of 136 samples submitted to indirect ELISA, the presence of Map was confirmed in 21 (15.45%) and suspected in 38 (27.95%). The presence of paratuberculosis in herds of dairy buffaloes in southern Brazil requires further investigation and the use of specific sanitary measures for its diagnosis, management and prevention.

INTRODUCTION

Johne's disease is a chronic disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map) characterized by granulomatous enteritis and lymph node enlargement. Amongst large ruminants, it affects dairy cattle, water buffaloes, bison, and cervids, in addition to pseudoruminants such as camelids (Buergelt et al., 1978, 2000; Tripathi et al., 2002).

Johne's disease (JD) in ruminants and Crohn's disease (CD) in humans has some characteristics in common: they are chronic and incurable intestinal diseases. The detection of Map DNA in samples of human intestine and lymph nodes suggests that this pathogen may be found in foods and other sources (Argueda et al., 2000; Naser et al., 2000; 2004).

Milk has been suggested as a medium for transmission of Map to humans (Hermon-Taylor, 1993), playing a similar role to that of dairy products in CD (Greenstein, 2003; Greenstein & Collins, 2004), or as a cofactor in some cases of CD (Rubery, 2002; Griffiths, 2006).

In countries where dairy buffaloes are raised, milk and its derivatives are important nutritional sources for humans. Paratuberculosis in these dairy animals, from which buffalo mozzarella is made, has great importance because raw milk is often used or because the

milk is submitted to inefficient pasteurization that is unable to eliminate Map (Millar et al., 1996; Gao et al., 2002).

There are few reports about paratuberculosis in water buffaloes in the world literature and practically no data about serology, prevalence, control and prevention measures in Brazil.

Lillini et al. (1999) conducted a serological survey in 15 herds of dairy buffaloes in central and southern Italy and found antibodies against Map. Of 1,321 samples tested by Elisa, three animals were positive for Map, but only a 5-year-old female buffalo had clinical signs associated with JD, such as progressive cachexia, weakness, intermittent chronic diarrhea, decreased milk production and infertility.

In northern India, paratuberculosis was confirmed in buffaloes by isolation of Map from tissue and lymph node samples, which were obtained from animals sacrificed at a slaughterhouse and then cultured on Herrold's egg yolk medium (HEYM). The histological lesions observed in naturally infected animals were classified into three categories, based on the type of cell infiltrate, development of granuloma and presence of alcohol-acid resistant band cells (Sivakumar et al., 2005; Sivakumar et al., 2006). The prevalence of Map was determined by ELISA in 28.6% and 29.8% of 1,425 samples obtained from buffaloes and domestic cattle, respectively (Singh et al., 2008).

In Brazil, Map was first described in buffaloes in the state of Pernambuco in a herd of 100 dairy animals, among which five had clinical signs of the disease and two revealed typical lesions at necropsy. The diagnosis was confirmed by histopathological and molecular analyses of intestinal and lymph node samples (Mota et al., 2010).

Isolation of Map in culture media is still a complex, time-consuming and expensive technique for which there is a lack of qualified personnel in most Brazilian laboratories. However, this technique is the gold standard for the diagnosis of infectious diseases even when there are numerous serological and molecular techniques with variable sensitivity and specificity.

The serological diagnosis by ELISA is an inexpensive, quick and easily performed method that is used worldwide to estimate, guide and help with control measures against Map (Gomes et al., 2002).

OBJECTIVES

The purpose of this study was to isolate Map from water buffaloes with JD.

MATERIAL AND METHODS

Five female water buffaloes aged four years or older presented with diarrhea, progressive weight loss, rapid decrease in milk production, but did not show loss of appetite. The animals were from a dairy property located in the Great Porto Alegre, Rio Grande do Sul State - South Brazil. Fecal and tissue samples (ileum, cecum and ileo-cecal valve) were collected at necropsy and sent to the Laboratory of Veterinary Bacteriology of the School of Veterinary Medicine of Universidade Federal do Rio Grande do Sul, southern Brazil, where they were immediately processed. Additionally, two bulk tank raw milk samples were collected from this infected herd and sent to the same laboratory for Map culture.

In the laboratory, the fecal and tissue samples (1 to 2 g) were treated with 0.9% HPC and processed according to the modified classic Cornell's technique, as described in Stabel (1997).

The three bulk tank raw milk samples were processed using the protocol recommended by Dundee et al. (2001). The samples (milk, stools, and tissues) were

inoculated into four tubes containing HEYM, two with mycobactin and two without mycobactin. The cultured samples were kept in a stove at 37°C and were checked fortnightly for 6 months.

The following criteria were used for Map identification: time of microbial growth, morphology, and mycobactin dependence (Manning & Collins, 2001; Gomes, 2002). All Ziehl-Neelsen-positive samples were submitted to polymerase chain reaction (PCR) for amplification of IS900 using P90-91 primers (Whittington et al., 1998) and confirmed by IS*Mav*2F / IS*Mav*2/B2 (Shin et al., (2004) and IS900BN1 / BN2 (Sivakumar et al., 2005).

A total of 136 serum samples were collected from the water buffalo herd over 3 years. Commercially available indirect ELISA (Allied Monitor, Fayette, MI, USA) with protoplasmic antigen (PPA-3) was used for the identification of antibodies against Map. The immunoassay was carried out according to the protocol supplied by the antigen manufacturer (Allied Monitor, Fayette, MI, USA).

RESULTS

Map was not isolated from any of bulk tank milk samples in one laboratory using a PCR method based on IS 900 P90-91 primers, but were positive in another laboratory using a PCR method based on IS900BN1/BN2 and *Mav*2F / *Mav*2B2 primers. The clinical signs were similar to those of bovine paratuberculosis. Map was isolated from the feces, ileum and intestinal lymph nodes of all sampled animals and cultured on HEYM up to 16 weeks.

The prevalence of Map was determined by submitting the whole herd (all cows aged > 3 years) to absorbed ELISA using PPA-3 antigen (Allied Monitor, Fayette, Missouri, USA). The ELISA test identified 21 positive animals (15.45%) among the 136 tested samples.

DISCUSSION

The clinical signs and lesions observed in domestic dairy cattle and buffaloes with paratuberculosis are similar (Lillini et al., 1999; Mota et al., 2010); however, the sanitary measures used for the control and prevention of Map in domestic cattle and buffaloes are probably different because the management of these animals is not the same.

Map colonies obtained from buffaloes and cultured on HEYM are apparently similar to those isolated from domestic cattle.

The infection in Map-positive buffaloes was detected by absorbed ELISA, being lower in this herd (15%) than in the domestic cattle (44.6%) even when the enzyme immunoassay is not validated in this species (Gomes et al., 2002). This difference is probably related to the health status of these two herds, as the infection may be associated with *M. bovis* in the 36 domestic cattle herds tested; therefore, the prevalence of Map may have been overestimated (Gomes et al., 2007).

Our study is the first one to report this disease in buffaloes in the state of Rio Grande do Sul. So far, clinical signs of paratuberculosis had been described in our state only for domestic dairy cattle (Ramos et al., 1986; Driemeier et al., 1999; Gomes et al., 2002; Gomes et al., 2005; Gomes et al., 2007).

The isolation of Map combined with clinical and pathological data validate the epidemiological diagnosis of paratuberculosis in our dairy buffalo herds.

CONCLUSIONS

Johne's disease in dairy buffaloes constitutes a challenge in terms of animal health care for our state and country. The prevalence of Map prevalence in the population of water buffaloes

is unknown; therefore, it is necessary to conduct further studies on this topic and to adopt control measures for the protection of dairy herds.

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The Canadian Johne's Disease Initiative

July 1, 2010 marked the first year of delivery of the Canadian Johne's Disease Initiative (CJDI). The Initiative is funded by Dairy Farmers of Canada (DFC) and the Canadian Cattlemen's Association (CCA). The CJDI priorities are:

- i) Education and awareness
- ii) Provincial program encouragement and coordination
- iii) Research support and facilitation

Informational articles related to Johne's disease (JD) were delivered in multiple CCA and DFC newsletters and agri-business magazines. In the fall of 2009, beef and dairy cattle JD control brochures were provided to individual Canadian producers. Canadian Johne's Disease Initiative Newsletters were prepared and emailed to Canadian industry and government leaders and contacts. The Canadian Animal Health Coalition's CJDI webpage (<http://www.animalhealth.ca/cjdi>) was also updated routinely.

Individual provinces enhanced or initiated JD control programs. For example, the Ontario Johne's Education and Management Assistance Program was launched in January. Features include herd risk assessment, MAP testing of individual cows and the removal of highly-positive cows from the food chain. Alberta recently initiated a new dairy JD control program that will also offer a JD herd certification program in 2011.

Canadian MAP scientists from each Canadian veterinary faculty provided technical advice to provincial programs via CJDI's Technical Committee, addressing priorities, such as veterinary training, risk assessment and herd management recommendations. Researchers from other universities are also important CJDI supporters and MAP research collaborators. The 3rd annual meeting of MAP researchers was successfully held in Banff, Alberta, on October 28 and 29, 2010 (>30 attendees).

CCA and DFC have extended their commitment to CJDI through 2011. JD awareness and control activities increased in Canada through 2010 as a result of significant industry and government support. Future CJDI goals include JD control activities by other species and in conjunction with more comprehensive on-farm disease bio-security programs.

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Comparative evaluation among 36 combinations of decontamination and isolation protocols for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) from bovine raw milk

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SUMMARY

Most protocols regarding samples decontamination for MAP isolation are based on the MAP detection from feces and not milk. Due to characteristics of each sample, different protocols must be followed. Therefore it is necessary to develop methods accordingly. MAP isolation also depends on the chemical decontamination of samples to inactivate microorganisms that could inhibit MAP growth. MAP presents very slow growth rate, thus, a balance between an efficient inactivation of undesirable microorganisms and low environment toxicity for MAP is needed. The choice of the best decontamination protocol is crucial to a successful MAP isolation. This study aimed at comparing 36 protocols combinations for samples decontamination and MAP isolation from bovine raw milk. A MAP K10 strain certified by genetic sequencing was grown in Middlebrook 7H9 supplemented with OADC and after that, inoculated into 40mL raw milk aliquots, collected from bulk tank from a historically paratuberculosis free farm, also tested negative for MAP presence by PCR. A total of 36 protocols combinations for MAP isolation presented in the literature were carried out on milk samples artificially contaminated and then they were inoculated into tubes with three culture media. Each treatment was performed in triplicate for each medium, in a total of 324 samples. Non-parametric statistical analysis was performed using the X2 test. Samples with unspecific contamination were excluded from the analysis and only 174 samples were evaluated. The protocol combination which provided higher MAP growth and lower unspecific contamination in a shorter period of time was considered the best. In this study, the protocol involving 0.9% HPC at room temperature for 24h, using centrifuge at 2500 × g for 15 minutes and antimicrobial solution immediately before inoculation into tubes with HEYM prepared with fresh egg yolk provided the greatest MAP isolation from bovine raw milk samples.

Serological survey of *Mycobacterium avium* subspecies *paratuberculosis* in dairy cattle in Espírito Santo state, Brazil

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This study aimed to verify the occurrence of antibodies to the MAP in dairy cattle from Espírito Santo state, located in Southeastern Brazil. We analyzed 1450 serum samples for antibodies to *Mycobacterium avium* subspecies *paratuberculosis* (MAP), using an ELISA test commercial kit (Milk ELISA Paratuberculosis Antibodies Screening - Institut POURQUIER, Montpellier, France). The animals were all dairy cattle, male and female, from four regions of Espírito Santo state. In total 165 (11.38%) samples were positive for antibodies to MAP, 33 (2.27%) were considered suspicious and 1252 (86.34%) were negative. In all regions we found seropositive animals indicating that the agent is present throughout the state, thereby posing a threat to the local dairy farming and neighboring states, as well as public health, since the MAP may be involved with Crohn's disease in humans. This result presents the first serological survey on antibodies to MAP in dairy cattle of Espírito Santo state of Brazil.

Improved diagnosis of paratuberculosis using the lipopentapeptide L5P, a specific antigen of *Mycobacterium avium* subsp. *paratuberculosis*

Background

Johne's disease or paratuberculosis, caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*), is a prevalent infectious disease problem notably in dairy cattle herds leading to significant economic losses for producers in most developed countries. In the United States alone, the cost is estimated to be as high as \$1.5 billion to the agriculture industry every year.

All control programs developed until now against this epizooty have failed due to the **lack of sensitivity and/or specificity of diagnostic assays**, and to the lack of an efficient vaccine. The high research priorities that are necessary to combat *Map* consist in developing an efficient diagnostic test essential for the **paratuberculosis control and eradication program**, and guaranteeing the food supplies to be *Map* free. Controlling paratuberculosis, which is suspected to be transmissible to human beings (Crohn's disease), is a challenge of increasing interest.

Description of the innovation

Scientists of the French National Institute for Agricultural Research (INRA), the French National Institute for Health and Medical Research (INSERM), the National Center for Scientific Research (CNRS) and the Pasteur Institute have addressed the question of improving the specific diagnosis of paratuberculosis in livestock using an ELISA system developed to detect, in infected animals, antibodies raised against a **lipopeptide, a *Map* specific metabolite** they identified. Following genetic and biochemical analysis of this novel cell-wall component, they proceeded to **the chemical synthesis of the lipopentapeptide L5P (LipidC₂₀-(D)Phe-(NMe)Val-Ile-Phe-Ala-OMe)** and variants, then assessed their immune reactivity against a panel of sera from animals infected either by *Map* (positive control), or *M. bovis*, *M. avium* (negative controls), as well as human sera from patients infected by *M. avium* or *M. intracellulare* (negative controls).

ELISA results demonstrated that major epitopes of the L5P are peptide-based, and that **the chemically synthesized L5P and derivatives thereof have many advantages** over the extracted/purified preparations which were proposed in the prior art for detection of *Map*:

- the large-scale preparation of these antigens by solid phase peptide synthesis is **convenient, inexpensive** and may be **standardized**;
- they allow to **avoid false-positive reactions** occurring due to contamination by compounds shared by other *mycobacterium* species;
- they are **far more specific** than crude cell-wall extract of *Map* currently employed as paratuberculosis diagnostic test, while being as **sensitive**, although they involve a single antigen.

Industrial application, IP and technology transfer

The diagnostic use of synthetic lipopentapeptide L5P, pentapeptide 5P and their analogues is protected under an international patent application (**WO2009/053844**), with INRA Transfert having rights to grant licenses for commercial applications. This patented technology will profit companies involved in animal healthcare, notably in **the development of various immunodiagnostic tests of paratuberculosis** in livestock.

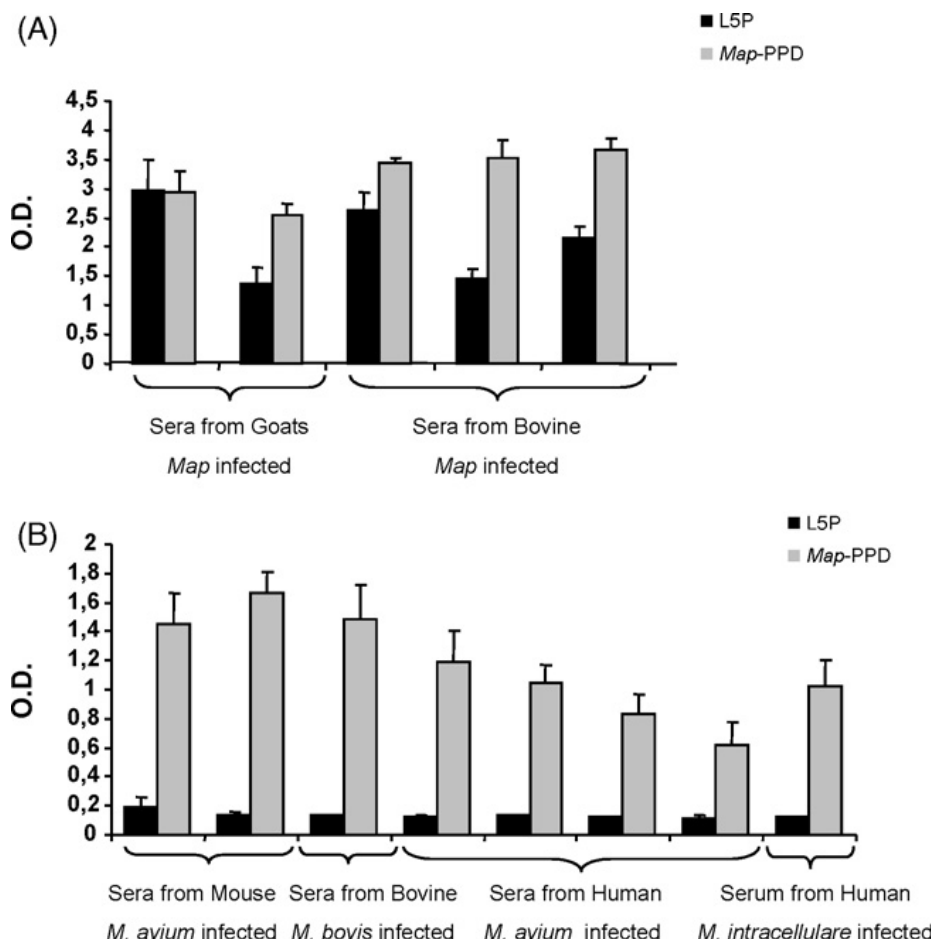


Figure 1. Immunogenicity of the lipopentapeptide L5P of *Map*. (A) ELISA performed on lipopentapeptide (L5P) and Purified Protein Derivatives (*Map*-PPD) using sera from *M. avium* subsp. *Paratuberculosis*-naturally infected bovines and goats. (B) ELISA performed on L5P and *Map*-PPD using sera from *M. avium* subsp. *Avium*-experimentally infected mouse, sera from *M. bovis*-naturally infected bovines, sera from *M. avium* subsp. *avium*- and *M. avium* subsp. *intracellulare*-naturally infected humans. The results are expressed as the means of triplicates (Biet et al., 2008).

Reference

Biet F, Bay S, Thibault VC, Euphrasie D, Grayon M, Ganneau C, Lanotte P, Daffé M, Gokhale R, Etienne G, Reytrat JM, 2008. Lipopentapeptide induces a strong host humoral response and distinguishes *Mycobacterium avium* subsp. *paratuberculosis* from *M. avium* subsp. *avium*. *Vaccine*. 26: 257-268.

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2. Comments & Opinions

Insanity

Gilles R. G. Monif, M.D.

“Insanity has been defined as doing the same thing and expecting a different result.”

In our collective pursuit of knowledge concerning Johne’s disease, we have embraced the following concepts:

1. *Mycobacterium avium* subspecies *paratuberculosis* (Map) is THE cause of the clinicopathological complex termed Johne’s disease;
2. The IS900 insertion sequence identified all pathogenic mycobacterium that cause Johne’s disease;
3. That identifying and removing infected animals, as in the case of brucellosis, will control the prevalence of infection and disease (test-and-cull); and
4. Crohn’s disease and Johne’s disease are etiologically unrelated.

For over two decades, the epidemiological foundation of our understanding of Map has rested with data derived from our serological and organism identification technology. The prevailing Map serological ELISA tests are predicated upon IS900 insertion sequence. Just how good is the comparative data derivable?

Collins et al. (2005) evaluated five antibody detection tests for the diagnosis of bovine paratuberculosis using serum samples from 359 dairy cattle in seven paratuberculosis-free herds and 2,094 dairy cattle in seven Map-infected dairy herds. Both the ParaChek® and HerdChek® (IDEXX) ELISA tests done in accordance with manufacturers’ instruction and interpreted as prescribed by the kit insert, identified less than 29% of fecal culture positive cows. Linear regression analysis of quantitative results showed a low correlation co-efficiency. Sockett et al. reported the sensitivity of commercial ELISAs for cattle to be 8.9 to 32.1% for low shedders and 47.1 to 62.9% for midlevel shedders (Sockett et al., 1992).

Sweeney et al. (2006) have suggested that commercial ELISAs might have a sensitivity rate lower than 13.5%. McKenna et al. (2006) tested sera collected from dairy cows at slaughter in assessing the agreement with documented infection of three commercially available Map ELISA tests which included Herschel® and Panache®. The investigators found a poor agreement between the three ELISA tests and infected cows.

Mycobacteria have been shown to be more readily identified within feces when the currently commercially available Map ELISA tests are in their projected positive diagnostic zones (Cocito et al., 1994). The high incidence of false negatives with the ParaChek® and/or HerdChek® ELISA tests has limited utility to identifying animals with an advanced disease state.

The most damning data comes from the Florida Johne’s Disease Dairy Herd Prevention Project in which 22 “heavy shedders” were identified. ParaChek® Map ELISA test identified only 2 of the 21 cows for which serological data existed (Monif et al., 2009). One other cow had a very high suspicious titer that became negative when retested 14 months later.

What we have learned is that a high titer Map ELISA predicts with 74% accuracy animals that will progress to clinical disease; but, a positive ELISA titer is not definitive in predicting outcome. By early removal of ELISA positive cows, the incidence of Johne's disease has been significantly reduced, but the herd prevalence of infection increased. Not fully addressed is the issue of whether a 25-26% false-positive rate is acceptable collateral damage.

A second epidemiological and herd management tool has been the use of quantitative assessment of the amount of Map present in an animal's feces. Neglected in making epidemiological and herd management assessments based upon quantitative data has been the fact that, unlike *M. bovis*, *M. avium* subspecies *avium* (Ma) and *M. avium* complex, (Mac), Map grows in clumps thus making sample error a genuine issue. When the issue of clumping is analyzed using divergent diagnostic technology, validation of "heavy shedding" was lacking in 45% of specimens identified as heavy shedders by culture (Monif et al., 2009).

Other than reducing the incidence of Johne's disease, two decades of epidemiological investigations have failed to lend clarity to public policy. Among the possible reasons why are:

1. Map is not THE cause of Johne's disease. Map is a cause of Johne's disease. Ma and Mac are documented causes of Johne's disease in horses and pigs. Rare cases exist of Ma having caused Johne's disease in cows have been described or inferred;
2. Map evolved from Ma. Not surprisingly between Ma and Map, pathogenic genomic variants exist that are not identified by IS900 insertion sequence-based tests;
3. If limiting infection due to Map and its polymorphic variants was the goal of test-and-cull, the net results are a cause for concern; and
4. Map DNA can be demonstrated predominantly in diseased tissue from Crohn's patients (Sechi et al., 2005), has been isolated from the breast milk of pregnant women with Crohn's disease (Naser et al., 2000), can be isolated from the blood of predominantly individuals with Crohn's disease (Naser et al., 2009), Map in its spheroplastic form can be isolated only from Crohn's patients (Mendoza et al., 2010). It will be left to individuals of average intelligence to determine whether Map and/or its genomic variants are causes of Crohn's disease.

A famous American comedian once described his periodic feelings concerning individual in leadership positions that might be applicable.

"I'm scared! I don't know whether the world is full of smart men bluffing
or imbeciles who mean it".

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3. Events

11th International Colloquium on Paratuberculosis 2012



The 11th International
Colloquium on Paratuberculosis 2012
5-10 February 2012 | Sydney | Australia



4. List of Recent Publications

- Aagaard C, Govaerts M, Meikle V, Gutiérrez-Pabello JA, McNair J, Andersen P, Suárez-Güemes F, Pollock J, Espitia C, Cataldi A, 2010. Detection of bovine tuberculosis in herds with different disease prevalence and influence of paratuberculosis infection on PPDB and ESAT-6/CFP10 specificity. *Prev Vet Med.* 96:161-9.
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