

# *Mycobacterium avium* subsp. *paratuberculosis*: pathogen, pathogenesis and diagnosis

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## Summary

Johne's disease, or paratuberculosis, is a chronic intestinal infection caused by *Mycobacterium avium* subsp. *paratuberculosis*. The usually fatal disease is characterised by cachexia, and in some species diarrhoea, after a long pre-clinical phase. Treatment is ineffective and economically impracticable. The infection primarily affects domestic and free-ranging ruminants, but has also been reported in primates, rabbits, stoats and foxes. Since paratuberculosis is often subclinical, under-reporting is suspected, even though the disease is notifiable in numerous countries. Herd prevalence of bovine paratuberculosis in Europe ranges from 7% to 55%. In the United States of America, herd prevalence is strongly associated with herd size; 40% of herds of more than 300 head were found to be infected. In Australia, reported dairy herd infection rates range between 9% and 22%. Paratuberculosis in domestic livestock entails significant economic losses due to several factors (e.g. reduced production, premature culling and increased veterinary costs). Free-ranging and captive wildlife are also at risk from paratuberculosis.

## Keywords

Diagnosis – Johne's disease – Microbial ecology – *Mycobacterium paratuberculosis* – Paratuberculosis – Pathogenesis – Zoonosis.

## Introduction

Johne's disease (pronounced 'YO-nees'), also known as paratuberculosis, is an intestinal mycobacterial infection that causes cachexia, and in some species diarrhoea, after a long pre-clinical phase (months to years). This ultimately fatal disease is caused by infection with *Mycobacterium avium* subsp. *paratuberculosis* (hereafter termed *M. paratuberculosis*). Primarily an infection of domestic and free-ranging ruminants, infection has also been reported in primates (86, 150), rabbits, weasels, foxes and a stoat (9). The 1998 report of status and control measures for paratuberculosis published by the Office International des Epizooties records the presence or suspicion of paratuberculosis in cattle in approximately half of the countries that provided reports (100). Although paratuberculosis is a notifiable disease in numerous countries, under-reporting of this often subclinical disease is likely to occur for many reasons. Published estimates of herd prevalence of bovine paratuberculosis in countries of Europe range from 7% to 55% (65). In the United States of America

(USA), a survey of dairy herds completed by the United States Department of Agriculture in 1996, reported that at least one animal was serologically positive for the infection in 22% of herds in the USA. The infection is distributed fairly uniformly across the USA, but herd prevalence is strongly associated with herd size: approximately 40% of herds of more than 300 animals were found to be infected (153). In Australia, the disease is not uniformly distributed; in the south-eastern portion of the country, dairy herd infection rates are 22% in the State of Victoria, and 9% in New South Wales (74).

Paratuberculosis is costly to domestic agriculture as the disease reduces production, forces premature culling and increases veterinary costs. The infection is also of concern for managers of free-ranging wildlife such as elk, bison, Bighorn sheep, etc. (17, 36, 67, 68, 160). Captive wildlife, such as non-domestic hoofstock held in zoological institutions, are also at risk. Up to one third of zoos accredited by the American Zoo and Aquarium Association reports at least one case of the infection since 1995 (93).

# Characteristics and ecology of *Mycobacterium paratuberculosis*

## Genetics

*Mycobacterium paratuberculosis* is a hardy, slowly growing member of the Mycobacteriaceae family. The organism is an intracellular pathogen surrounded by the complex tripartite lipid-rich cell wall characteristic of this bacterial family. *Mycobacterium paratuberculosis* shares over 99% deoxyribonucleic acid (DNA) homology with *M. avium* subsp. *avium*, a cause of tuberculosis in birds, and the 16S ribosomal ribonucleic acid (rRNA) sequences of these two organisms are identical (145). Given this genotypic similarity, a reclassification as *M. avium* subsp. *paratuberculosis* has been proposed (142). However, phenotypic differences between these two organisms are considerable, notably growth rate, mycobactin dependency *in vitro*, and pathogenicity. In recognition of these differences and for historical reasons, the name *M. paratuberculosis* continues to be widely used and is the nomenclature adopted by the authors in this paper. These circumstances also exist for *M. tuberculosis* and *M. bovis* whose names imply separate species, although by genetic criteria the organisms are not different species, but rather subtypes of the same species. The paper by Rastogi *et al.* in this issue provides further details (113).

The insertion sequence IS900 is the sole genetic element that distinguishes *M. paratuberculosis* from *M. avium*. Thus far, only *M. paratuberculosis* strains have IS900 (18, 30, 148) and all strains tested to date have fourteen to eighteen copies of IS900. Gene-based diagnostics capitalise on this difference. Recently, polymorphisms have been detected in another insertion sequence, IS1311, that allow differentiation between *M. avium* and *M. paratuberculosis* (158). This may provide the means to identify both of these closely related organisms with a single assay.

The close genetic similarity of *M. avium* and *M. paratuberculosis* is reflected in the large number of common antigens (19, 20, 40, 52, 98, 114, 155). This can confound accurate diagnosis of *M. paratuberculosis* infection when immunologically-based tests are used that rely upon antigen-based antibody or cytokine detection protocols. This mycobacterial cross-reaction problem can also be observed when skin testing for bovine tuberculosis (*M. bovis*): false-positive tuberculosis skin test results are reported in animals infected with *M. paratuberculosis* (R.M. Meyer, unpublished data).

Information about many aspects of *M. paratuberculosis* is lacking, due in large measure to the research impediments created by the extremely slow growth rate of this organism. *Mycobacterium avium* can be studied as a useful model for some aspects of the biology of *M. paratuberculosis*, given the close genetic similarity to *M. avium* and ease of study of

*M. avium*. The recent increase in research on *M. avium* (due to the frequency with which the organism occurs as an opportunistic infection in immunosuppressed patients infected with human immunodeficiency virus [HIV]) has improved the understanding of the biology of both *M. avium* and *M. paratuberculosis*. In some of the sections below, knowledge about *M. avium* is extrapolated to draw corollaries to *M. paratuberculosis*, when specific research data on the latter organism is lacking.

## Thermal tolerance

Mycobacteria are notoriously resistant to physical and chemical factors. *Mycobacterium paratuberculosis* seems to be among the most resistant of this group. This explains the ability of the organism to persist in the environment, a characteristic integral to the epidemiology of the infection. *Mycobacterium paratuberculosis* is more thermoresistant than *M. avium*, *M. chelonae*, *M. phlei*, *M. scrofulaceum* and *M. xenopi* (122, 135). The relevance of this finding is in the capacity of pasteurisation to kill the organism in dairy products, given that infected animals shed the organism in milk (133, 137, 140). A number of studies have assessed the effect of pasteurisation on *M. paratuberculosis* (24, 56, 76, 130, 135). The most recent study, reported to the Advisory Committee on the Microbiological Safety of Food, focused on raw and pasteurised milk obtained from dairy facilities in various areas of Great Britain. From the 679 samples for which data are currently available, viable *M. paratuberculosis* was found in 1.9% of the raw and 2.1% of the pasteurised milk samples (5). These data further stimulated the debate about whether *M. paratuberculosis* can survive pasteurisation, and thus be conveyed to humans through dairy foods.

## Resistance to ultraviolet light

The ultraviolet (UV) light doses required to inactivate bacteria and viruses are relatively low (e.g. 2 mW·s/cm<sup>2</sup> to 6 mW·s/cm<sup>2</sup> for 1 log<sub>10</sub> inactivation) (144). However, differences in water characteristics such as pH, hardness, turbidity and biological oxygen demand, can significantly affect the disinfection efficiency of UV light. Preliminary data demonstrated that *M. paratuberculosis* is equally susceptible to UV inactivation as other bacteria. When 10<sup>5</sup>-10<sup>6</sup> *M. paratuberculosis* were suspended in sterile deionised water, 4 mW·s/cm<sup>2</sup> UV light was sufficient to achieve a 1 log<sub>10</sub> reduction in viable counts, and at UV doses greater than 15 mW·s/cm<sup>2</sup>, complete disinfection was achieved (E.J.B. Manning and M.T. Collins, unpublished data). Earlier studies concerning the effects of natural sunlight on mycobacteria in the environment indicate that sunlight (presumably UV radiation) decreases the survival rate and that *M. paratuberculosis* is more resistant to the adverse effects of sunlight than *M. bovis*. However, recent work in Australia indicates that UV light has minimal effect on *M. paratuberculosis* viability in soil spiked with the organism (121).

## Survival in faeces and soil

The 1944 publication by Lovell *et al.* provides some of the best information regarding environmental survival of *M. paratuberculosis* (85). A series of studies using naturally contaminated bovine faeces was conducted, in which the infected faecal matter was exposed to a variety of natural conditions such as freezing, drying, sunlight, changes in ambient temperature, and rain, with regular attempts to re-isolate *M. paratuberculosis*. In general, *M. paratuberculosis* kept outdoors in faeces survived between 152 and 246 days, depending on specific conditions. Drying of soil appeared to shorten survival. The authors concluded that in considering the longevity of *M. paratuberculosis*, pastures should be considered as sources of infection for at least one year following contamination. This apparently supports the recommendations of earlier workers (1929-1933). The statement that *M. paratuberculosis* survives for one year on pastures, frequently found in the literature on Johne's disease, is likely to originate from this work, since few comparable studies have been published since the work of Lovell *et al.* (85).

Factors that may shorten the survival time of *M. paratuberculosis* in soil are drying, exposure to sunlight (81), pH above 7.0 and low iron content (69, 70, 71). Bovine urine is also detrimental to *M. paratuberculosis* survival, and increasing concentrations of bovine urine (2%-10%) caused a reduction in survival rates (at pH 6.3 to pH 6.6) (81). A study on *M. paratuberculosis* survival in spiked soils in Australia in 1999, revealed a shorter survival time in dry alkaline soils and no apparent effect of UV light (121).

Observations regarding soil pH, calcium or iron content and the incidence of paratuberculosis have a long history. The 1956 review article on Johne's disease by Doyle covers most of the early observations on the association between soil type and paratuberculosis incidence (42), and the 1997 review by Johnson-Ifearulundu and Kaneene (70) complements this by covering more recent literature. The observation of this association, in particular concerning soil pH, in England (126), France, the Netherlands (66), the USA (70, 80) and most recently Spain (115), adds credibility to the theory that soil composition and paratuberculosis are linked. Johnson-Ifearulundu and Kaneene demonstrated by careful epidemiological analysis that, in the State of Michigan in the USA, the application of lime to pastures (a practice that should increase soil pH) was associated with a ten-fold lower probability of a dairy herd being serologically test-positive for *M. paratuberculosis* infection (71). Interestingly, an association was also demonstrated between soil acidity and frequency of skin test reactions (to purified protein derivative from *M. bovis* [PPD-B]) in humans (14).

These epidemiological observations have led to speculation concerning the mechanisms by which soil pH, or the interaction of soil pH with soil calcium and iron content,

affect *M. paratuberculosis* survival. However, it should be noted that the relationships observed are indirect. The associations are merely between the incidence of clinical bovine paratuberculosis and crudely characterised soil type, and no laboratory studies have been undertaken to verify directly if soil type affects *M. paratuberculosis* survival or to explain the mechanism by which this may occur.

## Survival in water

Lovell *et al.* reported in 1944 that, after spiking sterilised pond water (pH 5.5) with 0.1 mg, 1.28 mg or 3.4 mg wet weight of *M. paratuberculosis* per 100 ml of water and holding the water at room temperature, the organism was recovered monthly for up to nine months (85). In 1956, Larsen *et al.* reported on the survival of *M. paratuberculosis* in spiked tap water of different pHs held at 38°C in the dark (81). In water of neutral pH (7.0), *M. paratuberculosis* was recovered up to seventeen months post inoculation, while at pH 5.0 and pH 8.5, *M. paratuberculosis* was isolated only for fourteen months post inoculation. Findings by Sung and Collins were consistent with these earlier reports, although these authors found that survival varied according to the strain of *M. paratuberculosis*. When *M. avium* was suspended in tap water and held at 4°C or 20°C, the organism survived beyond 485 days (149). This is of relevance to the increase in *M. avium* infections of immune-suppressed patients. Most investigations point to domestic water supplies as the source of the *M. avium*, due in part to the abundance in the environment and the resistance to chlorination of the species (47, 53). Under natural environmental conditions, warmer waters with lower pHs have been associated with higher levels of *M. avium* (38, 63, 77, 103). Similar factors could affect the survival of *M. paratuberculosis* in water.

## Chlorine resistance

*Mycobacterium avium* is more resistant to free chlorine than most other bacteria (139). At a concentration of 1 mg/ml, the time for a 1 log<sub>10</sub> reduction in viable *M. avium* counts is approximately 50 min (extrapolated from the data of Taylor *et al.* [139]). In contrast, the time to achieve a 1 log<sub>10</sub> reduction in *E. coli* was 28 seconds (extrapolated from the data of Taylor *et al.* [139]). Chlorine susceptibility varied according to the strain of *M. avium* tested. The slowest growing strains were most resistant. Water-grown *M. avium* cells were ten times more chlorine-resistant than cells grown in culture medium.

These laboratory findings are supported by epidemiological studies: for example, in Los Angeles, California, *M. avium* and other mycobacteria (but not *M. bovis* or *M. tuberculosis*) were isolated from water in 82% of fifty-five homes, and 100% of thirty-one commercial buildings and fifteen hospitals (8). Although comparable studies for *M. paratuberculosis* have not been reported, unpublished findings indicate that *M. paratuberculosis* is equally resistant to chlorine, if not more resistant than *M. avium* (M.T. Collins, unpublished data). This observation is consistent with the reported relationship between growth rate and chlorine resistance. As a point of

reference, a survey of disinfection practices in the USA found that water utilities maintain a median chlorine residual of 1.1 mg/ml and a median exposure time of 45 min before the point of first use in the distribution system (151).

### Resistance to low pH

Lovell *et al.* reported on the survival of *M. paratuberculosis* in sterile water spiked with 0.5 mg to 17 mg wet weight of cultured organism and held at 10°C-21°C (85). Isolation of *M. paratuberculosis* was attempted monthly and the pH of the water at each sampling was reported. Three types of water were tested: distilled water, tap water and pond water. The pH values were 6.4-6.8, 7.1-8.0 and 5.3-5.9, respectively over the course of the study. *Mycobacterium paratuberculosis* was re-isolated monthly until eight to nine months post inoculation from all three types of water. Over the range of pHs tested in water, pH had no apparent effect on *M. paratuberculosis* survival.

### Summary

Nutritional requirements, tendency to form large clumps and cell wall characteristics define the microbial ecology of *M. paratuberculosis* and distinguish the organism phenotypically from *M. avium*. Replication of *M. paratuberculosis* occurs primarily inside the macrophages of infected animal host cells, although the possibility of replication inside free-living amoeba has not been sufficiently studied. Once excreted from the host, *M. paratuberculosis* depends on the ability to persist in the environment until ingestion by another susceptible host. As the infection continues to spread within and among animal populations, the level of *M. paratuberculosis* also increases in raw foods of animal origin and in the environment. This may provide the opportunity for exposure to animal species, including humans, previously not considered to be possible hosts of *M. paratuberculosis*.

The epidemiology of paratuberculosis is sometimes viewed as a static situation, but this is an artifact of the long time frame over which changes in infection prevalence in animal populations occur. The situation is in fact dynamic; in addition to the natural spread of an infectious agent, the authors speculate that two global factors may be influencing the ecology of *M. paratuberculosis* and rising infection prevalence, namely: an increase in number of susceptible hosts and the acidification of the environment. Eradication of *M. tuberculosis* from humans and *M. bovis* from animals has possibly left an immunological void (i.e. animals of increased mycobacterial susceptibility) for *M. paratuberculosis* to occupy. This concept of competitive exclusion was demonstrated for species of *Salmonella* in poultry in England, Wales and the USA (111).

Environmental pollution leading to the acidification of soils and water is a global problem. The interplay of such changes on ecosystems and the ecology of pathogens such as *M. paratuberculosis* is little studied, but the effects could be

profound (46, 102, 152). Moreover, the data described herein is over fifty years old. Similar ecological studies performed with more sensitive methods of *M. paratuberculosis* detection could alter the conclusions.

## Infection susceptibility

Young animals (approximately less than six months of age) are thought to be most susceptible to infection through close contact with contaminated faeces. This idea is supported primarily by data from cattle (83), and the biological basis of this age-related susceptibility is not known. Whether the same window of susceptibility exists for other species and the exact infectious dose for animals of different ages have not been confirmed. Susceptibility to infection is likely to be linked to maturation of cellular immunity and the limited capability of very young animals to cope with intracellular pathogens such as *M. paratuberculosis*. Adult animals may also become infected if exposed to a sufficiently large and/or frequent dose of the organism (107, 112).

Anecdotal reports and one study indicate that a breed susceptibility may exist (Channel Island, Limousin and Shorthorn cattle; Scottish Blackface and Shetland sheep), but these reports may reflect breed popularity or exposure factors only (161). The genetic resistance to intracellular pathogens such as mycobacteria is ascribed at least in part to the *natural resistance associated macrophage protein 1* (NRAMP1) gene (2, 54, 55, 124). This gene may also be relevant to the control of the early phases of *M. paratuberculosis* infection, although comprehensive evidence for this has yet to be presented (78, 88).

## Transmission

Exposure to the organism can occur by a variety of routes. The most common is through nursing from an infected dam (via contaminated teats or direct shedding of the organism into the milk/colostrum) or ingestion of faecally contaminated solid feed and water. Infected cows and probably other species excrete *M. paratuberculosis* directly into milk during at least the later, disseminated, stages of the infection (133, 137, 140). Given the volume of milk consumed and the young age of animals drinking milk, this is probably the most frequent route of infection (136). *In utero* infection has been reported in clinically and subclinically infected cattle and may also occur in other species (43, 123, 138). The risk of transplacental exposure is greatest when the dam is in the final stages of clinical disease (43, 123, 138). While isolation of *M. paratuberculosis* from semen has been described, no studies have been completed to assess the likelihood of infecting either the dam or foetus via contaminated semen (82). The chance of transmission by this route is believed to be extremely low.

## Susceptible species

Infection has been reported in all common domestic agricultural species (cattle, goats, sheep, deer, bison, etc.). Johne's disease has also been diagnosed in a variety of captive and wild non-domestic animals of the order Artiodactyla, representing virtually all taxonomic families of ruminants (i.e. Cervidae, Antilocapridae, Bovidae; but not Giraffidae) and pseudoruminants (Camelidae) (90, 92, 95, 132). The transmission frequency from wildlife to domestic animals has not been documented. However, the spread of the infection from domestic animals to wildlife has been reported previously, as have a few cases of transmission to hind-gut fermentors such as rabbits (36, 60). The recent finding of lesions typical of Johne's disease in carnivores (foxes and stoats), together with IS900-positive mycobacteria isolated from the tissues of these animals, in an area of Scotland with *M. paratuberculosis*-infected dairy cattle, may indicate a new class of wildlife reservoirs or end hosts. The carnivores are believed to have acquired the infection by eating infected rabbits. This report demonstrates that *M. paratuberculosis* infection resulting in inflammatory gastrointestinal disease is not restricted to ruminant physiology and immunology. This unusual report of carnivore disease may be a function of the pathogenic characteristics of a particular strain of *M. paratuberculosis* (the strain found in the carnivores was the same as that infecting cattle and rabbits) or some other local factor that may not prevail elsewhere. The success of paratuberculosis control and eradication programmes for domestic agriculture may be affected by the ability of wildlife to acquire and then pass the infection back to domestic animals.

## Pathogenesis and pathology

Although some studies have indicated that the tonsil is the site of primary infection, at least through experimental oral challenge (107), other reports have not confirmed this conclusion (51). The organism is generally believed to cross from the lumen of the small intestine into the lymphoid system via the M cell (97). The bacterium is then taken up by epithelioid macrophages which, once activated, elicit T cell activation and clonal expansion (125). Two T helper cell subpopulations (TH1 and TH2) activate different host immune responses. *Mycobacterium paratuberculosis* infection appears to follow patterns observed with infections by *M. leprae*, *M. bovis* or *M. tuberculosis*. These patterns entail an initial TH1 response (referred to as 'tuberculoid') that is characterised by a tissue infiltrate distinguished primarily by lymphocytes with few if any detectable organisms (101). The TH1 response is also characterised by production of the cytokine gamma interferon (IFN $\gamma$ ), one of the earliest detectable reactions to *M. paratuberculosis* infection, in addition to interleukin 2 (IL-2) and tumour necrosis factor alpha (TNF- $\alpha$ ). These cytokines are believed to orchestrate the cell-mediated immune functions necessary to contain

such an intracellular infection. During the early, subclinical stage of *M. paratuberculosis* infection, the TH1 T cell activity appears to predominate. This subclinical phase of infection can last for months to years, as the bacilli are contained within macrophages and microscopic granulomas. Continued T cell memory and response is required to maintain these granulomas, control bacterial dissemination and minimise tissue damage.

Animals typically begin to exhibit the non-specific clinical signs of the *M. paratuberculosis* infection, such as weight loss, and in some species diarrhoea, as the animal enters the TH2, or lepromatous stage of infection (79, 129). The trigger for this transition is not known. The TH2 T cells stimulate production of cytokines co-ordinating a humoral (antibody) immune response (IL-4, IL-5, IL-6 and IL-10). This humoral response is not protective and does not halt the progression of *M. paratuberculosis* infection and pathology. The influx of inflammatory cells causes the intestinal wall to thicken until no longer functional, leading to a malabsorption and protein-losing enteropathy (104). A concomitant elevation of TNF- $\alpha$  may also contribute to emaciation through the stimulation of tissue catabolism (3, 4, 7, 12). At this phase of infection, the organism may disseminate within and beyond the gastrointestinal tract, as exhibited by lesions comprised of infiltrating macrophages packed with organisms in the kidney, liver, mammary gland, etc. (106). Clinically affected animals at this phase of Johne's disease usually succumb to the infection within weeks.

Although some authors have described these tuberculoid and lepromatous phases as mutually exclusive in mice (1), some overlap may occur in cattle and sheep (15, 27, 108). Another report postulates that in mice (12), the magnitude of the infecting dose received affects the type of immune response elicited (110). Although not confirmed, other artiodactylids are likely to display a sequence of immune responses similar to those observed in cattle.

## Clinical signs and pathological lesions

In cattle, weight loss despite adequate rations, accompanied by chronic diarrhoea are standard clinical signs of Johne's disease. Hypoproteinaemia and 'bottle jaw' or dependent mandibular oedema, are also reported in cases of advanced disease. However, in other species, clinical hints of the infection may be limited to the vague and non-specific finding of weight loss. In species with heavy coats (e.g. sheep and llamas), this single indicator may be easily missed. Diarrhoea is infrequently observed with paratuberculosis in sheep, goats, bison and perhaps other non-domestic hoofstock species. In the last phases of Johne's disease, animals of any species may become cachectic and too weak to rise.

A range of pathological lesions can be exhibited, depending upon the stage of infection at necropsy and the species in question. The classic lesions described in bovine cases include a corrugated and thickened ileum with enlarged and oedematous mesenteric lymph nodes in addition to distension of lymphoid channels. 'Classic' histopathological findings include an extensive granulomatous infiltrate of intestinal villi, abundant multinucleated giant cells and innumerable intracellular acid-fast bacilli (16). This florid microscopic presentation is in contrast to a more muted histopathological presentation observed in some cases of infection in bison or sheep, in which the only indication of infection may be a single giant cell with sparse or no intracellular bacilli (17). The broad array of histopathological findings in sheep has stimulated a number of classification methods (26). Analogous to the histopathological spectrum related to immunity as described for leprosy (116), Perez *et al.* have suggested that the various categories of lesions in both subclinically and clinically affected sheep may be linked to phases in the pathogenesis of *M. paratuberculosis* infection (109). These categories consider the presence and pattern of gross signs: lesion type, location and distribution (focal or diffuse); characteristics of the cellular infiltrate (primarily granulomatous or lymphocytic) and the quantity of bacilli. The authors propose that cases can correspond to a 'tuberculoid' form of infection, as characterised by sparse paucibacillary lesions limited to lymphoid tissue, and a 'lepomatous' form with a diffuse infiltrate of epithelioid macrophages containing abundant bacilli. 'Borderline' categories were also suggested for intermediate histological profiles that may represent cases in transition, profiles that may represent sequential stages of *M. paratuberculosis* infection. These authors accept the possibility that some animals can recover from infection or at least halt the progression of the infection through effective cellular immune responses.

In cervids, some cases of *M. paratuberculosis* infection have been reported to be indistinguishable from *M. avium* infection, with lymph node caseation and necrosis a not uncommon finding (87). The pathological findings in the recently reported cases in foxes and weasels were consistent with early phase infections. The lesions were mild and restricted to the mesenteric lymph nodes. Small numbers of intracellular acid-fast bacilli were present in single 'macrophage-like cells' and discrete granulomata had formed in the cortex and paracortex of the nodes in six of nine foxes and in one stoat (9).

In summary, microscopic lesions such as histiocytic or granulomatous inflammation including acid-fast bacilli in any tissue of the gastrointestinal tract or mesenteric lymph nodes are compatible with Johne's disease. Multinucleated giant cells or epithelioid (Langhans' type) macrophages (without the intracytoplasmic crystalline material, pigment or debris that indicates the cell is simply draining inert material from the tissue) in the lamina propria at any level of the gastrointestinal

tract should raise suspicions of *M. paratuberculosis* infection, even in the absence of acid-fast bacilli.

## Methods of diagnosis

Two basic methods exist for Johne's disease diagnosis, namely: finding the agent causing the disease (*M. paratuberculosis*) or finding an immunological response to the infection (such as cytokines, antibodies or lesions). Given the pathobiology of Johne's disease, with the end-stage or intermittent production of detectable immunological signs of infection, at the individual animal level, the positive test result provides the most useful diagnostic data. A negative test result does not prove that the animal is free of infection, it may simply mean that the particular sample collected did not contain the analyte in question (e.g. antibody or *M. paratuberculosis* organism).

### Finding and identifying the agent

#### Direct stain of faecal samples

Direct acid-fast staining of faecal samples may reveal mycobacterial bacilli, but the sensitivity of this method is low. An additional drawback is that accurately distinguishing *M. paratuberculosis* from the non-pathogenic mycobacteria (saprophytes) commonly occurring in such samples can be difficult even for experienced technicians, resulting in low specificity of this diagnostic method.

#### Direct stain of tissue impression slides

Slide impression smears of tissue collected at necropsy or through a biopsy of the ileum and mesenteric lymph nodes (e.g. via right flank laparotomy) may be useful. Staining of the slides with either an acid-fast stain (e.g. Ziehl-Neelsen) or Wright's stain can highlight the rod-shaped organism. Although neither method is specific for *M. paratuberculosis* and detection is unlikely in the paucibacillary form of infection, the process is rapid, simple and inexpensive.

#### Faecal and tissue culture

Given the successful history of isolating the organism in taxonomically diverse hoofstock, this method of diagnosis is believed to be effective for any host species. However, the sensitivity of the assay will vary, as some strains of *M. paratuberculosis* are more difficult to isolate than others (e.g. those from sheep or bison), requiring longer periods of culture or enriched media. Since a variety of other mycobacterial species can be found in faecal samples, all isolates should be confirmed as *M. paratuberculosis* through mycobactin dependency tests (growth only on media supplemented with mycobactin, an iron chelator required for in-vitro growth by *M. paratuberculosis*) or IS900 detection with a validated genetic probe (see the subsection entitled 'Isolate identification' below). Given the slow generation rate of the organism, isolations cannot be obtained rapidly. Both conventional (twelve to sixteen weeks) and radiometric (five to eight weeks) methods of culture are effective for isolating

the organism when conducted by experienced laboratories (33, 127, 156, 157). The time difference between the two methods is due to the process of detection. In the conventional method, slants of media streaked with the sample are inspected until a colony is visible. For radiometric culture, a machine monitors sample-inoculated bottles of media for  $^{14}\text{C}$ -labelled products of bacterial metabolism. Since metabolic products are detectable before the organism forms a colony sizeable enough to be observed on standard slants of media by the naked eye, the radiometric method is more rapid. Both the analytical and diagnostic sensitivity of the radiometric method is higher than that of conventional culture (33, 127, 156, 157). Other automated mycobacterial culture and detection systems, widely used for mycobacterial detection in human clinical laboratories, are being evaluated and may perform well for detection of *M. paratuberculosis*. An added advantage of these systems over the BACTEC system is that radioisotopes are not used.

The culture method is also useful for tissue samples. At necropsy, tissue samples should be collected for both culture and histopathological examination to maximise the likelihood of confirming a diagnosis of *M. paratuberculosis* infection. While the distribution of the organism may vary by case or perhaps by species, the ileum, jejunum, caecum, ileocaecal and mesenteric lymph nodes warrant sampling for isolation of *M. paratuberculosis* in animals with an ante-mortem diagnosis of Johne's disease.

## Isolate identification

### Phenotypic identification

This method relies upon the fact that *M. paratuberculosis* is essentially the only member of the acid-fast mycobacterial family to require mycobactin J, a siderophore necessary for obtaining iron from the environment, for in-vitro isolation. The inability of *M. paratuberculosis* to produce this compound has been utilised to distinguish among acid-fast organisms: the sample is streaked to media with mycobactin and to media without mycobactin. Patterns of growth for the two slants are compared: if slow growing acid-fast bacteria are noted only on the media with mycobactin, or a marked difference is observed in the degree of growth between the two slants, the organism is identified as *M. paratuberculosis*.

### Genotypic identification (polymerase chain reaction-based probes for insertion sequences)

This assay focuses on the insertion sequence IS900, considered unique to *M. paratuberculosis*. When applied to confirm the identity of acid-fast bacteria isolated from sample cultures, the sensitivity of the assay is 100% and the specificity approaches 100%. However, recent reports of false-positives due to non-specific primers warrant further analysis of discordant results (37). Attempts to apply polymerase chain reaction (PCR)-based probes directly to biological samples has been plagued with interference by components of the sample (154), but research is underway at a number of institutions to surmount this obstacle. Immunomagnetic separation

techniques have been used with milk samples and appear to be promising (58). Numerous laboratories are investigating different primer sets and protocols for IS900 detection. This method can also be applied to formalin-fixed tissue if lesions consistent with the infection have been noted and acid-fast bacilli are observed.

Another genetic target, IS1311, is under investigation. Although this insertion sequence occurs in both *M. avium* and *M. paratuberculosis*, certain loci allow *M. paratuberculosis* to be distinguished from the other closely related mycobacteria and may also permit classification of isolates as either sheep, cattle, or bison strains, based on point mutations and restriction enzyme patterns (158). The effectiveness of this procedure as a diagnostic tool is currently being researched.

### Molecular fingerprinting

Progress has been made in establishing a standard system for DNA fingerprinting of *M. paratuberculosis* strains using a variety of enzyme restriction fragment length polymorphism (RFLP) protocols. For instance, the method can be applied to strains from different hosts and different geographical locations to provide genetic profiles that can support analyses of the molecular epidemiology of paratuberculosis. A study of 1,008 strains of *M. paratuberculosis* from thirteen different animal host species obtained from twenty-two countries revealed a total of twenty-eight RFLP types using two restriction endonucleases (*Pst* I and *Bst* EII). No relationship was found between RFLP type and host species (105).

### Antigen 85 – monoclonal antibody immunoassay

Research is underway to develop an assay capable of detecting circulating antigen 85, a secreted product of actively replicating mycobacteria (159). Attempts have been made to capitalise on this secretory protein for diagnosis of *M. tuberculosis* infections in humans (11). In animals, a dot-blot immunoassay protocol was used, with encouraging results, to detect and quantify circulating antigen in a captive group of ruminants of various species with a documented history of *M. bovis* infection (89).

If a monoclonal antibody to *M. paratuberculosis* antigen 85 were to show high specificity and sufficient sensitivity, this ability to detect the infection without relying on an immune response (e.g. antibody production or cytokine release) from the host would be most beneficial. The assay would thus offer the potential of detecting animals at the earliest stages of infection, months before any histopathological or clinical evidence of Johne's disease would appear. Efforts are in progress to validate this assay for diagnosis of Johne's disease in both bison and captive wildlife. Confidence in the accuracy of this method would have to be substantial, as the diagnosis may be questioned if traditional post-mortem evidence, such as tissue lesions or isolation of the organism, is not found. Post-mortem findings would probably not support a diagnosis of Johne's disease at this early stage of infection.

The usefulness of antigen 85 may not be limited to diagnostic purposes. A recent paper presented data showing that guinea-pigs vaccinated with a strain of bacillus Calmette-Guérin (BCG) expressing a component of antigen 85 (A85B, a 30 kDa major secretory protein) were more resistant to oral challenge with a virulent strain of *M. tuberculosis* than guinea-pigs vaccinated with conventional BCG vaccine. To date, Johne's disease vaccines have not been particularly effective in preventing infection with *M. paratuberculosis* (72). Antigen 85 may be able to increase the potency of vaccines and improve efforts to control transmission of the infection (62).

## Finding an immunological response to infection

### Humoral immunity

Three standard assays exist for serum antibody detection, namely: agar gel immunodiffusion (AGID), complement fixation (CF) and enzyme-linked immunosorbent assay (ELISA). These assays have been validated primarily for domestic bovine, ovine or caprine species (50). Most authors have relied upon histopathological or faecal culture evidence of infection to categorise the true infection status against which to measure the performance of these assays. This approach, though logical, is problematic for *M. paratuberculosis* infections given the intermittent pattern of faecal shedding and the often undetectable nature of tissue lesions in early disease phases, in contrast to the production of antibody during late phases of infection. As a result, more than one sensitivity rate is often reported for these assays: one for subclinically and one for clinically affected animals. Lower sensitivity is expected from these assays for the former, and higher sensitivity for the latter cases.

Serological assays are best applied as surveillance tools to establish the infection status (i.e. presence or absence of infection) for entire herds or flocks of adult animals. The predictive values of the test results also vary, based on the infection prevalence within the group of animals being tested. The value of a result for an individual animal is therefore limited in the absence of herd prevalence information. Given the slowly evolving nature of the infection, designers of control programmes usually recommend that herd/flock serological screens be focused on animals of two years and older, to maximise the diagnostic value of the screen.

The ability of these assays to detect antibody produced in response to infection could be improved. Currently, most commercial ELISAs and AGIDs are based on a crude lysate of a laboratory-adapted, high passage *M. paratuberculosis* strain (39). Identifying and incorporating specific cell wall or secreted antigens linked to virulence or pathogenicity from clinical *M. paratuberculosis* strains may boost serological assay performance.

### Agar gel immunodiffusion test

The AGID has been used successfully in Johne's disease control programmes for cattle, sheep and goats (44, 131). For

cattle, the AGID is reported to be less analytically sensitive than the ELISA (more molecules of antibody are required to produce a positive result) (29, 128). However, for whole sheep flock surveillance programmes, the AGID has performed comparably to the ELISA (35). Test specificity may be higher for the AGID than for the ELISA (false-positive results may occur more often with the ELISA due to cross-reacting antibodies caused by infection with other organisms such as *Corynebacterium pseudotuberculosis*, the cause of caseous lymphadenitis, or other mycobacteria).

### Complement fixation

This assay detects complement-fixing antibodies to *M. paratuberculosis*, and is the assay often requested for international animal shipment health documents. In cattle, sensitivity and specificity are reported to be lower than both the AGID and ELISA (31, 128).

### Enzyme-linked immunosorbent assay

In cattle, the ELISA is analytically more sensitive than the other two serological assays. The assay is more specific than the CF test, due to an absorption step with *M. phlei* that removes many non-specific antibodies from the serum sample, such as those elicited by *Nocardia asteroides* and other closely related bacteria. For performance reasons and because of automation, this assay is probably the most widely used screening test. The absorbed ELISA method is used in Australia, the Netherlands and the USA for voluntary Johne's disease control programmes. To avoid having to submit the sample to the laboratory, 'cow-side' versions of this assay are now commercially available. Research efforts are currently focussing on the application of ELISA technology to the detection of antibody in non-domestic species, by using either a non-specific binding conjugate (e.g. protein G) or by developing a monoclonal/polyclonal conjugate that can bind antibody produced by all artiodactylids (91). If effective, the ELISA could then be used to screen all hoofstock for antibody produced in response to infection with *M. paratuberculosis*.

### Cellular immunity

#### Skin testing

A delayed-type hypersensitivity response based on a cell-mediated response (i.e. an increase in skin thickness of over 3 mm within 24 h-72 h of intradermal injection of purified protein derivative of *M. paratuberculosis*) is an indication of infection. While recent re-evaluation of skin testing on young cattle (six to twenty-four months old) indicates that the test may be useful for early diagnosis (K. Kalis, personal communication), this assay has not been recommended due to low specificity. In fact, some animals with *M. paratuberculosis* infection may be positive to the caudal fold tuberculosis skin test that uses PPD-B. Numerous antigens are shared among mycobacteria, and some of these are components of the tuberculin used for assessing animals for *M. bovis* infection. The non-specific response can be clarified, at least in cattle, through subsequent use of the comparative cervical test (CCT). The CCT employs both PPD-B and *M. avium* tuberculin (PPD-A), and the separate

responses of the animal to each are compared. Animals with Johne's disease are more likely to produce a stronger response to the *M. avium* tuberculin, given the close genetic similarity with *M. paratuberculosis* and greater number of shared antigens in comparison with *M. bovis*. While the CCT is not recommended for Johne's disease surveillance, animals producing a strong response to PPD-A should be investigated further for *M. paratuberculosis* infection.

### Gamma interferon

This cytokine is produced by sensitised lymphocytes as part of a cellular immune response to infection. Based on sandwich enzyme immunoassay protocols, this test can be used to compare the amount of IFN $\gamma$  produced by peripheral blood leukocytes in response to stimulation by *M. bovis* antigens, *M. avium* antigens (as a surrogate for *M. paratuberculosis* antigens), and to a non-specific stimulant such as phytohaemagglutinin used as a control to assess the viability of the cells (13, 117, 162). While this assay can be both informative and useful in some settings, non-specific reactions, different responses of species to the non-specific stimulant, the time sensitive and time consuming nature of the protocol, and interpretation issues have limited this assay to research rather than standard diagnostic usage. The assay is commercially available and a version for cervids will soon be released.

## Application of diagnostics

Many different situations require the use of laboratory tests for paratuberculosis, including confirmation of clinical diagnosis, confirmation of a positive serological test, estimation of prevalence of infection in herds/flocks, operation of a control programme, certification or classification of herds/flocks, screening herd replacements raised on farm, prepurchase testing, and export testing. Each of these requires tests with slightly different performance characteristics (sensitivity, specificity, speed, cost, ease of use). An understanding of the differences in tests is important when trying to diagnose *M. paratuberculosis* infection in individual animals, rather than in herds or flocks (31).

Predictive values for positive or negative test results are strongly influenced by *M. paratuberculosis* infection prevalence within a herd or among several herds. This is true for individual animal and herd level diagnosis of paratuberculosis, respectively, and must be considered when interpreting any test. For assay results expressed as continuous variables, such as for the ELISA (optical density or sample-to-positive [s/p] ratios) and even conventional culture (colony forming units), it is essential to appreciate that the magnitude of a test result is important. Quantitative results should be used as indicators of the severity or stage of infection. These data can be used to express diagnostic probabilities using likelihood ratios (119) and to rank animals for culling from herds or flocks with the goal of swift removal

of the most infectious animals (i.e. those shedding the greatest numbers of bacilli or with the highest ELISA results).

Other factors affecting choice of diagnostic test include the speed, cost, consequences of actions taken on positive or negative test results and the cost-benefit of those actions, both financial and otherwise, to the herd or flock owner. For these reasons, generalisation about the application of diagnostic tests for paratuberculosis is difficult. The specific situation requiring paratuberculosis diagnostics has to be considered, for the sake of the animal owner, preferably before the tests are used rather than after.

Diagnosis of mycobacterial infections is difficult. Hence it is important that as much information as possible is extracted and that all of the clinical epidemiological tools available are used to maximise the information gathered from diagnostic tests. Given the biology of the disease, tests for paratuberculosis will rarely, if ever, be definitive. Instead these tests will reflect some probability of infection diagnosis. Learning to cope with this reality will advance disease control programmes more rapidly than waiting for technological advances to deliver a perfect diagnostic test for paratuberculosis that is also rapid and affordable.

## Zoonosis

Public health issues have been raised about the transmission of *M. paratuberculosis* from animals to humans through animal products (dairy foods, meat, contaminated surface water) and the potential for subsequent infection and perhaps disease (32). Crohn's disease, a debilitating chronic inflammatory bowel disease, is thought by some to be linked to *M. paratuberculosis* (25). The studies reviewing a simple association between *M. paratuberculosis* and Crohn's disease are divergent and inconclusive (22, 61, 141, 146). No studies have moved beyond an association between the organism and Crohn's disease to focus on direct causality. The primary approach of the research has been to compare the detection rate of *M. paratuberculosis* DNA in tissue from Crohn's disease patients to that in control tissues (21, 23, 28, 41, 45, 49, 61, 73, 84, 96, 99, 118, 120, 134, 146, 147). In a recent study, serology corroborated PCR findings, but isolation of *M. paratuberculosis* from patients with Crohn's disease was not successful (34). A critical review of this controversial subject is beyond the scope of this paper. Moreover, medical opinion on this issue is rapidly changing as new information is published. Given that most mycobacterial pathogens discussed in this special issue, including the closest relative of *M. paratuberculosis* (*M. avium*), are capable of infecting both animals and humans and are classed as zoonotic agents, it is plausible that *M. paratuberculosis* is also zoonotic.

If a causal link exists between *M. paratuberculosis* and human disease, the possible routes for human exposure are multiple, including milk, meat and water. Milk is a common form of

nutrition in many countries, especially for children. If the pathogenesis of Crohn's disease is similar to that of Johne's disease, children may be more susceptible to infection than adults. Many studies have questioned whether pasteurisation is completely effective at killing *M. paratuberculosis* (5, 24, 57, 59, 94, 135). In response to preliminary reports of isolation of *M. paratuberculosis* from retail milk in the United Kingdom, in 1998, the Food Safety Authority of Ireland announced the following recommendations (48):

- a) 'Milk derived from animals diagnosed with Johne's disease, in the interim between diagnosis and removal of the animals, should be excluded from the milk supply. It should not be fed to calves but discarded.
- b) Raw milk from farms where Johne's disease is current should not be used for human consumption or for use, unpasteurised, in the making of cheese.'

While contamination of milk with *M. paratuberculosis* may offer a simple explanation of how humans not living on farms are exposed to this animal pathogen, this may not be the most significant route of exposure. A substantial portion of ground beef is made from culled dairy cattle. If the cattle are culled due to clinical signs subsequent to *M. paratuberculosis*, the infection is likely to be disseminated throughout the animal. Ante-mortem and post-mortem examinations at the slaughterhouse are unlikely to condemn such animals or carcasses. Hence, products made from such cattle under some preparation methods are likely to contain the organism and no published information is available on the cooking methods required to destroy it. Similarly, surface waters contaminated with *M. paratuberculosis* could theoretically enter domestic water supplies, and water treatments such as filtration and chlorination are not likely to remove or kill the organism (see discussion of chlorine resistance above). Domestic water is a common source of *M. avium*, and *M. paratuberculosis* has also been detected in domestic water on at least one occasion (96). Hence, if *M. paratuberculosis* becomes labelled as a potential human pathogen, a broader examination of the ecology of *M. paratuberculosis* is warranted before milk and other dairy products are unduly incriminated as the source of exposure. However, regardless of the route of distribution, the source of *M. paratuberculosis* must be infected animals, since these are the natural host of the organism. In most countries, due to the absolute number of animals and the prevalence of Johne's disease, dairy cattle will be the animals that contribute the largest amount of *M. paratuberculosis* to the environment.

## Control programmes

Control of paratuberculosis is warranted for animal health, welfare and productivity (economics). Additionally, should this agent be considered a food-borne infection of humans, effective and efficient control of this pathogen must commence on the farm (64). New national programmes have begun in the USA (cattle and goats) (143), Australia (cattle and sheep) (6, 74), and the Netherlands (cattle) (10).

Descriptions of these programmes can be found in the paper by Kennedy and Benedictus in this issue of the Review (75).

## Conclusions and future developments

Viewed from the broadest perspective, *M. paratuberculosis* is an emerging bacterial infection that, while initially and primarily focused on domesticated ruminants, is spilling over into other species, possibly even humans. Perhaps the largely successful *M. bovis* control and eradication campaigns in many countries have at the same time removed an organism from animal populations that conceivably competed with *M. paratuberculosis* for susceptible hosts. In the absence of organised control programmes, *M. paratuberculosis* is likely to continue spreading within and among animal species.

The level of public investment in paratuberculosis control will probably be strongly influenced by the outcome of research and debate in the medical community and perception of the public as to the significance of *M. paratuberculosis* to human health. Animal agriculture industries in co-operation with veterinarians should be encouraged to adopt cost-effective control measures before this infection spreads further.

Although differences may exist in aspects of *M. paratuberculosis* infection among species, the prevention, diagnosis and control of the infection is best approached conservatively. This includes assuming that transmission through faecal contamination of food, water, milk and colostrum can occur in any species. While different strains of *M. paratuberculosis* have been isolated from different host species ('sheep strain' versus 'bovine strain'), it is most productive from a control standpoint to assume that each strain is capable of infecting any species of artiodactylid. The most cost-effective method to control *M. paratuberculosis* infection is attention to biosecurity. Stringent sanitation to prevent exposure of young stock to the organism is critical.

Research is underway in a number of countries to improve the ability to understand and control this mycobacterial infection. Improved detection of the organism in biological samples such as milk, blood and manure, through immunomagnetic separation and PCR shows promise. Vaccines to limit the dissemination of the infection are also being developed. Identification and application of *M. paratuberculosis*-specific antigens may improve diagnostic assays. Countries with national animal identification and infection control systems in place have the best chance of halting the spread of *M. paratuberculosis* and even causing the prevalence of this infection to decline. Simply increasing awareness of the disease can contribute to control, thus education programmes should be supported as a first step in any Johne's disease management programme.

## *Mycobacterium avium* subsp. *paratuberculosis*: l'agent pathogène, sa pathogénie et son diagnostic

E.J.B. Manning & M.T. Collins

### Résumé

La maladie de Johne ou paratuberculose est une entérite chronique due à *Mycobacterium avium* subsp. *paratuberculosis*. La maladie, dont l'issue est généralement fatale, se caractérise par un état de cachexie et, chez certaines espèces, par de la diarrhée à l'issue d'une longue phase précliniqu. Le traitement est non seulement inefficace mais également impraticable du point de vue économique. L'infection concerne essentiellement les ruminants, mais elle a également été signalée chez les primates, le lapin, l'hermine et le renard. Comme la paratuberculose présente rarement des signes cliniques, elle n'est sans doute pas toujours signalée comme elle le devrait, bien qu'elle fasse partie, dans de nombreux pays, des maladies à déclaration obligatoire. En Europe, la prévalence troupeaux de la paratuberculose bovine dans les élevages varie de 7 % à 55 %. Aux États-Unis d'Amérique, elle est étroitement associée à la taille des troupeaux: 40 % de ceux qui comptent plus de 300 têtes de bétail ont été reconnus infectés. En Australie, le taux d'infection des troupeaux de vaches laitières va de 9 % à 22 %. La paratuberculose chez les animaux domestiques entraîne de lourdes pertes économiques pour plusieurs raisons (baisse de production, réforme prématurée et accroissement des frais vétérinaires, entre autres). La maladie constitue également une menace pour les animaux sauvages vivant en liberté ou en captivité.

### Mots-clés

Diagnostic – Écologie microbienne – Maladie de Johne – *Mycobacterium paratuberculosis* – Paratuberculose – Pathogénie – Zoonose.

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## *Mycobacterium avium* subsp. *paratuberculosis*: el patógeno, su patogenia y su diagnóstico

E.J.B. Manning & M.T. Collins

### Resumen

La enfermedad de Johne, o paratuberculosis, es una infección intestinal crónica provocada por *Mycobacterium avium* subsp. *paratuberculosis*. De curso normalmente fatal, esta enfermedad se caracteriza por estados de caquexia y, en algunas especies, diarrea, tras una prolongada fase preclínica. Su tratamiento resulta, además de ineficaz, económicamente inviable. La paratuberculosis afecta sobre todo a rumiantes, aunque también ha sido descrita en primates, conejos, armiños y zorros. Dado el frecuente carácter subclínico de la infección, se sospecha que se notifican menos casos de los que en realidad se producen, aun cuando se trate de una enfermedad de declaración obligatoria en muchos países. La prevalencia de rebaños infectados por la paratuberculosis bovina oscila en Europa entre un 7% y un 55%. En los Estados Unidos de América, la prevalencia de rebaños infectados guarda una estrecha correlación con el tamaño de los mismos: un 40% de los rebaños de más de 300 cabezas resultaron estar infectados. En Australia, las tasas de infección declarada entre rebaños lecheros oscilan entre un 9% y un 22%. La infección por paratuberculosis del ganado doméstico es causa de considerables pérdidas económicas, que

obedecen a factores diversos (por ejemplo la caída de la producción, la realización prematura de sacrificios selectivos y el incremento de los gastos veterinarios). Tampoco la fauna salvaje, libre o cautiva, se encuentra a salvo de la paratuberculosis.

#### Palabras clave

Diagnóstico – Ecología microbiana – Enfermedad de Johne – *Mycobacterium paratuberculosis* – Paratuberculosis – Patogenia – Zoonosis.

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## References

1. Abbas B., Murphy K. & Sher A. (1996). – Functional diversity of helper T lymphocytes. *Nature*, **383**, 787-793.
2. Abel L., Sanchez F.O., Oberti J., Thuc N.V., Hoa L.V., Lap V.D., Skamene E., Lagrange P.H. & Schurr E. (1998). – Susceptibility to leprosy is linked to the human NRAMP1 gene. *J. Infect. Dis.*, **177**, 133-145.
3. Adams J.L. & Czuprynski C.J. (1995). – Ex vivo induction of TNF- $\alpha$  and IL-6 mRNA in bovine whole blood by *Mycobacterium paratuberculosis* and mycobacterial cell wall components. *Microb. Pathogen.*, **19**, 19-29.
4. Adams J.L., Collins M.T. & Czuprynski C.J. (1996). – Polymerase chain reaction analysis of TNF-alpha and IL-6 mRNA levels in whole blood from cattle naturally or experimentally infected with *Mycobacterium paratuberculosis*. *Can. J. vet. Res.*, **60**, 257-262.
5. Advisory Committee on the Microbiological Safety of Food (2000). – Preliminary results from the national study on the microbiological quality and heat processing of cows' milk: *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Ref. ACM/485. Website: [http://www.foodstandards.gov.uk/pdf\\_files/papers/acm485.pdf](http://www.foodstandards.gov.uk/pdf_files/papers/acm485.pdf) (document accessed on 8 January 2001).
6. Allworth M.B. & Kennedy D.J. (1990). – Progress in national control and assurance programs for ovine Johne's disease in Australia. In Proc. 6th International Colloquium on paratuberculosis (E.J.B. Manning & M.T. Collins, eds), 14-18 February, Melbourne, International Association for Paratuberculosis, Madison, Wisconsin, 33-38.
7. Alzuherrri H.M., Woodall C.J. & Clarke C.J. (1996). – Increased intestinal TNF-alpha, IL-1 beta and IL-6 expression in ovine paratuberculosis. *Vet. Immunol. Immunopathol.*, **49**, 331-345.
8. Aronson T., Holtzman A., Glover N., Boian M., Froman S., Berlin O.G., Hill H. & Stelma G. Jr (1990). – Comparison of large restriction fragments of *Mycobacterium avium* isolates recovered from AIDS and non-AIDS patients with those of isolates from potable water. *J. clin. Microbiol.*, **37**, 1008-1012.
9. Beard P., Henderson D., Daniels M.J., Pirie A.A., Buxton D., Greig A., Hutchings M.R., McKendrick L., Rhind S., Stevenson K. & Sharp J.M. (2000). – Evidence of paratuberculosis in fox (*Vulpes vulpes*) and stoat (*Mustela erminea*). *Vet. Rec.*, **145**, 612-613.
10. Benedictus G., Verhoeff J., Schukken Y.H. & Hesselink J.W. (1990). – Dutch paratuberculosis programme: history, principles and development. In Proc. 6th International Colloquium on paratuberculosis (E.J.B. Manning & M.T. Collins, eds), 14-18 February, Melbourne, International Association for Paratuberculosis, Madison, Wisconsin, 2-15.
11. Bentley-Hibbert S.L., Quan X., Newman T., Huygen K. & Godfrey H. (1990). – Pathophysiology of antigen 85 in patients with active tuberculosis: antigen 85 circulates as complexes with fibronectin and immunoglobulin G. *Infect. Immun.*, **67**, 581-588.
12. Beutler B. & Cerami A. (1980). – The biology of cachectin/TNF- $\alpha$  primary mediator of the host response. *Annu. Rev. Immunol.*, **7**, 625-655.
13. Billman-Jacobe H., Carrigan M., Cockram F., Corner L.A., Gill I.J., Hill J.F., Milner A.R. & Wood P.R. (1992). – A comparison of the interferon gamma assay with the absorbed ELISA for the diagnosis of Johne's disease in cattle. *Aust. vet. J.*, **69**, 25-28.
14. Brooks R.W., Parker B.C., Gruft H. & Falkinham J.I. (1984). – Epidemiology of infection by nontuberculous mycobacteria. V. Numbers in eastern United States soils and correlation with soil characteristics. *Am. Rev. respir. Dis.*, **130**, 630-633.
15. Brown W.C., Rice-Ficht A.C. & Estes D.M. (1998). – Bovine Type I and Type II responses. *Vet. Immunol. Immunopathol.*, **63**, 45-55.
16. Buergelt C.D., Pool M.O. & Williams E. (1978). – Pathological evaluation of paratuberculosis in naturally infected cattle. *Vet. Pathol.*, **15**, 196-207.

17. Buergelt C.D., Layton A.W., Ginn P.E., Taylor M., King J.M., Haebecker P.L., Mauldin E., Whitlock R., Rossiter C. & Collins M.T. (2000). – The pathology of spontaneous paratuberculosis in North American bison (*Bison bison*). *J. vet. diagn. Invest.*, **37**, 428-438.
18. Bull T.J., Pavlik I., Hermon-Taylor J., El-Zaatari F.A. & Tizard M.L. (1990). – Study of IS900 loci in *Mycobacterium avium* subsp. *paratuberculosis* by multiplex PCR screening. In Proc. 6th International Colloquium on paratuberculosis (E.J.B. Manning & M.T. Collins, eds), 14-18 February, Melbourne, International Association for Paratuberculosis, Madison, Wisconsin, 265-274.
19. Camphausen R.T., Jones R.L. & Brennan P.J. (1985). – A glycolipid antigen specific to *Mycobacterium paratuberculosis*: structure and antigenicity. *Proc. natl Acad. Sci. USA*, **82**, 3068-3072.
20. Camphausen R.T., Jones R.L. & Brennan P.J. (1988). – Antigenic relationship between *Mycobacterium paratuberculosis* and *Mycobacterium avium*. *Am. J. vet. Res.*, **49**, 1307-1310.
21. Chiba M., Fukushima T., Horie Y., Iizuka M. & Masamune O. (1998). – No *Mycobacterium paratuberculosis* detected in intestinal tissue, including Peyer's patches and lymph follicles, of Crohn's disease. *J. Gastroenterol. Hepatol.*, **33**, 482-487.
22. Chiodini R.J. (1980). – Crohn's disease and the mycobacterioses: a review and comparison of two disease entities. *Clin. Microbiol. Rev.*, **2**, 90-117.
23. Chiodini R.J., Van Kruiningen H.J., Merkal R.S., Thayer W.R. Jr & Couto J.A. (1984). – Characteristics of an unclassified *Mycobacterium* species isolated from patients with Crohn's disease. *J. clin. Microbiol.*, **20**, 966-971.
24. Chiodini R.J. & Hermon T.J. (1993). – The thermal resistance of *Mycobacterium paratuberculosis* in raw milk under conditions simulating pasteurization. *J. vet. diagn. Invest.*, **5**, 629-631.
25. Chiodini R.J. & Rossiter C.A. (1996). – Paratuberculosis: a potential zoonosis? *Vet. Clin. N. Am. (Food Anim. Pract.)*, **12**, 457-467.
26. Clarke C.J. (1997). – The pathology and pathogenesis of paratuberculosis in ruminants and other species. *J. comp. Pathol.*, **116**, 217-261.
27. Clarke C.J. & Little C.L. (1996). – The pathology of ovine paratuberculosis: gross and histological changes in the intestine. *J. comp. Pathol.*, **114**, 419-437.
28. Clarkston W.K., Presti M.E., Petersen P.F., Zachary P.E. Jr, Fan W.X., Leonardi C.L., Vernava A.M., Longo W.E. & Kreeger J.M. (1998). – Role of *Mycobacterium paratuberculosis* in Crohn's disease – a prospective, controlled study using polymerase chain reaction. *Dis. Colon Rectum*, **41**, 195-199.
29. Colgrove C.S., Thoen C.O., Blackburn B.O. & Murphy C.D. (1980). – Paratuberculosis in cattle: a comparison of three serologic tests with results of fecal culture. *Vet. Microbiol.*, **19**, 183-187.
30. Collins D.M., Gabric D.M. & de Lisle G.W. (1980). – Identification of a repetitive DNA sequence specific to *Mycobacterium paratuberculosis*. *FEMS Microbiol. Lett.*, **60**, 175-178.
31. Collins M.T. (1996). – Diagnosis of paratuberculosis. *Vet. Clin. N. Am. (Food Anim. Pract.)*, **12**, 357-371.
32. Collins M.T. (1997). – *Mycobacterium paratuberculosis*: a potential food-borne pathogen? *J. Dairy Sci.*, **80**, 3445-3448.
33. Collins M.T., Kenefick K.B., Sockett D.C., Lambrecht R.S., McDonald J. & Jorgensen J.B. (1990). – Enhanced radiometric detection of *Mycobacterium paratuberculosis* using filter concentrated fecal specimens. *J. clin. Microbiol.*, **28**, 2514-2519.
34. Collins M.T., Lisby G., Moser C., Chicks D., Christensen S., Reichelderfer M., Hoiby N., Harms B.A., Thomsen O.O., Skibsted U. & Binder V. (2000). – Results of multiple diagnostic tests for *Mycobacterium avium* subsp. *paratuberculosis* in patients with inflammatory bowel disease and in controls. *J. clin. Microbiol.*, **38**, 4373-4381.
35. Condron R.J., Hope A.F., Kluver P.F., Jones S. & Millar H.W.C. (1999). – Performance of diagnostic tests to detect ovine Johne's disease. In Proc 6th International Colloquium on paratuberculosis (E.J.B. Manning & M.T. Collins, eds), 14-18 February, Melbourne, International Association for Paratuberculosis, Madison, Wisconsin, 503.
36. Cook W.E., Cornish T.E., Shideler S., Lasley B. & Collins M.T. (1997). – Radiometric culture of *Mycobacterium avium paratuberculosis* from the feces of tule elk. *J. Wildl. Dis.*, **33**, 635-637.
37. Cousins D.V., Whittington R., Marsh I., Masters A., Evans R.J. & Kluver P. (1990). – *Mycobacteria* distinct from *Mycobacterium avium* subsp. *paratuberculosis* isolated from the faeces of ruminants possess IS900-like sequences detectable by IS900 polymerase chain reaction: implications for diagnosis. *Molec. cell. Probes*, **13**, 431-442.
38. Covert T.C., Rodgers M.R., Reyes A.L. & Stelma G.J. (1990). – Occurrence of nontuberculous mycobacteria in environmental samples. *Appl. environ. Microbiol.*, **65**, 2492-2496.
39. Cox J.C., Drane D.P., Jones S.L., Ridge S. & Milner A.R. (1991). – Development and evaluation of a rapid absorbed enzyme immunoassay test for the diagnosis of Johne's disease in cattle. *Aust. vet. J.*, **68**, 157-160.
40. De Kessel M., Gilot P., Coene M. & Cocito C. (1995). – Composition and immunological properties of the protein fraction of A36, a major antigen complex of *Mycobacterium paratuberculosis*. *Scand. J. Immunol.*, **201**, 212.
41. Dell'sola B., Poyart C., Goulet O., Mougnot J.F., Sadoun-Journo E., Brousse N., Schmitz J., Ricour C. & Berche P. (1994). – Detection of *Mycobacterium paratuberculosis* by polymerase chain reaction in children with Crohn's disease. *J. infect. Dis.*, **169**, 449-451.
42. Doyle T.M. (1956). – Johne's disease. *Vet. Rec.*, **68**, 869-878.

43. Doyle T.M. (1958). – Foetal infection in Johne's disease. *Vet. Rec.*, **70**, 238-238.
44. Dubash K., Shulaw W.P., Bech-Nielsen S., Stills H.F.J. & Slemons R.D. (1996). – Evaluation of an agar gel immunodiffusion test kit for detection of antibodies to *Mycobacterium paratuberculosis* in sheep. *J. Am. vet. med. Assoc.*, **208**, 401-403.
45. Dumonceau J.M., Van Gossum A., Adler M., Fonteyne P.A., Van Vooren J.P., Deviere J. & Portaeis F. (1996). – No *Mycobacterium paratuberculosis* found in Crohn's disease using polymerase chain reaction. *Dig. Dis. Sci.*, **41**, 421-426.
46. Evans C.D. & Jenkins A. (2000). – Surface water acidification in the South Pennines. II. Temporal trends. *Environ. Pollut.*, **109**, 21-34.
47. Falkinham J.O. (1996). – Epidemiology of infection by nontuberculous mycobacteria. *Clin. Microbiol. Rev.*, **9**, 177-215.
48. Food Safety Authority of Ireland (1998). – Statement in relation to UK MAFF announcement. Press release: 11 August 1998. Website: [http://www.fsai.ie/press\\_releases/110898.htm](http://www.fsai.ie/press_releases/110898.htm) (document accessed on 8 January 2001).
49. Frank T.S. & Cook S.M. (1996). – Analysis of paraffin sections of Crohn's disease for *Mycobacterium paratuberculosis* using polymerase chain reaction. *Mod. Pathol.*, **9**, 32-35.
50. Gardner I.A., Hietala S. & Boyce W.M. (1996). – Validity of using serological tests for diagnosis of diseases in wild animals. In: *Wildlife husbandry and diseases* (M.E. Fowler, ed.). *Rev. sci. tech. Off. Int. Epiz.*, **15** (1), 323-335.
51. Gilmour N.J.L., Nisbet D.I. & Brotherston J.G. (1965). – Experimental oral infection of calves with *Mycobacterium johnei*. *J. comp. Pathol.*, **75**, 281-286.
52. Gilot P., De Kessel M., Coene M. & Cocito C. (1992). – Induction of cellular immune reactions by A36, an antigen complex of *Mycobacterium paratuberculosis*. Comparison of A36 and johnin components. *Scand. J. Immunol.*, **36**, 811-821.
53. Glover N., Holzman A., Aronson T., Froman S., Berlin O.G.W., Dominguez P., Kunkel K.A., Overturf G., Stelma G. Jr, Smith C. & Yakus M. (1994). – The isolation and identification of *Mycobacterium avium* complex (MAC) recovered from Los Angeles potable water, a possible source of infection in AIDS patients. *Int. J. Environ. Hlth Res.*, **4**, 63-72.
54. Gomes M.S. & Appelberg R. (1998). – Evidence for a link between iron metabolism and *Nramp1* gene function in innate resistance against *Mycobacterium avium*. *Immunology*, **95**, 165-168.
55. Govoni G. & Gros P. (1998). – Macrophage *NRAMP1* and its role in resistance to microbial infections. *Inflamm. Res.*, **47**, 277-284.
56. Grant I.R., Ball H.J., Neill S.D. & Rowe M.T. (1996). – Inactivation of *Mycobacterium paratuberculosis* in cows' milk at pasteurization temperatures. *Appl. environ. Microbiol.*, **62**, 631-636.
57. Grant I.R., Ball H.J. & Rowe M.T. (1998). – Effect of high-temperature, short-time (HTST) pasteurization on milk containing low numbers of *Mycobacterium paratuberculosis*. *Let. appl. Microbiol.*, **26**, 166-170.
58. Grant I.R., Ball H.J. & Rowe M.T. (1998). – Isolation of *Mycobacterium paratuberculosis* from milk by immunomagnetic separation. *Appl. environ. Microbiol.*, **64**, 3153-3158.
59. Grant I.R., Ball H.J. & Rowe M.T. (1999). – Effect of higher pasteurization temperatures, and longer holding times at 72 degrees C, on the inactivation of *Mycobacterium paratuberculosis* in milk. *Let. appl. Microbiol.*, **28** (6), 461-465.
60. Greig A., Stevenson K., Henderson D., Perez V., Hughes V., Pavlik I., Hines M.E., McKendrick I. & Sharp J.M. (1990). – Epidemiological study of paratuberculosis in wild rabbits in Scotland. *J. clin. Microbiol.*, **37**, 1746-1751.
61. Hermon-Taylor J., Bull T.J., Sheridan J.M., Cheng J., Steliakakis M.L. & Sumar N. (2000). – The causation of Crohn's disease by *Mycobacterium avium* subspecies *paratuberculosis*. *Can. J. Gastroenterol.*, **14**, 521-539.
62. Horowitz M.A., Harth G., Dillion B.J. & Maslesa-Galic S. (2000). – Recombinant bacillus Calmette-Guerin (BCG) vaccines expressing the *Mycobacterium tuberculosis* 30-kDa major secretory protein induce greater protective immunity against tuberculosis than conventional BCG vaccines in a highly susceptible animal model. *Proc. natl. Acad. Sci. USA*, **97**, 13853-13858.
63. Iivanainen E.K., Martikainen P.J., Vaananen P.K. & Katila M.L. (1993). – Environmental factors affecting the occurrence of mycobacteria in brook waters. *Appl. environ. Microbiol.*, **59**, 398-404.
64. International Dairy Federation (IDF) (1994). – The significance of pathogenic microorganisms in raw milk. IDF, Brussels, 218 pp.
65. International Dairy Federation (IDF) (2001). – *Mycobacterium paratuberculosis*. Monograph of the International Dairy Federation Task Force on *Mycobacterium paratuberculosis*. Bulletin of the International Dairy Federation, No. 362/2001. IDF, Brussels, 61 pp.
66. Jansen J. (1951). – Paratuberculosis. *J. Am. vet. med. Assoc.*, **112**, 52-55.
67. Jessup D.A., Abbas B., Behymer D. & Gogan P. (1981). – Paratuberculosis in tule elk in California. *J. Am. vet. med. Assoc.*, **179**, 1252-1254.
68. Jessup D.A. & Williams E. (1990). – Paratuberculosis in free-ranging wildlife in North America. In: *Zoo and wild animal medicine. Current therapy* (M.E. Fowler & R.E. Miller, eds). W.B. Saunders, Philadelphia, 616-620.

69. Johnson-Ifearulundu Y. & Kaneene J.B. (1990). – Distribution and environmental risk factors for paratuberculosis in dairy cattle herds in Michigan. *Am. J. vet. Res.*, **60**, 589-596.
70. Johnson-Ifearulundu Y.J. & Kaneene J.B. (1997). – Relationship between soil type and *Mycobacterium paratuberculosis*. *J. Am. vet. med. Assoc.*, **210**, 1735-1740.
71. Johnson-Ifearulundu Y.J. & Kaneene J.B. (1998). – Management-related risk factors for *M. paratuberculosis* infection in Michigan, USA, dairy herds. *Prev. vet. Med.*, **37**, 41-54.
72. Kalis C.H.J., Hesselink J.W. & Barkema H.W. (1990). – Long-term use of a killed vaccine does not prevent fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis*. In Proc. 6th International Colloquium on paratuberculosis (E.J.B. Manning & M.T. Collins, eds), 14-18 February, Melbourne, International Association for Paratuberculosis, Madison, Wisconsin, 153-156.
73. Kanazawa K., Haga Y., Funakoshi O., Nakajima H., Munakata A. & Yoshida Y. (1990). – Absence of *Mycobacterium paratuberculosis* DNA in intestinal tissues from Crohn's disease by nested polymerase chain reaction. *J. Gastroenterol. Hepatol.*, **34**, 200-206.
74. Kennedy D.J. & Allworth M.B. (1990). – Progress in national control and assurance programs for bovine Johne's disease in Australia. In Proc. 6th International Colloquium on paratuberculosis (E.J.B. Manning & M.T. Collins, eds), 14-18 February, Melbourne, International Association for Paratuberculosis, Madison, Wisconsin, 19-26.
75. Kennedy D.J. & Benedictus G. (2001). – Control of *Mycobacterium avium* subsp. *paratuberculosis* infection in agricultural species. In *Mycobacterial infections in domestic and wild animals* (E.J.B. Manning & M.T. Collins, eds). *Rev. sci. tech. Off. Int. Epiz.*, **20** (1), 151-179.
76. Keswani J. & Frank J.F. (1998). – Thermal inactivation of *Mycobacterium paratuberculosis* in milk. *J. Food Protec.*, **61**, 974-978.
77. Kirschner R.J., Parker B.C. & Falkinham J.I. (1992). – Epidemiology of infection by nontuberculous mycobacteria. *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum* in acid, brown-water swamps of the southeastern United States and their association with environmental variables. *Am. Rev. respir. Dis.*, **145**, 271-275.
78. Koets A.P., Adugna G., Janss L.G., van Weering H.J., Kalis C.H.J., Harbers A., Rutten V.P.M.G. & Schukken Y.H. (1990). – Genetic variation in susceptibility to *M. a. paratuberculosis* infection in cattle. In Proc. 6th International Colloquium on paratuberculosis (E.J.B. Manning & M.T. Collins, eds), 14-18 February, Melbourne, International Association for Paratuberculosis, Madison, Wisconsin, 169-175.
79. Koets A.P., Rutten V.P.M.G., Hoek A., Bakker D., van Zijderveld F., Müller K.E. & van Eden W. (1990). – The T(h) 1 to T(h) 2 shift in bovine paratuberculosis and the role of apoptotic cell death. In Proc. 6th International Colloquium on paratuberculosis (E.J.B. Manning & M.T. Collins, eds), 14-18 February, Melbourne, International Association for Paratuberculosis, Madison, Wisconsin, 585-592.
80. Kopecky K.E. (1977). – Distribution of paratuberculosis in Wisconsin, by soil regions. *J. Am. vet. med. Assoc.*, **170**, 320-324.
81. Larsen A.B., Merkal R.S. & Vardaman T.H. (1956). – Survival time of *Mycobacterium paratuberculosis*. *Am. J. vet. Res.*, July, 549-551.
82. Larsen A.B. & Kopecky K.E. (1970). – *Mycobacterium paratuberculosis* in reproductive organs and semen of bulls. *Am. J. vet. Res.*, **31**, 255-258.
83. Larsen A.B., Merkal R.S. & Cutlip R.C. (1975). – Age of cattle as related to resistance to infection with *Mycobacterium paratuberculosis*. *Am. J. vet. Res.*, **36**, 255-257.
84. Lisby G., Andersen J., Engbaek K. & Binder V. (1994). – *Mycobacterium paratuberculosis* in intestinal tissue from patients with Crohn's disease demonstrated by a nested primer polymerase chain reaction. *Scand. J. Gastroenterol.*, **29**, 923-929.
85. Lovell R., Levi M. & Francis J. (1944). – Studies on the survival of Johne's bacilli. *J. comp. Pathol.*, **54**, 120-129.
86. McClure H.M., Chiodini R.J., Anderson D.C., Swenson R.B., Thayer W.R. & Cook J.A. (1987). – *Mycobacterium paratuberculosis* infection in a colony of stump tail macaques (*Macaca arctoides*). *J. infect. Dis.*, **155**, 1011-1019.
87. Mackintosh C.G., Reichel M.P., Griffin J.F.T., Montgomery H. & de Lisle G.W. (1990). – Paratuberculosis and avian tuberculosis in red deer in New Zealand: clinical syndromes and diagnostic tests. In Proc. 6th International Colloquium on paratuberculosis (E.J.B. Manning & M.T. Collins, eds), 14-18 February, Melbourne, International Association for Paratuberculosis, Madison, Wisconsin, 449-457.
88. Mackintosh C.G., Qureshi T., Waldrup K., Labes R.E., Dodds K.G. & Griffin J.F. (2000). – Genetic resistance to experimental infection with *Mycobacterium bovis* in red deer (*Cervus elaphus*). *Infect. Immun.*, **68**, 1620-1625.
89. Mangold B.J. (1990). – Detection of elevated levels of circulating antigen 85 by dot immunobinding assay in captive wild animals with tuberculosis. *J. Zoo Wildl. Med.*, **30**, 477-483.
90. Manning E.J.B. & Collins M.T. (1990). – Paratuberculosis in zoo animals. In *Zoo and wild animal medicine. Current therapy* (M.E. Fowler & R.E. Miller, eds). W.B. Saunders, Philadelphia, 612-616.

91. Manning E.J.B., Kramsky J.A. & Collins M.T. (1990). – Johne's disease diagnosis for non-domestic hoofstock. In Proc. 6th International Colloquium on paratuberculosis (E.J.B. Manning & M.T. Collins, eds), 14-18 February, Melbourne. International Association for Paratuberculosis, Madison, Wisconsin, 458-461.
92. Manning E.J.B., Steinberg H., Russow K., Ruth G.R. & Collins M.T. (1998). – Epizootic of paratuberculosis in farmed elk. *J. Am. vet. med. Assoc.*, **213**, 1320-1322.
93. Manning E.J.B. & Ziccardi M. (2000). – Johne's disease and captive non-domestic hoofstock: prevalence and prevention. In Proc. American Association of Zoo Veterinarians (AAZV) and International Association for Aquatic Animal Medicine (IAAAM) Conference, 17 September, New Orleans, AAZV/IAAAM, Media, Pennsylvania, 432-434.
94. Millar D., Ford J., Sanderson J., Withey S., Tizard M., Doran T. & Hermon-Taylor J. (1996). – IS900 PCR to detect *Mycobacterium paratuberculosis* in retail supplies of whole pasteurized cows' milk in England and Wales. *Appl. environ. Microbiol.*, **62**, 3446-3452.
95. Miller D.S., Smith B.B., Gerros T., Collins M.T., Cooley J. & McClure J. (1990). – Paratuberculosis in llamas and alpacas. *Comp. Contin. Educ. Pract. Vet.*, **21**, 220-229.
96. Mishina D., Katsel P., Brown S.T., Gilberts E.C. & Greenstein R.J. (1996). – On the etiology of Crohn's disease. *Proc. natl Acad. Sci. USA*, **93**, 9816-9820.
97. Momotani E., Whipple D.L., Thiermann A.B. & Chevillat N.F. (1988). – Role of M cells and macrophages in the entrance of *Mycobacterium paratuberculosis* into domes of ileal Peyer's patches in calves. *Vet. Pathol.*, **25**, 131-137.
98. Mutharia L.M., Moreno W. & Raymond M. (1997). – Analysis of culture filtrate and cell wall-associated antigens of *Mycobacterium paratuberculosis* with monoclonal antibodies. *Infect. Immun.*, **65**, 387-394.
99. Naser S.A., Schwartz D. & Shafran I. (2000). – Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from breast milk of Crohn's disease patients. *Am. J. Gastroenterol.*, **95**, 1094-1095.
100. Office International des Epizooties (OIE) (1999). – World animal health in 1998. Part One: Reports. OIE, Paris, 410 pp.
101. Orme I.M. (1993). – Immunity to mycobacteria. *Curr. Opin. Immunol.*, **5**, 5.
102. Oxley T. & Allen P.M. (2000). – The dynamic inter-relationship between acid rain and eutrophication in the soil domain. *Water Air Soil Pollut.*, **117**, 371-400.
103. Papapetropoulou M., Tsintzou A. & Vantarakis A. (1997). – Environmental mycobacteria in bottled table waters in Greece. *Can. J. Microbiol.*, **43**, 499-502.
104. Patterson D.S., Allen W.M. & Lloyd M.K. (1967). – Clinical Johne's as a protein losing enteropathy. *Vet. Rec.*, **81**, 717-718.
105. Pavlik I., Horvathova A., Dvorska L., Bartl J., Svastova P., du Maine M.R. & Rychlik I. (1990). – Standardisation of restriction fragment length polymorphism analysis for *Mycobacterium avium* subspecies *paratuberculosis*. *J. microbiol. Meth.*, **38**, 155-167.
106. Pavlik I., Matlova L., Bartl J., Svastova P., Dvorska L. & Whitlock R. (1990). – Parallel faecal and organ *Mycobacterium avium* subsp. *paratuberculosis* culture showing organism distribution in different breeds of cattle. In Proc. 6th International Colloquium on paratuberculosis (E.J.B. Manning & M.T. Collins, eds), 14-18 February, Melbourne. International Association for Paratuberculosis, Madison, Wisconsin, 642-656.
107. Payne J.M. & Rankin J.D. (1961). – The pathogenesis of experimental Johne's disease in calves. *Res. vet. Sci.*, **2**, 167-174.
108. Perez V., Tellechea J., Corpa J.M., Gutierrez M. & Marin J.F.G. (1990). – Relation between pathologic findings and cellular immune responses in sheep with naturally acquired paratuberculosis. *Am. J. vet. Res.*, **60**, 123-127.
109. Perez V., Garcia M.J. & Badiola J.J. (1996). – Description and classification of different types of lesion associated with natural paratuberculosis infection in sheep. *J. comp. Pathol.*, **114**, 107-122.
110. Power C.A., Wei G. & Bretscher P.A. (1998). – Mycobacterial dose defines the Th1/Th2 nature of the immune response independently of whether immunization is administered by the intravenous, subcutaneous, or intradermal route. *Infect. Immun.*, **66**, 5743-5750.
111. Rabsch W., Hargis B.M., Tsolis R.M., Kingsley R.A., Hinz K.-H., Tschäpe H. & Bäumlner A.J. (2000). – Competitive exclusion of *Salmonella enteritidis* by *Salmonella gallinarum* in poultry. *Emerg. infect. Dis.*, **6**, 443-448.
112. Rankin J.D. (1961). – The experimental infection of cattle with *Mycobacterium johnei*. II: Adult cattle inoculated intravenously. *J. comp. Pathol.*, **71**, 6-9.
113. Rastogi N., Legrand E. & Sola C. (2001). – The mycobacteria: an introduction to nomenclature and pathogenesis. In *Mycobacterial infections in domestic and wild animals* (E.J.B. Manning & M.T. Collins, eds), *Rev. sci. tech. Off. int. Epiz.*, **20** (1), 21-54.
114. Reichel M.P., Kittelberger R., Penrose M.E., Meynell R.M., Cousins D., Ellis T., Mutharia L.M., Sugden E.A., Johns A.H. & de Lisle G.W. (1990). – Comparison of serological tests and faecal culture for the detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle and analysis of the antigens involved. *Vet. Microbiol.*, **66**, 135-150.
115. Reviriego F.J., Moreno M.A. & Dominguez L. (2000). – Soil type as a putative risk factor of ovine and caprine paratuberculosis seropositivity in Spain. *Prev. vet. Med.*, **43**, 43-51.
116. Ridley D.S. (1980). – The histopathological spectrum of mycobacterioses. In *The biology of the mycobacteria*, Vol. 2. Immunological and environmental aspects (C. Ratledge & J.L. Stanford, eds), Academic Press, London, 129-171.

117. Rothel J.S., Jones S.L., Corner L.A., Cox J.C. & Wood P.R. (1990). – A sandwich enzyme immunoassay for bovine interferon-gamma and its use for the detection of tuberculosis in cattle. *Aust. vet. J.*, **67**, 134-137.
118. Rowbotham D.S., Mapstone N.P., Trejdosiewicz L.K., Howdle P.D. & Quirke P. (1995). – *Mycobacterium paratuberculosis* DNA not detected in Crohn's disease tissue by fluorescent polymerase chain reaction. *Gut*, **37**, 660-667.
119. Sackett D.L., Haynes R.B. & Tugwell P. (1985). – The interpretation of diagnostic data. In *Clinical epidemiology. A basic science for clinical medicine*. Little, Brown and Company, Boston, 59-138.
120. Sanderson J.D., Moss M.T., Tizard M.L.V. & Hermon-Taylor J. (1992). – *Mycobacterium paratuberculosis* DNA in Crohn's disease tissue. *Gut*, **33**, 890-896.
121. Schroen C., Kluver P., McDonald W., Butler K., Condron R. & Hope A. (1990). – Survival of *M. paratuberculosis* in the environment. Report to Meat and Livestock Australia.
122. Schulze-Roebbecke R. & Buchholtz K. (1992). – Heat susceptibility of aquatic mycobacteria. *Appl. environ. Microbiol.*, **58**, 1869-1873.
123. Seitz S.E., Heider L.E., Hueston W.D., Bech-Nielsen S., Rings M. & Spangler L. (1980). – Bovine fetal infection with *Mycobacterium paratuberculosis*. *J. Am. vet. med. Assoc.*, **194**, 1423-1426.
124. Skamene E., Schurr E. & Gros P. (1998). – Infection genomics: *Nrampl* as a major determinant of natural resistance to intracellular infections. *Annu. Rev. Med.*, **49**, 275-287.
125. Slagame P., Abrams J.S. & Clayberger C. (1991). – Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. *Science*, **254**, 279-282.
126. Smythe R.H. (1951). – Some observations on Johne's disease. *Vet. Rec.*, **63**, 359-363.
127. Sockett D.C., Carr D.J. & Collins M.T. (1992). – Evaluation of conventional and radiometric fecal culture and a commercial DNA probe for diagnosis of *Mycobacterium paratuberculosis* infections in cattle. *Can. J. vet. Res.*, **56**, 148-153.
128. Sockett D.C., Conrad T.A., Thomas C.B. & Collins M.T. (1992). – Evaluation of four serological tests for bovine paratuberculosis. *J. clin. Microbiol.*, **30**, 1134-1139.
129. Stabel J.R. (1990). – Transitions in immune response to *M. a. paratuberculosis*. In *Proc. 6th International Colloquium on paratuberculosis* (E.J.B. Manning & M.T. Collins, eds), 14-18 February, Melbourne, International Association for Paratuberculosis, Madison, Wisconsin, 579-584.
130. Stabel J.R., Steadham E.M. & Bolin C.A. (1997). – Heat inactivation of *Mycobacterium paratuberculosis* in raw milk: are current pasteurization conditions effective? *Appl. environ. Microbiol.*, **63**, 4975-4977.
131. Stehman S.M. (1996). – Paratuberculosis in small ruminants, deer, and South American camelids. *Vet. Clin. N. Am. (Food Anim. Pract.)*, **12**, 441-455.
132. Steinberg H. (1981). – Johne's disease (*Mycobacterium paratuberculosis*) in a Jemela topi (*Damaliscus lunatus jemela*). *J. Zoo Anim. Med.*, **19**, 33-41.
133. Streeter R.N., Hoffsis G.F., Bech-Nielsen S., Shulaw W.P. & Rings M. (1995). – Isolation of *Mycobacterium paratuberculosis* from colostrum and milk of subclinically infected cows. *Am. J. vet. Res.*, **56**, 1322-1324.
134. Suenaga K., Yokoyama Y., Nishimori I., Sano S., Morita M., Okazaki K. & Onishi S. (1990). – Serum antibodies to *Mycobacterium paratuberculosis* in patients with Crohn's disease. *Dig. Dis. Sci.*, **44**, 1202-1207.
135. Sung N. & Collins M.T. (1998). – Thermal tolerance of *Mycobacterium paratuberculosis*. *Appl. environ. Microbiol.*, **64**, 999-1005.
136. Sweeney R.W. (1996). – Transmission of paratuberculosis. *Vet. Clin. N. Am. (Food Anim. Pract.)*, **12**, 305-312.
137. Sweeney R.W., Whitlock R.H. & Rosenberger A.E. (1992). – *Mycobacterium paratuberculosis* cultured from milk and supramammary lymph nodes of infected asymptomatic cows. *J. clin. Microbiol.*, **30**, 166-171.
138. Sweeney R.W., Whitlock R.H. & Rosenberger A.E. (1992). – *Mycobacterium paratuberculosis* isolated from fetuses of infected cows not manifesting signs of the disease. *Am. J. vet. Res.*, **53**, 477-480.
139. Taylor R.H., Falkinham J.O., Norton C.D. & LeChevallier M.W. (2000). – Chlorine, chloramine, chlorine dioxide, and ozone susceptibility of *Mycobacterium avium*. *Appl. environ. Microbiol.*, **66**, 1702-1755.
140. Taylor T.K., Wilks C.R. & McQueen D.S. (1981). – Isolation of *Mycobacterium paratuberculosis* from milk of a cow with Johne's disease. *Vet. Rec.*, **109**, 532-533.
141. Thompson D.E. (1994). – The role of mycobacteria in Crohn's disease. *J. med. Microbiol.*, **41**, 74-94.
142. Thorel M.-F., Krichevsky M. & Levy-Frebault V.V. (1990). – Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. nov., and *Mycobacterium avium* subsp. *silvaticum* subsp. nov. *Int. J. syst. Bacteriol.*, **40**, 254-260.
143. United States Animal Health Association (USAHA) (1990). – National Johne's Disease Working Group Certification Subcommittee U.S. voluntary Johne's disease herd status program for cattle. In *Proc. 6th International Colloquium on paratuberculosis* (E.J.B. Manning & M.T. Collins, eds), 14-18 February, Melbourne, International Association for Paratuberculosis, Madison, Wisconsin, 33-43.
144. United States Environmental Protection Agency (USEPA) (1999). – Ultraviolet radiation. In *EPA guidance manual. Alternative disinfectants and oxidants*. USEPA, Washington, 1-25.

145. Van der Giessen J.W.B., Eger A., Haagsma J., Haring R.M., Gastra W. & van der Zeijst B.A.M. (1992). – Amplification of 16S rRNA sequences to detect *Mycobacterium paratuberculosis*. *J. med. Microbiol.*, **36**, 255-263.
146. Van Kruijning H.J. (1990). – Lack of support for a common etiology in Johne's disease of animals and Crohn's disease in humans. *Inflam. Bowel Dis.*, **5**, 183-191.
147. Van Kruijning H.J., Chiodini R.J., Thayer W.R., Coutu J.A., Merkal R.S. & Runnels P.L. (1986). – Experimental disease in infant goats induced by a *Mycobacterium* isolated from a patient with Crohn's disease. A preliminary report. *Dig. Dis. Sci.*, **31**, 1351-1360.
148. Vary P.H., Andersen P.R., Green E., Hermon-Taylor J. & McFadden J.J. (1990). – Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. *J. clin. Microbiol.*, **28**, 933-937.
149. Von Reyn C.F., Maslow J.N., Barber T.W., Falkinham J.O. & Arbeit R.D. (1994). – Persistent colonization of potable water as a source of *Mycobacterium avium* infection in AIDS. *Lancet*, **343**, 1137-1141.
150. Walsh T.F., Barbiere R. & Collins M.T. (1995). – Enteric *Mycobacterium paratuberculosis* in a mandrill (*Papio sphinx*). In Proc. Joint Conference of the American Association of Zoo Veterinarians (AAZV), Wildlife Disease Association (WDA) and the American Association of Wildlife Veterinarians (AAWV), 12-17 August, Media, Pennsylvania. AAZV, Media, 262-264.
151. Water Quality Disinfection Committee (1992). – Survey of water utility disinfection practices. *J. Am. Water Works Assoc.*, **84**, 121-128.
152. Watt W.D., Scott C.D., Zamora P.J. & White W.J. (2000). – Acid toxicity levels in Nova Scotian rivers have not declined in synchrony with the decline in sulfate levels. *Water Air Soil Pollut.*, **118**, 203-229.
153. Wells S.J. & Wagner B.A. (2000). – Herd-level risk factors for infection with *Mycobacterium paratuberculosis* in US dairies and association between familiarity of the herd manager with the disease, of prior diagnosis of the disease and use of preventive measures. *J. Am. vet. med. Assoc.*, **216**, 1450-1457.
154. Whipple D.L., Kapke P.A. & Andersen P.R. (1992). – Comparison of a commercial DNA probe test and three cultivation procedures for detection of *Mycobacterium paratuberculosis* in bovine feces. *J. vet. diagn. Invest.*, **4**, 23-27.
155. White W.B., Whipple D.L., Stabel J.R. & Bolin C.A. (1994). – Comparison of cellular and extracellular proteins expressed by various isolates of *Mycobacterium paratuberculosis* and other mycobacterial species. *Am. J. vet. Res.*, **55**, 1399-1405.
156. Whittington R.J., Marsh L., McAllister S., Turner M.J., Marshall D.J. & Fraser C.A. (1990). – Evaluation of modified BACTEC 12B radiometric medium and solid media for culture of *Mycobacterium avium* subsp. *paratuberculosis* from sheep. *J. clin. Microbiol.*, **37**, 1077-1083.
157. Whittington R.J., Marsh L., Turner M.J., McAllister S., Choy E., Eamens G.J., Marshall D.J. & Ottaway S. (1998). – Rapid detection of *Mycobacterium paratuberculosis* in clinical samples from ruminants and in spiked environmental samples by modified BACTEC 12B radiometric culture and direct confirmation by IS900 PCR. *J. clin. Microbiol.*, **36**, 701-707.
158. Whittington R., Marsh L., Choy E. & Cousins D. (2000). – Polymorphisms in IS1311, an insertion sequence common to *Mycobacterium avium* and *Mycobacterium avium* subsp. *paratuberculosis*, can be used to distinguish between and within these species. *Molec. cell. Probes*, **12**, 349-358.
159. Wiker H.G., Harboe M., Bennedsen J. & Closs O. (1988). – The antigens of *Mycobacterium tuberculosis*, H37Rv, studied by crossed immunoelectrophoresis. Comparison with a reference system for *Mycobacterium bovis*, BCG. *Scand. J. Immunol.*, **27**, 223-239.
160. Williams E., DeMartini J. & Snyder S. (1985). – Lymphocyte blastogenesis, complement fixation, and fecal culture as diagnostic tests for paratuberculosis in North American wild ruminants and domestic sheep. *Am. J. vet. Res.*, **46**, 2317-2321.
161. Withers F. (2000). – Incidence of disease. *Vet. Rec.*, **71**, 1150-1153.
162. Wood P.R., Corner L.A. & Placejett P. (1990). – Development of a simple, rapid *in vitro* cellular assay for bovine tuberculosis based on the production of  $\gamma$ -interferon. *Res. vet. Sci.*, **49**, 46-49.