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BRIEF COMMUNICATION

Mycobacterium avium subsp *paratuberculosis*—Incidences in milk and milk products, their isolation, enumeration, characterization, and role in human health

Ami Patel*, Nihir Shah

Department of Dairy Microbiology, SMC college of Dairy Science, Anand Agricultural University, Anand, Gujarat, India

Mycobacterium avium subsp *paratuberculosis* (MAP), excreted in the feces and milk, is reported to be not easily inactivated by pasteurization and thermal treatments as other bacteria infecting humans and animals do. The *D* values of all MAP strains tested were considerably higher than those published for other pathogens. Culturing techniques for this organism are labor intensive. Although an increasing amount of scientific evidence suggests that this organism can be responsible for at least some cases of Crohn's disease (CD), there is controversy about MAP being a cause of CD in humans. In general, although some studies have described an association between the presence of MAP and CD, the role of Mycobacterium species and MAP in the etiology of this human disease remains unestablished. Although published reports indicate that it may not be completely inactivated by pasteurization of milk, the effectiveness of increasing the time or temperature in the pasteurization process has not been established and hence any potential benefit to human health cannot be determined. This article summarizes the incidences of MAP in milk and milk products with respect to human health and brief discussion of various serological as well as molecular techniques used for their isolation, enumeration, and characterization.
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 * Corresponding author. Department of Dairy Microbiology, SMC College of Dairy Science, Anand Agricultural University, Anand, Gujarat, India.
   E-mail address: amiamipatel@yahoo.co.in (A. Patel).

Introduction

Mycobacterium avium subsp *paratuberculosis* (MAP) is the etiologic agent of paratuberculosis, a disease with considerable economic impact, principally on dairy cattle herds.[62] MAP have been also causing considerable concern to the dairy industry worldwide in recent years because of unresolved issue of its potential role in Crohn's disease (CD) in humans.[28,33,46] Published reports indicate that it may not be completely inactivated by pasteurization of milk at 72°C for 15 seconds.[28,68] Determination of definitive thermal destruction characteristics of MAP does not represent a trivial undertaking because culturing technique of this organism is labor intensive and very slow, taking up to 16 weeks to produce visible colonies on appropriate microbiological media.[20,29]

Morphologically, they are gram positive, aerobic plump big rods, having $1-2$ μm length, and having an acid-fast nature.[35] The property of acid fastness is because of waxy materials in the cell walls and it is particularly important for recognizing them. MAP is an obligate intracellular pathogen; it lacks the iron-chelating compound mycobactin, and thus they can only survive in a host that can provide the iron required for growth.[23] It is closely related to other *M avium* bacteria, sharing some antigenic determinants and differentiated from the other mycobacterial species, which are common in the environment by its ability to cause bowel disease in cattle and other animal species.[6,17] It is found that anti-*M bovis* antibodies generated the cross-reaction to MAP. There is a paucity of information in relation to MAP in milk and milk products. This article encompasses the scientific literature relating to disease, diagnosis, effect of processing, detection, and enumeration of concerned organism under laboratory conditions.

Disease in animal

Paratuberculosis, commonly known as Johne's disease (JD), is caused by MAP in cattle and in other ruminants, such as goat, sheep, deer, elk, antelope, and camelids, whereas guinea pigs, rabbits, rats, and mice are not affected by MAP.[27,39,47] It is a transmissible disease and the infection may be chronic, progressive, and incurable. Typical signs are rapid weight loss, diarrhea that lasts for more than 3 days, and failure to respond medical treatment. Cattle with JD do not have a fever, continue to eat, and generally appear to feel well. As the infection progresses, excretion of MAP in feces and milk occurs. Infection of newborn calves occurs by ingestion of milk from infected cows. Typically, cattle develop clinical signs between 3 and 5 years of age that progress over a $3-6$ months period. Small ruminant species, such as sheep and goat, generally do not exhibit diarrhea, although the feces may be less formed than normal. The progression of disease is generally more rapid in these species than in cattle, with weight loss being the predominant sign.[74] In addition to the classic clinical signs attributed to JD, other secondary clinical effects have been reported in dairy cattle. Decreased milk production is considered a major economic consequence of this infection and has been documented for both subclinical and clinical MAP infection.[3,75] Increased mortality rates have been

identified in infected dairy herds,[57,60] whereas other reports have identified reduced reproductive performance in infected cows.[61] Several anecdotal reports suggest that MAP infection may predispose cows to the development of mastitis; however, in one epidemiologic study, MAP infection was associated with decreased incidence of mastitis.[75] Clinically affected animals shed as many as 5×10^{12} MAP cells per day in feces[10,11] and they can remain viable for several months in the environment. They have been also isolated from colostrums of subclinically infected cows.[67] Consequently, raw products originating from cattle may harbor MAP.[66]

Disease in humans

CD is a painful, chronic, often debilitating, inflammatory disease of the gastrointestinal tract that affects at least 500,000 Americans and millions more worldwide. Incidence of CD is increasing in developed countries.[1,43,49] Genetic and environmental factors seem to be involved in the etiology of CD as well as immune disregulation. CD is probably a multifactorial disease because although environmental factors (as infection or diet) and at least eight genetic loci have been implicated, none of them have been proven to cause the disease alone.[2,5] The fact that MAP can cause disease in several mammal species, including primates, is an argument in favor of the link between MAP and CD in humans.[9,11] Similarities between CD and JD point to a possible link to MAP infection.

The area first affected in the patient is the lymph nodes of the neck, which clearly indicates that the obvious route for the entry of pathogen-MAP is the mouth, that is, through consumption of MAP contaminated food. MAP in human assumes an obligate intracellular spheroplast form, residing in macrophages and dendritic cells, as other mycobacteria. Production of proinflammatory cytokines by MAP infected cells of susceptible host may contribute to generate the inflammatory process in CD.[2,9] The disease is autoimmune, causes immune system to attack, and inflame the body's own tissues of the gastrointestinal tract and also in other parts of the body.[59,71]

The association between MAP and human's CD is highly controversial.[4,50,55] In 1984, Chiodini et al[7] reported the first isolation of this organism from a CD patient. Further studies showed that these isolates were able to cause JD by oral inoculation in goats.[26,72] Since then a variety of studies have been conducted to determine whether there is a correlation between the presence of MAP or other mycobacterium species and human CD. Many studies have shown that a variety of mycobacterium species, including MAP, can be isolated from patients with CD. For example, Chiodini et al[8] isolated MAP from two of four patients with CD but from none of 26 controls samples. Although Sanderson et al[58] found that 65% of the intestinal samples from the CD patients ($n = 40$) tested positive for the presence of MAP DNA by polymerase chain reaction (PCR) as compared with 10% of the samples from controls (no inflammatory bowel disease or ulcerative colitis; $n = 63$), Fidler et al[25] reported that 4 of 31 CD tissues, but none of 30 control and ulcerative colitis derives samples, were positive for MAP DNA by PCR. Additional studies also found a higher

incidence of MAP DNA in tissues from CD patients as compared with control samples.[19] However, other studies did not find any evidence for the presence of mycobacterial DNA in tissues from CD patients using PCR.[57,76] MAP was detected by PCR and culture from intestinal tissue, blood, and breast milk of CD patients.[4,50,51] Recently, a report showed that administration of MAP to interleukin-10-deficient mice housed in a germfree environment led to colitis development; increase in serum tumor necrosis alpha, interferon gamma, and chemokines; and also an increase in lymphocyte proliferation and interleukin-2 production.[61] However, to enable consumer assurance of the microbial safety of their pasteurized dairy products, determination of thermal destruction characteristics of MAP is a very high priority for the dairy industry.

Milk and MAP

According to a study conducted by the United States Department of Agriculture in 1996, in the United States, between 20% and 40% of dairy cattle herds are infected with MAP, resulting in economic losses of at least US$1.5 billion each and every year.[6] Collins[15] stated that MAP affects approximately 33% of US dairy herds and dramatically reduces milk production, reproductive performance, and animal condition[66] and thus has a significant negative economical impact on the dairy industry. The herd prevalence of MAP infection in Western Europe and North America is reported to be in the range 21−70%.[3] These subclinically infected animals shed MAP in their milk and onto pastures.[36] It has been suggested that MAP-infected macrophages are present in lipid droplets on the cream layer of milk.[41] The mechanism of the shedding of MAP organisms into milk has not yet been well investigated. Presumably, the shedding of MAP into milk occurs by hematogenous or lymphatic spread.

Many researches have shown that milk-producing cows affected by JD may pass MAP into the milk.[26,31] If the animals show physical signs of JD, the milk is thrown away, but some animals can carry MAP without showing any sign. The milk from these animals goes into the general milk supply for pasteurization. MAP has also been detected in retail cheeses in the United States, the Czech Republic, and Greece by culture and PCR methods.[62]

There is a body of opinion which holds that although the incomplete inactivation of the bacteria by pasteurization, under laboratory conditions, results from clumping, which effectively protects at least some of the organisms from the full effects of the heat applied, the relative turbulence of the flow of milk through commercial pasteurizers prevents clumping and thus allows for a higher kill. This, coupled with the much lower burden of bacteria resulting from the dilution effect of bulking milk from a large number of cows, is one of the bases for an optimistic view of the risk involved.[31]

Effect of processing

MAP is affected by physical factors like processing and storage temperature, radiation treatment, and so on, as well as by some of the chemical and environmental factors, such as, NaCl, NaOH, and pH.[7,29,69] Currently, the most common method for destroying pathogenic microorganisms and for reducing or eliminating spoilage organisms in various dairy products is through pasteurization by high temperature short time (HTST) method that was introduced in the mid-19th century as a public health measure to destroy the most heat-resistant nonspore-forming human pathogens, such as *M tuberculosis* and *Coxiella burnetti*, that are likely to be present in raw milk.[35,38]

There are many evidences that indicate that MAP is not killed by the standard food processing techniques, such as cooking and pasteurization, that we rely on to protect ourselves from disease causing bacteria.[7,29,68] In one study to examine commercially pasteurized milk for the presence of MAP, Millar et al[46] found that at least 15 of 312 milk samples collected at retail stores contained viable MAP. These results do not necessarily prove that MAP survives commercial pasteurization procedures but could also suggest the possibility of postpasteurization contamination with this organism. Studies by Grant et al[32] suggested that homogenization increases the lethality of subsequent heat treatment to some extent with respect to MAP, but the extended 25-seconds holding time at 73°C was found to be no more effective at killing MAP than the standard 15-seconds holding time. This provides clear evidence that MAP bacteria in naturally infected milk are capable of surviving commercial HTST pasteurization if they are present in raw milk in sufficient numbers.

In one of the study, Grant et al[28] isolated MAP from milk samples (12 of 27) after heat treatment at 72.5°C, that is, a temperature close to the minimum pasteurization temperature specified by the legislation or from non-homogenized milk (13 of 27). It has been shown that viable MAP can be detected even after application of different levels of pressure in conjunction with pasteurization.[20] The survival of cells in clusters after pasteurization has been confirmed by the several studies.[35,40] Heat treatment with concurrent homogenization applied either as a separate process or in the dwell zone resulted in a significantly lower number of positive samples than pasteurization without homogenization.[28] Sung and Collins[69] found that MAP can survive in cheese (2% salt, pH 5.0). They also stated that MAP may survive HTST pasteurization when the initial organism concentration is greater than 10^1 cells/mL. The *D* values of all MAP strains tested were considerably higher than those published for Listeria (71.7°C/5 sec) and Coxiella sp (62°C/30 min) and estimated for *M bovis* (71.5°C/14 sec), indicating that MAP is more thermally tolerant and far most resistant to inactivation by low pH, such as 4.0.[31,69] According to UK Food Standards Agency, *D* values for MAP cited within most of the industry is found to be ranged between 71/72°C for 12−14 seconds. Collins et al[14] found that when 1−4% NaOH solution is mixed with suspension of MAP in water, 1% NaOH caused more than 2log$_{10}$ decrease in viable MAP within 15 minutes contact and concentration where as, 2% causes total killing of MAP in less than 60 seconds. However, some workers found that MAP got inactivated at laboratory scale pasteurization of milk.[37,66]

According to Richards and Theon,[56] bovine fecal samples infected naturally and experimentally showed significantly decreased viable MAP counts when stored at −70°C after 3 weeks. Continued refrigeration up to 15 weeks did not

decline further in viable cell count. When 10^5–10^6 MAP cells were suspended in sterile deionized water 4 mW-s/cm^2, UV radiation is sufficient to achieve a 1 log$_{10}$ reduction in total viable count, and at UV doses greater than 15 mW-s/cm^2, complete disinfection is achieved.[14] However, recent work in Australia indicates that UV light had minimal effect on MAP viability in spiked soil.[45] A study on irradiation of MAP suspended in bovine colostrums, however, found that if frozen bovine colostrums spiked with 10^4 MAP/mL is exposed to 10 kGy gamma radiation, 100% of the organisms are killed.[43]

Isolation of MAP from milk

The lack of an appropriate selective culture medium, the organism's slow growth rate compared with other milk microorganisms, and the low numbers of MAP in milk may significantly posses a number of difficulties in isolation of MAP either from milk or from other related products.[20] The successful isolation of MAP from a food or clinical specimen, as for other Mycobacterium sp, is currently reliant on chemical decontamination of the sample to inactivate undesirable microorganisms that could overgrow the slow-growing MAP cells during subsequent culture. A range of decontamination procedures have been used by different researchers.[16,46,70] Most decontaminants are bacteriostatic for MAP at low concentration and become bactericidal at higher concentration when kept in contact with the sample for longer time periods.[70]

Test sensitivity is a key consideration where the isolation of MAP from milk is concerned and it must be realized that decontamination protocols developed for the isolation of MAP from feces may not be appropriate for milk.[11,70] Because MAP are likely to be present in the feces of an infected cow with JD in much higher numbers [10^8 colony forming unit (CFU)/g] than are thought to occur in milk (2–8 CFU/50 mL). Such milk is aseptically obtained from infected cows.

Dundee et al[21] concluded that the decontamination protocol involving 0.75% (wt/vol) hexadecylpyridinium chloride for 5 hours at room temperature was the most sensitive for the recovery of MAP from liquid milk, although a decontamination step used to isolate MAP from cheese or other dairy products may sometime have implications for the accurate enumeration of MAP. For an example, Spahr and Schafroth[64] reported a loss of 60% in the colony forming units of MAP in decontaminated Swiss cheese samples compared with nondecontaminated samples. Therefore, monitoring the persistence of MAP in cheese may rely on culture methods without prior decontamination of the sample.

Decontamination step is usually followed by culturing of MAP on selective media. A range of solid and liquid culture media, with a variety of appropriate supplements, are being used for the culture of MAP from dairy products, including Herrold's egg yolk medium (solid), BACTEC radiometric medium (liquid), Dubos broth (liquid), and modified Middlebrook agar (solid).[21] Selectivity of these media has been improved by the incorporation of antibiotic mixtures, including polymyxin, amphotericin B, nalidixic acid, trimethoprim, and azlocillin.[20] Irrespective of the medium used, the plates are required to be incubated at 37°C for up to 18 weeks for the proper development of MAP colonies.[29]

Detection methods for MAP

Because of fastidious growth pattern of the microorganism and paradoxical immune response of the host animal to infection, diagnosis of this organism is very difficult. Detection and enumeration of MAP from milk and other infected matter is possible using various serological and molecular techniques or combination of thereof. In that, the major serological tests includes radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), agar gel immunodiffusion (AGID), and complement fixation (CF), and so on, which are easy to perform but at the same time not much sensitive,[12,42,60] whereas PCR, nucleic acid hybridization using probes, immunomagnetic separation (IMS), radiometric detection, and flow cytometry (FCM) are the widely used recent methods for the detection of MAP from contaminated materials.

Generally, it is very difficult to compare the sensitivities or specificities from different serological tests using different samples or carrying out different conditions. Different authors had reported higher specificities for different serologic tests using same grouped specimens, such as Sherman et al,[60] 97.4% for CF and 99.4% for AGID; Sockett et al,[63] 95.4% and 99% for two ELISA, 99% for CF and 100% for AGID. AGID represents an easy-performing and low-cost method; but because of its low sensitivity rate, it has shown to be unsatisfactory as a screening diagnostic method for subclinical herd infections.[24] Nevertheless, because it presented an acceptable specificity rate, it can be useful as a confirmatory diagnosis method of clinical disease suspect animals. CF is the official test in many countries for cattle. As cross reactions with other bacteria have been reported for this test in cattle, it is no longer considered sufficiently accurate for diagnostic purposes. In brief, a consistent, standardized, and quality-controlled serological test for evaluating same grouped specimens would be useful to discuss the advantages or disadvantages among different serological tests.

Malamo et al[44] developed modification of ELISA to detect antibodies specific to truncated 34 kDa proteins of MAP. The 34-kDa protein of MAP is immunodominant; and at the carboxyl terminus, it is reported to contain specific epitopes for MAP and therefore the method showed high specificity and sensitivity. Similarly Speer et al,[65] used formaldehyde and sonication with ELISA, so called SELISA and found that SELISAs prepared by treating MAP with 37% formaldehyde and then a 2-seconds burst of sonication produced the greatest difference (7×) between MAP-negative and MAP-positive serum samples. They concluded that the diagnostic sensitivity and specificity for JD by the SELISA were greater than 95%; and based on diagnostic sensitivity as well as specificity, the SELISA appears superior to the commercial ELISAs routinely used for the diagnosis of JD. Eda et al[22] compared results of the FCM with that of a commercially available ELISA and found that serum samples which had tested negative by ELISA were also found to be JD-positive by FCM. The probable reason may be that FCM is based on detection of DNA/RNA

from the cells rather than detecting antigens as in case with ELISA. The FCM is capable of distinguishing MAP-infected from MAP-noninfected cattle as well as MAP from *M scrofulaceum* and *M avium* subsp *avium*.

In IMS technique, rabbit polyclonal antibodies against radiation-killed intact MAP cells are required to coat sheep anti-rabbit IgG type M-280 Dynabeads, also known as immunomagnetic beads (IMB). The IMB were found to have a maximum binding capacity of 10^4-10^5 CFU of MAP. Grant et al[30] found that IMS technique selectively recovered MAP from inoculated milk containing as few as 10 CFU of MAP per milliliter when 10 μL of IMB (ca. 10^6 beads) was added to 1 mL of milk after incubation. Collins et al[13] in one experiment compared the radiometric technique with conventional methods and found that out of 75 positive specimens; the radiometric technique detected 92%, whereas conventional methods detected 60%. It suggests that radiometric detection method is useful tool for diagnosis and control of bovine paratuberculosis.

PCR makes it possible to detect small number of MAP against background of other DNA signals of human origin present in the sample. It is developed on the basis of detection of insertion sequence *900* (IS*900*) of MAP in raw milk.[52,54] The insertion sequence IS*900* appears to be found only in MAP and about 15–20 dispersed copies of IS*900* sequence are integrated into genome of MAP in a stable manner.[34,52,73] According to Pillai and Jayarao,[53] detection of MAP by IS*900* PCR was found to be consistent (24/24 PCR assays) when about 100 CFU/mL were present, whereas detection was variable (12/24 PCR assays) at concentrations as low as 10 CFU/mL. The results of their study also suggested that MAP can be detected directly from quarter milk and bulk tank milk by IS*900* PCR. However, sensitivity regarding low number of these organisms in milk samples could affect the results of IS*900* PCR.

Preparation of nucleic acid probe requires the identification of a species-specific DNA segment, which is then cloned and possibly sequenced. Only two species-specific DNA fragments have been identified in the MAP genome. One probe corresponds to a part of the IS*900* sequence and it was claimed to identify the DNA from 10^2 cells after amplification by PCR. The other probe F57 that is 620 base pairs long and able to recognize all of the tested MAP strains is isolated from paratuberculous animals and humans with CD.[47,48,52] The potential value of IMS method is as an aid for rapid detection of MAP in milk when it is used in conjunction with end point detection methods, such as IS*900* PCR or an ELISA.

Del Prete et al[18] carried out detection of MAP in stool samples of patients with inflammatory bowel disease using IS*900*-based PCR followed by identification involving a colorimetric detection procedure designed DNA enzyme immunoassay, based on the hybridization of the denatured DNA with a nonradioactively labeled interprimer-specific oligonucleotide probe. They suggested that newly developed PCR-DNA enzyme immunoassay technique have a higher sensitivity than cultural technique and being much more rapid, representing a useful tool for both epidemiological and therapeutic purposes.

It is reasonable to prohibit the use of milk derived from animals with clinical JD in the interim between their identification and their removal from the herd because the use of milk from such animals for calves is likely to facilitate spread of infection and prohibition of its use for this purpose may also be reasonable. Because culturing techniques used for the detection of MAP is time consuming, newly developed serological or molecular methods may provide better alternative because they reveal higher sensitivity than cultural technique and being much more rapid, representing a useful tool for both epidemiological and therapeutic purposes. Although we know that MAP may pass into the human food chain via cows' milk, we do not know if the bacteria contribute to CD in humans, although it has been reported that MAP has been detected more commonly in patients with CD than in the general population. Normal pasteurization of milk at 71.7°C for 15 seconds kills low levels of MAP; however, experimental work in laboratory conditions has shown that at high levels of MAP, normal pasteurization does not render all of the bacteria nonviable. The effectiveness of increasing the time or temperature in the pasteurization process has not been established and hence any potential benefit to human health cannot be determined.

References

1. Bernstein CN, Blanchard JF, Rawsthorne P, Wajda A. Epidemiology of Crohn's disease and ulcerative colitis in a central Canadian province: a population-based study. *Am J Epidemiol* 1999;**149**:916–24.
2. Brant SR, Shugart YY. Inflammatory bowel disease gene hunting by linkage analysis: rationale, methodology, and present status of the field. *Inflamm Bowel Dis* 2004;**10**:300–11.
3. Buergelt CD, Layton AW, Ginn PE, Taylor M, King JM, Habecker PL, et al. The pathology of spontaneous paratuberculosis in the North American bison (*Bison bison*). *Vet Pathol* 2000;**37**:428–38.
4. Bull TJ, McMinn EJ, Sidi-Boumedine K, Skull A, Durkin D, Neild P. Detection and verification of *Mycobacterium avium subsp. paratuberculosis* in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. *J Clin Microbiol* 2003;**41**:2915–23.
5. Chamberlin W, Graham DY, Hulten K, El-Zimaity HM, Schwartz MR, Naser S. Review article: *Mycobacterium avium subsp. paratuberculosis* as one cause of Crohn's disease. *Aliment Pharmacol Ther* 2001;**15**:337–46.
6. Chiodini RJ, Rossiter CA. Paratuberculosis: a potential zoonosis? *Vet Clin North Am* 1996;**12**:457–67.
7. Chiodini RJ, Hermon-Taylor J. The thermal resistance of *Mycobacterium paratuberculosis* in raw milk under conditions simulating pasteurization. *J Vet Diagn Invest* 1993;**5**:629–31.
8. Chiodini RJ. Crohn's disease and the mycobacterioses: a review and comparison of two disease entities. *Clin Microbiol Rev* 1989;**2**:90–117.
9. Clancy R, Ren Z, Turton J, Pang G, Wettstein A. Molecular evidence for *Mycobacterium avium subspecies paratuberculosis* (MAP) in Crohn's disease correlates with enhanced TNF-alpha secretion. *Dig Liver Dis* 2007;**39**:445–51.
10. Clark DL, Anderson JL, Koziczkowski JJ, Ellingson JL. Detection of *Mycobacterium avium subspecies paratuberculosis* genetic components in retail cheese curds purchased in Wisconsin and Minnesota by PCR. *Mol Cell Probes* 2006;**20**:197–202.
11. Cocito C, Gilot P, Coene M, Kesel M, Poupart P, Vannuffel P. Paratuberculosis. *Clin Microbiol Rev* 1994;**7**:328–45.
12. Colgrove CS, Thoen CO, Blackbum BO. Paratuberculosis in cattle: a comparison of three serologic tests with results of fecal culture. *Vet Microbiol* 1989;**19**:183–7.

13. Collins MT, Kenefick KB, Sockett DC, Lambrecht RS, McDonald J, Jorgensen JB. Enhanced radiometric detection of *Mycobacterium paratuberculosis* by using filter-concentrated bovine fecal specimens. *Clin Microbiol* 1990;**11**:2514—9.

14. Collins MT, Wells SJ, Petrini KR, Collins JE, Schultz RD, Whitlock RH. Evaluation of five antibody detection tests for diagnosis of bovine paratuberculosis. *Clin Vaccine Immun* 2005;**12**:685—92.

15. Collins MT. Mycobacterium paratuberculosis: a potential foodborne pathogen? *J Dairy Sci* 1997;**80**:3445—8.

16. Cornejo BJ, Shaguin-Ruiz A, Suarez-Guemes F, Thornton CG, Ficht TA, Adams LG. Comparison of C18-carboxypropylbetaine and glass bead DNA extraction methods for detection of *Mycobacterium bovis* in bovine milk samples and analysis of samples by PCR. *Appl Environ Microbiol* 1998;**64**:3099—101.

17. Cosma CL, Sherman DR, Ramakrishnan L. The secret lives of the pathogenic mycobacteria. *Annu Rev Microbiol* 2003;**57**:641—76.

18. Del Prete R, Quaranta M, Lippolis A, Giannuzzi V, Mosca A, Jirillole E, et al. Detection of *Mycobacterium paratuberculosis* in stool samples of patients with inflammatory bowel disease by IS900-based PCR and colorimetric detection of amplified DNA. *J Microbiol Meth* 1998;**33**:105—14.

19. Dell'Isola B, Poyart C, Goulet O, Mougenot JF, Sadoun- Journo E, Brousse N, et al. Detection of *Mycobacterium paratuberculosis* by polymerase chain reaction in children with Crohn's disease. *J Infect Dis* 1994;**169**:449—51.

20. Donaghy JA, Totton NL, Rowe MT. Evaluation of culture media for the recovery of *Mycobacterium avium subsp. paratuberculosis* from Cheddar cheese. *Lett Appl Microbiol* 2003;**37**:285—91.

21. Dundee L, Grant IR, Ball HJ, Rowe MT. Comparative evaluation of four decontamination protocols for the isolation of *Mycobacterium avium subsp. paratuberculosis* from milk. *Lett Appl Microbiol* 2001;**33**:173—7.

22. Eda S, Bannantine JP, Waters WR, Mori Y, Whitlock RH, Scott MC, et al. New method of serological testing for *Mycobacterium avium subsp. paratuberculosis* (Johne's disease) by flow cytometry. *Foodborne Pathog Dis* 2005;**2**:250—62.

23. El-Zaatari FAK, Osato SM, Graham DY. Etiology of Crohn's disease: the role of *Mycobacterium avium paratuberculosis*. *Trends Mol Med* 2001;**35**:173—5.

24. Ferreira R, Fonseca LS, Lilenbaum W. Agar gel immunodiffusion test (AGID) evaluation for detection of bovine paratuberculosis in Rio de Janeiro, Brazil. *Lett Appl Microbiol* 2002;**35**:173—5.

25. Fidler HM, Thurrell W, Johnson NM, Rook GA, McFadden JJ. Specific detection of *Mycobacterium paratuberculosis* DNA associated with granulomatous tissue in Crohn's disease. *Gut* 1994;**35**:506—10.

26. Florou M, Leontides L, Kostoulas P, Billinis C, Sofia M, Kyriazakis I, et al. Isolation of *Mycobacterium avium subspecies paratuberculosis* from non-ruminant wildlife living in the sheds and on the pastures of Greek sheep and goats. *Epidemiol Infect* 2008;**136**:644—52.

27. Glawischnig W, Steineck T, Spergser J. Infections caused by *Mycobacterium avium subspecies avium*, hominissuis, and paratuberculosis in free-ranging red deer (Cervus elaphus hippelaphus) in Austria, 2001-2004. *J Wildl Dis* 2006;**42**:724—31.

28. Grant IR, Williams AG, Rowe MT, Muir DD. Efficacy of various pasteurization time-temperature conditions in combination with homogenization on inactivation of *Mycobacterium avium subsp. paratuberculosis* in milk. *Appl Environ Microbiol* 2005;**71**:2853—61.

29. Grant IR, Ball HJ, Neill SD, Rowe MT. Inactivation of *Mycobacterium paratuberculosis* in cows' milk at pasteurization temperatures. *Appl Environ Microbiol* 1996;**62**:631—6.

30. Grant IR, Ball HJ, Rowe MT. Isolation of *Mycobacterium paratuberculosis* from milk by immunomagnetic separation. *Appl Environ Microbiol* 1998;**64**:3153—8.

31. Grant IR, Rowe MT, Dundee L, Hitchings E. *Mycobacterium avium ssp. paratuberculosis*: its incidence, heat resistance and detection in milk and dairy products. *Int J Dairy Tech* 2001;**54**:2—13.

32. Grant IR, Hitchings EI, McCartney A, Ferguson F, Rowe MT. Effect of commercial-scale high-temperature, short-time pasteurization on the viability of *Mycobacterium paratuberculosis* in naturally infected cows' milk. *Appl Environ Microbiol* 2002;**68**:602—7.

33. Grant IR, Rowe MT. Methods for the detection and enumeration of viable *Mycobacterium paratuberculosis* from milk and milk products. *Bull Int Dairy Fed* 2001;**362**:41—52.

34. Green EP, Tizard ML, Moss MT, Thompson J, Winterbourne DJ, McFadden JJ, et al. Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. *Nucleic Acids Res* 1989;**17**:9063—73.

35. Hammer P, Wiesner C, Walte H-G, Knappstein K, Teufel P. Heat resistance of *Mycobacterium avium subsp. paratuberculosis* in raw milk tested in pilot plant pasteuriser. *Kieler Milchwirtschaftlich* 2002;**54**:275—303.

36. Hermon-Taylor J, Quirke P. Mycobacterium avium subspecies paratuberculosis is a cause of Crohn's disease. *Gut* 2001;**49**:755—60.

37. Hope AF, Tulk PA, Condron RJ. Pasteurization of *Mycobacterium paratuberculosis* in whole milk. In: Chiodini RJ, Hines II ME, Collins MT, editors. *Proceedings of the Fifth International Colloquium on Paratuberculosis*. Rehoboth, Mass: International Association of Paratuberculosis; 1997. p. 377—82.

38. Jay JM. High temperature food preservation and characteristics of thermophilic microorganisms. In: *Modern food microbiology*. 4th ed. New York: Chapman & Hall; 1992. p. 335—55.

39. Judge J, Kyriazakis I, Greig A, Allcroft DJ, Hutchings MR. Clustering of *Mycobacterium avium subsp. paratuberculosis* in rabbits and the environment: how hot is a hot spot? *Appl Environ Microbiol* 2005;**71**:6033—8.

40. Keswani J, Frank JF. Thermal inactivation of Mycobacterium paratuberculosis in milk. *J Food Prot* 1998;**61**:974—8.

41. Koenig GJ, Hoffsis GF, Shulaw WP, Bech-Nielsen S, Rings DM, St Jean G. Isolation of *Mycobacterium paratuberculosis* from mononuclear cells in tissues, blood, and mammary glands of cows with advanced paratuberculosis. *Am J Vet Res* 1993;**54**:1441—5.

42. Kreeger J. Ruminant paratuberculosis—a century of progress and frustration. *J Vet Diagn Invest* 1991;**3**:373—83.

43. Loftus EV, Silverstein MD, Sandborn WJ, Tremaine WJ, Harmsen WS, Zinsmeister AR. Crohn's disease in Olmsted County, Minnesota, 1940-1993: incidence, prevalence, and survival. *Gastroenterology* 1998;**114**:1161—8.

44. Malamo M, Sakoda Y, Ozaki H, Kida H. Development of ELISA to detect antibodies specific to *Mycobacterium avium subsp. paratuberculosis* with truncated 34kDa proteins Jpn J. *Vet Res* 2006;**54**:90—107.

45. McDonald WL, O'Riley KJ, Schroen CJ, Condron RJ. Heat inactivation of *Mycobacterium avium subsp. paratuberculosis* in milk. *Appl Environ Microbiol* 2005;**71**:1785—9.

46. Millar D, Ford J, Sanderson J, Withey S, Tizard M, Doran T, et al. IS900 PCR to detect *Mycobacterium paratuberculosis* in retail supplies of whole pasteurized. *Appli Environ Microbiol* 1996;**62**:3446—52.

47. Moravkova M, Trcka I, Lamka J, Pavlik I. A mixed infection of *Mycobacterium avium subsp. paratuberculosis* and *M. a. hominissuis* in one red deer (Cervus elaphus) studied by IS900 BstEII and IS1245 PvuII RFLP analyses: a case report. *Veterinarni Medicina* 2008;**53**:445—51.

48. Moss T, Green EP, Tizard ML, Malik ZP, Hermon-Taylor J. Specific detection of *Mycobacterium paratuberculosis* by DNA

hybridization with a fragment of the insertional element IS900. *Gut* 1991;**32**:395—8.

49. Munkholm P, Langholz E, Nielsen OH, Kreiner S, Binder V. Incidence and prevalence of Crohn's disease in the county of Copenhagen, 1962-87: a sixfold increase in incidence. *Scand J Gastroenterol* 1992;**27**:609—14.

50. Naser SA, Ghobrial G, Romero C, Valentine JF. Culture of *Mycobacterium avium subspecies paratuberculosis* from the blood of patients with Crohn's disease. *Lancet* 2004;**364**:1039—44.

51. Naser SA, Schwartz D, Shafran I. Isolation of *Mycobacterium avium subsp. paratuberculosis* from breast milk of Crohn's disease patients. *Am J Gastroenterol* 2000;**95**:1094—5.

52. Nielsen SS, Thamsborg SM, Houe H, Bitsch V. Bulk-tank milk ELISA antibodies for estimating the prevalence of paratuberculosis in Danish dairy herds. *Prev Vet Med* 2000;**44**:1—7.

53. Pillai SR, Jayarao BM. Application of IS900 PCR for detection of *Mycobacterium avium subsp. paratuberculosis* directly from raw milk. *J Dairy Sci* 2002;**85**:1052—7.

54. Plante Y, Remenda BW, Chelack BJ, Haines DM. Detection of *Mycobacterium paratuberculosis* in formalin-fixed paraffin-embedded tissues by the polymerase chain reaction. *Can J Vet Res* 1996;**60**:115—20.

55. Ren Z, Turton J, Borody T, Pang G, Clancy R. Selective Th2 pattern of cytokine secretion in *Mycobacterium avium subsp. paratuberculosis* infected Crohn's disease. *J Gastroenterol Hepatol* 2008;**23**:310—4.

56. Richards WD, Thoen CO. Effect of freezing on the viability of *Mycobacterium paratuberculosis* in bovine feces. *J Clin Microbiol* 1997;**6**:392—5.

57. Rowbotham DS, Mapstone NP, Trejdosiewicz LK, Howdle PD, Quirke P. *Mycobacterium paratuberculosis* DNA not detected in Crohn's disease tissue by fluorescent polymerase chain reaction. *Gut* 1995;**37**:660—7.

58. Sanderson JD, Moss MT, Tizard MLV, Hermon-Taylor J. Mycobacterial diseases of the gut: some impact from molecular biology. *Gut* 1992;**33**:145—7.

59. Scanu AM, Bull TJ, Cannas S, Sanderson JD, Sechi LA, Dettori G. Mycobacterium avium subspecies paratuberculosis infection in cases of irritable bowel syndrome and comparison with Crohn's disease and Johne's disease: common neural and immune pathogenicities. *J Clin Microbiol* 2007;**45**:3883—90.

60. Sherman DM, Gay JM, Bouley DS. Comparison of the complement fixation and agar gel immunodiffusion tests for the diagnosis of subclinical bovine paratuberculosis. *Am J Vet Res* 1990;**51**:461—5.

61. Singh UP, Singh S, Singh R, Karls RK, Quinn FD, Potter ME, et al. Influence of *Mycobacterium avium subsp. paratuberculosis* on colitis development and specific immune responses during disease. *Infect Immun* 2007;**75**:3722—8.

62. Slana I, Paolicchi F, Janstova B, Navratilova P, Pavlik I. Detection methods for *Mycobacterium avium subsp.*

paratuberculosis in milk and milk products: a review. *Veterinarni Medicina* 2008;**53**:283—306.

63. Sockett DC, Carr DJ, Richards WD, Collins MT. A repository of specimens for comparison of diagnostic testing procedures for bovine paratuberculosis. *J Vet Diagn Invest* 1992;**4**:188—91.

64. Spahr U, Schafroth K. Fate of *Mycobacterium avium subsp. paratuberculosis* in Swiss hard and semihard cheese manufactured manufactured from raw milk. *Appl Environ Microbiol* 2001;**67**:4199—205.

65. Speer CA, Scott MC, Bannantine JP, Waters WR, Mori Y, Whitlock RH, et al. A novel enzyme-linked immunosorbent assay for diagnosis of *Mycobacterium avium subsp. paratuberculosis* infections (Johne's Disease) in cattle. *Clin Vaccine Immunol* 2006;**13**:535—40.

66. Stabel JR, Steadham EM, Bolin CA. Heat inactivation of *Mycobacterium paratuberculosis* in raw milk: are current pasteurization conditions effective? *Appl Environ Microbiol* 1997;**63**:4975—7.

67. Streeter RN, Hoffsis GF, Bech-Nielsen S, Shulaw WP, Rings DM. Isolation of *Mycobacterium paratuberculosis* from colostrum and milk of subclinically infected cows. *Am J Vet Res* 1995;**56**:1322—4.

68. Sung N, Collins MT. Thermal tolerance of *Mycobacterium paratuberculosis*. *Appl Environ Microbiol* 1998;**64**:999—1005.

69. Sung N, Collins MT. Effect of three factors in cheese production (pH, salt and heat) on *Mycobacterium avium subsp. paratuberculosis* viability. *Appl Environ Microbiol* 2000;**66**:1334—9.

70. Sweeney RW, Whitlock RH, Rosenberger AE. *Mycobacterium paratuberculosis* cultured from milk and supramammary lymph nodes of infected asymptomatic cows. *J Clin Microbiol* 1992;**30**:166—71.

71. Thompson DE. The role of mycobacteria in Crohn's disease. *J Medical Microbiol* 1994;**41**:74—94.

72. Van Kruiningen HJ, Chiodini RJ, Thayer WR, Coutu JA, Merkal RS, Runnels PL. Experimental disease in infant goats induced by a *Mycobacterium* isolated from a patient with Crohn's disease—a preliminary report. *Dig Dis Sci* 1986;**31**:1351—60.

73. Vary PH, Andersen PR, Green E. Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. *J Clin Microbiol* 1990;**28**:933—7.

74. Wilson DJ, Rossiter C, Han HR. Association of *Mycobacterium paratuberculosis* infection with reduced mastitis, but with decreased milk production and increased cull rate in clinically normal dairy cows. *Am J Vet Res* 1993;**54**:1851—7.

75. Whitlock R, Buergelt C. Preclinical and clinical manifestations of paratuberculosis (including pathology). *Vet Clin North Am Food Anim Pract* 1996;**12**:345—56.

76. Wu SW, Pao CC, Chan J, Yen TS. Lack of mycobacterial DNA in Crohn's disease tissue. *Lancet* 1991;**337**:174—5.