BRIEF COMMUNICATION

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

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*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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Introduction

*Mycobacterium avium* subsp paratuberculosis (MAP) is the etiologic agent of paratuberculosis, a disease with considerable economic impact, principally on dairy cattle herds. 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. Published reports indicate that it may not be completely inactivated by pasteurization of milk at 72°C for 15 seconds. 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.

Morphologically, they are gram positive, aerobic plump big rods, having 1–2 μm length, and having an acid-fast nature. 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. 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. 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 camelds, whereas guinea pigs, rabbits, rats, and mice are not affected by MAP. 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. 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.

Increased mortality rates have been identified in infected dairy herds, whereas other reports have identified reduced reproductive performance in infected cows. 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. Clinically affected animals shed as many as 5 × 10^12 MAP cells per day in feces and they can remain viable for several months in the environment. They have been also isolated from colostrums of subclinically infected cows. Consequently, raw products originating from cattle may harbor MAP.

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. 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. 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. 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. 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.

The association between MAP and human’s CD is highly controversial. In 1984, Chiodini et al 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. 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 isolated MAP from two of four patients with CD but from none of 26 controls samples. Although Sanderson et al 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 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. However, other studies did not find any evidence for the presence of mycobacterial DNA in tissues from CD patients using PCR. MAP was detected by PCR and culture from intestinal tissue, blood, and breast milk of CD patients. 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. 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. Collins stated that MAP affects approximately 33% of US dairy herds and dramatically reduces milk production, reproductive performance, and animal condition 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%. These subclinically infected animals shed MAP in their milk and onto pastures. It has been suggested that MAP-infected macrophages are present in lipid droplets on the cream layer of milk. 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. 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 cheesess in the United States, the Czech Republic, and Greece by culture and PCR methods.

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 bulkling milk from a large number of cows, is one of the bases for an optimistic view of the risk involved.

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. 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.

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. Studies by Grant et al. 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. 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. The survival of cells in clusters after pasteurization has been confirmed by the several studies. 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. Sung and Collins 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 107 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 more resistant to inactivation by low pH, such as 4.0.

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. found that when 1–4% NaOH solution is mixed with suspension of MAP in water, 1% NaOH caused more than 2log10 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.

According to Richards and Theon, 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. However, recent work in Australia indicates that UV light had minimal effect on MAP viability in spiked soil. 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.

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 possess a number of difficulties in isolation of MAP either from milk or from other related products. 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. 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.

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. 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 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 low numbers of MAP in milk may significantly possess a number of difficulties in isolation of MAP either from milk or from other related products. 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. 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.

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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). Selectivity of these media has been improved by the incorporation of antibiotic mixtures, including polymyxin, amphotericin B, nalidixic acid, trimethoprim, and azlocillin. 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.

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, 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, 97.4% for CF and 99.4% for AGID; Sackett et al, 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. 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 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, 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 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 specificity, the SELISA appears superior to the commercial ELISAs routinely used for the diagnosis of JD. Eda et al 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.52,54 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. 106 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 $900$ (IS900) of MAP in raw milk.52,54 The insertion sequence IS900 appears to be found only in MAP and about 15–20 dispersed copies of IS900 sequence are integrated into genome of MAP in a stable manner.34,52,73 According to Pillai and Jayarao,53 detection of MAP by IS900 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 IS900 PCR. However, sensitivity regarding low number of these organisms in milk samples could affect the results of IS900 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 IS900 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 IS900 PCR or an ELISA.

Del Prete et al.18 carried out detection of MAP in stool samples of patients with inflammatory bowel disease using IS900-based PCR followed by identification involving a colorimetric detection procedure designed DNA enzyme immunonassay, based on the hybridization of the denatured DNA with a nonradioactively labeled interprimer-specific oligonucleotide probe. They suggested that newly developed PCR-DNA enzyme immunonassay 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


Mycobacterium avium subsp. paratuberculosis in human health


