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Review

Assessment of Food as a Source of Exposure to *Mycobacterium avium* subspecies *paratuberculosis* (MAP)†‡

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NATIONAL ADVISORY COMMITTEE ON MICROBIOLOGICAL CRITERIA FOR FOODS

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ABSTRACT

The National Advisory Committee on Microbiological Criteria for Foods assessed the importance of food as a source of exposure to *Mycobacterium avium* subspecies *paratuberculosis* (MAP). MAP is the causative agent of Johne's disease, which affects primarily the small intestine of all ruminants. The significance of MAP as a human pathogen is unknown and is being investigated by several research groups. This document also reviews the efficacy of current detection methods, processing interventions, and MAP inactivation. Research needs related to MAP are provided. The Committee reached the following conclusions: current methods for detection of MAP have significant limitations, and a standard method for the detection of viable MAP cells is needed. Aside from MAP-infected domestic ruminant animals, the organism is found infrequently. If MAP in cattle is controlled, the source of MAP in other animals, food, and water may largely be eliminated. Milk, particularly raw milk, may be a likely food source for human exposure to MAP. Given the prevalence of MAP in U.S. cattle herds, ground beef may be a potential source of MAP. Although humans may be exposed to MAP through a variety of routes, including food and the environment, the frequency and amount of exposure will require additional research.

EXECUTIVE SUMMARY

The National Advisory Committee on Microbiological Criteria for Foods (NACMCF or Committee) was asked to assess the importance of food as a source of exposure to *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the causative agent of Johne's disease, which affects primarily the small intestine of all ruminants. The significance of MAP as a human pathogen is unknown and is being investigated by several research groups. The Committee was directed not to consider whether MAP is a human pathogen, and therefore did not evaluate the public health relevance of foodborne and other exposures to MAP. In the United States, dairy cattle represent the largest population of MAP-infected animals (Johne's disease–positive herds) and therefore are the most likely source of direct or indirect exposure to humans. There are several possible modes of MAP transmission to humans, including exposure to a contaminated environment, person-to-person transmission, direct contact with infected animals, as well as exposure to contaminated foods.

After examining the scientific literature, the Committee reached the following conclusions:

- Current methods for detection of MAP have significant limitations.
- A standard method for the detection of viable MAP cells needs to be developed and adopted by researchers in order to accurately determine the presence and numbers of MAP in foods and other potential sources of exposure.
- Aside from MAP-infected domestic ruminant animals, the organism is found infrequently. This may be a function of low prevalence and/or a consequence of the absence of reliable detection methods.

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§ Note to the reader: On 20 March 2009 the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) reviewed current scientific literature on *Mycobacterium avium* subspecies *paratuberculosis* relevant to this report. The NACMCF agreed to update this report with additional references as appropriate. The reference section of this report reflects the 20 March 2009 updates agreed upon by the NACMCF. The addition of references to this report and supporting text where appropriate leave the conclusions of the report adopted on 28 September 2007 unchanged.

- If MAP in cattle is controlled, the source of MAP in other animals, food, and water may largely be eliminated.
- Milk, particularly raw milk, may be a likely food source for human exposure to MAP.
- Thermal processes that deliver a 4- to 7-log reduction in the number of MAP cells should be adequate to inactivate the numbers of MAP estimated to be present in raw milk.
- A small percentage (<3%) of commercially pasteurized milk may contain small numbers of viable MAP cells.
- Although the data are limited, cheese made from pasteurized milk is probably not a significant source of exposure to MAP, but the potential for exposure to MAP from milk products made from raw milk is unknown.
- Given the prevalence of MAP in U.S. cattle herds, ground beef may be a potential source of MAP.
- MAP survives in cattle feces, water, and soil and is found in many wild animals; therefore, farm runoff may potentially contaminate irrigation water, which can come in contact with fruits and vegetables and result in human exposure.
- Although there is no information to indicate that municipal drinking water is a source of human exposure to MAP, further study is needed.
- Although humans may be exposed to MAP through a variety of routes, including food and the environment, determination of the frequency and amount of exposure will require additional research.

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease in dairy cows and other ruminants. According to the National Animal Health Monitoring System (NAHMS) 1996 Dairy Study, this transmissible disease has been estimated to affect approximately 21.6% of U.S. dairy herds *(137)*. MAP, which is excreted in feces and milk, is reportedly not inactivated by thermal treatments as easily as other bacteria of public health and animal health concern *(129)*. The Committee was charged with assessing the role of food in exposure of humans to MAP by answering six specific questions. The Committee was directed not to consider whether MAP is a human pathogen and therefore did not evaluate the public health relevance of foodborne and other exposures to MAP.

There are several potential modes of transmission of MAP to humans. These include exposure to a contaminated environment (soil, water), person-to-person horizontal transmission, direct contact with infected animals, and consumption of contaminated foods, including produce and food products originating primarily from dairy cattle but also from beef cattle, sheep, and goats. In fulfilling its charge, the Committee reviewed the pertinent available literature, with an emphasis on the most recent publications since these reflected more current methodologies and results.

WORK CHARGE AND BACKGROUND

Paratuberculosis, or Johne's disease, is an infectious bacterial disease in animals that is caused by MAP. Johne's disease has been spreading slowly through domestic livestock populations for nearly a century and has become endemic in many countries. MAP, the etiologic agent of Johne's disease, is being investigated as a pathogen of animals that may be naturally transmitted to humans. In the United States, dairy cattle represent the largest population of MAP-infected animals (Johne's disease–positive herds) and therefore the most likely source of direct or indirect exposure to humans.

MAP, like other members of the *M. avium* complex (MAC), is an opportunistic pathogen in immunocompromised persons. The question remains whether generally immunocompetent individuals can be infected with MAP and whether this leads to disease. Leaving aside whether or not MAP is a pathogen to humans, there are several possible modes of transmission, including exposure to a contaminated environment (soil, water), person-to-person horizontal transmission, direct contact with infected animals, and preharvest and postharvest contamination of foods, including produce and food products originating primarily from dairy cattle but also from beef cattle, sheep, and goats. Specifically, the presence of MAP in raw milk has raised concerns about whether MAP has potential public health significance.

Charge to the Committee. The Committee was asked to limit their deliberations to the consideration of a very specific set of questions. The Committee was not being asked to consider the question of whether or not MAP is a human pathogen.

The Committee was asked to consider the following questions during their deliberations:

1. What food, water, or environmental sources are of most concern with respect to exposure of humans to MAP?
2. What are the frequencies and levels of MAP contamination found in the above referenced sources?
3. What is the efficacy of the current methods of detection for MAP?
4. What processing interventions are available for the foods of concern to eliminate or reduce the levels of MAP contamination to an acceptable level or to ensure that MAP does not enter the food supply?
5. What are the research needs to determine:
   a. additional sources of MAP;
   b. the frequencies and levels of MAP contamination in specific sources of concern;
   c. potential processing interventions to eliminate or reduce the levels of MAP contamination; and
   d. potential processing interventions to prevent MAP from entering the food supply?
6. Additional research needs?

RESPONSE TO QUESTIONS IN THE CHARGE

The Committee agreed to change the order of the questions in order to allow for a more logical progression as follows below. The Committee chose to address detection methods for MAP first.

1. What is the efficacy of the current methods of detection for MAP?

The ability to culture MAP and detect the organism in various relevant sample matrices, including veterinary clinical samples (feces, tissues), milk, water, and other samples, has been limited by significant challenges. The Committee reviewed the pertinent current literature describing the detection of MAP in these sample types (summarized in Tables 1 through 5). A review compared the various methods, including their specificities and sensitivities, costs, and average time to obtain test results that can be used to screen, diagnose, and confirm MAP infection in animals *(19)*. Four general categories of methods for MAP detection were available: immunological, cultural, molecular, and histopathological methods, the last of which was restricted to veterinary diagnostic settings. A brief discussion of the other three categories is given below.

Immunological methods. Commercially available immunological methods detect antibodies against the organism that are present in blood and milk and hence are most often used in clinical screening or diagnosis of infection of livestock. The commercial assays generally have high specificity, although that varies with the particular matrix being screened. These tests are most accurate when diagnosing animals that have seroconverted in the final stages of the disease when antibody responses are well developed *(59)*. They have low sensitivity for detection of serum antibodies to MAP, especially in subclinical infections *(4, 59)*, detecting only about 30% of infected animals when they are in the preclinical disease stage *(4)*, and likewise perform poorly for detection of antibodies to MAP in milk *(19, 59, 83, 126)*. However, Nielsen *(94)* concluded that MAP antibodies were generally detected prior to bacterial shedding based on a 3-year study involving analysis of over 24,000 milk and 10,000 fecal samples. An evaluation of four commercial serum enzyme-linked immunosorbent assays (ELISAs) for detecting MAP infection in dairy cows in herds with a history of clinical paratuberculosis indicated that none of the ELISAs were effective for early detection of infection *(28)*. Nonetheless, immunological methods are widely used on-farm because they are rapid and inexpensive. New ELISAs show promise for greater sensitivity. For example, Shin et al. *(118)* developed a JTC-ELISA (a novel ELISA based on the MAP strain JTC303) using antigens secreted by early to mid–log phase culture of MAP strain JTC303 that had a significantly higher sensitivity and equivalent specificity compared to those of five commercial ELISA kits. The sensitivity of detection of low-level fecal shedders was 40% compared to 20% for the commercial kits.

Culture methods. There is no ''gold standard'' culture method that can be considered the definitive approach for identifying viable cells of MAP. All of the cultural methods available are cumbersome and time-consuming, requiring at best up to 16 weeks of incubation, which is often further extended (to 8 or more months) to ensure detection of injured cells that might occur due to heat or other treatments. The general steps for isolation of MAP include concentration, decontamination, culturing (solid or liquid media), and confirmation.

Concentration. Because MAP may be present in low numbers and grows slowly, especially in subclinically infected animals and food or environmental samples, a cell concentration step often precedes detection. This step also reduces total sample volume and may remove some matrix-associated inhibitors. In some protocols, sonication may be used to aid in the disintegration of clumps of cells. Sonication may, however, have a negative impact on assay detection limits *(44)*. The most common concentration methods are sedimentation, centrifugation, and immunomagnetic separation (IMS); filtration has been used but with limited success. Many protocols combine two or more of these approaches. Data indicate that MAP may segregate differentially during centrifugation, particularly in milk fat, which can cause losses during the concentration steps that precede detection *(87)*.

Decontamination. Due to the prolonged incubation required for propagation of MAP and the nature of most specimens that are tested (e.g., feces), rigorous sample decontamination methods are typically applied before transfer into growth media to reduce the number of unwanted organisms. Typical decontamination protocols expose the sample to antimicrobial agents such as hexadecylpyridinium chloride (HPC), amphotericin B, vancomycin, naladixic acid, carbenicillin, trimethoprim, sodium hydroxide, and zepharin, among others. Some of these compounds also may be added in the media to suppress competitive microbial growth during the incubation period. Use of decontaminants at high concentrations or for extended time periods may be bactericidal, and it is important to balance reduction of the growth potential of competitors with optimal recovery of MAP *(33)*. The true extent of such inhibition depends on the agent, time and temperature of treatment, and other factors *(42)*. Investigators have attempted to diminish this effect by optimizing combinations of the agents and/or their concentrations *(69)*, which may improve the sensitivity of cultural methods.

Culturing. Currently there are three solid media commonly used to culture MAP using standard plating methods. These media are Herrold's egg yolk agar (HEYA), Lowenstein-Jensen (LJ) agar, and Middlebrook 7H11 agar. MAP lacks the ability to produce the compound mycobactin J, an iron chelator required for growth; therefore, media used to grow MAP must be supplemented with mycobactin J. These media have been shown to support the growth of some but not all MAP isolates *(25)*. To avoid culture bias, investigators have begun using multiple media for isolation of MAP. For example, de Juan et al. *(25)* reported that some strains could be isolated using LJ or Middlebrook 7H11, but the use of these two agars in conjunction with HEYA was necessary to isolate all MAP strains. Better results were obtained when HEYA was supplemented with sodium

TABLE 1. Mycobacterium avium *subsp.* paratuberculosis *detection methods in water*[a]

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Develop an RT-PCR method to detect MAP, incorporating IAC with appropriate pre-PCR processing for application to drinking water and milk *(109)*.	Purified, seeded drinking water	Varying quantities of MAP were added to 20 ml of drinking water and centrifuged in sequence: 2,000 × *g* for 10 min, recover and suspend precipitate in 1 ml of PBS, 13,000 × *g* for 5 min, recover pellet by resuspending in 100 µl of PBS, DNA extract (Wizard kit), PCR. TaqMan probes for RT-PCR targeting IS*900* with independently amplified internal control; primers designed by investigators.	60 strains total: 18/18 (100%) MAP strains positive; 17/17 (100%) non-MAP *Mycobacterium* and 25/25 (100%) other bacterial species were negative. 100 CFU/20 ml, high degree of relative accuracy in drinking water.	Relative accuracy: percentage of the number of cells calculated by the Q-PCR assay versus the number of cells calculated by hemocytometer.
Develop a sensitive detection method for MAP in water by modifying and optimizing an existing IMS-PCR method *(143)*.	Sterile distilled water, spiked	50 ml of water was centrifuged (2,500 × *g*, 20 min) and the resulting pellet was resuspended in 1 ml of PBS with Tween 80. This was followed by IMS (polyclonal antibody–coated beads) and SDS–proteinase K DNA extraction method with ribolysing and IS*900* PCR. This was optimized by the addition of Tween 80 (0.4% being optimal). IMS-PCR targeting IS*900* with primers previously described *(68, 89)*.	Two strains of MAP used. Detection limit of 10 MAP/ml water in original 50 ml of water sample using 0.4% Tween 80.	The attempted filtration method for sample pretreatment was not successful.

[a] LOD, level of detection; RT-PCR, real-time PCR; MAP, *Mycobacterium avium* subsp. *paratuberculosis*; IAC, internal amplification control; PBS, phosphate-buffered saline; Q-PCR, quantitative PCR; IMS-PCR, immunomagnetic separation PCR; SDS, sodium dodecyl sulfate.

pyruvate. All studies with solid media have used the incubation temperature of 37°C for lengths of time ranging from 6 weeks to 40 months under aerobic conditions.

Automated liquid culture systems (including BACTEC, MGIT, TREK ESP II) use selective media in conjunction with indicator systems to detect growth. TREK (TREK Diagnostics, Cleveland, OH) uses a computer controlled incubation cabinet to continuously monitor changes in head space pressure within each individual sample vial. As bacteria grow, nutrients from the media are metabolized and the pressure inside the sample vial changes. This change in pressure is used as an indicator of bacterial growth. Although samples can be called suspect as soon as a change in head space pressure is detected, in order for a sample to be called negative or ''none detected'' it must remain in the incubation chamber for a minimum of 6 weeks.

The BACTEC (Becton Dickinson [B-D], Sparks, MD) system detects ^{14}C-labeled carbon dioxide as an indicator of bacterial growth. As bacteria metabolize ^{14}C-labeled palmitic acid in the media, they produce $^{14}CO_2$. The BACTEC is one of the most sensitive culture methods. However, due to its reliance on radioactive media, its use in laboratories is steadily declining. In response to these concerns, B-D has developed the MGIT system. This method uses the reduction or consumption of O_2 as an indicator of growth. Each sample tube is continuously monitored in a computer-controlled incubation chamber. As O_2 is depleted from the tube, an indicator substance in the bottom of the tube fluoresces when the tube is exposed to UV light. Both the BACTEC and MGIT systems also require lengthy incubation periods.

All three liquid culture systems use selective media to inhibit competing organisms. However, no medium is 100% selective. All three use time to detection to estimate cell number, based on the assumption that the higher the MAP load, the more rapid the detection endpoint. Liquid culture (BACTEC 12B) was found to be more sensitive than a solid medium (Middlebrook 7H10 agar with mycobactin J) for isolation of sheep strains from fecal and tissue samples and may be more sensitive for cattle strains as well *(147)*. Addition of ampicillin to BACTEC medium can reduce contamination with organisms other than MAP *(147)*. Shin et al. *(117)* developed an immunoassay to screen liquid mycobacterial cultures for evidence of MAC. This MAC-ELISA test was effective in determining cultures that warranted the resources to identify the organism by PCR.

Confirmation. A sample that gives a presumptive positive result using any of the culture systems must undergo further testing. Growth with and without mycobactin J, combined with acid-fast staining and/or PCR, are confirmatory tests for MAP. Although culture methods are cumbersome, they are the only ones available that result in isolation of a viable bacterial culture, which can be further characterized if so desired.

Molecular methods. As is the case for cultural methods, molecular amplification methods must also be preceded by sample concentration. An additional consideration is the need for nucleic acid extraction prior to detection. There is no single method of choice for this extraction, but as MAP cells are extremely difficult to disrupt, sonication or disruption with glass beads is usually recommended before DNA extraction.

Improvements in nucleic acid detection by PCR have accelerated rapid detection of MAP DNA, theoretically reducing the detection time to 1 to 3 days. The majority of these assays have targeted the IS*900* DNA base sequence (there are approximately 17 copies of this insertion sequence per cell) *(7, 9, 11, 37, 46, 66, 68, 76, 96, 98, 108, 109, 122, 126, 139, 143)*, which provides the potential for a higher level of sensitivity relative to assays based on detection of single copy genes. Single-copy gene targets include *hspX (5, 7, 11, 36, 37, 132, 139)* and F57 *(85, 119, 132)*. The recent completion of the MAP genome sequencing project has identified the ISMAP*02* sequence, which is present in six copies, as an alternative target *(122)*.

Limits of detection, sensitivity, and specificity vary with the targeted sequence and primer choice, matrix tested, and PCR format (conventional gel-based PCR, reverse transcriptase PCR, nested PCR, and real-time PCR). The real-time PCR format eliminates the subjective interpretation of gels, providing an objective determination of the presence or absence of MAP DNA and theoretically can be adapted to produce quantitative estimates of MAP concentration *(60, 109, 119)*. Incorporation of an internal amplification control to each reaction minimizes the likelihood of false-negative results in both conventional and real-time PCR formats *(7, 11, 98, 109, 132)*. Investigators have attempted nested amplification with little improvement in overall detection limits *(122, 139)*. PCR assays have a detection limit of 1 to 10 MAP per reaction. When applied to artificially contaminated sample matrices, detection limits are approximately 10^2 to 10^3 CFU per sample. Some, but not all, of the assays have been evaluated using naturally contaminated samples. A major concern about PCR-based diagnostics is that DNA-based methods do not discriminate between viable and nonviable MAP cells. The application of reverse transcriptase PCR may address this issue. Interestingly, a recent publication described a phage-PCR method for the differentiation of viable from nonviable MAP as applied to naturally contaminated raw milk samples *(127)*. This may be more practical than previous vital staining methods for demonstration of viable cells in clumps *(57)*. Application of robotic technology for both DNA and RNA extraction coupled with real-time PCR formats offers the possibility of high throughput.

Interpretation of results. Negative test results should be interpreted with caution, given the sensitivity and specificity of the test methods *(19)*. The cultural methods could be considered a ''reference'' standard, but they are not ideal. For example, decontamination and declumping steps may affect the accuracy of detection. After thorough review of the literature, it appears that the molecular methods hold the most promise, as this approach is rapid and sensitive when targeting multicopy genes (e.g., IS*900*). PCR is an excellent method for confirmation of cultures

TABLE 2. Mycobacterium avium *subsp.* paratuberculosis *detection methods in milk and dairy products*[a]

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Develop an RT-PCR method to detect MAP, incorporating IAC with appropriate pre-PCR processing for application to drinking water and milk *(109)*.	Semiskim milk, seeded	Varying quantities of MAP were added to 20 ml of drinking water and centrifuged in sequence: 2,000 × *g* for 10 min, recover and suspend precipitate in 1 ml of PBS, 13,000 × *g* for 5 min, recover pellet by resuspending in 100 μl of PBS, DNA extract (Wizard kit), PCR. TaqMan probes for RT-PCR targeting IS*900* with independently amplified internal control; primers designed by investigators.	60 strains total: 18/18 (100%) MAP strains positive; 17/17 (100%) non-MAP *Mycobacterium* and 25/25 (100%) other bacterial species were negative. Detection limit in artificially contaminated semiskim milk was 100 CFU/20 ml with low relative accuracy.	Relative accuracy defined as percentage of the number of cells calculated by the Q-PCR assay versus the number of cells calculated by hemocytometer.
Develop quantitative RT-PCR assay for detection and quantitation of MAP in milk *(96)*.	Whole, pasteurized milk, seeded	24-ml samples of milk were seeded with 10^4 MAP cells, incubated, precentrifuged for MAP concentration followed by DNA extraction using seven approaches: (1) proteinase K and lysis; (2) lysozyme and proteinase K; (3) chloroform-methanol; (4) basis lysis; (5) alternative buffer; (6) GITC lysis; (7) clarifying solution. FRET-based RT-PCR targeting IS*900*, primers designed by investigators.	Specificity not reported. Lower detection limit of 40 CFU/ml in seeded milk.	No IAC.
Comparison of four decontamination methods for recovery of MAP on HEYM *(33)*.	Raw milk spiked with 10^6–10^7 CFU/ml and dilutions of decontaminated milk samples were used.	Three different isolates were grown in Middlebrook broth with OADC enrichment and mycobactin J (7–8 wk), pelleted and resuspended in PBS to yield 10^6–10^7 MAP CFU/ml. 0.5 ml of suspension was added to 40 ml of raw milk. Samples were centrifuged (2,500 × *g*, 15 min) and the pellet was decontaminated by four different methods: (1) pellet resuspended in 0.75% HPC; (2) overnight incubation in BHI and 0.75% HPC, then addition of an antibiotic mixture; (3) method 2 but with 0.9% HPC; (4) use of CB-18. Samples inoculated onto HEYM, incubated (18 wk), then quantified.	Minimum detection limits per 40 ml of raw milk for four decontamination methods evaluated: (1) 30; (2) 10^5; (3) not determined; (4) 10^3. Recoveries from methods 2 and 3 were 2–3 log lower than for method 1.	Decontaminants are bacteriostatic at low concentrations and bacteriocidal at higher concentrations; therefore, a balance must be struck between efficient inactivation of undesirable microbes at the lowest possible toxicity for MAP. Even most effective decontamination method recovered a fraction (28.7%) of MAP present in milk. Extra centrifugation steps may also yield lower recoveries.
To develop rabbit polyclonal antibody–coated Dynabeads to selectively isolate MAP from milk samples to enable end-point detection by another method (e.g., IS*900* PCR or ELISA) *(46)*.	Spiked raw cow's milk from a ''healthy'' cow or pasteurized whole cow's milk; authors did not distinguish which was used in each individual test.	Sample prepared for analysis (centrifuged at 2,500 × *g* and resuspended in PBS); coated Dynabeads added to sample for IMS; inoculated onto HEYM (12 wk); also tested by IS*900* PCR.	Polyclonal IgG gave weak agglutination in the presence of *Hafnia* when undiluted; for *Pseudomonas* sp. and *Staphylococcus* sp. up to 1:10 dilution; for *M. avium* and *M. intracellulare* up to 1:1,000 dilution. There were moderate cross-reactions with three other *Mycobacterium* spp. and weak cross-reactions with other bacterial	Under the conditions used the beads had a maximum binding capacity of 10^4–10^5 CFU.

TABLE 2. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
			isolates from raw milk. Detection limit of 10 CFU/ml without centrifugation. Detection limit increased to 10 CFU per original sample volume with centrifugation.	
Evaluate the efficacy of various media to suppress the growth of cheese starter cultures and other interfering bacteria to enable enumeration of MAP *(31)*.	Cheddar cheese manufactured from pasteurized milk (and one unpasteurized sample) and various starter cultures and MAP strain NCTC 8578.	Cheddar cheese suspensions and dilutions of starter cultures were decontaminated (0.75% HPC, 5 h), centrifuged (2,500 × *g*, 15 min) then inoculated onto (1) HEYM, (2) HEYM + VAN, (3) Middlebrook 7H10 agar + PANTA supplemented with OADC and PANTA PLUS, or (4) BACTEC 12B radiometric medium supplemented with PANTA PLUS and incubated at 37°C (98.6°F) or up to 12 wk. Parallel samples not decontaminated prior to inoculation. MAP strain NCTC 8578 was added to pasteurized milk, mixed with starter cultures, and inoculated as above.	Without decontamination, starter cultures grew on HEYM at initial levels $>10^8$ CFU/ml but not on HEYM +VAN or HEYM + PANTA. BACTEC gave false-positive results in the presence of two starter cultures. Decontamination required for cheeses to prevent overgrowth of competitors on culture media. PANTA was significantly better than BACTEC 12B at suppressing growth of cheese microflora. PANTA allowed growth of ''contaminants'' once (identified as *Lactobacillus rhamnosus*). Growth was observed on all media from cheeses made from unpasteurized milk.	Study illustrates the critical need to suppress non-MAP microbes in complex samples bearing high numbers of other microbes. HEYM + PANTA appeared most effective for starter cultures and cheeses; BACTEC 12B was the least effective.
Evaluate sensitivity, specificity, and predictive value of IS*900* PCR assay for detection of MAP in raw cow's milk *(68)*.	Raw, bulk tank and pooled quarter milk samples, naturally contaminated.	50-ml milk samples, centrifugation (1,950 × *g*, 30 min), pellets were washed twice in PBS, decontaminated overnight at RT in 0.75% HPC, recentrifuged (1,600 × *g*, 10 min), resuspended in 0.75% HPC. Culture: inoculated into slants of HEYM supplemented with mycobactin J and incubated at 37°C (98.6°F) for 16 wk. Typical MAP colonies counted; confirmed by Ziehl-Neelsen staining. PCR: DNA extraction by Instagene DNA purification matrix with additional lysis by sequential incubation (56°C [132.8°F] for 30 min, 100°C [212°F] for 15 min), centrifugation (20,800 × *g*) and recovery of DNA-containing supernatant. PCR detection targeting IS*900*; primers of Moss et al. *(89)* yields a 229-bp fragment for sequence confirmation.	By PCR and compared to fecal culture, positive pooled-quarter milk samples tested by PCR displayed sensitivity, specificity, and predictive value of 0.87, 0.95, and 0.71, respectively. This is juxtaposed to 0.17, 0.98, and 0.67, respectively, for pooled-quarter milk samples tested by cultural methods.	Fecal cultures done by double incubation and centrifugation technique (Cornell method); fecal culture considered gold standard. True positives: culture-positive individual cows. Improved sensitivity, specificity, and detection limits when using individual cow samples compared to bulk tank milk samples.

TABLE 2. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
To determine if MAP is present in retail supplies of whole pasteurized cow's milk obtained over 19 mo in England and Whales *(87)*.	Milk, raw and pasteurized, seeded and naturally contaminated	For seeded samples, 15-ml milk samples were centrifuged (41,000 × *g*, 1 h at 4°C). Each fraction (whey, cream, and pellet) was recovered individually. 500-µl aliquots were boiled (20 min), centrifuged, and the supernatant was used in PCR amplifications. For naturally contaminated samples: culture confirmation was done on aliquots (whey, pellet, and cream) decontaminated with 0.1% BAC or 0.75% CPC (overnight at RT). After centrifugation and washing, samples were inoculated in Dubos broth supplemented with mycobactin J and incubated at 37°C (98.6°F) for 13–40 mo. Microscopic evaluation by Ziehl-Neelsen staining; aliquot of the culture supernatant processed for DNA extraction (GITC and heat) and tested by IS*900* PCR. PCR detection using IS*900* primers not previously referenced. Amplicons confirmed by internal probe hybridization (radiolabeled) produced by PCR product internal to the amplicon (226 bp).	Spiking trials: sensitivity of detection in milk was 200–300 MAP cells/ml. In naturally contaminated samples, 0–25% (total 22/312 or 7.05% positive) of cartons of retail milk were positive by IS*900*; difficult to confirm by culture due to overgrowth; IS*900* PCR on pellets of cultures were positive in 9/18 (50%) samples and negative in 6/36 (16%) samples tested.	MAP partitioned in fat and pellet fractions, not whey.
Improve procedure for isolating MAP from bulk tank raw milk. Specifically interested in (1) effects of different decontamination agents and procedures on recovery of MAP and (2) partition of MAP in milk fractions *(42)*.	Bulk tank (raw) milk and pasteurized milk, seeded	Seeded milk centrifuged (3,100 × *g*, 30 min); decontaminate pellet with 0.75% HPC or 0.75% HPC + half-strength BHI for 2–48 h with shaking. Samples centrifuged (1,000 × *g*, 15 min at RT), and pellet resuspended in sterile antibiotic brew (amphotericin B 50 mg/ml; vancomycin 100 mg/ml; nalidixic acid 100 mg/ml, BHI 18.5 mg/ml) and held at RT or 37°C (98.6°F) for up to 96 h. Plated on HEYM slants and LB plates. Incubated 1, 2, 4, 8, and 16 wk at 37°C (98.6°F).	Not reported.	Freshness of milk is critical; should be kept at 4°C for no more than 3 days. 0.75% HPC was more effective than HPC with half-strength BHI; optimal time of disinfection was 2–5 h at RT. Extensive incubation during decontamination reduced the number of culture-positive samples in both raw and pasteurized milk. High levels of MAP were recovered in the cream fraction; best to pool cream and pellet. 16.6% of input MAP recovered culturally using the optimized sample prep method.

TABLE 2. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Use of solid phase cytometry for rapid detection of MAP in milk *(27)*.	Retail pasteurized milk, seeded	50 ml of seeded milk treated as follows: 0.5% ammonia, 5% diethylether, 12% petroleum ether, and 0.1% SDS. Centrifuge (9,500 × *g,* 15 min), wash the pellet in Ringer solution, decontaminate with 0.75% CPC. After double washing, pellet resuspended in Ringer solution, incubated (RT, 1 h), homogenized, digested with 1 ml of 4% trypsin (37°C [98.6°F], 1 h), filtered through a polycarbonate membrane filter (0.4 μm). Bacteria eluted in 10 ml of Ringer solution, centrifuged (9,500 × *g,* 15 min). Pellet resuspended in 2 ml of 0.5 M NaCl, subjected to solid phase extraction in loose C_8 sorbent, washed with 2 ml of 0.5 M NaCl, and MAP eluted with 2 ml of 100% n-propanol and collected in 18 ml of physiological saline. Aliquots were filtered through a polyester membrane filter (0.4 μm), counterstained with 1 ml of Chemunex, stained with ChemChrome V6, and incubated at 30°C (86°F) for 45 min to label viable cells. The membrane filter was then subjected to automated laser scanning using a ChemScan.	Method combined, when applied to milk spiked at 10^2 and 10^4 CFU/50 ml; recovered 70 and 11%, respectively, for *M. smegmatis,* whereas the recovery for MAP (10^2 cells/50 ml) was 73%.	Most of the developmental work was done with *M. smegmatis,* not MAP. The test organism segregated in the pellet and not cream. About 0.1% of the inoculum was lost in the cream fraction. Total 5 min time in 0.75% CPC killed all seeded representative gram-positive and gram-negative bacteria but not *M. smegmatis*. Trypsin improved filterability of the milk pellet. 100% retention-adsorption of MAP on C8 hydrophobic beads. Feasibility of dual labeling approach using red and green fluorescent antibodies and fluorescent viability substrate (for cellular identity and viability) was demonstrated but needs additional optimization.
Compare detection capabilities of the nonradiometric MGIT and radiometric BACTEC 460TB culture systems for recovering MAP from milk *(51)*.	UHT milk, spiked	UHT milk seeded with 10^1–10^7 cells/ml was inoculated into the two systems with and without prior chemical decontamination in 0.75% CPC for 5 h. Time to detection of growth was measured in days from each system; in addition, counts were calculated from BACTEC readings using a published formula.	Both systems detected 10–100 MAP cells/ml in UHT milk in 30–40 days with no prior decontamination treatment. Only 10^2–10^3 cells/ml were detected with chemical decontamination. Correlation between the two methods was 0.6983.	The nonradioactive MGIT systems could be substituted for the radiometric BACTEC system without loss of detection sensitivity. Chemical decontamination before culture caused a significant reduction in the numbers of viable MAP. Detection time for the two systems was not significantly different.
Develop an RT-PCR assay for detection of MAP in milk that targets a more specific genetic marker (F57 gene) *(132)*.	Raw milk, spiked	10-ml samples of raw milk seeded with 10^6, 10^2, or 0 MAP cells/ml were treated with 100 μl of Triton X-100 and centrifuged twice (30 min at 4,500 rpm and 10 min at 14,000 rpm) with recovery of pellets. Pellets were stored at 20°C (68°F) until testing was performed. Three different DNA extraction approaches were applied: (1) precipitation	On purified DNA, assay could detect equivalent to two to three gene copies per reaction. Specific for MAP (10 of 10 [100%] MAP cultures positive), no cross-reactivity with 24 non-MAP *Mycobacterium* cultures or 9 representative gram-positive and gram-negative enterics.	Assay performance was not impacted by high levels of background DNA. Best DNA extraction was achieved with precipitation or column-based methods. Assay successfully detected MAP in raw milk obtained from two clinically confirmed cases. Assay was also applied to 80 individual cow's milk

TABLE 2. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
		(two commercial methods); (2) column based (two commercial methods); and (3) DNA binding beads (two commercial methods). Primers targeted the F57 region (254-bp amplicon), not previously reported, in RT-PCR method and IAC (400-bp amplicon, additional set of primers).	Detection limits in spiked raw milk: 10^3 and 10^2 MAP cells/ml (10/10 [100%] positive); 10 MAP cells/ml (7/10 [70%] positive); <10 MAP cells/ml (0/10 [0%] positive).	samples (pooled to 16 samples total) obtained from a farm with a history of MAP; 2 of 16 pooled samples were positive for MAP.
Rapid enumeration of viable MAP in milk *(27)*.	Milk	50-ml retail pasteurized milk samples were treated with 0.5% ammonia, 5% diethyl ether, 12% petroleum ether, and 0.1% SDS and centrifuged at 9,500 × *g* for 15 min. The resulting pellet was decontaminated with 0.75% CPC for 10 min. The pellet was double washed by homogenizing by aspiration through a syringe and needle. This procedure was followed by digestion with trypsin. Bacteria were collected by filtration, centrifuged, and resuspended in 2 ml of 0.5 M NaCl. The suspension was transferred to a column for solid phase extraction with Bio-Rad C_8 sorbent. The extraction was followed by a wash step with 0.5 M NaCl. MAP was eluted with 100% n-propanol. Rapid detection of viable cells was performed using solid phase cytometry.	Recovery for 10^2 cells/50 ml of milk was 73%.	MAP detected in a single working day. Investigation of dual labeling as a means of detection the presence of viable MAP, but optimization is needed, as well as testing on field samples.
To compare commercially available DNA extraction–IS*900* PCR assays and three culture-based methods (MGIT, HEYM, and BACTEC 12B radiometric medium) for detection of MAP in milk in multiple laboratories *(30)*.	Raw milk inoculated with naturally infected feces	For PCR assays, DNA was extracted by three methods (using combinations of magnetic beads [IMS and other], mechanical disruption [Ribolyser], phenol-chloroform extraction, and commercial extraction kits). Various decontamination methods were used, including centrifugation and HPC treatment; diethyl ether–petroleum–SDS–NH_3 followed by centrifugation; or sodium citrate clarification, centrifugation, and HPC treatment.	Commercial PCR method gave overall detection rates of 100, 90, 85, and 25%, respectively, for MAP concentrations of 300, 30, 3, and 0.3 copies of IS*900*/ml in raw milk. The Adiavent PCR method yielded 100, 88, 65, and 11% for the same concentrations of IS*900*. At 20 MAP cells/ml in raw milk, 100% of samples tested by MGIT were positive; 10/12 were positive by HEYM, and 0% were positive using BACTEC. Of the three cultural methods, the MGIT method showed the best performance, with consistent positive detection in samples inoculated with approx 20 MAP cells/ml.	RT-PCR had increased sensitivity over conventional endpoint PCR. PCR methods were more sensitive than cultural methods. Interlaboratory variability was surprisingly small.

TABLE 2. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Use of Pourquier ELISA for detection of antibodies against MAP in individual and bulk milk samples *(140)*.	Milk	ELISA	99.8% specificity for individual milk samples and 100% for bulk milk samples; 87–96% sensitivity depending on the level of shedding by the cows tested.	Method for detection of antibodies in milk from infected cows.
Evaluate phage-PCR method for live-dead differentiation and molecular characterization of MAP *(127)*.	Pure culture then applied to naturally contaminated raw milk samples	Bacteria in milk pelleted by low speed centrifugation, with the pellet subsequently used in the phage amplification assay (FASTPlaque TB, Biotech Laboratories, UK). Presumptive colonies tested by IS*900*-based PCR to confirm MAP.	10/14 milk samples from 14 animals with evidence of clinical infection were presumptively positive by phage amplification assay; all 10 confirmed as MAP by IS*900* PCR.	The phage amplification assay PCR method allows rapid and specific detection of viable MAP in milk in as little as 48 h.

[a] LOD, level of detection; RT-PCR, real-time PCR; MAP, *Mycobacterium avium* subsp. *paratuberculosis*; IAC, internal amplification control; PBS, phosphate-buffered saline; Q-PCR, quantitative PCR; GITC, guanidinium isothiocyanate; FRET, frequency resonance energy transfer; HEYM, Herrold's egg yolk medium; OADC, oleic acid, dextrose, catalase; HPC, hexadecylpyridinium chloride; BHI, brain heart infusion; CB-18, C_{18}-carboxypropylbetaine; ELISA, enzyme-linked immunosorbent assay; IMS, immunomagnetic separation; VAN, vancomycin, amphotericin B, nalidixic acid; PANTA, polymyxin, amphotericin B, nalidixic acid, trimethoprim, and azlocillin; PANTA PLUS, polymyxin, amphotericin B, nalidixic acid, trimethoprim, azlocillin, and 0.5% (vol/vol) glycerol; RT, room temperature; BAC, benzalkonium chloride; CPC, cetylpyridinium chloride; LB, Luria-Bertani; SDS, sodium dodecyl sulfate; MGIT, mycobacteria growth indicator tube; UHT, ultrahigh temperature.

thought to be MAP. However, the sensitivity of molecular-based methods as applied to complex samples is limited by matrix and sampling effects, which can result in false-negative results if internal controls are not used in the assay. DNA-based molecular methods are hampered by an inability to discriminate viable from nonviable MAP. This continues to pose concerns regarding the potential for false-positive detection. Reliance on molecular methods alone means that an isolate is not obtained for further characterization.

Pinedo et al. *(105)* analyzed feces, blood, and milk from 328 cows in four infected dairy herds and determined that the combined use of ELISA and fecal PCR has the potential to increase the overall sensitivity for detection of MAP infection. Currently, neither cultural nor molecular methods are able to provide accurate quantitative information about the numbers of MAP present in the sample. Finally, lack of method standardization complicates comparison among studies.

To avoid duplication, the Committee determined that the two questions below could best be answered concurrently:

2. **What food, water, or environmental sources are of most concern with respect to exposure of humans to MAP?**
3. **What are the frequencies and levels of MAP contamination found in the above referenced sources?**

MAP, which is shed in the feces of infected animals, can potentially enter the food supply through several routes, including fecal contamination of raw food products, contaminated water due to runoff containing feces, and mammary gland secretions. Therefore, potential sources of MAP include domestic livestock, wildlife, insects, protozoa, environmental contamination, water, milk (raw and pasteurized), milk products, meat, fish and shellfish, and produce. Since MAP is an extremely fastidious pathogen requiring mycobactin J for in vitro culture, it is unlikely to grow in the environment or food. However, it has been shown to persist in both.

Prevalence and Numbers of MAP in the Agricultural Environment

Domestic livestock. Cattle are the most significant source of MAP that could impact the U.S. food supply. There are several studies examining the prevalence of MAP in cattle, with variable results *(10, 23, 68, 82, 95, 111, 112, 134, 137, 138)*. Several of these studies indicate there are regional and seasonal differences in prevalence; however, seropositive animals are widely distributed geographically. The most comprehensive multistate, multiherd studies are those conducted by U.S. Department of Agriculture (USDA), Animal and Plant Health Inspection Service for NAHMS. According to the 1997 NAHMS Beef Cattle Study, it was estimated that 7.9% of the U.S. beef herds tested ($n = 380$) had one or more ELISA-positive animals, and 0.4% of all animals tested ($n = 10{,}371$) were positive

TABLE 3. Mycobacterium avium *subsp.* paratuberculosis *detection methods in pure culture*[a]

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
To improve specificity of PCR assays for MAP detection by multiple targets and nested approaches *(139)*.	190 pure mycobacterial cultures: 74 MAP, 116 non-MAP	DNA was extracted by GITC-lysis-alcohol precipitation. The researchers refined IS*900* primers and developed nested PCR as well as developed F57 region primers and nested PCR.	Detection limit of 1 CFU/PCR for nested reactions for both assays. 74/74 (100%) MAP strains were correctly identified by either assay; 116/116 (100%) non-MAP strains were negative by either assay.	Both assays performed equivalently.
Describes cloning and sequencing of a single-copy gene unique to MAP and application of an efficient nucleic acid diagnostic test that identifies mycobacterial infection, specifically MAP *(36)*.	Pure cultures 14 MAP and 27 non-MAP isolates	DNA was extracted from all isolates using a lipase digestion followed by lysozyme digestion and proteinase K plus SDS and purified with repeated phenol–chloroform–isoamyl alcohol extractions. MAP DNA was digested with *Pst*1, size fractionated by agarose gel electrophoresis, and screened using eight 15-bp DNA probes that contained in one open reading frame the nucleotide sequence encoding the RGD peptide adhesion motif.	Screening using degenerated RGD probes identified a unique sequence encoding 144 amino acid residues for a putative heat shock protein called *hspX*. Southern blot analysis using the sequence for *hspX* positively identified 100% of the MAP isolates and negatively identified 100% of non-MAP isolates.	*hspX* is a single-copy gene unique to MAP.
To detect viable MAP using plasmid and phage-based luciferase reporter constructs *(115)*.	FSL S2-02, 04, and 16 for phage study; eight MAP isolates for plasmid study	Grow pure culture, sonicate, infect with phage.	The lowest MAP numbers detected by luminescence were 10^3 CFU from whole milk or saline, 10^2 CFU from skim milk.	Luciferase reporter phage shows promise for rapid and sensitive detection of viable MAP cells in milk.
Compare Accu-Probe, serotyping, and single IS*900*, IS*901*, IS*1245*, and IS*901* flanking region PCR with internal standards *(7)*.	4,729 mycobacterial isolates	Isolates were grown on HEYM or LJ medium. A loopful of a single colony was resuspended in 50 ml of distilled water, boiled for 20 min, centrifuged for 5 min at 14,000 × *g*; 2 μl of supernatant was used for PCR amplification.	Sensitivity was 1×10^1 CFU/reaction for IS*900*, 5×10^3 CFU/reaction for IS*901*, 1×10^2 CFU/reaction for IS*1245*, and 1×10^3 CFU/reaction for ISFR*300*. Percent inhibition during PCR analysis ranged from 0 to 50% depending on the strain being tested.	PCR was completely inhibited by more than 50 ng of chromosomal DNA per reaction.
Detection of MAP by RT-PCR and differentiation from other mycobacteria using SYBR Green and TaqMan assays *(108)*.	6 MAP, 25 non-MAP strains	The template DNA used for real-time detection of target sequences was extracted with InstaGene matrix according to the manufacturer's protocol.	The TaqMan assay detects as low as 4 fg of MAP-specific DNA per assay (equivalent to 0.8 cells) and has the potential to detect even smaller quantities by increasing the amplification cycles. The SYBR Green assay yielded a minimum detectable limit of 0.07 cells (equivalent to 0.34 fg of DNA).	Multiple primer sets may be needed to detect all MAP.

TABLE 3. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Develop nested PCR assay using ISMAP02 *(122)*.	Fecal samples from cows naturally infected with MAP and negative control samples from noninfected cows	A previously published methodology was used that applied double-centrifugation, double-decontamination *(120)*.	Only MAP DNA was detectable after amplification with the ISMAP02 primers. The sensitivity of detection for the ISMAP02 element in conventional PCR or RT-PCR was <100 fg or 10^2 CFU/ml.	RT-PCR and conventional PCR were equivalent. Maximum recovery was obtained with IS*900* conventional PCR and RT-PCR.
Develop an internal control plasmid for MAP detection by RT-PCR *(11)*.	3 MAP, 13 non-MAP strains	The methodology for the extraction of DNA from the 16 bacterial strains was not provided.	The optimal plasmid amounts are 10 fg/reaction for *hspX* detection and 1 fg/reaction for IS*900* detection. Positive results were only observed for the MAP strains.	Internal control amplification was detected by fluorescence at 705 nm on the LightCycler. Amplification of IS*900* was detected by fluorescence at 640 nm on the LightCycler.

[a] LOD, level of detection; MAP, *Mycobacterium avium* subsp. *paratuberculosis*; GITC, guanidinium isothiocyanate; SDS, sodium dodecyl sulfate; HEYM, Herrold's egg yolk medium; LJ, Lowenstein-Jensen; RT-PCR, real-time PCR.

(23). The 1996 NAHMS Dairy Cattle Study *(137)* found a 21.6% apparent herd prevalence based upon two or more positive tests or one positive test with at least 5% of cull animals with clinical signs of disease. Serological testing may have underestimated the true incidence of positive cows in each herd, since seroconversion occurs at the later stages of the disease. Also, factors such as within-herd prevalence, sample size, test sensitivity and specificity, and the definition of a positive herd cause uncertainty in estimating herd prevalence. The 2002 NAHMS Dairy Study *(138)* was done to study positive herds ($n = 62$) and examine the prevalence of MAP within these herds. This study found 8.6% of the animals ($n = 7{,}272$) in positive herds tested positive for MAP. Because the study focused on positive herds, the 2002 NAHMS Dairy Study cannot be used to accurately estimate the true prevalence of positive herds or animals in the United States. A 2007 NAHMS study yet to be published may provide more accurate information on herd and animal prevalence of MAP.

Wildlife. Understanding the ecology of the host species can provide valuable information about disease risk to susceptible livestock. Multiple studies have demonstrated the presence of MAP in the feces and tissues of wild nonruminant animals, including feral cats, mice, foxes, stoats, weasels, crows, rooks, jackdaws, rats, wood mice, rabbits, hares, and badgers *(8, 21, 22, 54, 70, 99)*.

Two studies have documented histopathological changes in wild animals consistent with MAP infection *(8, 22)*. The finding of viable MAP organisms in the feces of nonruminant animals could indicate a potential for spread of MAP beyond the range of infected ruminant herds *(22)*. Molecular genotyping could not discriminate between rabbit and cattle isolates of MAP *(54)*. MAP has also been isolated from several species of wild ruminants, including red deer, roe deer, fallow deer, moufflons, and North American bison *(12, 101)*. There is evidence of the transmission of MAP between wild ruminant populations and free-range cattle *(101)*.

Insects. Insects may be vectors of MAP, but their significance is unknown *(38–40)*. Fischer et al. *(39, 40)* recovered MAP in the droppings from nymphs of the oriental cockroach and from the abdomen and head of blowflies experimentally infected with MAP. Another study performed in the Czech Republic found MAP in Diptera flies in areas occupied by MAP-infected cattle *(38)*.

Protozoa. Mycobacteria (MAP and non-MAP) have been described in protists *(74, 88, 133)*. In laboratory studies, growth of bovine and human MAP isolates in *Acanthamoeba polyphaga* as well as replication of clinical and environmental strains of *M. avium* (non-MAP) within *Acanthamoeba castellanii* suggest that protozoa may provide a reservoir for mycobacteria *(15, 92)*. Mura et al. *(92)* reported the growth and survival of MAP in *A. polyphaga* for up to 4 years using in situ hybridization and quantitative real-time PCR; however, these authors did not attempt to culture the organism. Others have reported survival of MAP within *Acanthamoeba* and demonstrated

TABLE 4. Mycobacterium avium *subsp.* paratuberculosis *detection methods in fecal matrices*[a]

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
To estimate sensitivity, specificity, and predictive value of an ELISA relative to fecal culture for fecal and milk samples *(126)*.	Fecal samples from 712 individual cows within 61 herds and bulk tank samples from 52 of the 61 herds	ELISA protocol was not described. For culture, fecal samples (2 g) were added to 35 ml of sterile deionized water, settled for 30 min; supernatant removed and centrifuged (1,700 × *g*, 20 min); pellet was resuspended in 0.9% HPC-BHI, held overnight at 37°C (98.6°F); centrifuged (1,700 × *g*, 20 min). Pellets were resuspended in 1 ml of antibiotics (naladixic acid, vancomycin, amphotericin B) and incubated overnight at 37°C (98.6°F). Samples inoculated onto HEYM with naladixic acid, vancomycin, and mycobactin J and incubated for 12 wk. Milk was centrifuged (2,000 rpm, 30 min), pellet was resuspended in 0.9% HPC-BHI and incubated overnight at 37°C (98.6°F). Samples were recentrifuged (2,500 rpm, 20 min); pellets were resuspended in 1 ml of antibiotic solution. Samples were inoculated onto HEYM with antibiotics and incubated for up to 12 mo at 37°C (98.6°F). For DNA extraction, 0.5 ml of composite milk sample was centrifuged (15,000 rpm, 5 min). Proteinase K was added to cell pellets, vortexed to resuspend the pellet, and incubated overnight at 50°C (122°F). TEN buffer and NaOH were added, mixed, and placed in a boiling water bath for 30 min, then extracted with phenol–chloroform–isoamyl alcohol, then centrifuged (10,000 rpm, 5 min). DNA was amplified by nested PCR targeting IS*900*. Positive PCR results were seen with spiked samples containing 10^2 or more MAP/ml of milk.	Only 25% of cows that were fecal-culture positive were also positive by fecal ELISA, and 6% of cows that were fecal-culture negative were positive by ELISA (25% sensitivity and 6% false-positive rate). 22% of ELISA-positive cows were fecal-culture positive (22% positive predictive rate). All milk samples cultured negative, but 68% of herds had positive PCR results from bulk tank samples, including 11/21 herds with negative fecal cultures.	Inability to culture MAP from bulk tank samples may reflect insensitivity of culture in conjunction with the very low levels present in the milk. The ELISA was a poor predictor of animal infection in this study.
Evaluate two different methods to recover mycobacterial DNA from bovine fecal specimens for PCR analysis *(98)*.	A total of 130 bovine fecal specimens were used; 70 samples were from MAP-infected farms and the remaining 60 samples were from uninfected farms.	Fecal specimens were processed for culture using conventional techniques. The direct dilution–centrifugation method was used to isolate cells from feces. The use of CB-18 was thought to aid in the isolation of MAP from fecal specimens. DNA was extracted with Qiagen modified method. The PCR target was IS*900* primers: 5′-CACGTGCT GTCCCCATCGGC-3′ and 5′-CTACGTCT TCGTGACCAAAG-3′. IAC targeted a universal plant chloroplast *rpoB* region. Primers were 5′-CCGTGATCAA TCAAATATTGCTAAGTC-3′ and 5′-ACCCATAGCTGATGATAGAACT-3′. Culture decontamination method used HPC (18–24 h), inoculated onto HEYM with mycobactin J.	Centrifugation method followed by PCR detection yielded a sensitivity of 92.6% and a specificity of 83.7%. CB-18 method followed by PCR detection yielded a sensitivity of 100% and a specificity of 53.5%. Sensitivity and specificity of both methods were calculated against culture results.	Low specificity of both methods was due to having a positive PCR result and a negative culture result within the same sample.

TABLE 4. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Compare two cultivation procedures with PCR confirmation for detection of MAP *(37)*.	Feces (USDA fecal check test kit samples)	Feces were decontaminated with 0.9% HPC in BHI overnight, centrifuged, and incubated with polymyxin B, amphotericin B, carbenicillin, and trimethoprim overnight. 200 μl was inoculated onto HEYM with mycobactin J, 200 μl into TREK ESP II vials, and confirmed with acid-fast staining and PCR. PCR direct extraction of feces with Qiagen QIAmp DNA stool mini kit, and PCR performed targeting IS*900* and *hspX*.	All negative via *hspX* primers. Some false positives with IS*900* primers. TREK ESP II had one false positive.	TREK ESP II missed one sample possibly because of reduced inoculation volume (normally 1 ml). Culture three of four positive. PCR prescreening lacked specificity. Combination of TREK culture and *hspX* PCR was 100% sensitive and specific.
Compare three ELISA methods for MAP using tissue and fecal culture as comparison standard *(83)*.	Fecal samples were collected from 994 cows.	2 g of feces was placed in 25 ml of DHOH, shaken (30 min), and let stand 30 min, and 5 ml was removed and placed into 25 ml of 0.75% HPC (24 h, 37°C [98.6°F]), centrifuged (900 × *g*, 30 min). Pellet was resuspended in half-strength BHI with naladixic acid, vancomycin, and amphotericin B for 12–14 h at 37°C (98.6°F). 1 ml was inoculated into TREK vials for 6 wk then acid-fast stained. If the sample was acid-fast positive it was subcultured to HEYM for 6 wk, then PCR confirmed.	The sensitivity of all three ELISA methods was 13.9–27.8%. The specificity was 90.1–97.1%.	The accuracy of all three ELISAs was very poor and of questionable value for screening food.
To determine if results from ELISAs for bovine milk and serum will correlate with bovine fecal culture samples *(59)*.	Fecal, blood, and milk samples were collected directly from 689 lactating cows.	Serum samples were tested in duplicate with a commercial ELISA at one lab. Milk samples were tested with an unspecified ''in-house'' ELISA at a second lab. Fecal samples were tested by a modified ''double incubation method.'' Feces (2 g) were added to 35 ml of sterile water and allowed to settle for 30 min. The top 5 ml was transferred to 25 ml of 0.9% HPC in BHI and held at 37°C (98.6°F) overnight. The sample was pelleted (900 × *g*, 30 min) and resuspended in 1 ml of an antibiotic solution (vancomycin, nalidixic acid, amphotericin B in BHI) and incubated (overnight at 37°C [98.6°F]). HEYM slants were inoculated and incubated for ~16 wk. Colonies confirmed by IS*900* PCR (primers neither referenced nor specified).	Fecal culture results were used as the ''gold standard.'' ELISA and culture results were compared. Agreement between milk and serum results were statistically ''moderate,'' although the *P* value from the McNemar test indicated significant differences in the proportions of positive results. Serum and fecal results had similar statistical outcomes. Agreement between milk and fecal samples was higher, and proportions of positive results were not different.	In all cases, most reliable results were obtained for cows classified as ''heavy shedders'' by the fecal culture technique.

TABLE 4. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Compare four culture methods for detection of MAP from cattle feces *(90)*.	240 bovine fecal samples	Sedimentation: 1 g of feces was added to 35 ml of DHOH, shaken for 30 min, and allowed to settle for 30 min. 5 ml was removed and added to 25 ml of 0.9% HPC and incubated overnight at RT. The resulting sediment was used to inoculate two plates of HEYM and one plate of HEYM with mycobactin J and incubated for 16 wk. Double centrifugation: 1 g of feces was added to 35 ml of DHOH, shaken for 30 min, and allowed to settle for 30 min. 25 ml was removed and centrifuged (4,000 × *g*, 20 min), pellet was suspended in 25 ml of 0.9% HPC in 25 ml of half-strength BHI and incubated overnight at 37°C (98.6°F). The mixture was centrifuged again and the pellet was suspended in half-strength BHI with amphotericin B, vancomycin, and naladixic acid and incubated overnight at 37°C (98.6°F). Inoculated onto two plates of HEYM and one plate of HEYM with mycobactin J for 16 wk. Double centrifugation was performed as above and used to inoculate MB/BacT broths. If growth occurred, 500 µl from the vial was inoculated to two plates of HEYM and one plate of HEYM with mycobactin J for 8 wk. Molecular detection: PCR was performed targeting IS*900* (L1 and L9 loci) plus 251-bp unique sequence.	Sensitivity method 1 = 37/240 Method 2 = 53/240 Method 3 = 65/240 Method 4 = 76/240	27 samples positive by all four methods, 76/81 positive samples detected by method 4. All but two of broth enriched–molecular assay positive confirmed by one of three other protocols. Indicated broth-molecular method (method 4) indicates true positives and other methods less sensitive.
Compare culture and RT-PCR for detection of MAP in cattle feces *(9)*.	Feces (bovine), 310 spiked samples	3 g of feces was homogenized in 50 ml of 4% NaOH, shaken 10 min, and centrifuged (3,000 × *g*, 15 min). The pellet was resuspended in 20 ml of 5% oxalic acid, vortexed, and shaken for 15 min. The pellet was resuspended in 4 ml of 0.15 M NaCl. 200 µl was transferred to three slants of LJ medium with mycobactin J and one LJ slant without mycobactin J and incubated 16 wk. RT-PCR performed targeting IS*900*.	Sensitivity 60% Specificity 93.4% LOD 500 CFU/g, 100% LOD 31 CFU/g, 71%	
Development of nested PCR targeting IS*900* and ISMAP*02* for detection of MAP *(122)*.	Feces (bovine naturally infected and spiked feces)	Cultured on HEYM with mycobactin J (double centrifugation, double decontamination procedure; no specifics on decontamination). DNA extracted for PCR targeting IS*900* and ISMAP*02*.	All methods equal in sensitivity; specificity not done; LOD 1–7 CFU.	ISMAP*02* PCR equal to nested conventional or RT-PCR with IS*900* target.

TABLE 4. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Evaluate DNA isolation method and RT-PCR for detection of MAP in feces *(66)*.	Feces (bovine, spiked)	Evaluated the use of Whatman FTA cards for isolation of DNA from fecal samples. Fecal samples were spiked with known amounts of MAP. PCR was performed using FRET. The target sequence was IS*900*.	Reported LOD in fecal samples was 10^2–10^4 CFU/g. No specificity reported.	Question whether spiking bovine feces would give an accurate estimate for limit of detection. (i.e., how were the feces determined to be MAP free prior to spiking with MAP).
Compare BACTEC 460 and MGIT 960 systems for detection of S strains of MAP in pooled ovine fecal samples. Also evaluate the use of ampicillin for control of contamination *(56)*.	Feces (ovine, naturally infected)	Pure culture of laboratory adapted S strain was used to inoculate both BACTEC 12B radiometric medium and MGIT ParaTB medium vials. Samples from four MAP-free and one MAP-positive animal were pooled together. All pooled fecal samples were decontaminated 24 h in HPC and then in vancomycin, naladixic acid, and amphotericin B for 48–72 h. Sample inoculated into BACTEC and MGIT culture vials. BACTEC vials also inoculated with MAP with and without ampicillin to determine effects of ampicillin on MAP growth. BACTEC vials also inoculated with MAP with and without vancomycin to determine effects of vancomycin on MAP growth.	Growth of pure culture was detected 2 wk earlier in BACTEC compared with MGIT culture vials. Analytical sensitivity was estimated to be 2.3×10^1 and 2.3×10^3 for MGIT and $<2.3 \times 10^1$ for BACTEC. Sensitivity of BACTEC on pooled fecal samples was 21.9%. When data were divided by the pathological lesions, when the positive sample in the pooled sample came from a sheep with multibacillary lesions the sensitivity was 100%, when the positive sample in the pooled sample came from a sheep with paucibacillary lesions the sensitivity was 17.8%. MGIT did not detect any of the pooled samples as positive. Ampicillin had to be added to cultures every 4 wk to prevent overgrowth of contaminants and delayed the growth of MAP 1–2 wk. Addition of vancomycin delayed growth of MAP (initial inoculum $= 2.3 \times 10^4$) 4 wk in BACTEC culture vials.	Animals were diagnosed during necropsy and considered infected with MAP if the terminal ileum had characteristic lesions or if MAP was cultured using BACTEC from the tissue or lymph node associated with the terminal ileum Inhibition experiments were conducted using a laboratory strain of S type MAP.

TABLE 4. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Develop new PCR for detection in feces *(71)*.	Ovine feces (naturally infected and spiked with laboratory strain of MAP)	DNA isolated using JohnePrep. 0.5 g of feces added to 10 ml of saline and allowed to settle 30 min. 1 ml of supernatant added to zirconia-silica beads, washed in lysis buffer and bead beating for 45 s. DNA precipitation with isopropanol.	Sensitivity determined three ways: plasmid containing portion of IS*900* = one copy; purified DNA from culture = 2 fg (approx 0.2 genomic equivalents); spiked feces = 10 CFU/g. Specificity = 100%.	Sensitivity calculated three ways, using cloned plasmid, purified DNA from culture, and spiked fecal samples. The authors tested 506 unknown samples with both culture and their PCR assay but did not report a sensitivity or specificity using the classical methods of calculation to compare their PCR assay with a ''gold standard.''
Comparison of ELISA testing of milk and serum using a commercial kit with fecal culture on HEYM in goats *(114)*.	Serum, milk, and feces from goats	Serum harvested via centrifugation. Milk centrifuged and sampled below cream layer. Feces decontaminated using ''Cornell method.''	Sensitivity and specificity of ELISA in serum = 74.3 and 98.6%; in milk = 60 and 99.3%.	Classification of low, medium, and high shedders based on culture results was elevated compared to most other studies. The authors categorized animals as low, <50 CFU/g; medium, 50–299 CFU/g; and high, >300 CFU/g.
Use of a radiometric method to detect MAP in pooled feces *(34)*.	Pooled feces from infected and noninfected cows	IS*900* PCR used to detect MAP in pooled fecal samples from infected and uninfected cows after HPC treatment, incubation for 20–26 h at 37°C (98.6°F), centrifugation, addition of VAN antibiotics, reincubation for 70–74 h, aliquot into BACTEC 12B radiometric medium containing egg yolk, mycobactin J, and PANTA antibiotics. Incubation at 37°C (98.6°F) for 12 wk and measured.	High-shedder cow feces could be diluted 1:30 and feces from low shedders 1:10 with feces from uninfected cows and detection still possible.	Detection of an infected cow population by examination of fecal material.
Comparison of RT-PCR with solid and liquid culture media and serum ELISA *(2)*.	Fecal and serum samples from dairy cows	RT-PCR per instructions with Tetracore VetAlert kit. Culture: HEYM and TREK ESP per Stabel et al. *(123)*.	Sensitivity of RT-PCR estimated to be 0.60; specificity 0.97, accuracy 0.90 (compared to 0.91 for solid culture, 0.93 for liquid culture, and 0.82 for ELISA).	Evaluated 143 animals.

TABLE 4. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Evaluate a triplex RT-PCR *(65)*.	Fecal samples from five calves experimentally exposed	1 g of feces was mixed with 20 ml of 5% SDS and allowed to sediment. The sediment was washed three times with PBS and centrifuged. The pellet was disrupted by boiling and freezing, followed by DNA extraction. PCR was performed with primers for F57, ISMAP*02*, and IS*900*.	LOD 2.5×10^2 MAP/g feces.	Tested 197 fecal samples collected over 3 yr during subclinical phase. Good agreement with culture. Used sequence analysis of IS*900* amplicons from three IS*900*-positive samples to confirm positivity.
Development of a rapid biosensor assay for live MAP *(75)*.	Spiked fecal samples	RNA was extracted using Trizol, treated with DNase and heat (95°C [203°F], 5 min). RNA was amplified using RT-PCR. The assay was performed with a lateral flow biosensor targeting IS*900*. Signal amplification occurred when target sequence hybridized to DNA probe coupled to liposomes encapsulating the dye sulforhodamine B.	Detects 10 viable cells.	No cross-reactivity with other mycobacteria.
Estimate the sensitivity and specificity of direct fecal PCR compared to HEYM; compare abilities of direct fecal PCR and serum ELISA to identify animals shedding MAP *(17)*.	Fecal samples, serum samples	2 g of feces was analyzed as follows. Culture: mixed with 35 ml of water and shaken to disrupt fecal material. After settling for 30 min, 5 ml was removed and decontaminated with 0.9% HPC in half-strength BHI and incubated overnight at 37°C (98.6°F). Material was centrifuged, and the resulting pellet was treated with antibiotics, incubated at 37°C (98.6°F), and cultured on HEYM up to 16 wk. PCR: mixed with 4 ml of water and vortexed. After settling for 60 min, 1.5 ml of liquid was removed to a tube with zirconia-silica beads. The mixture was centrifuged, incubated at 95°C (203°F) for 10 min, and bead beat (45 s, 6,500 rpm). Centrifuge and isolate DNA. PCR analysis with P90 and P91 primers. ELISA: Parachek Johne's Absorbed EIA.	Compared to culture ''gold standard'' Sensitivity: PCR, 70.2% (57.7–80.7) ELISA, 31.3% (20.6–43.8) Specificity: PCR, 85.3% (79.3–90.1) ELISA, 97.8% (94.5–99.4)	Two farms, one with suspected high prevalence and one with low prevalence. Of 250 animals, 26.8% were culturally confirmed to be shedding MAP; PCR detected 29.6% and ELISA 10.0%. A positive ELISA was more likely associated with a diseased animal but a negative ELISA was less likely to predict a nondiseased animal.

TABLE 4. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Describe a rapid effective method for the extraction of MAP DNA for fecal samples for PCR *(123)*.	Fecal samples	Culture using double centrifugation, double decontamination *(120)* and HEYM with mycobactin J and antibiotics. DNA extraction: 1 g of feces diluted with 9 ml of 1 × Tris-EDTA buffer, vortex 5 s, settle 2 min, revortex. Centrifuge. Transfer supernatant and dilute with Tris-EDTA buffer. Centrifuge, resuspend pellet in Tris-EDTA buffer, heat 100°C (212°F) for 10 min. Cool, treat with RNase, then run PCR.	Sensitivity: 81% when >70 CFU/g 45% when <1 CFU/g Reproducibility: 75% when >70 CFU/g 25% when <1 CFU/g	1,000 fecal samples. Sensitivity and reproducibility were highly dependent on the load of bacteria. Method can detect animals that are shedding <1 CFU/g.

[a] LOD, level of detection; ELISA, enzyme-linked immunosorbent assay; HPC, hexadecylpyridinium chloride; BHI, brain heart infusion; HEYM, Herrold's egg yolk medium; TEN buffer, 0.1 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), Sigma Chemical Co.; MAP, *Mycobacterium avium* subsp. *paratuberculosis*; CB-18, C_{18}-carboxypropylbetaine; IAC, internal amplification control; DHOH, sterile double-distilled water; RT, room temperature; MB/BacT, a modified Middlebrook 7H9 broth, bioMérieux; RT-PCR, real-time PCR; LJ, Lowenstein-Jensen; FRET, frequency resonance energy transfer; MGIT, mycobacteria growth indicator tube; VAN, vancomycin, amphotericin B, and nalidixic acid; PANTA, polymyxin, amphotericin B, nalidixic acid, trimethoprim, and azlocillin; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline.

that strains sequestered in protozoa were more resistant to chlorination than free-living noningested strains *(144)*. Whether *Mycobacterium* spp. colonize protozoans in their natural habitat is unknown.

Environmental contamination. Cattle manure can serve as a source of contamination for other foods. Runoff from cattle holding areas and improperly composted manure can spread the pathogen in the environment and contaminate water and food products *(78)*. Mycobacteria are known to survive for variable periods in the environment *(149, 150)*. In a study of New York herds ($n = 33$), spreading manure on fields used to grow forage for cattle was identified as a risk factor (odds ratio = 10.3) for on-farm transmission of MAP *(95)*. Grewal et al. *(55)* examined the persistence of MAP during simulated composting, manure packing, and liquid storage of dairy manure. Data indicated that MAP may survive at least 8 weeks in liquid slurry, but MAP was not detected in packed or composted manure beyond day 0.

Raizman et al. *(107)* examined 122 samples from cow alleyways on 66 Minnesota farms and found 77% of these farms were culture positive for MAP. The authors suggested that environmental sampling might be a useful means by which to identify positive herds.

Whittington et al. *(149)* isolated MAP from 25 of 146 soil, creek sediment, and fecal samples on farms with stock having a high prevalence of Johne's disease. Only 1 of the 22 originally positive soil sites was culture positive 5 months after the infected animals were removed from the area, and there were no culture-positive sites after 12 months. The authors noted a decreased recovery of MAP from mixed fecal and soil samples compared to fecal samples alone. They postulated that the reason for the decreased recovery was due to binding of MAP cells to soil particles, which are typically removed from the culture matrix during sample preparation. If soil reduces the recovery of MAP, MAP prevalence in soil may be underestimated.

It has been speculated that MAP may be disseminated via aerosols that land on pastures and are ingested by animals *(148)*. No studies have been reported that evaluate this route of exposure.

Presence of MAP in water. Since cattle and other ruminants are reservoirs of MAP, farm runoff may potentially contaminate water supplies *(78)*. MAP has been isolated from soil and water samples surrounding sheep farms *(149)*, and under experimental conditions MAP has been known to survive for months in both water and water sediments from the environment *(148)*. MAP also was detected in a river in England downstream from cattle- and sheep-grazed grasslands *(103)*. Under experimental conditions, MAP remained culturable in surrounding lake water samples for 632 days *(103)*.

MAP detection by PCR in a river in Wales peaked 5 to 7 days after rainfall, suggesting runoff from up-river cattle and sheep sources *(104)*. In the same study, MAP was not detected immediately after water treatment but was detected by PCR in 1 of 54 posttreatment domestic cold-water storage tanks, indicating the potential for MAP to access

TABLE 5. Mycobacterium avium *subsp.* paratuberculosis *detection methods in tissue matrices*[a]

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Test several of the available solid culture media for isolation of both bovine and caprine MAP isolates. Study the differential requirements for isolation depending on MAP type and not host *(25)*.	Tissue—homogenized ileocecal valve, intestine, and mesenteric lymph node	All samples were previously decontaminated with HPC before inoculation onto solid media. Each sample was inoculated onto HEYA with mycobactin J, HEYA with mycobactin J and sodium pyruvate, Middlebrook 7H11 medium with mycobactin J, and LJ with mycobactin J. All media contained polymyxin B, carbenicillin, amphotericin, and trimethoprim. Isolates of MAP classified as either type II or type I/III.	100% of type I/III were isolated from either LJ or Middlebrook 7H11 medium. All four media required to isolate 100% of type II isolates.	Demonstrates that even isolates of the same type may have different requirements for growth.
Describe different diagnostic procedures for detection of caprine paratuberculosis based on clinical signs, pathological lesions, conventional isolation and identification, and nested PCR *(5)*.	Tissue—mesenteric lymph node	Methods were limited to the PCR methods. Nested PCR was conducted targeting IS*1311*. First round PCR primers were 5′-TGAACGGAGCGCAT-CACGAA-3′ and 5′-TGCAGCTGGTGATCTCTGAT-3′. Second round primers were 5′-GCAAAGTCGACGATT-TGGTC-3′ and 5′-AAGTGCTCGGCTTCGACGTC-3′.	Pathology included thickening and corrugation of the mucosal folds of the ileum, jejunum, and colon. Mesenteric lymph node enlarged, pale, and edematous. Acid-fast bacilli found in both the ileum and sections of the mesenteric lymph node.	All tests performed on a single goat. Tissue source and DNA extraction method not described.
Evaluate ability of immunohistochemistry and in situ hybridization to detect MAP in formalin-fixed tissues *(26)*.	Tissue from six cows—ileum, ileocecal, lymph node	Tissues were fixed in 10% formalin and embedded in paraffin, sectioned and stained (hematoxylin and eosin), and tested with anti-MAP antibody. In situ hybridization was conducted with a 284-bp segment of IS*900* and alkaline phosphatate–streptavidin conjugate.	Both techniques were able to detect MAP, but immunohistochemistry produced higher intensity staining.	Four adult animals with clinical signs, two young animals from a disease-free herd. Immunohistochemistry was easier to perform. In situ hybridization was as effective as Ziehl-Neelsen staining.

[a] LOD, level of detection; MAP, *Mycobacterium avium* subsp. *paratuberculosis*; HPC, hexadecylpyridinium chloride; HEYA, Herrold's egg yolk agar; LJ, Lowenstein-Jensen.

domestic outlets *(104)*. A study in Northern Ireland involving 192 samples of untreated source water obtained from nine water treatment facilities found 9 of 192 samples positive by PCR and 8 of 192 positive by cultural methods; the combined prevalence using all methods was 7.8% *(145)*. Surveys of treated and untreated water from treatment facilities in Great Britain failed to detect MAP *(62, 141, 142)*. It is thought that municipal water treatment according to current U.S. Environmental Protection Agency (EPA) requirements will reduce the number of MAP present in water. Studies are needed to determine the extent and adequacy of reduction of MAP by municipal water treatment processes. Representatives of the U.S. EPA advised this Committee that there are no data relative to the prevalence of MAP in the U.S. water supply, but studies to address this issue are being planned.

Prevalence and Numbers of MAP in Food Sources

Milk. The vast majority of the reviewed literature regarding MAP in food dealt with raw (not heat treated) and pasteurized bovine milk. Given the large volume of milk consumed (USDA Economic Research Service estimates the annual per capita consumption of beverage milk to be 21 gal [79 liters]), the potential for exposure to MAP from milk is important to consider. Studies reporting the presence of MAP in milk are summarized in Table 6. Most of these studies only reported the prevalence of MAP in milk and provided no information on the number of cells that may be present. A few studies have provided estimates of the numbers of MAP cells in raw cows' milk using cultural methods; numbers typically were on the order of 1 cell per 10 ml *(49, 80, 106, 131)*. The number of MAP in raw milk may be underestimated due to the effects of clumping and inactivation or loss during decontamination steps used in testing for the presence of MAP *(49, 52)*.

Raw milk. A number of studies have evaluated the prevalence of MAP in raw milk and can be summarized as follows. The percentage of raw bovine milk samples positive for MAP when analyzed by PCR ranged from 7.8 to 32.5% *(6, 20, 49, 68, 97, 119)*. Prevalence of MAP in raw bovine milk samples analyzed by culture ranged from 0.25 to 11.1% *(49, 68, 97, 131)*. One study in Switzerland found that 23% of raw goat milk samples and 23.8% of raw ewe milk samples were MAP positive by PCR *(91)*.

Grant et al. *(49)* found that 19 of 244 (7.8%) 50-ml bulk milk samples from 241 approved dairy processing establishments throughout the United Kingdom were positive for MAP by IS*900* PCR, but only 4 of 244 (1.6%) were positive using culture techniques. In a Pennsylvania study *(41)*, 13.5 and 2.8% of quarter milk samples were positive for MAP by PCR and culture, respectively; 27.5 and 6.8% of bulk milk samples were also positive by PCR and culture, respectively. Slana et al. *(119)* analyzed 342 milk samples by IS*900* and F57 real-time quantitative PCR (qPCR); 13.7 and 18.7% were positive by the F57 and the IS*900* qPCR assay, respectively. No sample, however, was culture positive on HEYM plus mycobactin J.

Regional differences in the prevalence of MAP are known to exist, as indicated in a study by Corti and Stephan *(20)* from Switzerland that found differences in prevalence in bulk tank milk samples ranging from 1.7 to 49.2%, depending on region.

Pasteurized milk. The prevalence of MAP in pasteurized bovine milk samples analyzed using PCR ranged from 2 to 15% *(6, 35, 41, 49, 59, 87)*. Pasteurized bovine milk samples analyzed by culture ranged from 0 to 2.8% positive *(6, 35, 41, 49)*. A study conducted in England and Wales found 9 of 18 PCR-positive retail milk samples as well as 6 of 36 PCR-negative samples were culture positive *(87)*, demonstrating the typical variability in prevalence based on the detection and recovery method(s) used.

O'Reilly et al. *(97)* analyzed 389 raw and 367 pasteurized milk samples in Ireland using immunomagnetic separation–PCR (IMS-PCR) and found that 12.9 and 9.8%, respectively, were positive. Yet, only one positive raw milk sample was identified by bacterial culture. Cream and whole milk samples were more frequently PCR positive than skim milk samples. Positive samples also peaked in June, suggesting possible seasonal variations. Grant et al. *(49)* also found 67 of 567 (11.8%) commercially pasteurized milk samples were MAP positive by PCR, while 10 of 244 (1.8%) were positive by culture.

Another study in Canada found 110 of 710 (15%) of milk samples from retail stores ($n = 535$) and dairy plants ($n = 175$) were positive for MAP by nested IS*900* PCR; however, no MAP was isolated from broth and agar cultures of 44 PCR-positive and 200 PCR-negative retail milk samples *(41)*. Ellingson et al. *(35)*, using two different culture methods, found that 20 of 702 (2.8%) retail pasteurized milk samples from Minnesota, California, and Wisconsin were positive for MAP by one or both methods.

The presence of MAP in pasteurized milk may be due to postprocess contamination, underprocessing, or survival of the process. These issues are discussed further in the response to question number 4.

Milk products. Cheeses may be made from pasteurized, thermized, or unpasteurized milk. The aging process in cheese manufacturing has been shown to reduce pathogens originally present in milk that have not been inactivated by heat treatments. However, pathogens may not be completely eliminated by the aging process *(32, 120, 130)*. If MAP is not inactivated during the aging process, cheese could be a source of exposure to this organism.

A study conducted by Clark et al. *(16)*, in which 98 samples of cheese curds were analyzed for MAP, reported that 23% of the samples were positive by IS*900* PCR, 9% were positive by PCR detection of the *hspX* gene, and 5% of the samples were positive by both methods. No viable MAP could be obtained by culture, although 6 to 11% of rinsates of culture slants were positive for MAP by PCR *(16)*. Depending on brand, 0 to 85.7% and 0 to 14.3% of retail cheeses from Greece and the Czech Republic were positive

by IS*900* PCR and culturing, respectively *(63)*. The study did not report if the milk used to manufacture these cheeses had been heat treated.

A study conducted in Switzerland examined 143 retail raw milk cheeses (9 soft, 133 semihard, and 1 hard) by real-time PCR and BACTEC culture following immunomagnetic capture. The investigators found no positives by culture, but 4.2% of the samples showed evidence of MAP by real-time PCR, suggesting that MAP had been present in the raw material *(128)*.

In addition, there are three studies in which cheeses were manufactured using artificially contaminated milk to determine survival and persistence of MAP during ripening *(32, 120, 130)*. These studies are addressed below in question 4 on processing interventions. Although the numbers of MAP cells decreased during cheese manufacturing, the organism was not completely eliminated at the inoculum levels used and the conditions studied *(32, 120, 130)*. It should be noted that these studies were not conducted with naturally contaminated milk.

Hruska et al. *(61)* tested 51 samples (20-mg portions) of powdered infant formula from 10 manufacturers in seven countries using IS*900* PCR, the fragment F57 real-time PCR method, and culture. A total of 25 (49%) samples were positive for MAP by IS*900* PCR, and 18 (35%) samples were positive by fragment F57 real-time PCR. Cultural confirmation of MAP contamination could not be achieved for any sample. These findings warrant confirmation, particularly in light of the high percentage of PCR-positive samples, the lack of culture confirmation, and the small sample size.

Meat. Since MAP results in a disseminated infection in cattle, including lymphatic tissue and the liver *(64, 110)*, beef should be considered a potential source of exposure for MAP, especially ground beef and organ meats such as liver. The Committee reviewed the limited reports describing the presence of MAP on carcasses, in meat, and in meat products. An abstract provided by Rossiter and Henning *(110)* demonstrated a correlation between animals with disseminated infection (determined by culture of the ileocecal lymph node and feces) and the presence of MAP in the liver and in other lymph nodes (superficial cervical and popliteal lymph nodes) that could be incorporated into ground beef. The authors concluded that the prevalence of MAP in the superficial cervical and popliteal lymph nodes in the total market cow population was very low but should be further investigated. Nelli et al. *(93)*, using a real-time PCR test for IS*900*, detected MAP in the gastrocnemius muscle of cows and sheep.

A survey of ground (minced) beef samples ($n = 113$) collected over 4 months from a single meat processing plant in the Republic of Ireland failed to find viable MAP *(64)*. The absence of a detailed description of the methods used in this study precludes assessment of the suitability of the isolation and/or detection protocols. A Canadian survey of 300 carcasses reported that while 22 to 54% of beef and dairy cow skinned carcasses were IS*900* PCR positive for MAP by a nested PCR assay and 6 to 50% were positive after dressing (including pasteurization), the fractions of IS*900*-positive preparations positive for F57 (specific for MAP) ranged from 5 to 43%. Only two dressed and/or pasteurized carcasses were positive for IS*900* with a quantitative PCR, and none for F57 *(84, 85)*. A survey of retail ground beef samples ($n = 200$) in California failed to detect MAP using an IS*900* PCR assay *(67)*. Meadus et al. *(85)* indicated that the limited data available suggest that contamination of beef carcasses with MAP may not be a major source of human exposure.

Fish and shellfish. Since MAP is present in fecal material from cattle and runoff from fields containing manure may contaminate water sources, there is the potential for fish or molluscan shellfish harvested from waters contaminated with bovine feces to be contaminated. However, there have been no studies published examining fish or shellfish for the presence of MAP.

Produce. In an abstract published by Pavlík et al. *(100)*, MAP was reportedly recovered from lettuce, radishes, and tomatoes cultivated for 4 weeks in soil artificially contaminated with MAP. The Committee did not find any published studies for the presence of MAP on vegetables grown in naturally contaminated soil.

4. What processing interventions are available for the foods of concern to eliminate or reduce the levels of MAP contamination to an acceptable level or to ensure that MAP does not enter the food supply?

At this time the Committee could not determine what frequency and level of MAP in food and water are ''acceptable,'' since the impact of foodborne exposures on human health is unknown; therefore, the Committee is not expressing an opinion on the relative public health importance of different levels of exposure to MAP. The literature reviewed by the Committee suggested that raw milk represents a potential source of human exposure to MAP. Milk pasteurization is an effective mitigation strategy for reducing MAP in milk and dairy products, although the organism has been found in up to 2.8% of pasteurized milk samples tested *(35)*. Studies on the efficacy of pasteurization to inactivate MAP are complicated by a number of factors:

- The tendency of the organism to clump interferes with accurate enumeration.
- The hydrophobic nature of the organism causes cells to congregate at liquid surfaces and in films on the sides of glass tubes, which complicates the preparation of accurate dilutions to be used in enumeration *(43)*.
- Extremely slow growth prolongs the time to results. Because of slow growth (inactivation studies often incubate cultures 2 to 3 months and sometime up to 1 year to recover low numbers of injured survivors), other organisms often overgrow the cultures and may mask the detection of MAP.
- Decontamination of samples reduces interfering organisms but may reduce the efficiency at which MAP is recovered *(52, 80)*.

TABLE 6. *Summary of studies on the prevalence of* Mycobacterium avium *subsp.* paratuberculosis *in milk*[a]

Source	Findings	Method	Comments
Raw milk *(131)*.	11.1% (8/72) cows gave MAP-positive milk; 11.7% (9/77) milk samples MAP positive.	50-ml composite of all four quarters, ice transport to lab. Decontaminated (0.75% HPC, 18 h); HEYM culture (37°C, 16 wk).	Examined cow lymph nodes, antibodies (ELISA), feces.
Whole pasteurized milk; England *(87)*.	7% (22/312) retail samples (cartons and bottles) PCR positive.	PCR IS*900*.	
	82% (18/22) of PCR signal segregated to cream or pellet fraction, consistent with presence of intact mycobacteria (although they may not have been viable).	Culture: decontaminate (overnight at RT with 0.1% benzalkonium chloride or 0.1 or 0.75% HPC in PBS; cultured in Dubos broth with mycobactin J (37°C [98.6°F], 13 to 40 mo).	Seasonal peaks in January–March and September–November when approx 25% of samples positive.
	50% (9/18) PCR-positive samples and 17% (6/36) PCR-negative milk samples culture positive (13–40 mo incubation).	5-μl sample (cream, whey, pellet) screened by PCR.	
	Peaked in January–March and September–November when approx 25% retail pasteurized milk positive.		
	27.7% (15/54) retail samples culture positive; 17% (6/36) PCR-negative samples culture positive. No plating, no isolation.		
Bulk raw and commercially pasteurized milk; UK *(49)*.	814 samples.	IMS-PCR and culture: decontamination with 0.75% HPC (5 h, 21°C [69.8°F]).	March 1999–July 2000.
	Bulk raw: 7.8% (19/244) PCR positive; 1.6% (4/244) culture positive.	Cultured on HEYM and BACTEC 12B radiometirc medium (approx 18 wk).	Commercially pasteurized milk samples included: 228 whole; 179 semiskim; 160 skim.
	Commercially pasteurized: 11.8% (67/567) PCR positive; 1.8% (10/244) culture positive.	The 14 positives were on HEYM; 1 pasteurized milk positive on BACTEC.	
	Ten culture-positive pasteurized milk samples from 8/241 dairy processing establishments.		
	50-ml milk sample for PCR and culture.		
	PCR detection limit approx 1 in 50 ml.		
	Counts based on the number of colonies on HEYM slants, approx 4–20 CFU/50 ml. One sample positive on both HEYM and BACTEC had confluent colony growth on HEYM slants, indicating a higher number of MAP in the sample. Decontamination may underestimate prevalence.		
Bulk milk *(20)*.	19.7% (273/1,394) PCR positive; range 1.7–49.9%, depending on region.	PCR IS*900*. No culturing.	Switzerland: prevalence varied significantly between regions.
Raw and pasteurized cow's milk; Ireland *(97)*.	Raw milk: 12.9% (50/389) IMS-PCR positive; 1 culture positive.	IMS-PCR IS*900*.	Milk from all 27 approved pasteurizing plants:
	Pasteurized milk: 9.8% (35/357) IMS-PCR positive.	Culture: HEYM with mycobactin J and BACTEC 12B radiometric medium (supplemented with egg yolk, mycobactin J, and PANTA antibiotics) after decontamination with 0.75% HPC; incubated 18 wk; confirm by Ziehl-Neelsen, and IS*900* PCR.	Skim milk (0/8); semiskim milk (2/31); whole milk (25/277); cream (8/41). Peak in June; culture may underestimate; subculturing since did not always produce cultures for confirmation.

TABLE 6. *Continued*

Source	Findings	Method	Comments
		50 ml of milk tested by each method; sensitivity approx 1 CFU/50 ml. Centrifuged milk and tested pellet only.	
Raw milk; Canada *(59)*.	1.1% (77/689) milk samples were positive.	Milk samples tested with a commercial (AntelBio Systems) and in-house ELISA for antibodies against MAP; serum tested using IDEXX HerdChek MAP ELISA; feces cultured: decontamination using 0.9% HPC in BHI broth (overnight, 37°C [98.6°F]), centrifuged, pellet resuspended in BHI with antibiotics, incubated (overnight, 37°C [98.6°F]), inoculated onto HEYM (37°C [98.6°F], 15 wk); colonies confirmed as MAP using IS*900* PCR.	Milk samples preserved with bronopol; serum and fecal samples frozen (−20°C [−4°F]).
Raw milk; United States *(68)*.	Quarter milk sample: 2.8% (43/1,493) culture positive; 13.5% (201/1,493) PCR positive. Bulk tank milk: 6.8% (2/29) culture-positive; 27.5% (8/29) PCR positive	Milk samples centrifuged, pellets resuspended 500:1 in PBS; half used for DNA extraction and IS*900* PCR, half decontaminated overnight at RT in 15 ml of 0.75% HPC; centrifuge, resuspend 300:1 in 0.75% HPC, and six drops used to inoculate two plates of HEYM with mycobactin J; incubated (37°C [98.6°F], 16 wk). Colonies confirmed using Ziehl-Neelsen staining.	1,524 lactating cows in 29 herds in Pennsylvania (1,493 complete sets of feces, serum, and quarter milk). 16/29 herds had history of Johne's from July 1999–June 2002.
Raw milk *(119)*.	111/345 milk samples (32.5%) were positive by RT-PCR; none were positive by culture.	72 cows sampled (all four teats; from farm with known history of paratuberculosis) on days 1, 5, 6, and 13. Milk samples from 53 cows taken on day 82. Continuous tank milk samples taken on days 1 and 6, and bulk tank milk samples taken after 11 h of mixing on each day. Samples were centrifuged, pellet was resuspended in 0.75% HPC and decontaminated for 5 h. Pellet was centrifuged, resuspensed in water, and inocuated onto HEYM with mycobactin J, 37°C (98.6°F) for 18–30 wk. RT-PCR for IS*900* and F*57*.	February–April 2007
Commercially pasteurized cow's milk; Czech Republic. Locally pasteurized milk. Raw milk *(6)*.	4/244 bottles and cartons (1.6%) Herd A (low level of MAP infection): 2/100 (2%); herd B (high level of MAP infection): 2/100 (2%); herd C (not infected): 0/100 (0%). From fecal culture-positive animals of herd A: 1/10 (10%); from fecal culture-positive animals of herd B: 13/66 (19.7%).	HEYM (32 wk); confirmed by morphology, Ziehl-Neelsen staining, mycobactin J dependency, and IS*900* PCR.	

TABLE 6. *Continued*

Source	Findings	Method	Comments
Retail pasteurized milk; United States *(35)*.	702 samples. 2.8% (20/702) positive by one or both methods; 7/233 in California; 9/234 in Minnesota; 4/235 in Wisconsin. 12/22 brands yielded at least one MAP-positive sample.	(1) HEYM evaluated at 8 and 16 wk; (2) TREK ESP II Culture System; PCR confirmation of IS*900* and *hspX*.	Seasonal peak July–September.
Retail milk; Canada *(41)*.	15% (110/710) retail milk samples from retail store (535) and dairy plants (175) PCR positive; no MAP isolated from the broth and agar cultures of 44 PCR-positive and 200 PCR-negative retail milk samples.	Nested IS*900*; all PCR-positive and a subset of PCR-negative samples screened for MAP by culture on Middlebrook broth and agar (37°C [98.6°F], 5 to 6 mo), and BACTEC 12B radiometric culture (16 wk).	Southwest Ontario
Goat and ewe's milk; Switzerland *(91)*.	23% (79/344) goat's milk (range, 21.76–32.06%) 23.8% (15/63) ewe's milk (range, 13.98–36.21%)	10 ml; PCR IS*900*; no culture confirmation.	403 farms; milk cooled, tested within 24–48 h; 20 ml frozen (−20°C) before testing. Regional prevalence of IS*900*: 17% north, 34% south. Centrifuged milk and tested pellet only.
Retail cheese; Greece and Czech Republic *(63)*.	84 samples. PCR: 30.9% (26/84; range, 0–85.7%) Culture: 3/84 (3.6%; range, 0–14.3%)	Homogenized; decontaminate (0.75% HPC, 5 h); inoculate HEYM with mycobactin J (approx 8 mo); IS*900* PCR.	Most popular brands. Five brands of feta from Greece; three brands (one each soft, hard, and semihard) from Czech Republic. pH, salt conc, temp during manufacture may inhibit MAP, reducing viability. Decontamination may yield false-negative results on culture when PCR is positive.
Cheese curds; United States *(16)*.	Prescreening: 23% (23/98) positive for IS*900* or P90/P91; 59% (9/98) positive for *hspX*; 5% (5/98) positive by both assays; 0% (0/98) culture positive. PCR confirmatory slant rinse: 11% (11/98) positive for IS*900* or P90/P91; 6% (6/98) positive for *hspX*; 1% (1/98) positive by both assays. Conclusion: unable to detect viable MAP; presence of MAP-specific DNA indicates viable MAP once present in component of cheese curds.	Culture: HEYM (16 wk); confirm by acid-fast staining; PCR: IS*900*, P90, P91, and *hspX*.	Wisconsin and Minnesota; approx five samples from four regions for 6 mo (April–September 2004); P90/P91 are portions of IS*900* detected using LightCycler RT-PCR. 5-g samples tested.

[a] MAP, *Mycobacterium avium* subsp. *paratuberculosis*; HPC, hexadecylpyridinium chloride; HEYM, Herrold's egg yolk medium; ELISA, enzyme-linked immunosorbent assay; RT, room temperature; PBS, phosphate-buffered saline; IMS-PCR, immunomagnetic separation PCR; PANTA, polymyxin, amphotericin B, nalidixic acid, trimethoprim, and azlocillin; BHI, brain heart infusion; RT-PCR, real-time PCR.

- Populations of MAP include dormant and metabolically active cells, which may vary considerably in their susceptibility to inactivation *(57, 58)*.
- Inactivation studies conducted with laboratory-grown strains may not be representative of natural contamination. Laboratory-adapted strains may have altered heat resistance, growth characteristics, or ability to clump, all of which can lead to over- or underestimation of inactivation *(106)*. For example, Sung and Collins *(129)* indicated that low-passage clinical strains seem more sensitive to killing by heat treatment than high-passage laboratory strains.
- When MAP is present as a natural contaminant, the number of cells is too small for accurate evaluation of inactivation *(106)*. On the other hand, MAP in artificially contaminated milk may not respond the same as naturally occurring cells. By way of illustration, Grant et al. *(50)* found that increasing holding time at 73°C (163.4°F) from 15 to 25 s did not affect inactivation in naturally infected cow's milk, but another study by Grant et al. *(48)* did find a difference in milk inoculated with laboratory cultures heated at 72°C (161.6°F).
- PCR assays detect a sequence of DNA rather than viable cells and thereby may overestimate the number of surviving cells. In addition, culture-positive samples have sometimes shown PCR-negative results *(87)*.

Thermal inactivation studies. A number of studies have examined the effect of heat on the inactivation of MAP. The results have been quite variable, with several studies reporting survival of MAP at many time and temperature combinations. Studies by Pearce et al. *(102)*, Stabel and Lambertz *(124)*, Rademaker et al. *(106)*, and Lynch et al. *(80)* indicate that pasteurization conditions (72 to 74°C [161.6 to 165.2°F] for 15 s) should be adequate to ensure the absence of viable MAP (>4- to 7-log reduction), whereas other studies such as those by Grant et al. *(50, 53)* and Hammer et al. *(58)* indicate that viable MAP cells can be recovered from milk following pasteurization treatments that meet regulatory requirements. Grant et al. *(47)* indicated that high-temperature, short-time (HTST) pasteurization in a laboratory unit was only completely effective at eliminating the organism when ≤10 MAP/ml were present in the raw milk.

Laboratory studies on the heat resistance of MAP have been conducted in a static mode using a batch process *(14, 72)*; however, most commercial milk pasteurization is done in continuous flow processing systems using laminar flow. Klijn et al. *(73)* indicated that the way heat is applied may influence inactivation. Chiodini and Hermon-Taylor *(14)* reported that methods simulating HTST pasteurization were more effective than batch pasteurization, but Gao et al. *(41)* showed the opposite. The degree of turbulence and the distribution of residence times for fastest and slowest milk particles are different for pilot scale and commercial scale pasteurizing equipment. This also complicates study design and interpretation of results *(106)*.

Several studies have calculated thermal *D*-values for MAP in milk *(72, 80, 102, 129)*. Using a capillary tube technique, Keswani and Frank *(72)* calculated $D_{60^\circ C(140^\circ F)}$-values ranging from 8.6 to 11 and 8.2 to 14.1 min for clumped and declumped cells, respectively. $D_{63^\circ C(145.4^\circ F)}$-values ranged from 2.7 to 2.9 and 1.6 to 2.5 min for clumped and declumped cells, respectively. Sung and Collins *(129)* determined *D*-values for human and bovine strains of MAP in milk at 62, 65, 68, and 71°C (143.6, 149, 154.4, and 159.8°F) using sealed vials. The *D*-value for clinical strains of MAP in milk at 71°C (159.8°F) was 11.7 s. Lynch et al. *(80)* obtained a $D_{65^\circ C(149^\circ F)}$-value of 20 s for one strain of MAP (ATCC 19698) in raw milk, while Pearce et al. *(102)* reported a $D_{66^\circ C(150.8^\circ F)}$-value of 5 s for the same strain. Both *D*-values were obtained in pilot plant pasteurizers. Interpretation of *D*-values determined by cultural enumeration of survivors is complicated by methodological considerations described previously. The use of *D*-values to establish a recommended process generally assumes first-order inactivation, which likely is not the case for MAP over a range of temperatures.

An excellent review of a number of heat resistance studies, including some of the reasons for the disparities, is found in Gould et al. *(43)*. A summary of a number of recent studies on the heat resistance of MAP in milk is found in Table 7; these focus on studies using pilot and commercial pasteurizers. The interpretation and comparison of the experiments and results is complicated due in part to the different experimental conditions employed *(106)*. Some key factors that could impact the results include laboratory versus natural contamination, the degree of turbulent flow, presence or absence of a resuscitation step after heating, the use of chemical decontamination after heating, and the volume of milk tested *(50, 53)*.

Although pasteurization appears to achieve at least a 4- to 7-log reduction of MAP, whether pasteurization completely eliminates MAP from the milk supply remains unresolved. Lund et al. *(79)* suggested that more controlled laboratory studies, with particular attention given to time and temperature of the heat treatment and the presence of clumps of cells, are needed to determine whether the heat treatment required for pasteurization ensures destruction of MAP. Studies also point out the risk of postprocess contamination *(35, 79)* as well as the potential for laboratory contamination during sample analysis *(86)*.

The detection of MAP in surveys of retail milk samples *(6, 35, 49)* is consistent with studies suggesting that low levels of MAP may be present after pasteurization at 71.7 to 72°C (161.06 to 161.6°F) for 15 s *(50, 53, 58)*; these findings are also consistent with postprocess contamination. Moreover, studies on heat inactivation are conducted in artificially contaminated products with levels of MAP far exceeding those which might be expected in naturally contaminated raw milk, making it difficult to determine if complete inactivation of the organism would routinely occur in a real-world setting. Since the organism is unlikely to replicate in food once it leaves the host, the number of any potential survivors should not increase.

TABLE 7. *Heat resistance of* Mycobacterium avium *subsp.* paratuberculosis *in milk heated in pilot and commercial pasteurizers*[a]

Product and processing procedures	Inoculum (MAP/ml)	Recovery methods	Results, reduction	MAP survival postpasteurization[b]	Comments
Raw milk, including milk from two dairy herds fecally positive for MAP. Pilot plant pasteurizer (580 liters/h; Reynold's no. 10,348), with and without homogenization. 16 trials at 72.5°C (162.5°F), 27 s *(80)*.	10^2–10^5 CFU/ml	Samples stored at 4°C (39.2°F) for 24–48 h prior to assay; no decontamination; pellet and resuspend in PBS; plate on HEYM plus mycobactin J (37°C [98.6°F] up to 6 mo).	No MAP survivors were detected in any of the 16 trials with laboratory strains, fecal material, or naturally contaminated milk, indicating a 4–5-log kill.	No	MAP reference strains, bovine field isolate; two natural isolates from feces and milk. MAP detected in milk from Johne's-positive herds on one of six occasions (<1 CFU/ml). Homogenization resulted in at most a 0.5-log increase in nos. Careful attention to characterizing pasteurizer performance. Decontamination after heating avoided to prevent inactivation of sublethally injured cells,and resuscitation step included.
Raw milk, raw skim milk, cream (30% fat, heated 15–20 s at 105°C). Continuous flow thermal pasteurization. Reynold's no. 2816-7005 depending on holding section used *(58)*.	Similar to Hammer et al. *(57)*, but used a five-MAP strain cocktail inoculum.	Same as Hammer et al. *(57)*.	Skim milk. 67–90°C (152.6–194°F) for 15–60 s: 53/93 (57.0%) positive, 3–6-log reduction by direct plating, 5–7-log reduction by enrichment. Whole milk. 95–100°C (203–212°F) for 15–60 s: 8/30 (26.7%) positive, 5–6-log reduction by direct plating and enrichment. Cream. 85–100°C (185–212°F) for 15–60 s: 9/57 (15.8%) positive, 6-log reduction by direct plating, 5–7-log reduction by enrichment.	Yes	Care taken to prevent cross-contamination between raw and processed milk. In comparative studies with whole, skim, and cream processed at 105–135°C (221–275°F) for 2–5 s, 5–7-log reductions observed. Relative to skim, 4-fold increase in survival in cream and 10-fold increase in survival in whole milk. For skim milk, the no. of tests with survivors tended to decrease with increasing temp and increasing holding time, but not significantly. Lower chance of detecting survivors at higher initial counts could not be explained. Double pasteurization of whole milk provided a 5–7-log reduction, with more positive samples after second treatment and failure to eliminate MAP.

TABLE 7. *Continued*

Product and processing procedures	Inoculum (MAP/ml)	Recovery methods	Results, reduction	MAP survival postpasteurization[b]	Comments
					Whole milk homogenization trials (72°C [161.6°F] for 15 and 30 s, and 75°C [167°F] for 15 and 30 s with various homogenization pressures [0–700 bar]), logistic regression demonstrated no significant effects of homogenization, either upstream or downstream and irrespective of pressure, on inactivation. However, heating temp had a significant influence, with a 167-fold higher chance of detecting survivors after heating at 75°C (167°F) versus 72°C (161.6°F).
Raw milk. Pilot-scale turbulent flow pasteurizer. 60–90°C (140–194°F) for 6–15 s (Reynold's no. 3405). With and without homogenization *(106)*.	10^2 to 3.5×10^5 CFU/ml	Clear milk with sodium citrate. Pellet cells. Decontaminate using 0.75% HPC, 5h, 37°C (98.6°F); MPN (detection limit 0.023 cells/ml) using dilution series in MGIT with M7H9 broth with OADC enrichment, PANTA antibiotics, and mycobactin J (37°C [98.6°F], 16–50 wk). Confirm with two-target IS*900*-based real-time PCR.	No survivors at 72°C (161.6°F) for 6 s or 70°C (158°F) for 10 s or more stringent conditions (>4.2- to >7.1-log reduction, varies with inoculum concentrations).	No	Feces of Johne's-infected cows used as inoculum.

TABLE 7. *Continued*

Product and processing procedures	Inoculum (MAP/ml)	Recovery methods	Results, reduction	MAP survival postpasteurization[b]	Comments
Fresh, raw milk. Commercial-scale pasteurizer, turbulent flow (Reynold's no. 61,112). 72, 75, or 78°C (161.6, 167, or 172.4°F) for 15, 20, or 25 s, respectively, with homogenization *(81)*.	4.7×10^3 to 1.4×10^5 CFU/ml	Three decontaminations: 4 h HPC (H); 4 h HPC + VAN (HV); overnight HPC + VAN (OHV). Centrifuge milk, resuspend pellet in 0.75% HPC for 4 h at RT or 18–24 h 37°C (98.6°F). Centrifuge and resuspend. For HV and OHV, 18–24 h at 37°C (98.6°F). BACTEC 12B medium without PANTA PLUS (H only); BACTEC 12B with PANTA PLUS and HEYJ (all treatments).	>6-log reduction in 17/20 pasteurization trials at 72, 75, and 78°C (161.6, 167, or 172.4°F) for 15, 20, and 25 s (viable MAP not detected in 1.5 liters of pasteurized milk inoculated at 4×10^2 to 8×10^3 CFU/ml. MAP recovered from three trials: 72°C (161.6°F) for 15 s, 75°C (167°F) for 25 s, and 78°C (172.4°F) for 15 s at concentrations of 0.004 and 0.002 CFU/ml (4–6-log reduction). Recover by H and BACTEC 12B with and without PANTA PLUS.	Yes	MAP field isolates of RFLP strain C1 ($n = 4$) or RFLP strain C3 ($n = 5$).
Raw milk and pasteurized milk. Pilot-scale pasteurizer with turbulent flow (Reynold's no. not reported). Temp: 72.5–84.5°C. Hold times: 15, 25, 60 s. Homogenizer: none, upstream, in-hold *(53)*.	Estimated 10^1–10^5 CFU/ml; MAP not enumerated in untreated raw milk due to overgrowth of other organisms.	BACTEC 12B medium with mycobactin J and PLANTA PLUS. HEYM agar with mycobactin J and VAN. Incubated for up to 18 wk at 37°C (98.6°F). Colonies confirmed by PCR (IS*900*).	>4- to 5-log reduction. In-hold homogenization more effective in reducing counts. No differences between holding times at same temperature.	Yes	

TABLE 7. *Continued*

Product and processing procedures	Inoculum (MAP/ml)	Recovery methods	Results, reduction	MAP survival postpasteurization[b]	Comments
Ultrahigh temp pasteurized whole homogenized milk. Slug-flow pasteurizer unit and laboratory scale pasteurizer unit. (Reynold's no. not reported). Five time, temp combinations: 62.7°C (144.9°F), 30 min; 65.5°C (149.9°F), 16 s; 71.7°C (161.1°F), 15 s; 71.7°C (161.1°F), 20 s; 74.4°C (165.9°F), 15 s *(124)*.	10^8 and 10^5 CFU/ml with one of three different MAP field strains.	Low-speed centrifugation to pellet bacteria; plate to HEYM (37°C [98.6°F], up to 52 wk) and to supplemented BACTEC medium (37°C [98.6°F], 8 wk). HEYM colonies confirmed by IS*900* PCR, subculture to M7H9, plating to blood agar, and acid-fast staining; BACTEC growth confirmed by acid-fast staining. Enrichment in Dubos broth and subsequent inoculation on HEYM slants and BACTEC medium with confirmation.	Survivors found in 17/45 (38%) of slug-flow pasteurization samples and 12/45 (27%) of HTST trials with inoculum of 10^8 MAP/ml. At 10^5 CFU/ml, survivors present in 9/45 (20%) slug-flow and 8/45 (17.7%) HTST pasteurization samples.	Yes	Log inactivation ranged from <1 to >8, with an average of 5.0- and 7.7-log kill for low and high inoculum levels. Most experiments demonstrated efficient kill except for 65.5°C for 16 s (milk treatment for cheese production), for which inactivation was variable and less effective.
Raw milk and pasteurized milk. APV HXP commercial scale pasteurizer (capacity 2,000 liters/h; Reynold's no. 39,226). 12 trials. In each trial, milks subjected to four pasteurization treatments (73°C [163.4°F] for 15 or 25 s, with and without prior homogenization) *(50)*.	Naturally contaminated	Pellet bacteria, culture, and IMS-PCR. For culture, resuspended pellet decontaminated with CPC the day after treatment, inoculated into HEYM with mycobactin J and supplemented BACTEC Middlebrook 12B radiometric medium (37°C [98.6°F], 18 wk). Presumptive colonies confirmed by acid-fast staining, IS*900* PCR, requirement of mycobactin J for growth. IMS of original pellet, nucleic acid extraction, and PCR.	Viable MAP cultured from 4/60 (6.7%) of raw milk samples and 10/144 (6.9%) of pasteurized samples. MAP DNA was detected in 30/144 (20.8%) pasteurized samples. Fewer pasteurized samples contained viable MAP if milk homogenized prior to pasteurization, although not statistically significant.	Yes	MAP in naturally infected milk survives commercial scale pasteurization (73°C for 15 and 25 s) with and without prior homogenization if bacteria present in sufficient numbers before heat treatment.

TABLE 7. *Continued*

Product and processing procedures	Inoculum (MAP/ml)	Recovery methods	Results, reduction	MAP survival postpasteurization[b]	Comments
Raw milk. Continuous flow thermal pasteurization at various time, temp combinations: 68.1–90°C (154.6–194°F) for 17.6–60 s *(57)*.	Free MAP and MAP internalized in macrophages; 10^3–10^5 CFU/ml	Pellet bacteria; (1) enrich in Dubos broth (3–6 mo, 37°C [98.6°F]), detection limit 0.02 CFU/ml; (2) plate to HEYM (6–8 mo, 37°C [98.6°F]), detection limit 0.1 CFU/ml; confirm MAP by viability staining and IS*900* PCR.	68.1–79.1°C (154.6–174.4°F), 18 s: 77/282 (27.3%) experiments positive, survival was always below 1 CFU/ml, 2–6-log reduction; holding time, temp, and inoculum nos. significant, strain insignificant with respect to survival. 72–75°C (161.6–167°F), 18–30 s: 45/45 (100%) experiments positive, 3–6-log reduction; increased holding time did not enhance inactivation. 80–90°C (176–194°F), 18–19 s: 53/53 (100%) experiments positive, 4–6-log reduction. 72–90°C (161.6–194°F), 40–60 s: 48/48 (100%) experiments positive, 4–6-log reduction; holding time and heating temp were negatively correlated (significantly) with survival.	Yes	Five MAP field strains used. Internalization did not significantly influence inactivation; evidence that MAP was heat inactivated at >74°C for >33 s. Survival was independent of initial MAP count and time, temp of heating.
Raw milk. Pilot-scale pasteurizer (Reynold's no. 11,050). 63, 66, 69, and 72°C (145.5, 150.8, 156.2, and 161.6°F) for 15 s, duplicate trials *(102)*.	0.7×10^3 to 16×10^3 CFU/ml	Centrifugation. Pellet treated with 1% CPC (RT, 50 min), centrifuge, inoculate HEYM (20 wk, 37°C [98.6°F]) and BACTEC medium supplemented with PANTA PLUS, egg yolk, and mycobactin J. Confirmation: acid-fast staining, presence of IS*900*, mycobactin dependence.	No survivors at 72°C (161.6°F), 15 s; one strain survived 69°C (156.2°F) for 15 s (1.5 CFU/ml) in one of two trials. $D_{63^\circ C} = 15 \pm 2.8$ s $D_{66^\circ C} = 5.9 \pm 0.7$ s $D_{72^\circ C} = <2.03$ s (>7- log kill at 72°C, 15 s) $z = 8.6^\circ C$ >4-log reduction in milk inoculated (3.25×10^3) with fecal material	No	ATCC 19698 (type strain), ATCC 43015 (Linda, human isolate), and three bovine isolates. Properly maintained and operated equipment should ensure absence of viable MAP in pasteurized dairy products. Additional safeguard is commercial practice of operating 1.5–2°C (34.7–35.6°F) above 72°C (161.6°F).

TABLE 7. *Continued*

Product and processing procedures	Inoculum (MAP/ml)	Recovery methods	Results, reduction	MAP survival postpasteurization[b]	Comments
			from high MAP shedder with Johne's (1.3×10^7 CFU/g) and heated at 72°C (161.6°F) for 15 s. In milk spiked (20–32 CFU/ml) with feces from moderate shedder, MAP isolated (0.4 CFU/ml) after 69°C (156.2°F) for 15 s in one of two trials.		

[a] MAP, *Mycobacterium avium* subsp. *paratuberculosis*; PBS, phosphate-buffered saline; HEYM, Herrold's egg yolk medium; HPC, hexadecylpyridinium chloride; MPN, most probable number; MGIT, mycobacteria growth indicator tubes; M7H9, Middlebrook 7H9 medium; OADC, oleic acid, dextrose, catalase; PANTA, polymyxin, amphotericin B, nalidixic acid, trimethoprim, and azlocillin; VAN, vancomycin, amphotericin B, and nalidixic acid; RT, room temperature; PANTA PLUS, polymyxin, amphotericin B, nalidixic acid, trimethoprim, azlocillin, and 0.5% (vol/vol) glycerol; HEYJ, Herrold's egg yolk medium with mycobactin J; RFLP, restriction fragment length polymorphism; HTST, high temperature short time; IMS-PCR, immunomagnetic separation PCR; CPC, cetylpyridinium chloride.

[b] Treatments meeting legal requirements for pasteurization, e.g., 72°C (161.6°F) for 15 s.

Explanations proposed for survival have included clumping of cells, physiological adaptation resulting in acquisition of thermotolerance, or a change in the physicochemical environment of the organism *(43)*. MAP has a natural tendency to form clumps of cells due to the hydrophobic nature of the cell wall. The organism is likely to be present in clumps in raw milk contaminated with feces from dairy cattle *(1)*. Clumps may be created or dispersed during processing; and this can have an effect on the determination of the initial number of cells as well as the number of cells recovered, which impacts the accuracy of thermal inactivation calculations. Detection of survivors after thermal inactivation is influenced by the enumeration method(s) used. For example, Hammer et al. *(57)* reported that sonication and decontamination resulted in lower recovery of MAP. Grant et al. *(50, 53)* indicated that homogenization increased the lethality of subsequent pasteurization, although Hammer et al. *(58)* found no effect of either upstream or downstream homogenization.

It has been postulated that clumping is responsible for the nonlinear thermal inactivation kinetics in laboratory studies *(45, 48)*. Using viability staining, Hammer et al. *(57)* found surviving MAP only in clumps. The significance of the size and shape of clumps has not been assessed *(58, 80)*. However, heat penetration calculations suggest that the time to reach equilibrium temperature in bacterial clumps is only a few hundredths of a second *(24)*. Klijn et al. *(73)* have suggested that survival may be related to the fraction of clumps with large cell numbers. The number of cells in clumps in fecal material (a potential source of MAP in milk) is low, but clumps from liquid cultures used in thermal resistance studies may have many layers of cells *(58)*. Fecal contamination should be negligible in commercial milk produced under the Pasteurized Milk Ordinance in the United States.

However, clumping alone is unlikely to account for the survival observed in many thermal inactivation studies *(43)*. Adaptation to heat stress has been postulated to account for survival at the high temperatures reported in some studies. However, survival at 90°C (194°F) would require a 1,000-fold increase in heat resistance, which is unlikely *(43)*. Another mechanism for survival could be related to the fact that vegetative bacteria can survive high heat under reduced moisture conditions, which occurs when cells are present in a lipid matrix, e.g., fat globules in milk. One or more of these hypotheses may explain the observed thermal resistance of MAP, but the true mechanism remains elusive.

It has been postulated that microorganisms may be more resistant to heat when sequestered in macrophages. Stabel *(121)* harvested mammary gland macrophages from noninfected control animals and infected them in vitro with MAP. Infected macrophages were subsequently added to raw milk that was treated for 15 s at 72°C (161.6°F). Culturing of this milk after treatment failed to yield viable MAP. Further experiments by Hammer et al. *(57)* utilized an experimentally infected mouse phagocytic cell line; macrophages containing MAP were added directly to milk samples, utilizing an identical aliquot as a control. The

results of this study demonstrated no significant difference between the survival of MAP in macrophages and the control (free bacterial cells) after treatment for 17.6 to 18.8 s at 70.7 to 76.6°C (159.26 to 169.88°F). As with other proposed survival mechanisms, survival of MAP at high temperatures cannot be entirely explained by protection of bacterial cells present in macrophages.

In summary, thermal inactivation studies on MAP have shown conflicting results, while MAP has been detected in retail samples. As noted by Hammer et al. *(58),* "despite all uncertainties regarding complete inactivation of [MAP] during heat treatment and possible reasons for survival, a reduction of at least 5 to 7 $\log_{10}$ cycles could be demonstrated in whole milk, skim milk and cream. In the framework of the Codex Alimentarius for samples of pasteurized milk, this should be fully sufficient." Pearce et al. *(102),* using data from raw milk obtained in New Zealand, the United Kingdom and the Netherlands along with inactivation data from an earlier study *(64),* applied a mathematical modeling approach to assess the probability of MAP occurring in pasteurized milk. These investigators reported in an abstract *(64)* that good milking practices combined with properly operated and validated pasteurizers treating milk at 72°C (161.6°F) for 15 s will result in an extremely low probability of viable MAP being present in the pasteurized product. A publication by Cerf et al. *(13)* described a quantitative model that estimates the probability of finding MAP in pasteurized milk (50-ml samples) in industrialized countries as less than 0.54%, even if interherd and intraherd prevalence are high, provided that MAP numbers in raw milk are low (mean concentration 10 MAP/ml). Thus, it appears that regardless of whether MAP is present in pasteurized milk due to survival of the process or due to recontamination, both the prevalence and the numbers of MAP in pasteurized product will be low. The public health significance of low numbers of survivors or any level of MAP contamination is not known. At this time, there are insufficient data to conclude that increasing the pasteurization times and temperatures would reduce the presence of MAP in milk.

MAP inactivation during cheese manufacture. Spahr and Schafroth *(120)* found that MAP survived for at least 120 days in semihard cheeses manufactured with raw milk inoculated with MAP. The number of organisms present decreased in both hard (Swiss Emmentaler) and semihard (Swiss Tisliter) cheeses over the ripening period. The *D*-value in the hard cheese was 27.8 days, with an expected 10^4 reduction at 120 days. The *D*-value was 45.5 days in semihard cheese, with approximately a 10^3 reduction over the same 120-day period.

Cheddar cheese was made from pasteurized milk that was artificially contaminated with either high (10^4 to 10^5 CFU/ml) or low (10^1 to 10^2 CFU/ml) numbers of MAP and cultured over 27 weeks *(32)*. If levels >3.6 log CFU were present in 1-day cheeses, then in all cases MAP was culturable at 27 weeks. The calculated *D*-values ranged from 90 to 107 days. At low levels of contamination, cheese inoculated with one of three strains of MAP was positive for MAP at 27 weeks.

Sung and Collins *(130)* evaluated rates of MAP inactivation in Hispanic-style, soft white cheese (queso fresco). Pasteurized milk was inoculated with 10^6 MAP/ml (non–heat treated or heat treated at 62°C [143.6°F] for 240 s), and the curd was sampled weekly for up to 4 weeks. MAP was cultured using BACTEC and confirmed by PCR. The investigators reported that the *D*-value in queso fresco was 36.5 days for heat-treated MAP and 59.9 days for non–heat-treated MAP; however, the final counts (4.12 $\pm$ 0.55 log CFU/ml) differed from the initial count (4.64 $\pm$ 0.06 log CFU/ml) by less than 1 log for non–heat-treated samples and for heat-treated samples (4.79 $\pm$ 0.14 log CFU/ml initial and 3.96 $\pm$ 0.16 log CFU/ml final).

Many cheeses in certain countries are made with raw milk, while in the United States cheeses are primarily made with either pasteurized or with thermized milk. Stabel and Lambertz *(124)* studied the effect of thermization (65.5°C [149.9°F] for 16 s) of milk inoculated with 10^5 or 10^8 CFU/ml using a slug-flow pasteurization unit and with an HTST pasteurizer. Survival was observed for the three strains of MAP studied at both inoculum levels (<1- to 4-log reduction with the slug-flow pasteurizer and 2- to 6-log reduction with the HTST unit). Survival was also observed following pasteurization, but to a much lesser extent (depending on inoculum level, 4- to >8-log reduction with the slug-flow pasteurizer and >5- to >8-log reduction with the HTST unit). However, there are a number of factors other than heat treatment that affect microbial survival during cheese manufacture, including salt content, pH, ripening period, and ripening temperature *(120, 124, 130)*.

Inactivation by other processes. Other processes have been explored to determine if they can effectively inactivate MAP. Using high hydrostatic pressure, MAP was reduced by an average 4 log CFU/ml after treatment with 500 MPa for 10 min at 5°C (41°F) and at 20°C (68°F) *(77)*. Donaghy et al. *(29)* obtained a reduction of 6.52 log CFU/ml with 500 MPa for 10 min at 20°C (68°F) and a reduction of 5.03 log CFU/ml for a 5-min treatment.

Stabel et al. *(126)* have shown that viable MAP could not be recovered from milk inoculated with 10^4 and 10^8 CFU/ml and exposed to either 5 or 10 kGy of radiation. Altic et al. *(3)* studied the effects of UV irradiation on the elimination of MAP from whole and semiskim cow's milk. These authors determined only 0.5- to 1.0-log reduction occurred at a dose of 1,000 mJ/ml, which is thought to be the maximum dose before unacceptable organoleptic changes occur in milk. Even doses as high as 2,860 mJ/ml resulted in only a maximum 2.8-log reduction. Thus, the use of UV light treatment of milk is not an acceptable alternative to current pasteurization processes for liquid milk.

Rowan et al. *(113)* studied the effect of pulsed electric fields (PEF) on inactivation of MAP inoculated into sterile cow's milk. PEF treatment at 50°C (122°F) (2,500 pulses at 30 kV/cm) resulted in a 5.9-log reduction, compared to a 0.01-log reduction observed for heating at 50°C (122°F) for

25 min; heating at 72°C (161.6°F) for 25 s (extended HTST pasteurization) resulted in a 2.4-log reduction.

Chlorine inactivation. Given that MAP may contaminate water sources that could be used in foods and food processing, chlorination of water may be an important intervention for reducing exposure. Whan et al. *(146)* determined that when high numbers (10^6 CFU/ml) of MAP were exposed to free chlorine concentrations of up to 2 μg/ml for 15 or 30 min, survivors remained. In this case, log reductions ranged from 1.32 to 2.85 depending on strain, chlorine level, contact time, and the presence or absence of organic matter.

5. What are the research needs?

Based on a review of the literature, including the research needs identified by other groups *(43, 136),* the Committee identified the following research needs. An underlying requirement to facilitate meeting future research needs is the development and standardization of rapid, sensitive, and specific methods to detect and enumerate MAP in a variety of matrices. This means that molecular-based assays will probably be the detection approach of choice. Specifically, the following issues should be addressed: (i) detection of viable versus nonviable cells; (ii) sample preparation prior to detection (concentration and decontamination); (iii) the most efficient manner by which to lyse the cells and extract the nucleic acid(s); (iv) interpretation of positive and negative test results; and (v) standardization and determination of a ''gold standard.''

The Committee decided to combine the two questions below and identified several supporting research needs:

a. What are the additional sources of MAP?
b. What are the frequencies and levels of MAP contamination in specific sources of concern?

The estimated MAP prevalence in U.S. beef herds is 7.9% *(23)*. Beef cattle are the source of the majority of ground beef. MAP prevalence in U.S. dairy herds is estimated at approximately 22% *(137)*. Approximately 10% of ground beef comes from culled dairy cows. Studies are needed to determine the prevalence, numbers of MAP, and thermal inactivation of MAP in meat products. Statistically rigorous studies proportionately representing the herds in the major beef and dairy states are needed to determine herd prevalence.

Of additional interest are cheeses made from raw milk. Studies are also needed on the frequencies and numbers of MAP in fresh produce (fruits and vegetables) and fish from areas of high and low domestic ruminant prevalence.

Water contaminated with MAP can serve as a transport mechanism on-farm to spread infection among domestic ruminants and exposed wildlife and between farms to spread infection to downstream areas and watersheds. Along with direct manure application, water can contaminate animal forage and produce intended for human food at considerable distance from infected herds. Research is needed to clarify the importance of each of these potential water-associated mechanisms in human exposures to MAP. The suitability of agricultural water for its intended use (e.g., animal drinking water, irrigation water, water for application of pesticides) needs to be studied in areas of high and low MAP prevalence and with different soil filtering characteristics.

In addition, the role of water as a direct source of MAP to humans is unknown. For example, there is a need for a large-scale prevalence study of U.S. municipal water supplies to determine the prevalence and number of MAP both pre- and posttreatment. There are no studies that have determined if well water and other ground water sources may serve as potential sources of MAP; however, unprotected wells have been known to become contaminated with pathogens from surface waters. In addition, there are no data on the prevalence or number of MAP in recreation waters, including lakes, ponds, swimming pools, and water parks.

Insects and rodents have been found contaminated with or infected with MAP, but their role in transmitting and/or maintaining the presence of MAP on a premise is unknown and should be further investigated.

Information is needed on the presence and survival of MAP in food processing facilities. If MAP becomes incorporated in biofilms, this could provide a source of pre- or postprocess contamination, but this has not yet been studied.

Additional information is needed about the physiology and metabolic states of MAP in various environments and in response to physical conditions contributing to survival and persistence. The significance of amoebae as a reservoir for MAP should be further investigated.

c. What are the potential processing interventions to eliminate or reduce the levels of MAP contamination?

There is a need for well-controlled studies that will determine the effect of pasteurization on naturally occurring MAP using equipment and conditions representative of commercial processing using standardized laboratory procedures. In this regard, note the Committee's statements on factors that have complicated previous studies.

Standardization of methods used to document efficacy of processes for interlaboratory studies is needed.

If it is determined that municipal water is a source of exposure for humans to MAP, studies are needed to determine effective decontamination procedures.

d. What are the potential pre- and postharvest interventions to prevent MAP from entering the food supply?

Preharvest controls include ways by which to eliminate the transmission of MAP within cattle populations. Identification and quarantine of infected animals is ideal; however, commercial detection methods are much more adept at detecting animals that are clinically infected and/or shedding at high levels. More sensitive clinical detection

methods are necessary. Research on the sensitivity and specificity of various cell-mediated immune diagnostic tests is indicated. Related to this are difficult issues of sampling strategies at both the herd and individual animal level *(19)*.

Strategies to protect newborn and newly acquired calves from contaminated feces, colostrum, milk, and environments have been developed, and following these strategies rigorously has been documented to reduce frequency of infection *(18)*. However, additional work on appropriate implementation strategies is necessary, and additional progress awaits better diagnostic techniques. Identification of methods to control environmental spread (composting, decontamination, water treatment) are needed. A promising control technology would be vaccination, but this is currently in the early stages of implementation. Finally, a standard animal model will be necessary in order to facilitate evaluation of many of the emerging preharvest control strategies.

Filtration is an emerging technology for the production of fluid milk. Currently filtration, especially ultrafiltration, is more commonly used for the treatment of raw milk for ice cream and cheese making. While data are not available specifically for the influence of these technologies on MAP contamination, they have been documented to reduce contamination by other microflora *(116)*.

6. Additional research needs?

The USDA Agricultural Research Service currently is engaged in using information from the MAP genome sequencing project to conduct research on genetic variability, identification of antigens, and transcriptional profiles. This could lead to progress in identification of epitopes from which antibodies or other ligands could be developed for bioseparation strategies that would improve detection and enumeration as well as validate processing interventions to remove MAP from foods and water *(135)*.

The process of risk assessment provides information on the extent and characteristics of the risk attributed to a hazard. An important step preceding a quantitative risk assessment is completion of a risk profile, a document intended to provide background information about the food, the hazard, and the potential risk posed. At the very least, there is a need to complete a risk profile for MAP in milk due to the current availability of data. Similar risk profiles should be conducted for meat, water, produce, fish, and shellfish when data become available. If warranted this could lead to more formal risk assessment efforts in the future. A risk assessment approach to the MAP problem will aid in the identification of information gaps, prioritization of research needs, and, if quantitative, can be used to evaluate, on a preliminary basis, the potential value of candidate intervention strategies.

CONCLUSIONS

The Committee determined that current methods of detection of MAP suffer from significant limitations. A standard method for detection of viable MAP needs to be developed and adopted by researchers in order to accurately determine the presence and numbers of MAP in foods and other potential sources of exposure.

The body of evidence indicates that absent infected domestic ruminant animals, MAP is rarely found. Thus, as with brucellosis, if MAP in cattle is controlled, the source of MAP in other animals, food, and water may largely be eliminated. Based on a comprehensive review of the current literature, the Committee concludes that milk, particularly raw milk, is a potential food source for human exposure to MAP. Thermal processes have been demonstrated to achieve a 4- to 7-log reduction in the number of MAP cells when using milk inoculated with high numbers of MAP. This level of thermal inactivation should be adequate to inactivate the numbers of MAP estimated to be present in raw milk. Nevertheless, several studies, including one using naturally contaminated raw milk, have detected survivors after time and temperature combinations used in commercial pasteurization in the United States and Europe. Several credible published reports described the isolation of MAP from commercially pasteurized milk. At this time, it is not known if such findings are due to MAP surviving the pasteurization process or to postpasteurization contamination. No studies examined retail raw milk for MAP; however, there are several reports that document the presence of MAP in bulk raw milk tanks. Thus, based on current methodology and available studies, it can be concluded that a small percentage (<3%) of commercially pasteurized milk may contain small numbers of culturable MAP cells.

Based on the current detection methodology, cheese made from pasteurized milk is unlikely to be a significant source of exposure to MAP. The Committee believes that pasteurization and ripening combine to greatly reduce the presence of viable MAP in pasteurized milk cheeses. The Committee was unable to assess exposure from raw milk cheeses. The Committee could not find any peer-reviewed data on the presence of MAP in other milk products such as butter, ice cream, cottage cheese, and yogurt. However, if these products are made from pasteurized milk, they are unlikely to be a significant source of MAP.

Ground beef may be another potential food source of MAP; the limited studies available have not detected viable MAP in ground beef. However, culled dairy cattle likely have a significantly higher prevalence of MAP infection compared to beef cattle. The Committee was unable to obtain precise figures for the contribution of meat from culled dairy cattle to the U.S. ground beef supply; however, one source estimated that culled dairy cows account for up to 10% of the ground beef supply. Neither of the studies detected viable MAP in ground beef, and there are no studies on the potential for MAP survival after cooking. The potential for exposure to MAP by consumption of ground beef, particularly containing lymph nodes, needs further study.

Numerous studies report that MAP is prevalent in the environment. It survives in water and soil and is found in many wild animals. Since cattle appear to be a significant source of MAP in the environment, farm runoff may

potentially contaminate irrigation water. For this reason, some food items such as fruits and vegetables that come in contact with an MAP-contaminated environment could result in exposure in humans. The significance of fruits and vegetables as a source of human exposure needs to be further investigated.

There is no information to indicate that treated municipal drinking water is likely to be a significant source of human exposure to MAP; however, further study is needed. Limited studies of water supplies in other countries have not detected MAP in treated water, although MAP has been detected in untreated source water. There are no studies on MAP in the U.S. water supply.

Although it is clear that humans may be exposed to MAP through various routes, including foods and the environment, the frequency and amount of exposure are not known, and research to address these issues is needed. Such research would be critical if it is determined that MAP presents a risk to public health at the likely levels of exposure.

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