SYMPOSIUM: HEALTH AND SAFETY ON THE DAIRY FARM

Johne's Disease and Milk: Do Consumers Need to Worry?

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ABSTRACT

Mycobacterium paratuberculosis, an acid-fast bacillus that causes enteritis in ruminants, has been suggested as an etiological agent of Crohn's disease in humans. The mode of transmission is unclear; however, some evidence suggests that humans may become infected via contaminated milk. Currently, it is not known whether commercial pasteurization effectively kills *M. paratuberculosis* in contaminated raw milk. Using a laboratory-scale pasteurizer unit designed to simulate the high-temperature, short-time method (72°C, 15 sec) currently used by commercial dairies, we previously demonstrated that treatment of raw milk inoculated with 10^4 to 10^6 cfu of *M. paratuberculosis*/ml reduced numbers to an undetectable level. However, *M. paratuberculosis* is an intracellular pathogen that resides within the macrophages of the host and evades destruction. We subsequently performed further experiments examining heat treatment of milk inoculated with mammary gland macrophages containing ingested *M. paratuberculosis*. Heat treatment of these samples under high-temperature, short-time conditions demonstrated that the macrophage does not protect the organism because we were unable to recover any viable *M. paratuberculosis* from the samples. Conversely, other researchers have demonstrated that a residual population of *M. paratuberculosis* may survive heat treatment of milk. In addition, a recent news report stated that viable *M. paratuberculosis* organisms have been cultured from retail-ready milk in Ireland. A summary of past and current studies concerning this issue along with a discussion of methodologies used to recover *M. paratuberculosis* from experimentally inoculated milk will be presented in this paper.
(**Key words:** *Mycobacterium paratuberculosis*, Johne's disease, milk, pasteurization)

Abbreviation key: HEYM = Herrold's egg yolk medium; **HTST** = high-temperature, short-time.

INTRODUCTION

The first significant recognition of paratuberculosis (Johne's disease) in cattle was described by Johne and Frothingham in 1895 (15) when they successfully isolated acid-fast bacilli from sections of diseased intestine from cattle with severe enteritis. Subsequently, the causative agent in paratuberculosis, a chronic, granulomatous enteritis of ruminants, was demonstrated to be the acid-fast bacterium, *M. paratuberculosis* (30, 31). Clinical paratuberculosis is characterized by profuse, nontreatable diarrhea, emaciation, and eventual death. During the course of the disease, the intestine becomes thickened and corrugated, disallowing proper absorption of nutrients. Although cattle usually become infected as calves by ingestion of feces and milk contaminated with *M. paratuberculosis* or by in utero transmission from infected dams, clinical signs of the disease may not manifest themselves until the animals reach 3 to 5 yr of age (6, 17). Indeed, not all infected cattle will develop clinical disease. Stressors such as parturition, lactation, dietary deficiencies, or parasitemia may precipitate clinical disease. Paratuberculosis represents a significant economic loss to the dairy industry since incidence of this disease has been reported in 20 to 40% of dairy herds in the United States (32). In addition to losses incurred by culling of clinical animals from the herd, producers are faced with further economic loss from cows with subclinical paratuberculosis through an increased incidence of mastitis, decreased milk production, and increased calving intervals (24).

DISCUSSION

It is unclear at this time if *M. paratuberculosis* is a human pathogen and what potential danger it may present to consumers exposed to dairy or meat products from infected animals. The questions raised by this issue have been: do cows shed enough *M. paratuberculosis* in their milk to be a significant threat to consumers; does *M. paratuberculosis* survive pasteurization; and is *M. paratuberculosis* a zoonotic agent and the causative factor in Crohn's disease in human beings? To address the first question: cows with clinical disease or asymptomatic cows with heavy fecal shedding shed viable *M. paratuberculosis* in their milk, albeit at low concentra-

tions (5 to 8 cfu/50 ml of milk) (28, 29). In addition, a significant dilution of the milk from cows shedding the organism can occur between the individual animal to the bulk tank to the holding tank at the processing plant where milk from many farms may be mixed together. In 1996, our laboratory conducted an add-on study to the National Animal Health Monitoring System Dairy Survey that included a comprehensive study of the prevalence of Johne's disease in dairy herds in the United States. Based on the serologic analysis, 69 dairy herds that had tested positive for paratuberculosis were included in the add-on study. Bulk tank milk samples were collected every 2 wk for a 6-wk period and shipped frozen to us for culture and PCR analysis. After 12 mo of culture we were unable to detect any *M. paratuberculosis* in these milk samples and, furthermore, were unable to pick up any positive PCR signal verifying the presence of *M. paratuberculosis* in these samples. Our conclusions were that dilution of *M. paratuberculosis* organisms in milk was too great to allow detection by either of these methods. Fecal contamination from cows that are shedding high numbers of *M. paratuberculosis* and remain on the milking line may be a more significant factor in contamination of the milk supply (20).

Several species of mycobacteria, including *M. fortuitum*, *M. avium-intracellulare*, *M. cheloni*, and *M. kansasii* have been found in intestinal biopsy tissue from Crohn's disease patients. *Mycobacterium paratuberculosis* has also been identified by primary isolation from intestinal tissue as well as PCR analysis of DNA specific for this organism. Because the clinical symptoms of Crohn's disease closely mimic those found in animals with paratuberculosis in the late stages of disease it has been proposed by a number of investigators that *M. paratuberculosis* may be the causative agent of this disorder in human beings. A summary of the studies that have evaluated the potential association between Crohn's disease and *M. paratuberculosis* through PCR analysis of tissues from human beings with inflammatory bowel disease is presented in Table 1. Of the studies that have been published to date, only two have described the definitive detection of *M. paratuberculosis*-specific DNA in Crohn's disease patients and not in samples from noninflammatory bowel disease control patients run concurrently (7, 11). In fact, most of these studies demonstrate a failure to detect *M. paratuberculosis* DNA in patients with Crohn's disease, or positive detection was observed for both Crohn's disease patients and the nondiseased controls (1, 3, 4, 8, 10, 12, 18, 21, 22, 23, 26). These data would suggest a lack of a definitive association between *M. paratuberculosis* and the incidence of Crohn's disease in human beings.

The potential relationship between Crohn's disease and *M. paratuberculosis* has become an issue for the

Table 1. Detection of *Mycobacterium paratuberculosis* DNA by PCR in intestinal samples from Crohn's disease patients and nondisease control patients.

	Positive samples	
Investigator	Crohn's disease	Controls
Al-Shamali et al., 1997 (1)	0/10	
Cellier et al., 1998 (3)	0/47	0/20
Chiba et al., 1998 (4)	0/30	
Clarkston et al., 1998 (7)	1/11	0/3
Dell'Isola et al., 1994 (8)	13/18	6/24
Dumonceau et al., 1996 (10)	0/36	0/23
Fidler et al., 1994 (11)	4/31	0/20
Frank and Cook, 1996 (12)	0/27	0/11
Lisby et al., 1994 (18)	11/24	3/28
Rowbotham et al., 1995 (22)	0/68	1/26
Riggio et al., 1997 (21)	0/7	0/12
Sanderson et al., 1992 (23)	26/40	5/40
Suenaga et al., 1995 (26)	10/10	14/16

dairy industry since the publication of a 1994 report by a group in the United Kingdom that suggested that viable *M. paratuberculosis* were present in pasteurized milk purchased from retail markets (19). Careful analysis of this report reveals that these researchers were unable to culture the viable organism even after 40 mo of incubation. They were able to demonstrate that 9 of 18 milk samples that were previously positive for PCR and 6 of 36 milk samples that were previously negative for PCR were confirmed PCR positive after the long-term culture period. The finding that samples previously negative for PCR yielded positive signal after incubation was interpreted by the authors to mean that a slow-growing residual population of *M. paratuberculosis* survived the pasteurization process. This work was preceded by the first publication that reported *M. paratuberculosis* could survive heat treatment at 65°C for 30 min or 72°C for 15 s in laboratory experiments (5). Results from these studies were highly controversial because of the potential characterization of *M. paratuberculosis* as a zoonotic pathogen due to its association with Crohn's disease and sparked a flurry of studies to determine optimal time and temperature conditions for heat inactivation of *M. paratuberculosis* in raw milk.

The first report to follow this work evaluated the effects of two methods of pasteurization: 1) the standard holder method (63.5°C for 30 min) and 2) the high-temperature, short-time method (**HTST**; 71.7°C for 15 s) on killing of *M. paratuberculosis* (14). In this study raw milk was inoculated with 10^4 or 10^7 cfu of *M. paratuberculosis*/ml then subjected to heat treatment. For each of the methods, the numbers of *M. paratuberculosis* were significantly reduced; however, in each series of experiments a residual population of viable organisms could be cultured from the milk. The authors suggest that this population of survivors may represent a heat-

resistant fraction of the *M. paratuberculosis* inoculum; that theory, however, remains untested. More likely, the surviving population of *M. paratuberculosis* resulted from inadequate heat penetration of a portion of the milk during the laboratory experiments. The Franklin laboratory-scale pasteurizer unit utilized for the HTST pasteurization experiments was designed to simulate the heating and cooling profiles of commercial pasteurization units. However, heat exchange was performed by placing the unit in a circulating water bath heated to specified temperature and milk remained static within the unit during heat treatment. Commercial pasteurizers employ a series of tubes and plates through which the milk product passes during heat treatment generating a turbulent flow of the product and more uniform penetration of heat through the product. A more recent study published by this group (13) evaluated the effectiveness of HTST pasteurization using the Franklin laboratory-scale pasteurizer unit on inactivation of raw milk containing lower levels of *M. paratuberculosis* (10 to 10^3 cfu/ml of milk). In this study, viable *M. paratuberculosis* were detected by culture in either liquid medium or on solid medium from raw milk initially spiked with 10^2 or 10^3 cfu/ml after heat treatment at 72°C for 15 s. However, no viable organisms were isolated from HTST-treated milk spiked with either 10 cfu/ml or 10 cfu/50 ml of milk. These authors determined that growth in liquid medium (BACTEC Middlebrook 12B radiometric medium) was more sensitive and more rapid than conventional solid agar medium (Herrold's egg yolk medium; **HEYM**). The main conclusion from this study (13) was that *M. paratuberculosis* may survive HTST pasteurization if present in numbers greater than 10^2 cfu/ml but will be inactivated by HTST if present in numbers lower than 10 cfu/ml of milk.

Further comparison of the holder method (65°C for 30 min) and HTST (72°C for 15 s) was performed by another laboratory (25). Again, raw milk was inoculated with high levels (10^4, 10^6, or 10^8 cfu/ml of milk) of *M. paratuberculosis* and subjected to various time and temperature combinations. Results from experiments evaluating the holder method using snap-cap polystyrene test tubes immersed in a shaking water bath demonstrated that a residual population of viable bacteria remained after treatment at 65, 72, 74, or 76°C for 0 to 30 min. These results were similar to those observed by Grant et al. (13) when a stoppered glass test tube model was employed to simulate the holder method. In contrast, experiments conducted with the laboratory-scale pasteurizer unit demonstrated that treatment of raw milk at 72°C for 15 s effectively killed all *M. paratuberculosis*. The laboratory-scale pasteurizer unit used in this study was designed to simulate the flow-through

technology of commercial pasteurizer units in which the milk flows through a series of stainless steel plates which rapidly heat or cool the product. The holding tube is designed in a curved fashion to encourage turbulent flow of the product during the 15-s heat treatment period. Results with these two different methodologies would indicate that active flow of the milk product during the heating process is critical for adequate killing of contaminating *M. paratuberculosis*.

In an effort to determine *D* values for *M. paratuberculosis* in milk (decimal reduction time; the time required to kill 1 log concentration of bacteria), both human and bovine strains were subjected to heat treatment at 62, 65, 68, and 71°C for 30 min (27). A *D*-value is used by the industry to estimate killing efficiency of different loads of bacterial contamination in milk. Methodology utilized in this study involved preheating the milk to the desired holding temperature, transferring it to Wheaton vials, inoculating with 10^6 cfu of *M. paratuberculosis*/ml of milk and sealing the vials. The vials were then immersed in a water bath and removed at various time points during heating. Viable *M. paratuberculosis* were estimated using BACTEC Middlebrook 12B radiometric culture medium. Thermal death curves indicated *D* values of 38.9 and 12.9 s to kill 1 log of *M. paratuberculosis* at 65 and 72°C, respectively. These results suggest that *M. paratuberculosis* would be effectively killed by the holder method of pasteurization but would survive current HTST pasteurization if present in numbers greater than 1 log of bacteria, similar to findings previously reported by Grant et al. (13). However, use of vials to perform HTST pasteurization is not fully acceptable and does not adequately simulate heat penetration that occurs during continuous-flow HTST processing in commercial dairy processing plants.

The effects of clumping or declumping of *M. paratuberculosis* on the rate of inactivation by heat treatment was also determined experimentally (16). In its natural state, *M. paratuberculosis* is found in large clumps and not as a singular bacteria so it was a concern whether this clumping would disallow efficient heat penetration during pasteurization. The investigators used a syringe and passed the bacterial suspension through a 26-gauge needle 20 times to declump before adding it to UHT milk for a final concentration of 10^6 cfu/ml. The authors used UHT milk to avoid confounding growth of contaminants present in raw milk. The thermal resistance of clumped or declumped cell suspensions was determined in sealed glass capillary tubes immersed in a water bath at 55, 58, 60, 63, and 72°C. Both clumped and declumped bacterial suspensions survived heat treatments in milk at 55, 58, and 60°C for up to 12 min and for 6 min at 63°C. The D values of clumped and

declumped *M. paratuberculosis* suspensions did not differ significantly in this study. Further, clumping of *M. paratuberculosis* did not affect the survival rate after heat treatment at 63°C for 30 min or 72°C for 15 s with no growth observed after culture on Middlebrook 7H11 agar for 4 mo or HEYM for 2 mo.

Because *M. paratuberculosis* is an intracellular pathogen which resides within the host macrophage it has been questioned whether this provides some protection of the organism from heat penetration during the pasteurization process. We have conducted studies in which bovine mammary gland macrophages were harvested from noninfected control animals, infected in vitro with *M. paratuberculosis* and subsequently added to raw milk and the milk was run through the laboratory-scale pasteurizer unit (unpublished data). We were unable to culture any viable *M. paratuberculosis* from the milk after heat treatment at 72°C for 15 s indicating that the organism does not escape heat inactivation simply because of its intracellular nature. Similar findings have been observed for *Listeria monocytogenes*, another intracellular pathogen which can be found in milk and milk products (9).

Thus far these reports are conflicting and difficult to interpret. Criticisms of each of these studies can and have been made by other researchers, the dairy industry as well as concerned consumers, yet each of the methodologies employed by these researchers have been previously utilized and established as acceptable methods by other laboratories evaluating heat resistance of pathogens in milk. Experimental protocols to simulate the holder method of pasteurization in these studies can probably provide us with reliable estimates of the heat inactivation of *M. paratuberculosis* in raw milk. The majority of studies presented here suggest that *M. paratuberculosis* are adequately killed with holder methods. However, the methods employed to simulate HTST pasteurization are not fully acceptable. To meet current standards for HTST pasteurization, laboratory-scale units must employ a series of stainless steel plates to form a regeneration section, a heating section and a cooling section. In addition, a curved holding tube of specific dimensions must be an integral part of the apparatus to generate turbulent flow of the milk product during the heating process. The laboratory-scale pasteurizer unit used in studies by Stabel et al. (25) complies with these specifications and most closely simulates HTST pasteurization as it occurs in US dairy processing plants today. Studies with this unit have shown that HTST pasteurization effectively kills all *M. paratuberculosis* present in raw milk.

Sensitivity of methods used to recover viable *M. paratuberculosis* from heat-treated milk has been another concern. Most of the laboratory studies utilized HEYM to enumerate viable bacterial counts; however, two laboratories also reported use of the BACTEC liquid medium. Each of these laboratories independently reports that this culture method is more sensitive than solid medium. We attempted to use this method in our laboratory as well with little success because the results were confounded by a significant overgrowth by other organisms present in the milk. Both of the other laboratories utilized some form of decontamination protocol or introduced antibiotics into the medium to retard some of this nonspecific growth. A newer technology has been developed and is currently being evaluated for use in the isolation of *M. paratuberculosis* from milk (13). This method involves capture of the bacteria on immunomagnetic beads coated with a polyclonal antibody against *M. paratuberculosis* followed by release of bacterial DNA from the beads and use in a PCR test. The immunomagnetic beads had a maximum binding capacity of 10^5 cfu of *M. paratuberculosis* and a sensitivity threshold of 10 cfu of *M. paratuberculosis*/ml. This method is about 10-fold more sensitive than current culture technology and circumvents the associated problem with necessary decontamination of raw milk samples; however, the test fails to differentiate dead or live *M. paratuberculosis* in the milk sample. Currently, studies are continuing in a number of laboratories to improve the sensitivity of detection of viable *M. paratuberculosis* in milk.

Lastly, a press release recently announced the successful isolation of viable *M. paratuberculosis* from 10 of 31 raw milk samples and 6 of 31 pasteurized milk samples obtained from local dairy processors in the United Kingdom (2). Due to the significant overgrowth by fungi or other nonacid-fast bacteria in culture, the immunomagnetic separation-PCR test was used to confirm the presence of *M. paratuberculosis* in milk. Efforts to subculture and further identify suspect cultures have not been successful at this point. However, due to the concern for public safety a nationwide survey of the microbiologic quality of raw and pasteurized cow's milk has been initiated in the United Kingdom. This survey is expected to take at least 18 mo to complete, with over 1000 milk samples collected and examined for a range of bacterial contamination.

In the United States, the USDA continues to collaborate with the dairy industry over this concern. We will continue to improve the sensitivity of our detection methods for *M. paratuberculosis* in milk. In addition, we are initiating studies with local dairy farms with confirmed Johne's disease to evaluate the degree of shedding of *M. paratuberculosis* into the milk on an individual animal basis and to evaluate effectiveness of on-farm heat treatment of milk and colostrum fed to neonate calves. Results from these studies will help

improve on-farm management of Johne's disease and reduce the spread of infection to young calves.

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