Development of improved method for isolation of *Mycobacterium avium* subsp. *paratuberculosis* from bulk tank milk: Effect of age of milk, centrifugation, and decontamination

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Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of Johne's disease in cattle and it has been suggested that this organism may be associated with Crohn's disease in humans. Cows at the advanced stage of the disease shed this organism into both their milk and feces. The objective of this study was to develop a more efficient procedure for isolating MAP from bulk tank raw milk. Bulk tank raw milk (50 mL) samples 3 to 13 d old after collection without spiking were investigated to evaluate the effects of milk age on the efficacy of decontamination. Milk samples, 2 to 3 d old, were seeded with MAP at levels of 50 to 200 colony forming units/mL in experiments involving factorial design to evaluate 1) the effects of different decontaminating reagents and decontamination procedures on recovery of MAP, and 2) partition MAP in milk fractions after centrifugation in raw milk. Decontamination in 20 mL of 0.75% hexadecylpyridinium chloride (HPC) at room temperature (22°C) for 2 to 5 h, with shaking, at intervals was found to be the most effective procedure for decontaminating milk 2 to 3 d old. Prolonged exposure to decontaminants, additional incubations in antibiotics, or at higher temperature (37°C) significantly reduced recovery of live MAP. Enhanced growth of microbial contaminants was noticed in samples decontaminated overnight at room temperature compared to those decontaminated for 2 to 5 h. Decontamination of 6 d old milk samples required extra incubation in antibiotic brew. Decontamination of milk samples that are 8 d and older was not effective in removing microbial contaminants. The MAP cells preferentially partitioned into the cream fraction after centrifugation, and combining the milk cream and pellet fractions enhanced recovery of MAP. A recovery rate of 16.6% was estimated with the use of our improved protocol.

Résumé

Mycobacterium avium *ssp.* paratuberculosis *(MAP) est l'agent étiologique de la maladie de Johne chez les bovins et il a été suggéré qu'il pourrait être associé à la maladie de Crohn chez l'humain. Dans les stades avancés de la maladie les bovins excrètent ce micro-organisme dans leur lait et leurs fèces. L'objectif de l'étude était de développer une procédure plus efficace pour isoler MAP du lait cru de réservoir. Des échantillons (50 mL) de lait de réservoir âgés de 3 à 13 j après la récolte ont été étudiés pour évaluer les effets de l'âge du lait sur l'efficacité de la décontamination. Des échantillons de lait âgés de 2 à 3 j ont été inoculés avec 50 à 200 cellules de MAP/mL dans des expériences visant à évaluer 1) les effets de différents agents de décontamination et procédures de décontamination sur le ré-isolement de MAP, et 2) la répartition de MAP dans les fractions du lait après centrifugation du lait cru. Une décontamination dans 20 mL de chlorure d'hexadecylpyridinium (HPC) 0,75 % à la température de la pièce (22 °C) pour 2 à 5 h, avec agitation à intervalle, s'est avérée être la procédure la plus efficace pour décontaminer du lait de 2 à 3 j. Une exposition prolongée aux décontaminants, une incubation additionnelle avec des antibiotiques, ou une température plus élevée (37 °C) a réduit de manière significative le recouvrement de MAP vivant. Une croissance plus abondante de contaminants microbiens a été notée dans des échantillons décontaminés durant la nuit à la température de la pièce comparativement à une décontamination de 2 à 5 h. La décontamination d'échantillons de lait âgés de 6 j a nécessité une incubation prolongée dans un mélange d'antibiotiques. La décontamination d'échantillons âgés de 8 j ou plus n'était pas efficace pour éliminer les contaminants microbiens. Les cellules de MAP se retrouvaient de manière préférentielle dans la fraction crème après la centrifugation, et en combinant la crème et le culot on augmentait le recouvrement de MAP. Un taux de recouvrement de 16,6 % était estimé en utilisant le protocole amélioré.*

(Traduit par Docteur Serge Messier)

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a recognized pathogen that affects many species of ruminant and non-ruminant animals (1). It is the etiological agent of Johne's disease (JD) in cattle. Infected animals with clinical disease and subclinical infections shed MAP bacteria in both milk and feces. Detectable levels of this organism have been found in milk from both clinically infected cattle and asymptomatic carriers (2–4). Economical losses due to JD was estimated to be US$ 200 to 250 million annually in US dairy industry (5). There are suggestions that this organism may be associated with Crohn's disease (CD) in humans and that contaminated milk may be

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Received June 18, 2004. Accepted October 21, 2004.

a source of exposure to the organism (6). Live MAP organisms were recovered from some samples of retail pasteurized milk, and several studies reported survival after exposure to high temperature short time pasteurization (7–9). *Mycobacterium avium* subsp. *paratuberculosis* has been cultured from the breast milk of patients with active CD (10). Although the role of this organism in CD is unclear, cow milk can be a source of human exposure to the organism (11). An efficient method for detection of MAP bacterium in bulk tank raw milk is important for assessment of this risk factor and the determination of MAP status of dairy farms in a JD control program.

Several methods are reported for isolation of MAP from milk and feces in the past decades (1–4,12–14). A basic procedure for isolation of MAP from milk includes centrifugation to collect pellet fraction, chemical decontamination, and culture on slants containing antibiotics and other supplements. However, the conditions for centrifugation and decontamination procedures varied significantly between research teams. For example, to separate the milk pellet, centrifugation methods include the use of an ultracentrifuge at 41 000 × *g* for 1 h (15), high-speed centrifuge at 13 000 × *g* for 30 min (12), 7000 × *g* for 10 min (14), and bench-top centrifuge at 900 × *g* for 30 min (3) and all at different temperatures. Raw milk normally contains a high level of microbial contaminants (16). *Mycobacterium avium* subsp. *paratuberculosis* is an extremely slow growing organism and it takes 2 to 4 mo of incubation for visible colonies to appear on slants. Chemical decontamination, used to selectively reduce and inhibit fast growing microbial flora in milk, is one of the most critical procedures for successful recovery of MAP. Dundee et al (17) compared 4 decontamination procedures and showed that decontamination in 0.75% hexadecylpyridinium chloride (HPC) for 5 h was most effective in reducing background contamination and recovery of MAP. Many studies reported the use of 0.75% HPC decontamination with samples exposed to HPC for between 4 h to overnight and between room temperature to 37°C (2,3,12). There is little information on how these conditions affected the recovery of MAP from milk samples. The objective of this study was, therefore, to develop an efficient procedure for isolation of MAP from raw and heat treated milk by investigating: 1) the efficiency of decontaminants, exposure time, and temperature in reducing the background microflora in milk; 2) the effect of decontamination protocols on the recovery of live MAP; and 3) the partition of MAP bacteria in pellet, cream, and whey fractions after centrifugation.

Materials and methods

Milk sample and MAP strain

Bulk tank raw milk samples from dairy farms delivered to Laboratory Services Division of University of Guelph within 48 h of collection were kept at 4°C until tested. Broth culture of MAP ATCC19698 at logarithmic phase of growth was centrifuged at 1000 × *g* for 20 min. The cell pellet was suspended in Middlebrook 7H9 broth (Difco, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA), containing glycerol (Difco, Becton, Dickinson and Company), 5 mL; mycobactin J (Allied Monitor, Fayette, Missouri, USA), 2 mg; penicillin G (Sigma Chemical Company, St Louis, Missouri, USA), 100 000 units; chloramphenical (Sigma Chemical Company), 50 mg; and OADC (Difco, Becton, Dickinson and Company), 100 mL, in every liter (9). Sterile soda lime glass beads (3 mm diameter) (Fisher Scientific, Hampton, New Hampshire, USA) were added and the pellet vortexed for 45 s to break up clumps of MAP. The suspension was allowed to stand at room temperature for 20 min to settle clumps of cells. The supernatant was carefully removed and optical density ($OD_{600\ nm}$) determined. A OD value of 1.0 corresponded to approximately 1.2×10^8 colony forming units (CFU)/mL based on colony count on Herrold's egg yolk medium (HEYM) slants (containing per liter: mycobactin J, 2 mg; amphotericin B [Sigma Chemical Company], 150 mg; vancomycin [Sigma Chemical Company], 50 mg; nalidixic acid [Sigma Chemical Company], 50 mg) (n = 21). The supernatant was serially diluted and used for spiking experiments. Raw milk samples (50 mL) were spiked with MAP to approximately 50 to 200 CFU/mL in the spiking experiments. At the same time, 2 to 3 slants were inoculated with 100 CFU of MAP to monitor the spiking level.

Decontamination of raw milk samples

A record of the age of milk sample was kept after collection. To evaluate the decontamination protocols, 6-day-old raw milk was subjected to decontamination using different reagents and time combinations in a factorial design (Table I). A total of 16 tubes containing 50 mL milk in each tube were used. The milk samples were centrifuged at 3100 × *g* for 30 min to collect the pellet. Pellets of tubes 1 through 8 were decontaminated in 20 mL of 0.75% hexadecylpyridinium chloride (HPC; Sigma Chemical Company), while the tubes 9 through 16 were in 20 mL of 0.75% HPC containing 1/2 strength of brain heart infusion (BHI; Difco, Becton, Dickinson and Company) (17) with shaking at intervals. Tubes 1, 2, 9, and 10 were removed after 2 h; tubes 3, 4, 11, and 12 after 5 h; tubes 5, 6, 13, and 14 after 24 h; and tubes 7, 8, 15, and 16 after 48 h. The samples were then centrifuged immediately at 1000 × *g* for 15 min at room temperature. The pellet was suspended in 2 mL of sterile antibiotic brew (per liter: amphotericin B 50 mg, vancomycin 100 mg, nalidixic acid 100 mg, BHI 18.5 g). Tubes in odd number (1, 3, 5, etc…) were plated out on HEYM slant immediately after suspension (0 h), and after 24, 48, 72, and 96 h at room temperature, while the tubes in even number (2, 4, 6, etc…) were plated on Luria-Bertani (LB) plates. A 100 μL sample was plated on each slant and 3 slants were used each time. The whole experimental procedure lasted 7 d and a total number of 120 HEYM slants and 120 LB plates were plated out. The HEYM slants were incubated at 37°C at slanted position in 1st wk with loosened cap. After the extra liquid was evaporated, the caps were tightened and checked on 1st, 2nd, 4th, 8th, and 16th wk after inoculation. The LB plates were packed into plastic bag together with wet tissue paper inside to prevent moisture loss during the long-term incubation.

In additional experiments, milk samples 3, 6, 8, 11, and 13 d old were subjected to decontamination in 0.75% HPC for 5 and 24 h, followed by antibiotic brew incubation for 0 and 24 h at room temperature. The samples were cultured on LB plates containing antibiotics (per liter: amphotericin B 50 mg, vancomycin 100 mg, nalidixic acid 100 mg), and incubated at 37°C to determine the relationship between decontamination procedures and the age of milk samples.

Table I. Number of contaminated slants out of 3 plated slants in the trial to decontaminate 6-day-old bulk milk

Slants	Time in antibiotic brew[a] (h)	Time in 0.75% HPC[b] (h)				Time in 0.75% HPC + 1/2 BHI[c] (h)			
		2	5	24	48	2	5	24	48
HEYM[d]	0	3	2	3	0	1	2	3	3
	24	0	2	0	1	1	3	3	0
	48	1	0	3	0	2	0	3	0
	72	0	0	0	0	3	0	3	0
	96	0	0	0	0	3	0	3	3
LB	0	3	3	3	0	0	0	3	3
	24	1	3	3	0	1	1	0	3
	48	0	0	3	0	0	0	3	3
	72	0	0	3	0	1	0	3	3
	96	3	0	3	0	2	0	0	2

LB — Luria-Bertani plate

[a]per liter: amphotericin B 50 mg, vancomycin 100 mg, nalidixic acid 100 mg, brain-heart infusion (BHI) 18.5 g

[b]0.75% hexadecylpyridinium chloride (HPC)

[c]0.75% HPC containing 1/2 strength of BHI

[d]Herrold's egg yolk medium (HEYM) slant with mycobactin J and antibiotics

Effect of decontamination protocols on the recovery of MAP

In another factorial design, we examined the effect of the decontaminant used, the time of exposure to decontaminant, and the length of incubation in antibiotic brew at room temperature on the survival and recovery of MAP. A total of 16 tubes, 8 tubes of raw milk (3 d old) and 8 tubes of heat-treated milk (3 d old, heated to 90°C for 5 min and cooled to room temperature), were seeded with MAP to a final concentration of 120 CFU/mL. After centrifugation, the pellet from tubes 1 through 4 and 9 through 12 was decontaminated in 20 mL of 0.75% HPC and that from tubes 5 through 8 and 13 through 16 in 20 mL of 0.75% HPC in 1/2 strength of BHI for 5 h (tubes 1, 2, 5, 6, 9, 10, 13, 14) and 24 h (tubes 3, 4, 7, 8, 11, 12, 15, 16) at room temperature. Samples were centrifuged at 1000 × *g* for 15 min. The pellet was then suspended in 1 mL of antibiotic brew, incubated for 0 h (tubes 1, 3, 5, 7, 9, 11, 13, 15, plated immediately) and 48 h (tubes 2, 4, 6, 8, 10, 12, 14, 16) at room temperature. A 100 μL sample was plated on each of the 3 HEYM slants for each sample. Slants were incubated at 37°C, as described above. See Table II for detailed treatment of each tube in the experiment.

To determine the effect of temperature on the recovery of MAP in decontamination, 8 tubes containing 50 mL of raw milk (2 d old) were used. Tubes 1 to 6 were seeded with MAP to about 100 CFU/mL. Tubes 7 and 8 were negative control. After centrifugation at 3100 × *g* for 30 min to collect the pellet, 20 mL of 0.75% HPC was added to tubes 1 through 3 and 7 and incubated at room temperature, while preheated 0.75% HPC (37°C) was added to tubes 4 through 6 and 8 and incubated at 37°C. Tubes 1 and 4 were centrifuged after 1 h, tubes 2, 5, 7, and 8 after 4 h, and tubes 3 and 6 after 18 h. Pellets were suspended in 1 mL of Middlebrook 7H9 broth and 100 μL was plated on each HEYM slant immediately. Four slants were plated for each tube and incubated at 37°C as describe above.

Partition of MAP organisms in milk fractions after centrifugation

Six tubes containing 50 mL of raw milk (3 d old) in each tube were set up. Tubes 1 through 4 were seeded with approximately 200 CFU/mL of MAP. Tubes 5 and 6 were negative controls. After centrifugation at 3100 × *g* for 30 min, pellet and cream were processed separately for tubes 1, 3 (duplicate), and 5, while the pellet and cream were processed together for tubes 2, 4 (duplicate), and 6. The samples were then decontaminated in 20 mL of 0.75% HPC for 4 h. After centrifugation at 1000 × *g* for 15 min, the pellet was resuspended in 1.0 mL of Middlebrook 7H9 broth. A 100 μL sample was plated on each HEYM slant immediately. Three slants were used for each tube. This experiment was conducted 3 times and 2 slants were plated out for each tube in the last experiment.

Data management and statistical analysis

Data obtained from experiments were managed (Microsoft Excel 2000; Microsoft Corporation, Redmond, Washington, USA) and analyzed using computer software (SAS; SAS Institute, Cary, North Carolina, USA). Correlation was conducted to analyze the association between time of exposure to decontaminants and number of contaminated slants. A chi-square test was used to analyze the independency between decontamination agents and the number of contaminated slants obtained from the decontamination study. Factorial analysis of variance (ANOVA) (general linear model procedure) was used to determine the effects of the factors evaluated in the decontamination protocols for recovery of MAP and "effect up to 2-way interaction" was employed in analyzing the interactions between the factors investigated. Graphs were drawn using computer software (SigmaPlot for Windows, version 4.01; SPSS, Chicago, Illinois, USA).

Table II. Factors effecting recovery of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from raw and heat-treated milk samples spiked with 120 colony forming units (CFU)/mL in 50 mL samples

	Factors investigated				
Tube	Milk sample	Decontaminant	Hours of decontamination	Hours in antibiotic brew	CFU recovered on HEYM slants (average of 3)
1	Raw	HPC[a]	5	0	20
2	Raw	HPC	5	48	10
3	Raw	HPC	24	0	11
4	Raw	HPC	24	48	2
5	Raw	HPC + BHI[b]	5	0	19[c]
6	Raw	HPC + BHI	5	48	8
7	Raw	HPC + BHI	24	0	11
8	Raw	HPC + BHI	24	48	4
9	Heat-treated[d]	HPC	5	0	117
10	Heat-treated	HPC	5	48	80
11	Heat-treated	HPC	24	0	88
12	Heat-treated	HPC	24	48	25
13	Heat-treated	HPC + BHI	5	0	52
14	Heat-treated	HPC + BHI	5	48	42
15	Heat-treated	HPC + BHI	24	0	25
16	Heat-treated	HPC + BHI	24	48	24

HEYM — Herrold's egg yolk medium

[a]0.75% hexadecylpyridinium chloride (HPC)

[b]0.75% HPC in 1/2 strength of brain-heart infusion (BHI) broth

[c]Count from 1 slant. The other 2 slants overwhelmed with background flora

[d]Heated to 90°C for 5 min and cooled to room temperature before seeding with MAP

Results

Decontamination of raw milk

Results of the decontamination of 6-day-old milk are listed in Table I. Of the 60 HEYM slants decontaminated in 0.75% HPC, 15 were overgrown by background bacteria, while 36 out of 60 HEYM slants were overgrown in the samples that were decontaminated in 0.75% HPC containing 1/2 strength of BHI. The chi-square analysis showed that the difference in the number of contaminated slants was associated with the decontaminant used (chi-square = 15.04, df = 1, $P < 0.001$). The 0.75% HPC controlled the contamination better than 0.75% HPC containing 1/2 strength of BHI. The difference between the numbers of contaminated slants treated in 0.75% HPC and that in 0.75% HPC containing 1/2 strength of BHI was not significant in samples evaluated using the LB plate (Table I). No significant correlation was recognized between the number of contaminated slants and the length of time of exposure to decontaminants in the time range we investigated (Pearson correlation coefficient = 0.0521, $P = 0.65$, $n = 80$). Of the 240 slants, 107 were contaminated, and 92 (86%) of the 107 showed up in 1st wk after the experiment. No additional contaminated slants appeared after 2 wk.

For 3-day-old milk samples, decontamination in 0.75% HPC for 2 to 5 h without incubation in antibiotic brew was sufficient in controlling background contaminants (data not listed). For the 6-day-old milk, effective decontamination required up to 5 h in 0.75% HPC followed by incubation in antibiotic brew for 48 h (Table I). For samples 8, 11, and 13 d old, none of the decontamination protocols tested were effective.

Factors affecting the recovery of MAP

Factorial ANOVA demonstrated that all the factors, such as the decontaminant, the length of time of decontamination, and the length of time in the antibiotic brew, affected the recovery of the MAP organism significantly (Tables II and III). Decontamination for a period of 24 h killed a significantly higher number of MAP cells (45%) compared to the 5 h decontamination. Incubation for 48 h in antibiotic brew killed about 42% of MAP cells compared to samples not subjected to the antibiotic brew treatment. Recovery from heat-treated (before spiking) milk sample was about 4 times higher than that in the raw milk. Significant interactions between some of these factors were observed (Table III). For example, more MAP colonies were recovered from samples that were treated with 0.75% HPC than those treated with the 0.75% HPC in 1/2 strength of BHI in the heat-treated milk, while MAP recovered from samples treated with 0.75% HPC were similar to those treated with 0.75% HPC in 1/2 strength of BHI in raw milk. In the case of heat-treated milk samples that were decontaminated in 0.75% HPC in 1/2 strength of BHI, no significant difference was recognized between the samples that were incubated in antibiotic brew for 48 h and those not incubated in antibiotic brew (Table II).

In the experiment to examine the effect of temperature and length of time of decontamination on the recovery of MAP (Table IV),

Table III. Summary of factorial analysis of variance (ANOVA) for factors affecting recovery of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from milk, based on the data in Table II

Source	DF	Sum of squares	F-value	*P*-value
Model	4	49033.10	35.85	< 0.0001
A. Milk sample (raw versus heat-treated)	1	24964.95	182.52	< 0.0001
B. Decontaminant (HPC versus HPC + BHI)	1	5641.07	41.24	< 0.0001
C. Hours of decontamination (5 versus 24 h)	1	3711.27	27.13	< 0.0001
D. Hours in antibiotic brew (0 versus 48 h)	1	3220.29	23.54	< 0.0001
Interaction: A × B	1	4164.95	30.45	< 0.0001
A × C	1	2055.93	15.03	0.0004
A × D	1	1228.50	8.98	0.0050
B × C	1	559.08	4.09	0.0509
B × D	1	1741.07	12.73	0.0011
C × D	1	105.93	0.77	0.3848
Error	35	4787.33	—	—
Corrected total	45	53820.43	—	—

DF — Degree of freedom; HPC — hexadecylpyridinium chloride; BHI — brain-heart infusion

Table IV. Effect of temperature and length of incubation in the decontamination step on the recovery of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from raw milk spiked at about 100 colony forming unites (CFU)/mL

	Room temperature (22°C)				37°C			
Decontamination[a]	1[b]	2	3	4	1	2	3	4
1 h	31	51	77	75	23	Cont	26	24
4 h	27	61	43	94	13	20	36	43
18 h	Cont	Cont	Cont	Cont	2	1	3	3

Cont — Contaminated

[a]Decontaminated in 0.75% hexadecylpyridinium chloride

[b]Ordinal number of slant

approximately 30% and 50% MAP were recovered from samples decontaminated at 37°C for 1 and 4 h, respectively, compared with those decontaminated at room temperature, while the overnight decontamination at 37°C inactivated most of the MAP. Unfortunately, all 4 of the slants decontaminated at room temperature for 18 h were contaminated. The difference between 1 h and 4 h of decontamination was not significant.

Suspect MAP colony was confirmed by IS 900 polymerase chain reaction (PCR), as described previously (19), except that cells from isolated colonies were transferred to the PCR tube directly.

Partition of MAP bacteria in milk fractions after centrifugation

Higher numbers of MAP organisms were recovered from cream than from the pellet milk fraction. The best recovery was obtained from the pooled cream and pellet sample (Figure 1). Data from a few slants were missing due to contamination so the sample size was less than the expected 16.

Discussion

Decontamination and the age of raw milk

Our data suggest that the freshness of a milk sample is an important factor for successful decontamination of milk when screening for MAP contamination. Samples should be stored at 4°C after collection and processed within 3 d for optimal recovery of MAP. The decontamination procedure should not kill or inhibit all of the background bacteria since this may result in the inhibition of MAP as well. Decontamination in 0.75% HPC was more effective than in 0.75% HPC containing 1/2 strength of BHI, as evaluated on the HEYM slant (Table I). To control the background organisms not killed by decontamination, antibiotics should be added to HEYM slants. Slants without antibiotics were often contaminated with fast growing organisms, as a result, MAP could not be detected. Our results suggest that the optimal time of decontamination was between 2 to 5 h when using 0.75% HPC at room temperature for milk samples stored at 4°C within 3 d after collection. Although it is assumed that a longer time of decontamination would reduce background microbial growth, treatment for 18 h or longer in 0.75% HPC consistently resulted in higher levels of contaminated HEYM slants (Table I and IV). The results indicate that longer incubation of milk samples in the decontaminant medium might selectively enhance the growth of HPC-resistant organisms.

Factors affecting the recovery of MAP

Grant and Rowe (20) recently reported that HPC decontamination resulted in a significant reduction in the number of culture-positive milk samples recovered from heat-treated milk. This is consistent with our results from raw milk that both the treatment in HPC and

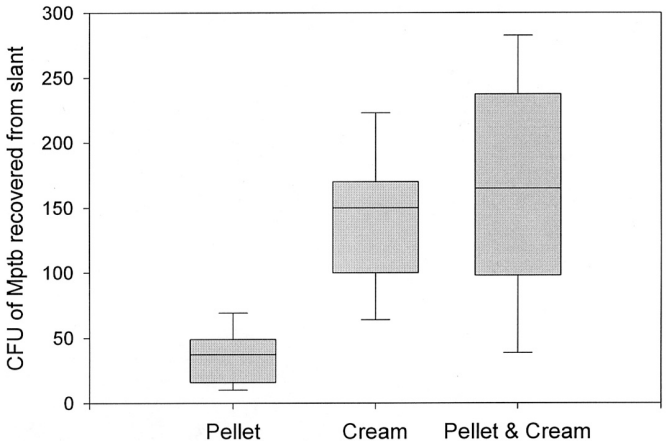

Figure 1. Recovered *Mycobacterium avium* subsp. *paratuberculosis* (MAP) organisms in different processing methods: Pellet (n = 14), cream (n = 14), and pooled pellet and cream (n = 12) samples. Marked are 10% (lower adjacent), 25% (lower hinge), 50% (median), 75% (upper hinge), and 90% (upper adjacent) percentiles.

incubation in antibiotic brew reduced the survival and recovery of MAP (Tables II and III). We also found that higher temperatures enhanced the detrimental effect of HPC on MAP survival (Table IV). Therefore, overnight incubation at room temperature should be avoided and overnight incubation at 37°C is not recommended for raw milk samples. Recovery of MAP from heat-treated milk (before spiking) was about 4 times higher than that of raw milk (Table II). Applying 90°C heat to the milk sample for 5 min before centrifugation significantly increased the sensitivity of the IS*900* PCR assay, with the template DNA prepared using the bead beating method (19). It is possible that heating altered the chemical and physical characteristics of the raw milk and subsequently improved the partitioning of MAP into the pellet fraction. Because of this, caution is needed in the application of a sample preparation protocol developed for heat-treated milk to the preparation of a raw milk sample.

Partition of MAP in milk fractions after centrifugation

Grant et al (21) evaluated the partition of MAP in milk fractions. They found the majority (69.4%) of the MAP cells present in a 10-mL milk sample segregated into the pellet, after centrifugation at 2500 × g for 15 min. Only 13.0% and 17.6% of the MAP partitioned into the cream and whey fractions, respectively (22). The present study demonstrated that MAP organisms recovered from the cream fraction was almost 3 times higher than that recovered from the pellet, and the best recovery was obtained from the pooled cream and pellet fractions (Figure 1). We recommend that the pellet and cream be processed together rather than the pellet only. We did not succeed in recovering MAP from the whey fraction, as the addition of HPC to the whey resulted in an insoluble glue-like precipitate.

Recovery rate of MAP in raw milk

In the experiments studying the partition of MAP in milk fractions after centrifugation (Figure 1), the total seeding level was about 10 000 CFU in 50 mL (200 CFU/mL). The number of CFU recovered from the slant corresponded to about 1/10 of the seeding. A recovery of 1000 CFU on a slant theoretically corresponds to a recovery rate of 100%. In our study, the recovery rate of MAP was estimated to be about 16.6%, when both the pellet and cream were processed using the optimized protocol (Figure 1). Factors that contributed to low recovery include: MAP cells fractionating to the whey fraction, killing during decontamination, and clumping of the cells. Unlike centrifugation, immunomagnetic bead separation (IMS) allows direct recovery from the whole milk sample. Grant et al (22) showed that IMS (using 10 μL beads in 1 mL milk) recovered 37.1% of the seeded MAP, suggesting that IMS is more efficicent (21). But the protocol was not sufficiently sensitive to detect samples contaminated with only a few MAP/mL of milk, unless a protocol for large sample volumes is developed. Milk from asymptomatic cows infected with MAP is estimated to contain only 2 to 8 CFU per 50 mL of sample (3). Although milk could be concentrated by centrifugation, most of the target organisms would be lost in whey and cream fractions if only the pellet was included for further processing (21,23). For example, to recover MAP cells from milk containing only 1 CFU/mL, samples of at least 50 mL should be used. When using centrifugation and extraction from the combined cream and pellet, we estimate a recovery rate of 16% or approximately 8 CFU per slant, based on findings from this study.

In this paper, we describe a centrifugation protocol using a large sample volume and combining the cream and pellet fractions for enhanced MAP recovery from milk. Decontamination and assay conditions for enhanced recovery of MAP from raw milk samples are described below.

Suggested protocol for isolation of MAP from raw milk

The following procedures are suggested for isolation of MAP from raw milk.

1. Collect and store milk at 4°C.
2. Test milk as soon as possible within 3 d from collection.
3. Use a volume of ≥ 50 mL to facilitate detection of low numbers of organisms.
4. Centrifuge milk at a minimum of 3100 × g for 30 min to separate the cream, whey, and pellet. Remove whey and process the cream and pellet fractions together.
5. Decontaminate the sample in 20 mL of 0.75% HPC (50 mL milk sample) at 22°C for 2 to 5 h with shaking at intervals.
6. Centrifuge sample at 1000 × g for 15 min and suspend the pellet in 0.5 to 1.0 mL of Middlebrook 7H9 broth containing mycobactin J and antibiotics. Plate in duplicates of at least 100 to 200 μL on each HEYM slant containing mycobactin J and antibiotics.
7. Incubate inoculated tubes at 37°C in a slanted position with a loose cap until the extra liquid has evaporated (about 1 wk). Tighten caps and incubate for at least 16 wk or until suspect colonies of MAP become visible. Pick suspect colonies for confirmation by IS*900* PCR.

Acknowledgments

The authors acknowledge the financial support received from the Special Research Fund Program of Ontario Ministry of Agriculture and Food (OMAF) and from the Dairy Farmers of Ontario (DFO).

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