Short communication: Application of an *N*-acetyl-L-cysteine-NaOH decontamination method for the recovery of viable *Mycobacterium avium* subspecies *paratuberculosis* from milk of naturally infected cows

L. Bradner,* S. Robbe-Austerman,† D. C. Beitz,*‡ and J. R. Stabel‡§¹

*Roy J. Carver Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames 50010

†USDA-Animal and Plant Health Inspection Service (APHIS), Veterinary Services, Ames, IA 50010

‡Department of Animal Science, Iowa State University, Ames 50010

§USDA-Agricultural Research Service (ARS), National Animal Disease Center, Ames, IA 50010

ABSTRACT

Mycobacterium avium ssp. *paratuberculosis* (MAP) is shed into the milk of cattle affected by Johne's disease and, therefore, is a route of transmission for infection in young stock in dairy herds. The objective of this study was to validate a decontamination and culture protocol for the recovery of MAP from individual bovine milk samples from known infected herds. Decontamination of milk samples ($n = 17$) with either 0.75% hexadecylpyridinium chloride for 5 h or *N*-acetyl-L-cysteine-1.5% sodium hydroxide (NALC-1.5% NaOH) for 15 min before culture in BACTEC 12B (Becton Dickinson, Franklin, NJ), *para*-JEM [Thermo Fisher Scientific (TREK Diagnostic Systems, Inc.), Cleveland, OH], and Herrold's egg yolk (HEY; Becton Dickinson) media was compared. Treatment with NALC-NaOH resulted in a lower percentage (6%) of contaminated samples than did treatment with hexadecylpyridinium chloride (47%), regardless of culture medium. The decontamination protocol (NALC-1.5% NaOH) was then applied to milk samples ($n = 144$) collected from cows at 7 US dairies. Recovery of viable MAP from the milk samples was low, regardless of culture medium, with recovery from 2 samples cultured in BACTEC 12B medium, 1 sample cultured in *para*-JEM medium, and no viable MAP recovered on HEY medium. However, 32 cows were fecal culture positive and 13 milk samples were positive by direct PCR, suggesting that several cows were actively shedding MAP at the time of milk collection. Contamination rates were similar across media, with 39.6, 34.7, and 41.7% of samples contaminated after culture in BACTEC 12B, *para*-JEM, and HEY media, respectively. Herd-to-herd variation had a major effect on sample contamination, with the percentage of contaminated samples ranging from 4 to 83%. It was concluded that decontamination of milk with NALC-

1.5% NaOH before culture in BACTEC 12B medium was the most efficacious method for the recovery of viable MAP from milk, although the ability to suppress the growth of contaminating microorganisms varied greatly between herds.

Key words: *Mycobacterium avium* ssp. *paratuberculosis*, milk, decontamination, culture

Short Communication

Johne's disease (JD) is a slowly progressive, chronic granulomatous enteritis of the small intestine caused by *Mycobacterium avium* ssp. *paratuberculosis* (MAP; Whitlock and Buerge, 1996). The dairy industry continues to struggle to control this disease, with the US dairy herd-level prevalence today estimated to be 91.1% (Lombard et al., 2013). In 1996, costs to the US dairy industry were reported at more than \$200 to \$250 million, based upon a herd prevalence of 22%, suggesting that current costs to the dairy industry have increased tremendously (Ott et al., 1999). As JD progresses, MAP can disseminate from intestinal tissues throughout the body and MAP has been isolated from milk, supramammary lymph nodes, and lymph fluid from the udder (Sweeney et al., 1992; Chiodini, 1996; Khol et al., 2012). Milk and colostrum from infected cows may be consumed by susceptible neonates and young stock, thereby potentiating the spread of infection within the herd. With this in mind, producers in the dairy industry need to have accurate information on the quantity of MAP shed into milk and colostrum.

Exogenous contamination (outside of the udder) of raw milk occurs primarily from dirty udders and improperly cleaned and sanitized milking equipment (Bramley and McKinnon, 1990). Milking facilities use different procedures to clean and sanitize milking equipment, leading to different levels of microbial contamination in raw milk postmilking. Some microorganisms commonly found in bulk tank milk include *Bacillus* spp., coliforms, *Enterococcus* spp., *Lactococcus* spp., *Micrococcus* spp., noncoliforms, *Pseudomonas* spp., *Staphylococcus* spp.,

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¹Corresponding author: judy.stabel@ars.usda.gov

Streptococcus spp., other gram-positive rods, and yeasts (Holm et al., 2004). For this reason, it is necessary to include a decontamination step in the protocol to culture MAP from raw milk. The present study was designed to compare efficacy of chemical decontamination, using treatment with hexadecylpyridinium chloride (HPC) or *N*-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) before culture of milk. Further, 3 culture media: BACTEC 12B (Becton Dickinson, Franklin, NJ), *para*-JEM [Thermo Fisher Scientific (TREK Diagnostic Systems, Inc.), Cleveland, OH], and Herrold's egg yolk (HEY; Becton Dickinson), were evaluated for recovery of viable MAP from raw milk samples collected from cows in JD-positive herds.

To determine the optimal decontamination protocol, milk was collected into sterile containers from a small JD-positive herd ($n = 17$) located at the National Animal Disease Center (Ames, IA). To prevent exogenous contamination of milk during collection, teats of each selected cow were cleaned with alcohol before the normal milking procedure. Milk samples were taken from the flow meter, thereby representing a composite sample of all 4 quarters. A 20-mL aliquot of each milk sample was centrifuged at $5,600 \times g$ for 30 min at 4°C. The whey layer was discarded and the cream and pellet layers were retained in the tube and subjected to decontamination with either NALC-NaOH or HPC as previously described (Bradner et al., 2013a,b). For the NALC-NaOH decontamination protocol, an equal volume of 0.5% NALC-3.0% NaOH-1.45% sodium citrate solution was added (final concentration of 1.5% NaOH) to cream and pellet fractions and allowed to incubate for 15 min at room temperature (22°C). Immediately after the incubation, 15 mL of PBS was added to dilute the NALC-NaOH solution. Samples subjected to the HPC decontamination protocol were resuspended in 5 mL of 1.05% HPC (0.75% final concentration) and allowed to incubate at room temperature for 5 h. After chemical exposure, all samples were centrifuged at $5,600 \times g$ for 30 min at 4°C, the chemical layer was discarded, and the cream and pellet were resuspended in 1 mL of PBS for inoculation of the media.

Additional milk samples ($n = 144$) were collected from 7 dairy herds across the United States that were selected on the basis of known JD status and willingness of the producer and assisting veterinarians to collect samples. Again, alcohol disinfection of the teats was performed before collection of composite milk samples from the flow meter. Cows were selected for sampling based upon a previous test-positive result by fecal culture. Feces and milk from each cow were collected into sterile containers and each container was placed into a separate plastic zip-lock bag to avoid cross-contamination during shipment. Samples were immediately

shipped on ice to the National Animal Disease Center and then stored at -80°C until processing.

To assess the disease status of the cow at the time of milk collection, fecal samples were cultured for MAP. The decontamination protocol was adapted from a previously published protocol where 2 g of feces was incubated overnight in 0.75% HPC (Sigma-Aldrich, St. Louis, MO) at 39°C and then incubated overnight in an antibiotic cocktail consisting of 100 μg of vancomycin/mL, 50 μg of amphotericin B/mL, and 100 μg of nalidixic acid/mL (Rajeev et al., 2007). The samples were then inoculated into *para*-JEM medium as described below. Milk samples were subjected to the NALC-NaOH decontamination protocol and inoculated into the 3 media as detailed below.

The BACTEC 12B medium was supplemented with 0.5 mL of sterile unmodified raw egg yolk (in house), 0.2 mL of PANTA Plus (Becton Dickinson), and 0.1 mL of 50 μg of mycobactin J/mL, and then inoculated with 200 μL of decontaminated milk sample and placed in a 37°C incubator for 84 d. The *para*-JEM medium was supplemented according to the manufacturer's instructions, inoculated with 500 μL of decontaminated milk sample and placed into the ESP Culture System II machine (Trek Diagnostics Inc., Westlake, OH) for up to 65 d. All bottles were subjected to MAP growth confirmation procedures of Ziehl-Neelsen acid-fast (AF) stain for MAP colonies and PCR of the *IS900* gene target as previously described (Bradner et al., 2013b). A sample was classified as positive if both the AF and PCR results confirmed the positive readout from the machine. If the machine signaled positive but AF and PCR confirmatory tests were negative, the sample was considered either contaminated or false positive, depending upon the presence of non-MAP microorganisms in the sample. Samples were classified as negative if the machine signal was negative and both the AF and PCR confirmatory tests were negative. A sample was considered a false negative if the AF and PCR results were both positive, following a negative machine signal.

The HEY medium was inoculated with 100 μL of decontaminated milk sample, incubated at 39°C for 12 wk, and colonies counted. *Mycobacterium avium* ssp. *paratuberculosis* growth was confirmed on the HEY medium slants by colony morphology and growth characteristics. All colonies were compared with those present on the positive-control sample slants. If any unusual colonies appeared, confirmatory protocols were performed as stated above. A HEY medium slant was deemed contaminated if any non-MAP microorganisms were present. Acid-fast positive, PCR-negative organisms were identified by partial 16S rRNA gene and *rpoB* gene sequencing (Toney et al., 2010; Higgins et al., 2011). A sample was classified as positive or nega-

tive only if both the AF and PCR results confirmed the readout.

The presence of MAP in milk samples was also ascertained by direct PCR. The milk sample (10 mL) was centrifuged at $5,600 \times g$ for 30 min at 4°C. The whey layer was removed and the remaining cream and pellet fractions in the tube were resuspended in 800 µL of PBS and transferred to a sterile 1.5-mL tube containing 200 µL of 10 mg/mL proteinase K (Qiagen Inc., Valencia, CA). The samples were incubated at 56°C for 1 h and then centrifuged at $15,000 \times g$ for 15 min. The liquid fraction was removed and the cream and pellet were resuspended in 200 µL of PBS. An aliquot (175 µL) was transferred to a MagMAX Total Nucleic Acid Isolation Kit bead beat tube (Life Technologies, Carlsbad, CA) and the manufacturer’s protocol was followed by using the MagMAX Express machine (Leite et al., 2013). Real-time PCR targeting the *IS900* gene was performed as before, except for the use of TaqMan Environmental master mix 2.0 (Life Technologies). All samples with a threshold cycle (C_t) ≤ 42 were considered positive.

Statistical analysis was performed by using PROC GLM of SAS (SAS Institute Inc., Cary, NC). The model included the fixed effects of herd, media, and herd $\times$ media interaction.

Treatment of the milk samples with NALC-NaOH resulted in lower ($P < 0.01$) contamination rates than did treatment with HPC across all 3 media (Table 1). After decontamination with HPC, culture of milk samples in BACTEC 12B medium yielded the least number of contaminated samples (3/17). In contrast, culture in either *para*-JEM or HEY media resulted in higher ($P < 0.01$) numbers of contaminated samples (11/17 and 10/17, respectively). When samples were treated with NALC-NaOH, negligible levels of contamination were noted in milk samples, regardless of culture medium, resulting in the selection of the NALC-NaOH decontamination protocol for application to milk samples in the second portion of this study.

A comparison of BACTEC 12B, *para*-JEM, and HEY media for the recovery of MAP from raw milk samples after decontamination with NALC-1.5% NaOH is shown in Table 2. Of the 144 cows sampled in 7 herds, 32 cows were shedding MAP in their feces. Because asymptomatic cows may not shed MAP into their feces, cows that were fecal-culture negative cannot conclusively be described as JD negative (Whitlock and Buergelt, 1996). It is noteworthy that the sample set is not an accurate representation of animal-level prevalence within a herd because animals that were either known or suspected to have JD were targeted for sampling.

Only 2 of the milk samples were positive for MAP via culture, regardless of culture medium. This find-

ing was not surprising because it is speculated that the amount of MAP shed into milk is highly correlated with advanced disease (Sweeney et al., 1992). Once a cow manifests clinical symptoms of JD, it is likely the cow will quickly be culled from the herd. Therefore, it is probable that most, if not all, of the milk samples were collected from asymptomatic cows, leading to a low likelihood of MAP being shed into the milk. If MAP is shed into the milk at preclinical stages of the disease, it is shed at very low concentrations (2–8 cfu of MAP/50 mL) and this may have been beyond the detection limits of these culture methods (Sweeney et al., 1992). *Mycobacterium avium* ssp. *paratuberculosis* was isolated from the feces of only 1 of the 2 cows that had positive milk samples. This inconsistency can be explained, as MAP is shed into the feces intermittently throughout the progression of JD (Whitlock and Buergelt, 1996). At the time of sampling, this cow may not have been actively shedding MAP into the feces but could still have been infected. Because little research has been conducted as to when and how MAP is shed into milk, it is difficult to speculate whether a cow sheds MAP into feces and milk at the same time or whether they are independent of one another (Pithua et al., 2011). It is also possible that external contamination of the milk sample occurred, resulting in a false-positive sample, but this is less likely due to the rigorous cleaning regimen before sample collection.

Of the 2 positive samples, only 1 was positive in both BACTEC 12B and *para*-JEM media. No samples were positive on HEY medium, as culture detection thresholds (10^4 cfu/mL; Bradner et al., 2013b) are much higher for MAP on this medium. The sample that was positive for both liquid media was detected at 49 d in BACTEC 12B medium and 57 d in *para*-JEM medium. The sample that was positive for MAP growth only in BACTEC 12B medium was detected in the last week of incubation (77–84 d), which is indicative of very

Table 1. Number of contaminated milk samples after treatment with either hexadecylpyridinium chloride (HPC) or *N*-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) and cultured in BACTEC 12B (Becton Dickinson, Franklin, NJ), *para*-JEM [Thermo Fisher Scientific (Trek Diagnostic Systems Inc.), Cleveland, OH], or Herrold’s egg yolk (HEY; Becton Dickinson) medium¹

Chemical decontaminant	Culture medium		
	BACTEC 12B	<i>para</i> -JEM	HEY ²
0.75% HPC	3 ^a	11 ^b	10 ^{ab}
NALC-1.5% NaOH	1	0	1

^{a,b}Values within a row with different superscript letters are significantly different ($P < 0.05$).

¹Number of milk samples = 17.

²At least 1 of the 3 slants was contaminated.

Table 2. Isolation of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) from milk collected from US dairy herds by culture in BACTEC 12B (Becton Dickinson, Franklin, NJ), *para*-JEM [Thermo Fisher Scientific (Trek Diagnostic Systems Inc.), Cleveland, OH], and Herrold's egg yolk (HEY; Becton Dickinson) media and contamination rate after decontamination with *N*-acetyl-L-cysteine-sodium hydroxide

Herd location	Cows (no.)	Fecal culture positive	Milk PCR positive	Milk culture					
				BACTEC 12B		<i>para</i> -JEM		HEY	
				Positive	Contaminated ¹	Positive	Contaminated ¹	Positive	Contaminated ^{1,2}
South Carolina	18	2	0	0	9	0	15	0	11
Ohio ³	20	1	0	0	6	0	9 ^a	0	2 ^b
Michigan	20	10	1	1	12 ^a	1	3 ^b	0	15 ^a
Iowa A	20	0	0	0	13 ^a	0	3 ^b	0	16 ^a
Iowa B	25	11	7	1	1	0	5	0	6
North Dakota	20	4	3	0	3	0	3	0	2
Colorado	21	5	2	0	13	0	11	0	7
Total	144	33	13	2	57	1	49	0	59

^{a,b}Values within a row with different superscript letters are significantly different ($P < 0.05$).

¹Number of contaminated samples.

²At least 1 of 3 slants contaminated.

³Herd was vaccinated for Johne's Disease.

low numbers of MAP (Bradner et al., 2013b). Both of these samples are consistent with previous studies in our laboratory where growth detection occurred earlier in BACTEC 12B medium than in *para*-JEM medium (Bradner et al., 2013b). This shorter incubation time for *para*-JEM (65 d) compared with BACTEC 12B medium (84 d) may have contributed to the failure to detect this sample. Interestingly, the BACTEC 12B medium was more adept at isolating other species of mycobacteria from milk samples than was *para*-JEM and HEY media. Some of these non-MAP mycobacteria were identified as *Mycobacterium fortuitum*, *Mycobacterium moriokaense*, and *Mycobacterium smegmatis*, all soil- or water-dwelling bacteria (Kamala et al., 1994).

The frequency of MAP detection in the milk samples was much higher via direct PCR (13 of 144 samples) than by culture (2 of 144 samples). This higher frequency can be explained by shedding of both viable and nonviable bacteria into the milk. The higher detection by PCR compared with culture may also be a factor of the decontamination procedure applied to the milk samples before culturing. Although the NALC-NaOH decontamination protocol minimizes detrimental effects on MAP viability compared with other chemical decontaminants, it may still affect MAP growth in culture, resulting in false-negative samples.

Overall, the 3 media performed equally in the ability to suppress contaminating microorganisms, with 39.6, 34.0, and 41.0% contamination recorded for samples cultured in BACTEC 12B, *para*-JEM, and HEY media, respectively. Examples of contamination from milk samples that were decontaminated with NALC-1.5% NaOH and cultured on HEY medium are shown in Figure 1. It is important to suppress the growth of all

contaminating organisms, as their rapid growth rate relative to MAP rapidly overwhelms the culture medium, rendering it ineffective for the growth and detection of MAP. A highly significant ($P < 0.01$) herd effect existed on the number of contaminated milk samples, with samples obtained from the Iowa B, North Dakota, and Ohio herds having the lowest degree of contamination across all 3 media. In addition, a significant ($P < 0.01$) herd-medium interaction was detected, as contamination rates ranged from 4% of milk samples from the Iowa B herd cultured in the BACTEC 12B medium to 83% of milk samples from the South Carolina herd cultured in the *para*-JEM medium. The variation in contamination rates was likely affected by the sample collection method and level of cleanliness of the milking equipment. Milk residues left by poor sanitation of the milking equipment supports the growth of a variety of microorganisms (Holm et al., 2004). Water temperature ($\geq 35^\circ\text{C}$) during sanitation of milking equipment was the most influential factor in lessening the bacterial load present in bulk-tank milk (Bava et al., 2011). In the present study, the milking parlor designated as Iowa B routinely monitors the water temperature during wash cycles of the milking equipment. This attention to hygiene likely contributed to the low level of sample contamination observed during culture of milk samples of all 7 dairy herds.

In conclusion, the present study validated the efficacy of the NALC-NaOH decontamination before culture for the recovery of viable MAP from raw milk samples. The liquid media performed better for culture of MAP from milk, but the number of positive samples was very low for this sample set. In addition, this study provided valuable information that proper sampling technique is

Figure 1. Examples of contaminated Herrold's egg yolk medium (Becton Dickinson, Franklin, NJ) slants after inoculation with raw milk. Slants A through E demonstrate various contaminants. Slant A was identified as *Mycobacterium hiberniae*, slant B as an *Enterobacter* species, and slants C through E as *Pseudomonas aeruginosa*. Slant F was a positive control demonstrating *Mycobacterium avium* ssp. *paratuberculosis* (MAP) growth upon isolation from a spiked milk sample.

necessary to ensure that milk samples have the lowest concentration of contaminating bacteria possible before culture for MAP.

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