Chemical Decontamination with *N*-Acetyl-L-Cysteine–Sodium Hydroxide Improves Recovery of Viable *Mycobacterium avium* subsp. *paratuberculosis* Organisms from Cultured Milk

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Mycobacterium avium subsp. *paratuberculosis* is shed into the milk and feces of cows with advanced Johne's disease, allowing the transmission of *M. avium* subsp. *paratuberculosis* between animals. The objective of this study was to formulate an optimized protocol for the isolation of *M. avium* subsp. *paratuberculosis* in milk. The parameters investigated included chemical decontamination with *N*-acetyl-L-cysteine–sodium hydroxide (NALC-NaOH), alone and in combination with antibiotics (vancomycin, amphotericin B, and nalidixic acid), and the efficacy of solid (Herrold's egg yolk medium [HEY]) and liquid (Bactec 12B and *para*-JEM) culture media. For each experiment, raw milk samples from a known noninfected cow were inoculated with 10^2 to 10^8 CFU/ml of live *M. avium* subsp. *paratuberculosis* organisms. The results indicate that an increased length of exposure to NALC-NaOH from 5 to 30 min and an increased concentration of NaOH from 0.5 to 2.0% did not affect the viability of *M. avium* subsp. *paratuberculosis*. Additional treatment of milk samples with the antibiotics following NALC-NaOH treatment decreased the recovery of viable *M. avium* subsp. *paratuberculosis* cells more than treatment with NALC-NaOH alone. The Bactec 12B medium was the superior medium of the three evaluated for the isolation of *M. avium* subsp. *paratuberculosis* from milk, as it achieved the lowest threshold of detection. The optimal conditions for NALC-NaOH decontamination were determined to be exposure to 1.50% NaOH for 15 min followed by culture in Bactec 12B medium. This study demonstrates that chemical decontamination with NALC-NaOH resulted in a greater recovery of viable *M. avium* subsp. *paratuberculosis* cells from milk than from samples treated with hexadecylpyridinium chloride (HPC). Therefore, it is important to optimize milk decontamination protocols to ensure that low concentrations of *M. avium* subsp. *paratuberculosis* can be detected.

Mycobacterium avium subsp. *paratuberculosis*, the causative agent of Johne's disease (JD) in ruminants, is transmitted primarily through the ingestion of contaminated feces, but it can also be transmitted through milk and *in utero* (1, 2). As the infection progresses, *M. avium* subsp. *paratuberculosis* can disseminate throughout the body, and it has been isolated from milk, supramammary lymph nodes, and lymph fluid samples from the udder (3–5). Contaminated colostrum or milk may be consumed by highly susceptible neonates and young stock, leading to possible infection with *M. avium* subsp. *paratuberculosis* and the persistence of JD within a herd. Studies enumerating the amount of *M. avium* subsp. *paratuberculosis* cells shed into milk have reported concentrations from <1 to 560 CFU/ml (3, 6). A meta-analysis of *M. avium* subsp. *paratuberculosis* in milk found that the prevalence rates of *M. avium* subsp. *paratuberculosis* in bulk tank milk samples collected from known infected herds and herds of unknown status were 6% and 1%, respectively, as detected by culture, and 68% and 22%, respectively, as detected by IS900 PCR (7). The huge discrepancies in the rates of prevalence between culture and PCR detection methods are because PCR detects both live and dead bacteria, whereas culture recovers only viable microorganisms.

Because current PCR assays have not been developed to differentiate between viable and nonviable *M. avium* subsp. *paratuberculosis* organisms, culture methods must be used to enumerate the *M. avium* subsp. *paratuberculosis* organisms present in milk. Unfortunately, the culture of *M. avium* subsp. *paratuberculosis* from milk presents many difficulties because of the organism's low growth rate and fastidious nature compared with other organisms

present in milk. Therefore, a decontamination and culturing protocol must be developed that will thwart the growth of more-rapidly growing microorganisms present in raw milk, allowing time for the detection of low concentrations of *M. avium* subsp. *paratuberculosis* cells that are naturally present in milk. Hexadecylpyridinium chloride (HPC) is the common chemical of choice for milk decontamination prior to culture, even though it has been shown to decrease the viability of *M. avium* subsp. *paratuberculosis* organisms (8–11). Therefore, other chemicals need to be investigated for their effects on the recovery of viable *M. avium* subsp. *paratuberculosis* organisms in conjunction with their ability to decrease the presence of other contaminating microorganisms. The chemical combination consisting of *N*-acetyl-L-cysteine–sodium hydroxide (NALC-NaOH) is primarily used for sputum decontamination for the isolation of *Mycobacterium tuberculosis* (12). The mucolytic agent NALC is useful as a liquefying agent for samples with high protein content, as it reduces the disulfide bonds in proteins and allows for the application of lower concentrations of NaOH (13). Like sputum, milk has a high protein content, so

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NALC-NaOH decontamination also may be applicable to the milk matrix.

In conjunction with decontamination protocols, the medium used for the culture of *M. avium* subsp. *paratuberculosis* has an impact on the sensitivity of *M. avium* subsp. *paratuberculosis* detection in milk. In a recent study, the Bactec 12B medium was found to be the superior medium for culturing *M. avium* subsp. *paratuberculosis* from milk after decontamination with HPC (11). Because it is not known whether the chemical used for decontamination had an influence on this outcome, the same commonly used media, Bactec 12B, *para*-JEM, and Herrold's egg yolk (HEY), were evaluated in the present study for their efficacy to recover *M. avium* subsp. *paratuberculosis* (14–16).

The objectives of this study were to examine the effects of treating raw milk samples spiked with *M. avium* subsp. *paratuberculosis* with NALC-NaOH and antibiotics on the recovery of viable *M. avium* subsp. *paratuberculosis* organisms. In addition, liquid (Bactec 12B and *para*-JEM) and solid (HEY) media were compared for their ability to support and accurately detect the growth of *M. avium* subsp. *paratuberculosis* and to suppress the growth of other microorganisms.

MATERIALS AND METHODS

Milk. Milk used in these experiments was obtained from a healthy cow (without JD) in a small dairy herd maintained at the National Animal Disease Center (NADC) in Ames, IA. The noninfected control cows in the herd were characterized by repeated fecal cultures that were negative for *M. avium* subsp. *paratuberculosis*, performed biannually over a 5-year period, and had been purchased from herds with no recent history of Johne's disease. In addition, these animals were negative by any serologic assays (i.e., production of antibodies specific for *M. avium* subsp. *paratuberculosis* and gamma interferon [IFN- γ]) performed during that period. All procedures performed on the animals were approved by the Institutional Animal Care and Use Committee (NADC, Ames, IA). Milk from the cow was hand stripped into sterile collection containers after thorough disinfection of the udder and teats with iodine teat dip and 70% ethanol. Large volumes of milk were collected at one time and stored at -80°C until use. For each individual experiment, one container of milk (1 liter) was thawed at 4°C and gently mixed prior to execution of the experiment.

Bacterial preparation. Milk was experimentally spiked with *M. avium* subsp. *paratuberculosis* strain 167 that was obtained from a clinical cow housed at the NADC. A frozen aliquot of *M. avium* subsp. *paratuberculosis* 167 was inoculated into 450 ml of Middlebrook 7H9 broth (Becton, Dickinson, Franklin Lakes, NJ) supplemented with 50 ml of oleic acid, albumin, dextrose, and catalase enrichment (OADC) (BD), mycobactin J (2 mg/liter) (Allied Monitor, Fayette, MO), and 1.5 ml Tween 80 (Sigma-Aldrich, St. Louis, MO) and cultured at 39°C until it reached logarithmic growth phase (optical density at 540 nm [OD₅₄₀], 0.2 to 0.4). The cultures were centrifuged at $10,000 \times g$ for 30 min to pellet the bacteria. Pellets were washed twice with phosphate-buffered saline (PBS) (1 mM [pH 7.4]; Sigma-Aldrich) and then resuspended in 10 ml of PBS. The suspension was sonicated briefly with a Tekmar sonic disruptor to break up any clumps of bacteria (40 W, three 10-s intervals) (Tekmar, Mason, OH). The OD₅₄₀ was measured, and the suspension was diluted to achieve an OD of 1.15, corresponding to 10^9 CFU/ml. Aliquots of the final suspension were frozen at -80°C in snap-cap tubes. For each of the experiments, an aliquot of bacterial suspension was thawed and sonicated briefly as described to break up clumps, and the suspension was diluted serially in PBS (10^3 to 10^9 CFU/ml). Then, 100 μl of each dilution was inoculated onto Herrold's egg yolk medium slants (BD) containing mycobactin J (2 $\mu\text{g/ml}$), amphotericin B (50 $\mu\text{g/ml}$), nalidixic acid (50 $\mu\text{g/ml}$), and vancomycin (50 $\mu\text{g/ml}$) and were incubated at 39°C for 12 weeks. Colonies on

the slants were counted every 4 weeks during the 12-week incubation period.

Inoculation of milk. For each experiment, triplicate samples of 18 ml milk and 2 ml of the respective dilution of bacterial suspension were prepared to yield final *M. avium* subsp. *paratuberculosis* concentrations of 10^2 , 10^4 , 10^6 , and 10^8 CFU/ml of milk. A positive control consisted of 10^6 CFU *M. avium* subsp. *paratuberculosis*/ml in PBS, and a negative control consisted of milk with no bacteria. A control consisted of 10^6 CFU of *M. avium* subsp. *paratuberculosis*/ml milk and was not subjected to the chemical treatment. Each of these controls was prepared in triplicate. After the addition of *M. avium* subsp. *paratuberculosis* to either milk or PBS, samples were mixed by gentle rocking for 10 min on a laboratory rocker (Daigger, Bethesda, MD). Each sample was subjected to the basic milk-processing protocol, with a single variable change between experiments. Within each protocol, the samples (20 ml) were centrifuged at $1,865 \times g$ for 30 min at 4°C to partition the milk. The whey layer was discarded, and the cream and pellet layers were retained for the experimental procedure.

Preparation of NALC-NaOH solution. Equal volumes of 2.9% sterile sodium citrate (Sigma-Aldrich, St. Louis, MO) and sterile NaOH (4 times the final concentration; Mallinckrodt, Hazelwood, MO) were mixed. Immediately prior to use, *N*-acetyl-L-cysteine (NALC) (Sigma-Aldrich) was added to yield a concentration of 0.5%.

NALC-NaOH concentration and exposure time. The cream and pellet layers were resuspended in PBS to a final volume of 5 ml. Then, 5 ml of NALC-NaOH solution containing 1.0, 2.0, 3.0, or 4.0% NaOH was added to the tubes and briefly mixed, yielding final NaOH concentrations of 0.50, 1.00, 1.50, and 2.00%, respectively. The tubes were incubated for 15 min at room temperature (RT) (22 to 25°C). Immediately after the incubation, 15 ml of PBS was added to dilute the NaOH and then was centrifuged at $1,865 \times g$ for 30 min at 4°C . The chemical layer was discarded, and the cream and pellet were resuspended in 1 ml PBS for inoculation of the media. To investigate the effects of different exposure times, the same protocol was performed, except 5 ml of NALC-NaOH solution containing 2.0% NaOH was added to the samples, yielding a final concentration of 1.0% NaOH. Samples were then incubated for 5, 15, or 30 min at RT.

Addition of antibiotics for decontamination. The milk samples were spiked in triplicate with *M. avium* subsp. *paratuberculosis* to final concentrations of 10^2 , 10^4 , and 10^6 CFU/ml. Positive and negative controls (as described above) also were used. Samples were decontaminated by using the protocol optimized in the aforementioned experiments with 1.5% NALC-NaOH for 15 min at RT. After centrifugation at $1,865 \times g$, replicate samples from each decontamination procedure were resuspended in either 1 ml of an antibiotic solution containing 100 $\mu\text{g/ml}$ vancomycin, 50 $\mu\text{g/ml}$ amphotericin B, and 100 $\mu\text{g/ml}$ nalidixic acid (VAN), or 1 ml of PBS. Samples resuspended in the antibiotic solution were incubated overnight at 39°C and then inoculated into the media. Samples resuspended in PBS were inoculated into the media immediately.

Media for recovery of *M. avium* subsp. *paratuberculosis* organisms. Two liquid culture media, Bactec 12B and *para*-JEM, and one solid medium, HEY, were compared for their ability to recover *M. avium* subsp. *paratuberculosis* organisms from spiked milk samples. Bactec 12B medium (BD) was supplemented with 0.5 ml of sterile unmodified raw egg yolk (in-house), 0.2 ml PANTA (BD), 0.1 ml of 50 $\mu\text{g/ml}$ mycobactin J, and 1.2 ml sterile deionized water. This supplemented medium will be referred to as Bactec 12B medium. Bactec 12B bottles were each inoculated with 200 μl of decontaminated milk sample and placed in a 37°C incubator. The growth index (GI) of microorganisms in the bottles was measured by the Bactec 460 machine (BD) twice per week for the first 2 weeks, and then once per week for the following 10 weeks. The bottles were considered positive when the GI reached 300, and they were then removed from incubation. At the end of the 12-week (84-day) incubation period, all bottles were subject to *M. avium* subsp. *paratuberculosis* growth confirmation. The *para*-JEM medium (Thermo Fisher Scientific [TREK Diagnostic Systems, Inc.], Cleveland, OH) was supplemented according to the manufacturer's instructions. Each bottle was inoculated

TABLE 1 Classification of the final interpretation of each milk sample based upon machine indication, acid-fast stain, and IS900 PCR results

Test	Test results indicating a classification of a milk sample as ^a :				
	Positive	Contaminated	False positive	No growth	False negative
Machine indicator	Positive	Positive	Positive	Negative	Negative
Acid-fast stain	Positive	Negative; non-AF organisms present	Negative	Negative	Positive
IS900 PCR	Positive	Negative	Negative	Negative	Positive

^a Results of all three tests were used to arrive at this classification.

with 500 μ l of decontaminated milk sample and placed into the ESP culture system II incubator for up to 65 days, according to the manufacturer's instructions. When the bottles indicated a positive result and indicated a positive growth curve, the bottles were removed for *M. avium* subsp. *paratuberculosis* growth confirmation. At the end of the incubation period, all bottles were subject to the *M. avium* subsp. *paratuberculosis* growth confirmation procedures noted below. Before a medium was removed from the bottles for growth confirmation, the bottles were shaken on a VXR IKA Vibrax shaker (IKA Works, Inc., Wilmington, NC) for 2 min to dislodge *M. avium* subsp. *paratuberculosis* cells from the sponges in the bottles. *M. avium* subsp. *paratuberculosis* organism recovery from milk also was evaluated on HEY medium (BD) and compared with recoveries from both liquid media. HEY medium containing mycobactin J and VAN was inoculated with 100 μ l of decontaminated milk sample. The slants were allowed to incubate at 39°C for 12 weeks. The colonies were counted at 4, 8, and 12 weeks postinoculation.

***M. avium* subsp. *paratuberculosis* growth confirmation.** A Ziehl-Neelsen acid-fast stain was performed on all bottles that were subject to *M. avium* subsp. *paratuberculosis* growth confirmation. One drop of Acid Fast Mycobactin cell adhesive (Wescor, Inc., South Logan, UT) was placed onto a microscope slide. About 100 μ l of sample was added to each slide and mixed thoroughly with the Mycobactin. The slides were allowed to air dry before being fixed in 100% methanol for 15 min. Slides were then stained with a standard Ziehl-Neelsen stain, followed by methylene blue counterstain. DNA was extracted from the samples for confirmation by PCR by incubating 500 μ l of sample with 100 μ l of proteinase K (10 mg/ml; Qiagen, Germantown, MD) at 50°C overnight in an orbital water bath, followed by centrifugation at 15,000 $\times$ g for 15 min to separate the fractions of the sample. The supernatant was removed, and the pellet was resuspended in 175 μ l of phosphate buffer from the MagMAX total nucleic acid isolation kit (Life Technologies Corporation, Carlsbad, CA). DNA was extracted from the pellet using the protocol provided by the kit on the MagMAX Express machine (Life Technologies). The extracted DNA was assayed for the presence of *M. avium* subsp. *paratuberculosis* cells using real-time PCR with the *M. avium* subsp. *paratuberculosis*-specific IS900 target. The primer and probe sequences for IS900 were as follows: 200 nM forward primer, 5'-CCGCTAATTGAGAGATGCGATTGG-3', 200 nM reverse primer, 5'-AATCAACTCCAGCAGCGCGGCCTCG-3', and 100 nM probe, 5'-TCCACGCCCGCCAGACAGG-3' with a 5' fluorescent 6-carboxyfluorescein (FAM) label and a 3' 6-carboxy-tetramethylrhodamine (TAMRA) quencher (Integrated DNA Technologies, Coralville, IA). Quantitative PCR (qPCR) was conducted in an Applied Biosystems 7500 fast real-time PCR system (Life Technologies) by using TaqMan fast advanced master mix (Life Technologies). Each qPCR plate also contained a standard curve ranging from 10⁻⁵ to 1 ng/ μ l that was generated from *M. avium* subsp. *paratuberculosis* strain K10 genomic DNA, no-template negative controls, and a positive control of *M. avium* subsp. *paratuberculosis* strain 19698 genomic DNA. Amplification conditions were as follows: stage 1, 95°C for 20 s; stage 2, 94°C for 3 s; and 66°C for 30 s, repeated for 40 cycles. All samples with a cycle threshold (C_T) of ≤ 37 were considered positive. *M. avium* subsp. *paratuberculosis* growth was confirmed on the HEY medium slants by colony morphology and slow-growing characteristics. All colonies were compared with those that were present on the positive-control sample slants. If any unusual colonies appeared, confirmatory protocols were performed, as stated above.

Classification of final culture results. The final interpretation of the positive or negative status of each milk sample after culture is shown in Table 1. A sample was classified as positive if both the acid-fast (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-*M. avium* subsp. *paratuberculosis* 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 false negative if the AF and PCR results were both positive following a negative machine signal.

Statistical analysis. Statistical analysis was performed by using the GLIMMIX procedure of the Statistical Analysis System (SAS Institute, Cary, NC). The model included the fixed effects of treatment (concentration of NaOH, time, culture medium, and antibiotics), sample (spiked milk), and the treatment-sample interaction. When significant effects ($P < 0.05$) because of treatment, sample, and the treatment-sample interaction were detected, the least square means separation was computed by the Tukey-Kramer *post hoc* test.

RESULTS

The effects of increasing concentrations of NaOH as a component of the NALC-NaOH decontamination protocol on the recovery of viable *M. avium* subsp. *paratuberculosis* cells from experimentally inoculated raw milk are shown in Fig. 1. Data from milk samples were collapsed across concentrations of *M. avium* subsp. *paratuberculosis* (10² to 10⁸ CFU of *M. avium* subsp. *paratuberculosis*/ml), as the pattern of results was similar within each level of inoculum. Within the milk matrix, *M. avium* subsp. *paratuberculosis* demonstrated significant resistance to NaOH as a chemical decontaminant, as subjection of the samples to NaOH concentrations ranging from 0.50% to 2.00% had no effect ($P = 0.34$) on the time to recovery, regardless of *M. avium* subsp. *paratuberculosis* concentration in the milk (Fig. 1A). Although the overall time to recovery was earlier for samples cultured in Bactec 12B medium than with samples cultured in *para*-JEM medium by an average of 3.2 days, the lack of NaOH-related lethality for *M. avium* subsp. *paratuberculosis* was noted for both media.

To assess the effects of increased time of exposure of *M. avium* subsp. *paratuberculosis* in milk to chemical decontamination with NALC-NaOH, only one concentration of NaOH (1%) was used (Fig. 1B). Increasing the time of exposure from 5 to 30 min did not affect the time to recovery of *M. avium* subsp. *paratuberculosis* in either medium tested. Once again, the Bactec 12B medium decreased recovery time compared with *para*-JEM by an average of 4.8 days (Fig. 1B). Although the concentration of NaOH and exposure time did not affect the viability of *M. avium* subsp. *paratuberculosis*, there were notable effects on the amount of contaminating microorganisms (Fig. 2). The incidence of contamination in samples cultured in Bactec 12B medium decreased sharply ($P < 0.01$) from 86.7 to 13.3% after treatment with NaOH concentrations of 0.50 and 1.00%, respectively. Sample contamination de-

FIG 1 Time to recovery of viable *Mycobacterium avium* subsp. *paratuberculosis* cells in raw milk after treatment with *N*-acetyl-L-cysteine (NALC) and different concentrations (0.50, 1.00, 1.50, and 2.00%) of sodium hydroxide (NaOH) for 15 min (A) or with NALC-1.00% NaOH for 5, 15, or 30 min (B). Treated samples were cultured in Bactec 12B and *para*-JEM media. Data were collapsed across all *M. avium* subsp. *paratuberculosis* concentrations (10^2 to 10^8 CFU/ml). Data are expressed as means $\pm$ standard errors of the mean (SEM) ($n = 12$). Significant differences ($P < 0.05$) between the two media within a treatment are represented by an asterisk.

creased to nondetectable levels when NaOH concentrations of 1.50 and 2.00% were used. A similar decrease in contamination level was observed when the time of exposure to NaOH was increased (Fig. 2). Contamination of raw milk samples decreased from 26.7% to 13.3% with exposure times of 5 and 15 min, respectively.

When samples were treated with 1.00% NaOH for 30 min, the presence of contaminating microorganisms was negligible. Distinct thresholds in both NaOH concentration and exposure time were observed for samples incubated in Bactec 12B, where NaOH was able to effectively suppress contamination. These thresholds

FIG 2 Percentage of samples cultured in Bactec 12B medium overgrown with contaminating microorganisms after treatment with *N*-acetyl-L-cysteine (NALC) and 0.50, 1.00, 1.50, and 2.00% sodium hydroxide (NaOH) for 15 min or treatment with NALC-1.00% NaOH for different exposure times (5, 15, or 30 min). Significant differences between treatments are represented by asterisks: **, $P < 0.01$; *, $P < 0.05$.

FIG 3 Comparison between Bactec 12B and *para*-JEM media in time to recovery for *Mycobacterium avium* subsp. *paratuberculosis* cells in raw milk spiked with 10² to 10⁸ CFU/ml after exposure to *N*-acetyl-L-cysteine–sodium hydroxide (NALC-1.00% NaOH) and hexadecylpyridinium chloride (HPC) (0.75%) chemical decontaminants. Data are expressed as means $\pm$ SEM; $n = 36$ for HPC, $n = 21$ for NALC-NaOH. Significant differences ($P < 0.01$) between the two media within a chemical treatment are represented by two asterisks, and significant differences ($P < 0.01$) between the two chemicals and within a medium type are represented by two number signs.

consisted of treatment of samples with either NALC-1.50% NaOH for 15 min or treatment with NALC-1.00% NaOH for 30 min. The *para*-JEM medium demonstrated a greater ability to suppress contaminating microorganisms, with no significant amounts of contamination detected, regardless of the concentration of NaOH or the time of exposure (data not shown). Contamination rates in nontreated control milk samples inoculated with 10⁶ CFU of *M. avium* subsp. *paratuberculosis*/ml approached 100% in all experiments, regardless of the type of culture medium.

Bactec 12B, *para*-JEM, and HEY media differed significantly in their capacity and efficiency to recover *M. avium* subsp. *paratuberculosis* from decontaminated milk samples. It quickly became apparent that HEY medium (HEYM) was inferior to the liquid media for the recovery of *M. avium* subsp. *paratuberculosis* cells from milk, with estimated limits of detection of 10⁴ CFU/ml. Because recovery rates were so poor for HEY medium, they are not presented here; however, it is important to note that contamination rates declined from 11.1% to 0% for milk samples (10⁴ CFU/ml) treated with NALC-0.50% NaOH and NALC-1.00% NaOH for 15 min, respectively. Contamination did not contribute to the lack of sensitivity of samples cultured on HEYM. There were no significant differences observed in the sensitivity of detection between the liquid culture media across all spiking concentrations, though there was a tendency ($P = 0.17$) for the Bactec 12B medium to recover *M. avium* subsp. *paratuberculosis* faster than the *para*-JEM medium (Fig. 3). Both media were able to recover all of the samples inoculated, including the lowest concentration of *M. avium* subsp. *paratuberculosis* inoculated into milk (10² CFU/ml). Compared with previous studies where the effects of HPC concentration and the time of exposure were explored, the NALC-NaOH protocol significantly decreased ($P < 0.01$) the time to recovery in both Bactec 12B and *para*-JEM media, as seen in Fig. 3. This difference became more pronounced with lower inoculation levels. On average, decontamination with NALC-NaOH decreased the time to detection by 18.3 and 28.4 days in Bactec 12B and *para*-JEM media, respectively, relative to decontamination with HPC. In addition, the accuracy of the systems of detection differed, as the *para*-JEM medium yielded two incidences of false machine signaling, whereas the Bactec 12B medium yielded none.

Overnight exposure of milk samples to the antibiotic cocktail VAN, after decontamination with NALC-NaOH, had detrimental effects on the viability of *M. avium* subsp. *paratuberculosis*. A significant ($P < 0.01$) increase in the time to recovery was observed after additional exposure to VAN for the samples cultured in both Bactec 12B and *para*-JEM media (Fig. 4). Further, an increase in undetectable samples was observed for samples cultured in the *para*-JEM medium, as two of the triplicate samples within the 10²-CFU/ml inoculation level were undetected after treatment with VAN, whereas all the replicates at this concentration were detected without exposure to VAN. The Bactec 12B medium was able to detect all of the samples inoculated with 10² CFU of *M. avium* subsp. *paratuberculosis*/ml, but there was a significant ($P < 0.01$) increase in the time to recovery of 19 days after treatment with VAN. The effects of VAN treatment were negligible because the rate of contamination in samples was low regardless, but contamination in the Bactec 12B medium was decreased from 1/12 to 0/12 samples after incubation with VAN. No contamination was seen in samples treated with either NALC-NaOH alone or NALC-NaOH plus VAN when cultured in *para*-JEM medium.

DISCUSSION

The present study examined the effects of exposure to NALC-NaOH alone and in combination with VAN on the recovery of viable *M. avium* subsp. *paratuberculosis* organisms from experimentally spiked raw milk. The application of the NALC-NaOH decontamination procedure to sputum samples prior to culture for *M. tuberculosis* was first reported in 1963 (12). In that study, the addition of the mucolytic agent NALC in conjunction with NaOH for the decontamination of sputum samples permitted a decrease in the concentration of NaOH from 4.0 to 1.0% without increasing the contamination rate. The decrease in NaOH concentration increased the number of sputum samples that tested positive for *M. tuberculosis* from 53/129 to 81/129. It is speculated that the liquefaction of sputum by NALC improved contact between the contaminating microorganisms and NaOH, thereby permitting the use of lower concentrations of NaOH. This liquefaction also may have increased the recovery of *M. avium* subsp.

FIG 4 Effects on the time to recovery of *Mycobacterium avium* subsp. *paratuberculosis* cells in milk spiked with 10² to 10⁶ CFU/ml and cultured in Bactec 12B and *para*-JEM media after treatment with *N*-acetyl-L-cysteine–sodium-hydroxide (NALC-NaOH) alone and in combination with an antibiotic cocktail (100 µg/ml vancomycin, 50 µg/ml amphotericin B, and 100 µg/ml nalidixic acid [VAN]). Data are expressed as means ± SEM (*n* = 3). Significant differences between NALC-NaOH alone or NALC-NaOH plus VAN within each medium type are represented by asterisks (**, *P* < 0.01; *, *P* < 0.05).

paratuberculosis cells from milk samples in the present study by facilitating better recovery of the mycobacterium during centrifugation. Because milk and sputum have similarly high protein content, we hypothesized that NALC would have the same liquefaction effect on milk samples, permitting the use of NaOH concentrations of <4.0%.

The present study demonstrates that the decontamination of milk with NALC-NaOH effectively controlled the growth of contaminating microorganisms while causing minimal damage to viable *M. avium* subsp. *paratuberculosis* cells. We found that neither increasing concentrations of NaOH from 0.5% to 2.0% in the NALC-NaOH decontamination protocol nor increasing the length of exposure to the chemical treatment from 5 to 30 min significantly affected the viability of *M. avium* subsp. *paratuberculosis* cells. Our finding is contrary to other studies that have found that NaOH both alone and in combination with NALC can be detrimental to the viability of mycobacteria (17, 18). When sputum samples were cultured for mycobacteria, an increase in NALC-NaOH concentration from 1.00 to 1.25% with a 15-min exposure time caused the frequency of positive cultures to decrease from 21 to 11%, respectively (18). Likewise, another study on *Mycobacterium bovis* suspended in tissue homogenate reported a decrease in survival rate from 89.0 to 4.1% when the concentration of NaOH is increased from 0.001 to 10.0% and exposed for 30 min (17). When NaOH has been used in a singular fashion to decontaminate samples, it has shown potential to be lethal to mycobacteria, depending upon the concentration and the time of exposure.

Different species of mycobacteria, however, have variable levels of tolerance to NaOH. A study compared the effects of the decontamination of sputum with 1.7% NaOH and 3.0% sulfuric acid on the recovery of *Mycobacterium* spp. (19). When the samples were decontaminated with NaOH, a higher number of samples were positive for *Mycobacterium intracellulare* than when decontaminated with sulfuric acid. Conversely, a higher number of samples were positive for *Mycobacterium lentiflavum* when decontaminated with sulfuric acid than with NaOH. This observation demonstrates that *M. intracellulare* has a higher tolerance for exposure to NaOH than does *M. lentiflavum*. In our protocol, we chose to neutralize the NaOH by adding excess amounts of PBS, as

it was a quick and simple method of neutralization. Another option would have been to use a pH indicator and acid to neutralize the solution. We chose not to use this option because it not only substantially increased the labor time, but the resulting samples would be opaque, an effect that could interfere with the machine indicators during culture.

Increased concentrations of and length of exposure to NALC-NaOH were effective in controlling the presence of contaminating microorganisms in milk samples. We observed threshold values of NaOH concentration and length of exposure that allowed for very few contaminating microorganisms to survive. These threshold values were NALC-1.50% NaOH with an exposure time of 15 min, or a lower concentration of NALC-1.00% NaOH with the exposure time extended to 30 min. Either of these threshold combinations would likely be an acceptable option as a decontamination protocol. Because there was a larger decrease in the incidence of contamination as the concentration of NaOH was increased than with increased exposure times, we selected the former as the optimal protocol.

Hexadecylpyridinium chloride has been the most common chemical used for the decontamination of milk for the isolation of *M. avium* subsp. *paratuberculosis*, even though it has been shown to be detrimental to the viability of *M. avium* subsp. *paratuberculosis* cells. In a previous study evaluating the effects of HPC decontamination on *M. avium* subsp. *paratuberculosis*, the mean recovery of *M. avium* subsp. *paratuberculosis* cells in milk after exposure to 0.75% HPC for 5 h was estimated to be 28.7% (10). When the time of exposure to HPC was increased to 24 h, 45% more *M. avium* subsp. *paratuberculosis* cells were killed than at 5 h exposure (8). In the present study, we found that treatment with NALC-NaOH always led to a faster time to recovery regardless of *M. avium* subsp. *paratuberculosis* concentration or the medium used for culture compared with treatment with HPC. The faster time to recovery after NALC-NaOH decontamination might be multifactorial. First, *M. avium* subsp. *paratuberculosis* might be more resistant to NaOH than to HPC, as the two chemicals disrupt the cells via different mechanisms (20, 21). Interestingly, NaOH in conjunction with oxalic acid is less detrimental to *M. avium* subsp. *paratuberculosis* during the decontamination of fecal samples than is HPC (22). The number of fecal samples from which *M. avium*

subsp. *paratuberculosis* was isolated increased from 3/186 when decontaminated with 0.75% HPC followed by incubation with VAN to 15/186 when decontaminated with 4.0% NaOH followed by incubation with 5.0% oxalic acid. Second, there might be more effective neutralization and less carryover of the chemical into culture with NALC-NaOH treatment than with HPC treatment because the NaOH is neutralized by dilution, whereas no steps are taken to neutralize the HPC. Lastly, the reduction of disulfide bonds by NALC causes liquefaction of the milk sample that may allow for better partitioning of *M. avium* subsp. *paratuberculosis* into the cream and pellet fractions during centrifugation, leading to a higher rate of recovery.

We had previously encountered deficiencies with the use of HEY medium for the culture of *M. avium* subsp. *paratuberculosis* from raw milk after decontamination with HPC. Many of the same deficiencies were encountered with milk that was decontaminated with NALC-NaOH. These deficiencies include a high threshold of detection, around 10^4 CFU of *M. avium* subsp. *paratuberculosis*/ml, and technical issues with inoculation of the medium and enumeration of the colonies.

Although the Bactec 12B and *para*-JEM media performed considerably better than HEYM, there were still marked differences in the abilities of these two media to recover low concentrations of *M. avium* subsp. *paratuberculosis* organisms from milk samples. A direct comparison between the Bactec 12B and *para*-JEM media for the recovery of mycobacteria from clinical specimens had previously been conducted, demonstrating that isolates of the *M. avium* complex (MAC) were recovered more efficiently using *para*-JEM medium (23). Although *M. avium* subsp. *paratuberculosis* has been associated with MAC, *M. avium* subsp. *paratuberculosis* isolates were not specifically identified within the study, and the clinical samples did not include milk. In the present study, Bactec 12B medium consistently detected growth of the samples faster than did the *para*-JEM medium, though the difference was not statistically significant. Faster detection might translate to lower thresholds of detection, which is a key factor in optimizing the recovery of *M. avium* subsp. *paratuberculosis* organisms from milk. The Bactec 12B medium had significantly higher contamination rates at low concentrations of NaOH and shorter exposure times than did the *para*-JEM medium. The contamination that was observed in the samples cultured in the Bactec 12B medium was primarily of a fungal nature. This result is contrary to previous studies in our laboratory that evaluated the use of HPC chemical decontamination in which contamination occurred in both media and was found to be primarily bacterial, with *Staphylococcus*, *Bacillus*, and *Pseudomonas* species isolated.

The varied performance of the media might be caused by differences in media formulation and growth detection. Major formulation differences include the formulation of egg yolk supplements, use of various antibiotics, and the addition of other modifying agents to the medium. The methods of detecting growth by each medium are also quite different, as the Bactec 12B medium detects evolved radioactive carbon dioxide after bacteria oxidize ^{14}C -labeled palmitic acid and the *para*-JEM medium detects a decrease in the headspace pressure of the bottle. Interestingly, samples treated with HPC had a higher incidence of false signals than those treated with NALC-NaOH. Collectively, the Bactec 12B medium achieved results that were superior to the *para*-JEM medium in regards to both detection thresholds and time to detection.

Antibiotic cocktails frequently are used in conjunction with chemical decontaminants to control the growth of contaminating microorganisms during culture. The VAN antibiotic cocktail is frequently used, even though studies have noted that VAN in concentrations used in the present study is detrimental to the viability of *M. avium* subsp. *paratuberculosis* organisms, with the suggestion to decrease the concentration of vancomycin and nalidixic acid to 50 $\mu\text{g}/\text{ml}$ (8, 24–26). In the present study, VAN exposure had detrimental effects on the viability of *M. avium* subsp. *paratuberculosis* cells and contaminating microorganisms. The lethal effects of VAN that were observed may be compounded by subsequent exposure to NALC-NaOH. NALC-NaOH can damage the *M. avium* subsp. *paratuberculosis* bacterium and may cause it to be more vulnerable to the antibiotics to which it is normally resistant. The marked decrease in viability is not a reasonable accommodation for the decrease in contaminants, especially when the goal is to define a protocol that is able to detect low concentrations of *M. avium* subsp. *paratuberculosis* present in milk. Further studies should investigate whether different antibiotics might have similar effects on *M. avium* subsp. *paratuberculosis* or whether decreasing the concentration of each antibiotic in the cocktail would decrease the detrimental effects observed for VAN on *M. avium* subsp. *paratuberculosis* as found in this study.

The primary focus of the current study was the comparison of NALC-NaOH and HPC for chemical decontamination prior to the culture of *M. avium* subsp. *paratuberculosis* from milk. Within this study, *M. avium* subsp. *paratuberculosis* 167 was the only *M. avium* subsp. *paratuberculosis* isolate used to inoculate milk samples. Strain 167 was originally isolated from a cow with clinical disease at the NADC and is aligned both genomically and phenotypically with the K-10 cattle strain that was used for sequencing the complete genome of *M. avium* subsp. *paratuberculosis* (27). This suggests that other cattle strains might demonstrate similar effects with chemical decontamination and culture. However, it is uncertain if differences exist between cattle and sheep strains of *M. avium* subsp. *paratuberculosis*, especially since it has been reported that growth curves between S-type (sheep and goat) strains were dissimilar from those of C-type strains (cattle and bison) in liquid culture (28). Furthermore, the growth of sheep strains of *M. avium* subsp. *paratuberculosis* has previously been shown to require the addition of supplemental egg yolk to the medium, suggesting phenotypic differences between the C- and S-type strains of *M. avium* subsp. *paratuberculosis* (29, 30). Studies should be conducted to determine the optimal conditions for the culture of *M. avium* subsp. *paratuberculosis* from the milk of small ruminants.

In conclusion, we recommend decontaminating raw milk with NALC-1.50% NaOH for 15 min at RT to maximize the recovery of *M. avium* subsp. *paratuberculosis* organisms from raw milk while minimizing the amount of contaminating microorganisms. The Bactec 12B medium had the lowest limit of detection and the fastest time to detection of the three media we evaluated. Although Bactec 12B medium is no longer commercially available, we hope that other products that will show similar efficacies for the recovery of viable *M. avium* subsp. *paratuberculosis* organisms from milk will be forthcoming. The ability to accurately assess the shedding of viable *M. avium* subsp. *paratuberculosis* organisms into the milk of naturally infected cattle is crucial for providing producers with credible information about the risk of transmitting *M. avium* subsp. *paratuberculosis* to calves via the consumption of milk from infected cows.

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