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Growth of *Mycobacterium avium* subsp. *paratuberculosis* in the Presence of Hexadecylpyridinium Chloride, Natamycin, and Vancomycin

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ABSTRACT

A design-of-experiments approach was used to examine the effect of hexadecylpyridinium chloride (HPC), alone or in combination with the antibiotics vancomycin and natamycin, on the growth of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). At concentrations above 74.4 μg/ml, HPC had a highly significant detrimental effect on the growth of MAP, whereas natamycin at 10.8 and 21.6 μg/ml and vancomycin at 5.2 and 10.4 μg/ml did not have such an effect. Titration of the amount of HPC tolerated by MAP indicated that growth can occur in the presence of 24.8 μg/ml or lower. Processing of bovine fecal specimens indicated that reducing the concentration of HPC from 32.22 to 1.07 mg/ml during decontamination may improve detection when cultures are grown on solid medium but not when cultures are grown in liquid medium. Further investigation into optimizing HPC concentration during processing of fecal samples is warranted. Natamycin, in conjunction with vancomycin, may be useful for controlling fungal contamination during isolation of MAP from fecal samples.

Johne's disease is a chronic enteropathy caused by the intracellular pathogen *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Clinical Johne's disease has been reported worldwide from a wide variety of ruminants, including cattle, sheep, goats, deer, bison, and llamas. Animals are typically infected as neonates, although clinical signs such as wasting do not appear until 2 to 5 years later. This lag time makes early detection of infected animals difficult. Diagnosis of Johne's disease is also made difficult by intermittent shedding of the organism and a poor correlation between an antibody response (measured with enzyme-linked immunosorbent assay) and fecal shedding *(13, 20).*

MAP is a slowly growing fastidious organism, and its in vitro culture can take up to 16 weeks on solid medium. Many of the currently employed fecal culture methods involve a decontamination step that includes incubation of the sample in the detergent hexadecylpyridinium chloride (HPC) *(2, 12, 18, 19).* Although HPC can reduce bacterial and fungal contamination, its carryover into the inoculum can cause cellular damage and inhibit the growth of MAP *(7, 9).* Many of the fecal culture protocols also include steps that initiate germination of bacterial and fungal spores in the sample and then introduce antimicrobial agents to kill any cells that have become vegetative. Commonly used antibiotics in these cocktails are vancomycin, amphotericin B, and nalidixic acid *(1, 2, 4).* The interaction of antibiotics, particularly vancomycin, with MAP cells in the presence of residual amounts of HPC may affect the culture of the organism. However, the interplay of decontaminating agents with antimicrobials and their effects on MAP growth has not been rigorously examined.

The use of antifungal agents that effectively suppress fungal growth is critical for the isolation of MAP, especially given the long culture periods required. Amphotericin B and cycloheximide have been used, but contamination rates are typically around 7 to 10% of cultures and may be much higher depending upon the local flora of the animals being tested and the culture method used *(4, 18)* (unpublished results). Natamycin is a fungicide that has been reported to be effective for suppressing fungal but not bacterial growth *(10).* Natamycin has higher antifungal activity than cycloheximide and lacks the toxicity associated with cycloheximide use. The fungicidal effects of natamycin are exerted through its binding to sterols in the fungal cell membrane, which makes the membrane leaky *(6).* Bacteria are not susceptible to natamycin because their cell membranes lack sterols *(16).* Natamycin is an attractive antimicrobial for use in fecal culture of MAP because of its safety and effectiveness and the natural resistance of bacteria.

Vancomycin at 10 μg/ml has been included as part of an antibiotic cocktail to control the growth of nonmycobacterial fecal microflora during culture for the isolation of MAP *(1).* However, some MAP isolates are inhibited by vancomycin concentrations this high *(17),* and organisms that have been injured during fecal specimen processing with HPC may also be more sensitive to vancomycin.

In this study, a design-of-experiments approach was used to examine the effects of HPC, vancomycin, and natamycin on the growth of MAP and to assess the potential

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for natamycin to improve fungal suppression in fecal cultures.

MATERIALS AND METHODS

Bacteria and culture conditions. *M. avium* subsp. *paratuberculosis* ATCC 19698 (American Type Culture Collection, Manassas, Va.) was used in these studies to inoculate Middlebrook 7H9 medium supplemented with oleic acid, bovine albumin, dextrose, and catalase (OADC; BBL, Becton Dickinson, Sparks, Md.). Cultures were grown in 30-ml polystyrene tissue culture flasks (Corning Inc., Corning, N.Y.) at 37°C without shaking. Growth was monitored weekly by measuring the optical density at 600 nm (OD_{600}) with a DU series 500 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Cell clumps were disrupted prior to determining absorbance by extended vortexing in the presence of 2-mm sterile glass beads.

Antibiotics. Vancomycin (Van; Sigma, St. Louis, Mo.) was used as a 10 mg/ml stock solution. Natamycin (Nat; Sigma) was used as a 2.5 mg/ml stock solution.

Inhibitory concentration of HPC. The starting concentration of HPC was based on an estimate of its carryover into an inoculum that was obtained with a centrifugation–double incubation fecal sample processing method *(18).* The decontamination procedure started with a 2-g fecal specimen in 35 ml of water, and 5 ml of this solution was transferred into 25 ml of 32.22 mg/ml HPC in 0.5× brain heart infusion (BHI) broth. We estimated that the pellet remaining after centrifugation would be 0.1 to 0.2 ml in volume. When this pellet is then resuspended in 1 ml of an antibiotic brew containing 100 μg/ml Van, 100 μg/ml nalidixic acid, and 50 μg/ml amphotericin B *(18),* the HPC concentration is about 2.094 mg/ml, and this amount would be present upon inoculation onto a solid medium. HPC prepared in 0.5× BHI was added to tissue culture flasks with Middlebrook 7H9 medium containing OADC such that the final concentration of HPC was 0, 2.094, 0.67, or 0.224 mg/ml or 74.4, 24.8, or 8.4 μg/ml in a 15-ml volume. The inoculum was prepared by scraping a plate of freshly grown MAP and placing it into a tube containing 1 ml of Middlebrook 7H9 medium. Disruption of cell clumps and determination of absorbance were performed as described above. The cells were then diluted to a density of 10^4 CFU/ml in Middlebrook 7H9 medium. The cell density was verified by plating serial 10-fold dilutions of the inoculum on solid Middlebrook 7H9 medium supplemented with OADC.

Cultures were prepared in triplicate and incubated at 37°C without shaking. Growth was monitored weekly for 6 weeks by determining the OD_{600} after first disrupting cell clumps as described above. Plate counts were used to verify cell densities.

Experimental design. Design-Ease version 6.0 software (Stat-Ease, Minneapolis, Minn.) was used to examine the effects of Nat, HPC, and Van on MAP growth and any interactions between or among these agents. This software randomizes the experimental set up to reduce user bias and allows the user to determine whether significant interactions have occurred between or among the factors. Different concentrations of HPC, Van, and Nat were examined in a 3 × 3 factorial design, creating 27 unique treatments of the three agents. HPC was tested at 0 mg/ml and 74.4 and 24.8 μg/ml based on the concentration of HPC tolerated by MAP. Van was used at 0, 5.2, and 10.4 μg/ml, and Nat was used at 0, 10.8, and 21.6 μg/ml *(1, 10).* HPC and the two antibiotics were added to flasks containing 15 ml of Middlebrook 7H9 medium with OADC. Duplicate flasks were then inoculated with MAP at 100 CFU/ml using 150 μl of an inoculum prepared as described above.

Statistical analysis was performed with Design-Ease 6.0 and its analysis of variance (ANOVA) to examine the interactions among the three antimicrobial agents. The highest CFU per milliliter obtained for each culture condition was determined based on the OD_{600}, given that an OD_{600} of 1 was equivalent to 10^8 CFU/ml, and verified using plate counts obtained after plating serial 10-fold dilutions of the culture on solid Middlebrook 7H9 medium supplemented with OADC. The ANOVA was then performed using these determinations after log transformation of the data. Paired *t* tests were used to compare contamination and bacterial recovery within a shedding level for fecal specimens processed with different concentrations of HPC. Differences were considered significant at $P < 0.05$.

Fecal processing. To determine whether lower concentrations of HPC could improve the recovery of MAP from bovine fecal specimens but still provide adequate contamination control, a pilot fecal sample processing study was performed. Thirty-five previously characterized bovine fecal specimens were each processed with 32.22, 3.22, and 1.07 mg/ml HPC in 0.5× BHI using a previously described method *(2, 19).* Based on their prior characterization, the specimens were classified as being from animals of high (>300 CFU/0.1 g of feces), moderate (30 to 300 CFU/0.1 g of feces), or low (<30 CFU/0.1 g of feces) shedding status *(5).* For each sample, 2 g of feces was placed into a 50-ml sterile conical tube containing 35 ml of sterile water. The samples were mixed and allowed to settle for 30 min. The upper 5 ml of each sample was then transferred to a tube containing HPC in 0.5× BHI as indicated above. The samples were incubated overnight at 37°C and centrifuged at 900 × *g.* The supernatant was decanted, and the pellet was resuspended in 1.0 ml of an antibiotic brew containing 100 μg/ml Van, 100 μg/ml nalidixic acid, and 50 μg/ml amphotericin B prepared in 0.5× BHI. After overnight incubation at 37°C, 100-μl samples were inoculated onto four Herrolds egg yolk (HEY) agar slants (Becton Dickinson) with mycobactin J and one HEY slant without mycobactin J. The HEY slants were incubated at 37°C and examined using a stereomicroscope at ×25 magnification at 4, 8, 12, and 16 weeks to determine the number of colonies present and the degree of surface contamination. Samples (200 μl) were also inoculated into MGIT paraTB medium (Becton Dickinson), supplemented as recommended by the manufacturer, and grown using a BACTEC MGIT 960 system (Becton Dickinson). Colonies arising on HEY were acid-fast stained with auramine-rhodamine *(3)* and an Aerospray slide stainer-cytocentrifuge according to the manufacturer's recommendations (Wescor, Inc., Logan, Utah). The colonies were considered to be MAP if they were acid-fast positive and demonstrated mycobactin J dependence. Samples that were positive for MAP growth in the BACTEC MGIT 960 instrument were acid-fast stained with auramine-rhodamine as described above, MAP was confirmed with a TaqMan-based *IS*900 PCR assay *(5).* At the end of the recommended 42-day incubation period, any samples that had not indicated positive MAP growth were removed from the instrument, and the presence of MAP was determined by acid-fast staining and PCR assay.

RESULTS

Growth inhibition by HPC. Although HPC is commonly used for decontamination of fecal samples during attempted recovery of MAP, the optimum HPC concentration that will inhibit the growth of contaminating microflora yet allow the growth of MAP is unknown. We sought to determine what concentration of HPC could be present in the culture and still permit MAP growth. A series of three-

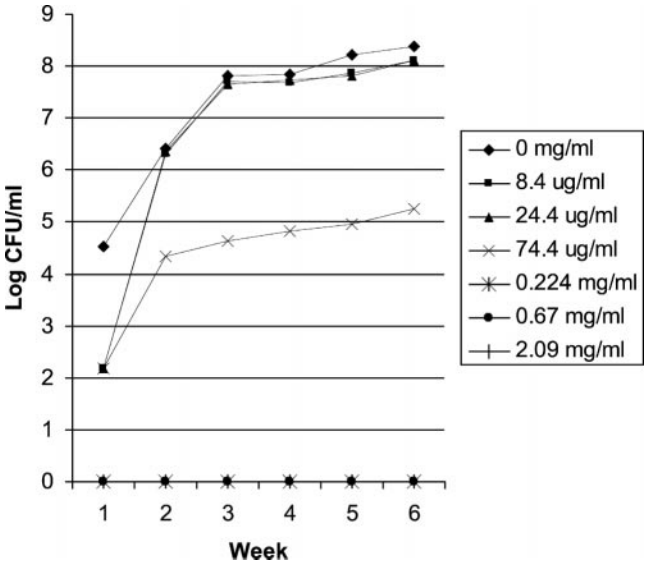

FIGURE 1. *Growth of MAP in the presence of decreasing concentrations of HPC. Middlebrook 7H9 medium containing different concentrations of HPC was inoculated with MAP. Growth (log CFU per milliliter) was monitored (OD_{600}) weekly for 6 weeks.*

fold dilutions were prepared and tested in triplicate to titrate the amount of HPC tolerated by MAP. Figure 1 indicates that MAP was able to grow well in the presence of 24.8 and 8.4 μg/ml HPC. Slight growth was observed in the presence of 74.4 μg/ml HPC, but no growth was seen in the presence of 0.224, 0.67, or 2.094 mg/ml HPC. To determine whether the effects of HPC could be reversed, bacteria from cultures containing the two highest concentrations of HPC were pelleted after 6 weeks of incubation, washed three times in Middlebrook 7H9 medium, and then recultured using fresh Middlebrook 7H9 containing OADC. No bacterial growth was observed after 12 weeks of incubation (data not shown).

Interaction of HPC, Van, and Nat. Figure 2A through 2E shows the results of determining growth in the presence of the 27 different combinations of the three agents. Figure 2A and 2B indicates that MAP growth is unaffected when Van or Nat are included in the medium, either alone or in combination, whereas growth occurs more slowly in the presence of 24.8 μg/ml HPC. Although some viable organisms (one or two colonies) were detected by plate counts after 4 weeks of growth, MAP growth was not observed in the presence of 74.4 μg/ml HPC. Figure 2C, 2D, and 2E indicates that the various combinations of HPC and Van, HPC and Nat, or all three agents do affect the growth of MAP more than the presence of HPC alone.

The results of the statistical analysis indicate that HPC has a highly significant detrimental effect on the growth of MAP ($P < 0.0001$). Neither Van nor Nat had a significant effect on the concentration of CFUs obtained for cultures containing these antibiotics ($P > 0.1$). Van and Nat also did not have any synergistic effects on the growth of MAP ($P > 0.1$).

Fecal processing. The results of processing 35 specimens with different HPC concentrations revealed that when HEY was used as the culture medium, 23 of 28 specimens previously characterized as positive for MAP were detected when they were processed with 32.22 mg/ml HPC, whereas 25 of 28 and 26 of 28 specimens containing MAP were detected when processing was performed with 3.22 and 1.07 mg/ml HPC, respectively (Table 1). When the specimens were categorized by shedding status, 7 of 7 and 8 of 8 MAP-positive specimens from animals of moderate and high shedding status, respectively, were detected by culture on HEY for each of the HPC processing concentrations tested. However, for specimens from animals of low shedding status, HPC processing at 32.22 mg/ml HPC resulted in the detection of 8 of 13 specimens, while processing in 3.22 and 1.07 mg/ml led to the detection of 10 of 13 and 11 of 13 MAP-positive samples, respectively.

When culture was performed in MGIT paraTB broth (Table 2), 23 of 28 MAP-positive specimens were detected when HPC processing concentration was 32.22 mg/ml. These 23 specimens included 9 of 13, 7 of 7, and 7 of 8 specimens from animals of low, moderate, and high shedding status, respectively. When HPC processing with 3.22 mg/ml was performed, 22 of 28 MAP-positive specimens were detected: 7 of 13, 7 of 7, and 8 of 8 from animals of low, moderate, and high shedding status, respectively. Processing using 1.07 mg/ml HPC resulted in the detection of 20 of 28 MAP-positive specimens: 7 of 13, 7 of 7, and 6 of 8 from animals of low, moderate, and high shedding status, respectively.

Although the average time to detection for specimens processed with 1.07 mg/ml HPC tended to be shorter than that observed for those processed with 32.22 and 3.22 mg/ml HPC, overall and within each shedding level, these differences were not significant (Table 3). In addition, no significant differences were observed in time to detection for specimens processed with 32.22 or 3.22 mg/ml HPC. However, time to detection for specimens from animals of low shedding status was significantly higher than that for animals of high shedding status for all HPC concentrations tested. Specimens from animals of moderate shedding status also had a significantly longer time to detection than did specimens from animals of high shedding status when processing was performed with 32.22 mg/ml HPC.

Comparison of contamination rates for cultures grown on HEY indicated that there were no significant differences between specimens processed with 32.22 or 3.22 mg/ml HPC through 12 weeks of incubation (Table 4). By 16 weeks of incubation, however, specimens processed with 3.22 mg/ml HPC had a significantly higher contamination rate ($P = 0.004$). There were no significant differences in contamination rates for specimens that had been processed with 3.22 or 1.07 mg/ml HPC through 8 weeks of incubation. However, after 12 weeks of incubation, the specimens that had been processed with 1.07 mg/ml HPC had a significantly higher contamination rate ($P = 0.045$). At 16 weeks of incubation, contamination was not significantly different between specimens processed with 3.22 and 1.07 mg/ml HPC. Specimens that had been processed with 1.07 mg/ml HPC had significantly higher contamination rates at

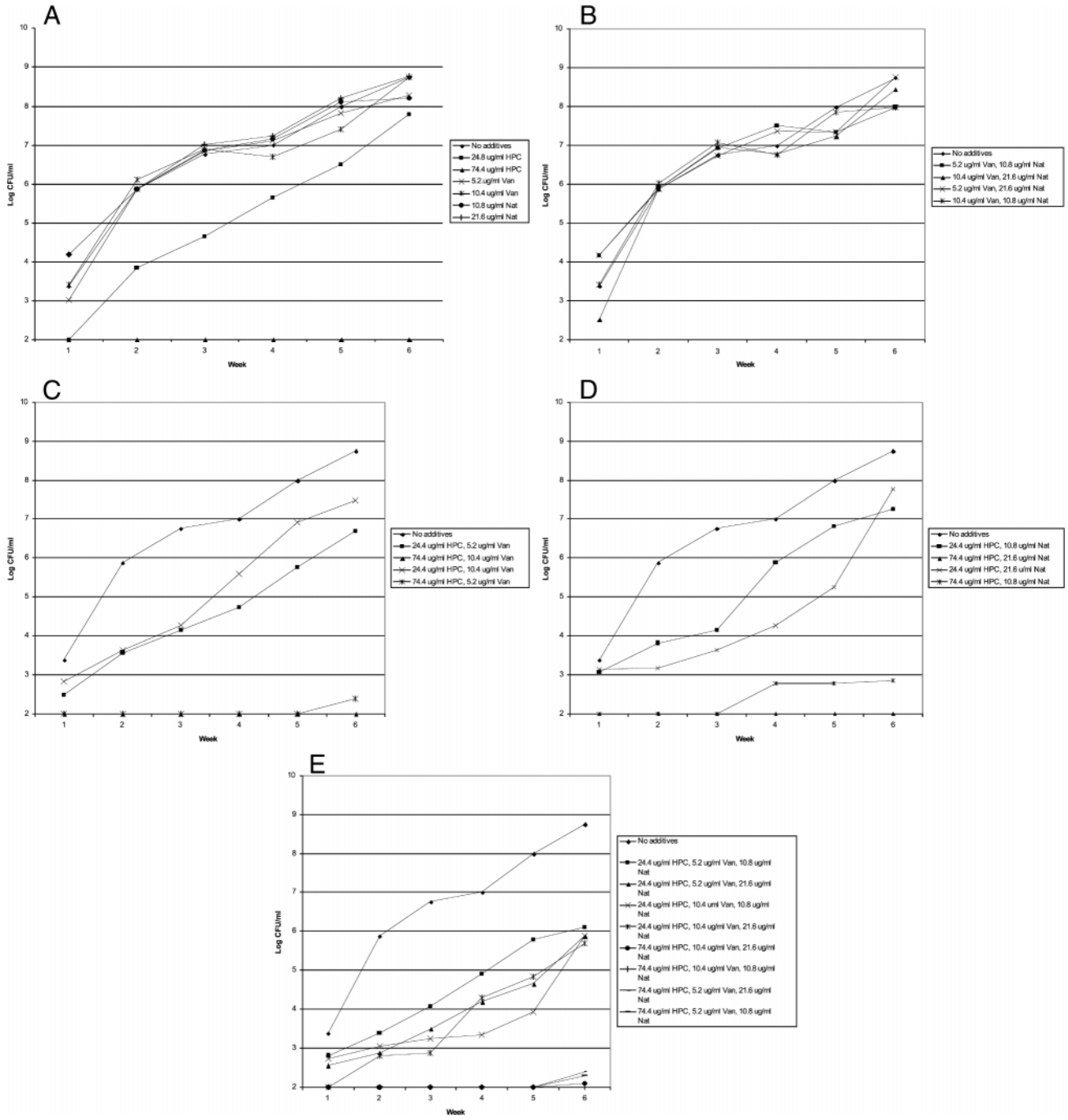

FIGURE 2. *Growth of MAP in the presence of HPC, vancomycin, and natamycin. Growth (log CFU per milliliter) in Middlebrook 7H9 broth with 27 different combinations of HPC, vancomycin (Van), and natamycin (Nat) was monitored in the presence of each agent individually (A), different concentrations of Van and Nat (B), different concentrations of HPC and Van (C), different concentrations of HPC and Nat (D), and different concentrations of all three agents (E).*

8, 12, and 16 weeks of incubation than those that had been processed with 32.22 mg/ml HPC ($P \leq 0.045$).

DISCUSSION

Recent reports have indicated that significant losses of bacteria occur during fecal sample processing for isolation of MAP *(11, 15).* For example, Stich et al. *(15)* reported a 2-log loss of seeded organisms during fecal sample processing; they attributed this loss to bacteria adhering to fecal matter that was discarded in the first step of processing or to detrimental effects of HPC on MAP. To examine the effects of HPC on the growth of MAP and investigate the effects of Van and Nat, a design-of-experiments approach was used. The findings of this study support the view that HPC toxicity could reduce recovery of MAP from fecal specimens. HPC completely inhibited growth of MAP at concentrations above 74.4 mg/ml, and fairly robust MAP growth occurred at HPC concentrations of less than or equal to 24.4 μg/ml, although the initial growth rate was slower. The toxic effect of HPC is most likely exerted during the overnight incubation step of the decontamination process. However, the toxic effect may be somewhat miti-

TABLE 1. *Comparison of fecal MAP results with different hexadecylpyridinium chloride concentrations for decontamination and culture on Herrolds egg yolk agar*

MAP status of specimens	*n*	No. of MAP-positive specimens with HPC concn (mg/ml) of:		
		32.22	3.22	1.07
Positive	28	23	25	26
Negative	7	12	10	9
Total	35	35	35	35
Sensitivity (%)		82.1	3.22	1.07

gated by the presence of organic material in the specimen, and inoculation into medium containing egg yolk may further neutralize the toxicity of HPC toward MAP *(8)*. Thus, although the results of this study indicate that small concentrations of HPC are toxic to MAP, the concentration of HPC that is active after processing and inoculation is difficult to estimate.

To the best of our knowledge, this is the first study of the effect of Nat on MAP growth. Neither Nat nor Van had an effect on the growth of MAP at concentrations commonly used to suppress growth of fungi *(10)* or other nonmycobacterial organisms *(1)*. There was no synergistic interaction between Nat and Van, indicating that these antibiotics can be used in combination to suppress the growth of contaminants during the isolation of MAP from fecal specimens.

A pilot processing study was performed to examine the effects of reduced MAP concentration on bacterial recovery and contamination. Because it could not be determined a priori whether the greatly reduced concentrations of HPC employed in this pilot study would prevent overgrowth of the sample by contaminants, a small number of fecal specimens was used. Although not significant in this small study, the results suggest that reducing the amount of HPC used during processing may improve detection when culture is performed on a solid medium. The results also suggest that reducing the HPC concentration may decrease the time to detection in liquid culture, although in some specimens processed with the lowest concentration of HPC contaminants appeared to outcompete MAP. However, the shorter average time to detection (4 to 5 days less) for specimens processed with 1.07 mg/ml HPC compared with those processed with 32.22 or 3.22 mg/ml HPC was not significant. The shorter time to detection for samples from animals of high and moderate shedding status compared with those for animals of low shedding status is presumably due to the higher numbers of organisms present in these specimens, resulting in higher numbers in the inoculum and a shorter lag period. The inability to detect MAP in samples previously characterized as positive, particularly those from animals of low shedding status, was most likely due to a loss of MAP viability associated with freezing and thawing *(12)*.

Additional studies of the effects of reduced HPC processing concentrations on MAP recovery and contamination in fecal samples are warranted. The use of Nat as a substitute for or in conjunction with amphotericin B *(1, 2, 4)* may help control contamination during the isolation of MAP; Nat could be incorporated into the antibiotic brew at the incubation step and/or into the culture medium.

TABLE 2. *Comparison of fecal MAP results with different hexadecylpyridinium chloride concentrations for decontamination and culture in MGIT paraTB broth*

MAP status of specimens	*n*	No. of MAP-positive specimens with HPC concn (mg/ml) of:		
		32.22	3.22	1.07
Positive	28	23	22	20
Negative	7	12	13	15
Total	35	35	35	35
Sensitivity (%)		82.1	78.6	71.4

TABLE 3. *Time to detection for confirmed MAP-positive fecal specimens cultured in MGIT paraTB broth*[a]

Shedding status	Time (days) to detection with HPC concn (mg/ml) of:		
	32.22	3.22	1.07
Low	32.2 ± 14.9[b]	34.8 ± 14.2[b]	30.3 ± 15.5[b]
Moderate	30.6 ± 7.3[c]	29.8 ± 11.7	28.3 ± 15.4
High	16.6 ± 7.3	17.8 ± 12.3	12.3 ± 12.0
Overall	27.6 ± 13.4	27.0 ± 14.21	24.7 ± 15.6

[a] Samples were determined to be positive for MAP by acid-fast staining and *IS*900 PCR assay with or without an instrument signal. Values are mean ± standard deviation.

[b] Significantly longer average time to detection for specimens from animals of low shedding status than for specimens from animals of high shedding status for all HPC concentrations ($P < 0.05$).

[c] Significantly longer average time to detection for specimens from animals of moderate shedding status than for specimens from animals of high shedding status for 0.9% HPC concentration ($P < 0.05$).

TABLE 4. *Effect of different hexadecylpyridinium chloride processing concentrations on contamination of cultures grown on Herrolds egg yolk agar*

Incubation wk	% tubes with visible contamination for HPC concn (mg/ml) of:		
	32.22	3.22	1.07
4	0	0.6	1.3
8	3.2	3.9	5.8[a]
12	16.1	16.8	19.4[b]
16	23.2	28.4[a]	27.1[a]

[a] Significantly higher contamination rate than samples processed with 0.09% HPC ($P < 0.05$).

[b] Significantly higher contamination rate than samples processed with 0.9 and 0.09% HPC ($P < 0.05$).

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