

Treatment with Antibiotics is Detrimental to the Recovery of Viable *Mycobacterium avium* subsp. *paratuberculosis* Cultured from Milk and Colostrum of Dairy Cows

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Summary and Implications

Antibiotic cocktails are frequently used as secondary decontaminants prior to the culture of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). This study investigated whether secondary incubation with an antibiotic cocktail containing vancomycin, nalidixic acid, and amphotericin B after primary exposure to *N*-acetyl-L-cysteine-1.5% sodium hydroxide affected the recovery of viable MAP from milk experimentally spiked with 10^5 to 10^6 cfu/ml. Results indicated that incubation with this antibiotic cocktail did decrease the incidence of contamination in culture media but it was also highly detrimental to the recovery of viable MAP. This effect was not advantageous given the low numbers of MAP naturally shed into milk and colostrum of infected cows. These results demonstrate that secondary incubation with antibiotics during the decontamination procedure could potentially lead to false-negative culture results because of their detrimental effect on the viability of MAP.

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP), the causative agent of Johne's Disease (JD), is an enteric pathogen that is primarily shed in the feces. JD is a slowly progressive wasting disease that does not manifest physical symptoms in ruminants until late in the disease, making early diagnosis difficult and costly. It is estimated that 91.1% of US dairy herds are currently infected with this pathogen. The primary route of exposure for neonates is fecal-oral. However, MAP is also shed into the milk and calves can be exposed to this pathogen by suckling the dam or being fed colostrum or waste milk from infected cows. Despite this possible event, there is little information in the literature to document the shedding of MAP into the colostrum and milk of infected dams, particularly, the bacterial load and how this relates to the infection status of the dam and the stage of lactation. This is in part because of the difficulty in culturing the organism from a complex moiety such as milk. Yet, if producers could understand the

association of disease with bacterial load in the milk, they might be more willing to make critical management decisions to further prevent dissemination of infection within the herd.

To obtain this information, an effective culture protocol must be established. Because of the presence of nonspecific microorganisms found naturally in milk, a decontamination protocol must be determined to inhibit the growth of the non-target microorganisms while minimally affecting the viability of MAP. Frequently, antibiotic cocktails are used in conjunction with a chemical decontamination to decrease the viability of contaminating microorganisms. The objective of this study was to determine if a secondary incubation with vancomycin, nalidixic acid, and amphotericin B affected the viability of MAP after a primary incubation with *N*-acetyl-L-cysteine-1.5% sodium hydroxide (NALC-1.5% NaOH).

Materials and Methods

Milk collected from a non-infected cow was inoculated with live MAP (strain 167 from a clinical cow) to achieve final concentrations of 10^6 , 10^4 , 10^2 cfu/ml. Non-inoculated milk was used for a negative control and 10^6 cfu/ml in phosphate buffered saline (PBS) was used as a positive control. Milk samples were partitioned by centrifugation, and the whey layer was discarded, leaving the pellet and the cream. Milk was exposed to a solution consisting of 0.25% NALC, 0.725% sodium citrate, and 1.5% NaOH for 15 minutes. The samples were centrifuged again, discarding the aqueous layer and retaining the cream and the pellet. The cream and pellet were resuspended either in PBS or a cocktail (VAN) containing 100 µg/ml vancomycin, 50 µg/ml amphotericin B, and 100 µg/ml nalidixic acid. If the sample was resuspended in PBS, it was inoculated into media immediately, whereas, if the sample was resuspended in VAN, it was incubated overnight at 39°C. Samples were inoculated into two liquid media, BACTEC 12B and *para*-JEM. The BACTEC 12B medium was supplemented with egg yolk, mycobactin J, and PANTA antibiotic mixture, and samples were incubated at 37°C and monitored biweekly for two weeks post-inoculation and then weekly for 12 weeks. The *para*-JEM medium was supplemented according to the manufacturer's instructions (proprietary information). The samples were incubated for up to 65 days. MAP growth was confirmed with an acid-fast stain and real-time PCR targeting a species specific genetic element, *IS900*.

Results and Discussion

Overnight exposure of milk samples to the antibiotic cocktail, VAN, following decontamination with NALC-1.5% NaOH, was detrimental to the viability of MAP. A significant ($P < 0.01$) increase in time to recovery was observed after additional exposure to VAN for samples cultured in both BACTEC 12B and *para*-JEM media (Figure 1). Further, an increase in undetectable samples was observed for samples cultured in the *para*-JEM medium, as two of the triplicate samples within the 10^2 cfu/ml inoculation level were undetected after treatment with VAN. In contrast, all replicates at this concentration were detected in samples without VAN treatment. The BACTEC 12B medium was able to detect all of the samples inoculated with 10^2 cfu of MAP/ml, but there was a significant ($P < 0.01$) increase in time to recovery of 19 days after treatment with VAN. The effects of VAN treatment on contamination were negligible because the rate of contamination in samples was low regardless, but contamination in the BACTEC 12B medium was decreased from 1/12 samples to 0/12 samples after incubation with VAN. No contamination was seen in samples treated either with NALC-NaOH or NALC-NaOH plus VAN when cultured in *para*-JEM medium.

The lethal effects of VAN observed may be compounded by subsequent exposure to NALC-NaOH. NALC-NaOH can damage the MAP bacterium and may cause it to be more vulnerable to the antibiotics to which it is normally resistant. Also, the lethal effects of VAN may be increased in the *para*-JEM medium because it already contains vancomycin, which is known to have detrimental effects on the viability of MAP. BACTEC 12B does not contain additional vancomycin. The marked decrease in viability is not a reasonable accommodation for the decrease in contaminants, especially when the goal is to define a culture protocol to detect low concentrations of MAP present in milk. The decrease in MAP viability associated with antibiotic treatment prior to culture could lead to inferior detection thresholds allowing for the possibility of false-negative culture results. This will ultimately give dairy producers incorrect information on the threat of infecting youngstock with MAP in milk and colostrum.

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Figure 1. Effect of secondary exposure to 100 µg/ml vancomycin, 50 µg/ml amphotericin B, and 100 µg/ml nalidixic acid (VAN) on the viability of *Mycobacterium avium* subsp. *paratuberculosis* measured in time to detection in BACTEC 12B and *para*-JEM media

