Optimization of Methods for the Detection of *Mycobacterium avium* subsp. *paratuberculosis* in Milk and Colostrum of Naturally Infected Dairy Cows

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Summary and Implications

Two decontamination chemicals, hexadecylpyridinium choride (HPC) and N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH), were compared for their efficacy of reducing the growth of non-specific microorganisms in milk while minimally affecting the recovery of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). In addition three culture mediums, Bactec 12B and Trek-ESP *para*-JEM, and Herrold's egg yolk media (HEYM), were compared for the ability to suppress growth of non-specific microorganisms as well as their sensitivity of detection of low levels of MAP in milk. Results indicated that exposing the milk to 1.5% NALC-NaOH for 15 minutes most effectively reduced non-target microorganisms without reducing MAP viability. In addition, the Bactec 12B medium detected the lowest levels of MAP more rapidly and more consistently than the other two mediums.

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP), the causative agent of Johne's Disease (JD), is an enteric pathogen that is shed in the feces. JD is slow progressing 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 68% of dairy herds are infected with the disease. 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, 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 due in part to 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 readily willing to make critical management decisions to further prevent dissemination of infection within the herd.

In order 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. In addition, various culture mediums, including liquid and solid mediums, must be investigated for their efficacy to determine the superior medium. Together, the decontaminating protocol and the efficacy of the mediums will be combined to ensure that the bacteria detection thresholds are as low as possible.

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 10^8, 10^6, 10^4, 10^2 cfu/ml. Non-inoculated milk was used for a negative control and 10^6 cfu/ml in PBS was used as a positive control. Milk samples were separated by centrifugation and the whey layer was discarded leaving the pellet and the cream. Two different decontaminating chemicals were investigated: hexadecylpyridinium chloride (HPC) and N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH). Milk was treated with either HPC at concentrations of 0.75, 1.00, 1.25, and 1.50% for 5, 24, and 48 hours, or with NALC-NaOH solution consisting of 0.25% NALC, 0.725% sodium citrate, and 0.5, 1.0, 1.5, and 2.0% NaOH for 5, 15, and 30 minutes. After each chemical treatment samples were centrifuged again, discarding the chemical layers and retaining the cream and the pellet. The cream and pellet were resuspended in PBS for media inoculation.

Each decontaminated milk sample was inoculated into three mediums: Bactec 12B, Trek-ESP *para*-JEM, and Herrold's Egg Yolk media (HEYM). Bactec 12B media 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. Trek-ESP *para*-JEM was supplemented according to the manufacturer's instructions (propriety information). The samples were incubated for up to 65 days. Samples were also inoculated onto three HEYM slants and incubated at 39˚C f or 12 weeks, with colony counts performed at 4, 8 and 12 weeks of incubation. Only results from Bactec 12B and Trek-ESP are presented here.

Results and Discussion

Results suggest that the concentration or time of exposure to HPC does not influence the level of non-target microorganisms in milk samples and recoveries of MAP in the milk were also not affected. However, high concentrations of HPC (1.25%) and time of exposure (48 hours) may reduce the recovery of MAP. Therefore, optimal decontamination conditions for milk samples were determined to be 0.75% HPC for 5 hours. Experiments to determine optimal conditions for treatment of samples with NALC-NaOH demonstrated that increased length of exposure and increasing concentration of NaOH did decrease the level of contaminating microorganisms. In contrast to HPC, increasing concentrations of NaOH did not appear to influence the recovery of MAP. Optimal decontamination conditions for NALC-NaOH were determined to be 1.5% NaOH for 15 minutes. HPC was superior at reducing fungal contamination and NALC-NaOH was superior at reducing bacterial contamination. A comparison of MAP recovery from the two liquid medium systems showed that Bactec 12B media was superior to Trek-ESP *para*-JEM in sensitivity of MAP detection and the speed of recovery from inoculated milk samples (Fig. 1). The liquid media each have their own strengths with regard to the abilities to suppress the growth of non-target microorganisms. The Bactec 12B media was superior in anti-bacterial properties and the Trek-ESP *para*-JEM was superior in anti-fungal properties. The NALC-NaOH decontamination protocol also appears to result in more rapid recovery of MAP than the HPC decontamination protocol (Fig. 1). The culture protocols defined in these studies will be used to quantify the amount of MAP shed into milk and colostrum of naturally infected dairy cows.

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Figure 1. Comparison of Decontamination Protocols and Liquid Culture Mediums on the Recovery of *Mycobacterium avium* subsp. *paratuberculosis* from Milk.