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Viable *Mycobacterium avium* ssp. *paratuberculosis* isolated from calf milk replacer

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ABSTRACT

When advising farmers on how to control Johne's disease in an infected herd, one of the main recommendations is to avoid feeding waste milk to calves and instead feed calf milk replacer (CMR). This advice is based on the assumption that CMR is free of viable *Mycobacterium avium* ssp. *paratuberculosis* (MAP) cells, an assumption that has not previously been challenged. We tested commercial CMR products (n = 83) obtained from dairy farms around the United States by the peptide-mediated magnetic separation (PMS)-phage assay, PMS followed by liquid culture (PMS-culture), and direct IS*900* quantitative PCR (qPCR). Conventional microbiological analyses for total mesophilic bacterial counts, coliforms, *Salmonella*, coagulase-negative staphylococci, streptococci, nonhemolytic *Corynebacterium* spp., and *Bacillus* spp. were also performed to assess the overall microbiological quality of the CMR. Twenty-six (31.3%) of the 83 CMR samples showed evidence of the presence of MAP. Seventeen (20.5%) tested positive for viable MAP by the PMS-phage assay, with plaque counts ranging from 6 to 1,212 pfu/50 mL of reconstituted CMR (average 248.5 pfu/50 mL). Twelve (14.5%) CMR samples tested positive for viable MAP by PMS-culture; isolates from all 12 of these samples were subsequently confirmed by whole-genome sequencing to be different cattle strains of MAP. Seven (8.4%) CMR samples tested positive for MAP DNA by IS*900* qPCR. Four CMR samples tested positive by both PMS-based tests and 5 CMR samples tested positive by IS*900* qPCR plus one or other of the PMS-based tests, but only one CMR sample tested positive by all 3 MAP detection tests applied. All conventional microbiology results were within current standards for whole milk powders. A significant association existed between higher total bacterial counts and presence of viable MAP indicated by either of the PMS-based assays. This represents the first published report of the isolation of viable MAP from CMR. Our findings raise concerns about the potential ability of MAP to survive manufacture of dried milk-based products.
Key words: *Mycobacterium avium* ssp. *paratuberculosis*, milk replacer, calf health, Johne's disease, infectious disease control

INTRODUCTION

Milk replacer has been fed to calves since at least the 1950s, although formulations have changed over the years in terms of percentage of fat and protein. Calf milk replacers (**CMR**) are generally made with by-products originating from milk processing industries, such as whole milk powder, skim milk powder, casein, whey, and whey protein, although protein sources other than milk by-products such as soy products, dried egg, fish protein concentrates, and single cell protein may also be used (FAO, 2011; Bovine Alliance on Management and Nutrition, 2014). Most dairy calves in the United States are fed milk replacer before weaning for reasons of convenience, biosecurity, and economics (Costello, 2012; Bovine Alliance on Management and Nutrition, 2014). Calves are particularly susceptible to infectious diseases, and some infectious agents such as *Mycobacterium avium* ssp. *paratuberculosis* (**MAP**), the cause of Johne's disease (**JD**), bovine viral diarrhea virus, bovine leukosis virus, *Pasteurella multocida*, *Salmonella* sp., and *Mycoplasma bovis* can be transmitted from cow to calf through feeding unpasteurized milk (Costello, 2012). Feeding CMR, as an alternative to feeding waste unpasteurized milk or farm-pasteurized milk, is a common practice in the United States. The latest statistics from the National Herd Monitoring Scheme indicate that 49.9% of all US dairy operations (of all sizes) fed

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some kind of CMR to pre-weaned heifers during 2014; 16.4% of operations fed nonmedicated CMR and 37.6% fed medicated CMR (USDA, 2016).

As mentioned above, calves may be fed CMR to prevent diseases such as JD, caused by MAP, which is shed in the milk and feces of infected cows. Transmission of MAP is considered to be an early event in a calf's life, and there are recognized risk factors for transmission of MAP to calves within dairy herds (Doré et al., 2012). A focus of JD control programs is calf-related interventions as part of herd management plans. Avoiding feeding waste milk and feeding CMR instead is a key recommendation within JD control programs worldwide (Doré et al., 2012; Garcia and Shalloo, 2015; Pieper et al., 2015). As stated by Cooper and Watson (2013), the assumption has always been that the risk of viable MAP organisms in commercial CMR powders is negligible because CMR is invariably pasteurized and often highly processed, but is that really the case? Seemingly, to date, no one has ever challenged that assumption.

Demonstrating the existence of viable MAP in processed milk or dairy products, such as pasteurized milk, cheeses, yogurt, milk powders, and powdered infant formula, has proven difficult because, until recently, culture always necessitated inclusion of a chemical decontamination step to inactivate non-MAP contaminants; the latter is known to adversely affect the viability of some or all of the MAP cells present in milk, potentially leading to negative culture results (Dundee et al., 2001; Gao et al., 2005). However, detection methods for viable MAP in milk and dairy products have improved considerably over recent years with the advent of immunomagnetic separation (Grant et al., 1998; O'Brien et al., 2016) and subsequently peptide-mediated magnetic separation (**PMS**; Stratmann et al., 2002, 2006; Foddai et al., 2010; O'Brien et al., 2016), which permit selective capture, separation, and concentration of whole MAP cells from a sample before culture or PCR, and novel mycobacteriophage-based methods of MAP detection (Stanley et al., 2007; Foddai et al., 2010; Swift et al., 2013; Botsaris et al., 2016), which require the MAP cells to be viable to obtain a positive result (plaques in a lawn of fast-growing *Mycobacterium smegmatis*). In particular, a method combining PMS and a phage amplification assay to detect MAP (PMS-phage assay), developed and optimized by Foddai et al. (2009, 2011), and used in combination with an optimized milk sample preparation protocol (Foddai and Grant, 2015), is proving to be a very sensitive method of detecting viable MAP in cow milk. The optimized PMS-phage assay was recently reported to have a limit of detection 50% of ~1 MAP cell per 50 mL of milk, making it a more sensitive detection method than existing quantitative PCR for MAP and conventional culture methods (Foddai and Grant, 2017).

As time passes and the novel optimized PMS-phage assay is applied to test various milk and dairy products, new information on the presence and numbers of viable MAP in these foods is emerging (Foddai and Grant, 2017). We previously reported the outcome of testing of powdered infant milk formula (**PIF**) by the PMS-phage assay (Grant et al., 2014). Of 68 PIF samples tested, 30 (44.1%) samples tested positive for viable MAP by the PMS-phage assay, with viable MAP numbers ranging from 4 to 678 pfu/50 mL of reconstituted PIF indicated by the plaque counts obtained. Because PIF and CMR are similar milk-based, powdered dairy products, probably with not dissimilar production processes, our viable MAP in PIF findings led us to query whether testing of CMR by the PMS-phage assay might also yield similar results with respect to the presence of viable MAP. Preliminary testing of a small number of CMR samples sourced in Wisconsin by the PMS-phage assay (carried out before the CMR testing reported here) found that 1 (12.5%) of 8 CMR samples tested positive for viable MAP. We hypothesized that viable MAP may be more widely prevalent in commercial powdered CMR products, so we decided to carry out a larger study. The objectives of the study were (1) to test commercial CMR products sourced from within the United States using standard culture methods, 2 PMS-based methods (PMS-phage assay and PMS plus liquid culture) and IS*900* quantitative PCR (**qPCR**) to detect the presence of viable MAP and MAP DNA, respectively, and (2) to assess the overall hygienic quality of the CMR samples by performing conventional microbiological analyses, to determine if the presence of any hygiene indicator microorganism might correlate with detection of viable MAP. An optimized method for detecting MAP in powdered dairy products has yet to be published, so during this study multiple methods, including several published and unpublished cultural and qPCR approaches (detailed below), were employed in the 2 CMR testing laboratories to maximize chances of detecting low numbers of viable MAP, if present, in the CMR samples.

MATERIALS AND METHODS

Acquisition of CMR Samples

The CMR samples were acquired in 2 stages. In the first stage, 50 samples were acquired during the summer of 2014 from dairy farms in southern Wisconsin by author Tarrant. Hygiene precautions were taken to avoid on-farm contamination. Samples were col-

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