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Grant, I. R., Foddai, A. C. G., Tarrant, J. C., Kunkel, B., Hartmann, F. A., McGuirk, S., ... Collins, M. T. (2017). Viable Mycobacterium avium subsp. paratuberculosis isolated from calf milk replacer. Journal of Dairy Science, 100(12). DOI: 10.3168/jds.2017-13154

Published in:
Journal of Dairy Science

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal

VIABLE MAP ISOLATED FROM CALF MILK REPLACER

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

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INTERPRETIVE SUMMARY

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

By Grant et al. Calf milk replacer (CMR) is widely used for feeding calves to control infectious diseases, notably Johne's disease. This practice assumes that CMR is free of viable *Mycobacterium avium* ssp. *paratuberculosis* (MAP). We tested 83 commercial CMR products obtained from US dairy farms. Seventeen (20.5%) CMR samples tested positive for viable MAP by a novel PMS-phage assay and 12 (14.5%) by PMS-culture. Seven (8.4%) tested positive for MAP DNA by IS*900* qPCR. Conventional microbiological results were within the guidelines for whole milk powders. These findings highlight concerns about the ability of MAP to survive manufacture of dried milk-based products.

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 USA by the Peptide-mediated magnetic separation (PMS)-phage assay, PMS followed by liquid culture (PMS-culture), and direct IS*900* qPCR. Conventional microbiological analyses for total mesophilic bacterial counts, coliforms, *Salmonella*, coagulase-negative staphylococci, streptococci, non-hemolytic *Corynebacterium* spp. and *Bacillus* spp. were also performed in order 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 6-1,212 PFU/50 mL 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 five 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 three 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 (p=0.024). 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.

Keywords: *Mycobacterium avium* ssp. *paratuberculosis* (MAP), 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 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, single cell protein may also be used (FAO 2011, Bovine Alliance on Management and Nutrition 2014). Most dairy calves in the United States of America (**USA**) are fed milk replacer prior to 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 unpasteurised milk (Costello 2012). Feeding CMR, as an alternative to feeding waste unpasteurised milk or farm-pasteurized milk, is a common practice in the US. The latest statistics from the National

77 Herd Monitoring Scheme (NAHMS) indicate that 49.9% of all US dairy operations (of all
78 sizes) fed some kind of CMR to pre-weaned heifers during 2014; 16.4% of operations fed
79 non-medicated CMR and 37.6% fed medicated CMR (United States Department of
80 Agriculture 2016).

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

92 Demonstrating the existence of viable MAP in processed milk or dairy products, such as
93 pasteurized milk, cheeses, yoghurt, milk powders, and powdered infant formula, has proven
94 difficult because, until recently, culture always necessitated inclusion of a chemical
95 decontamination step to inactivate non-MAP contaminants; the latter is known to adversely
96 impact the viability of some or all of the MAP cells present in milk, potentially leading to
97 negative culture results (Dundee et al. 2001; Gao et al. 2005). However, detection methods
98 for viable MAP in milk and dairy products have improved considerably over recent years with
99 the advent of immunomagnetic separation (**IMS**, Grant et al. 1998; O'Brien et al. 2016) and
100 subsequently peptide-mediated magnetic separation (**PMS**, Stratmann et al. 2002, 2006;
101 Foddai et al. 2010; O'Brien et al. 2016), which permit selective capture, separation and
102 concentration of whole MAP cells from a sample prior to culture or PCR, and novel
103 mycobacteriophage-based methods of MAP detection (Stanley et al. 2007, Foddai et al.
104 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 optimised by Foddai et al. (2009, 2011), and used in combination with an optimised milk sample preparation protocol (Foddai and Grant, 2015), is proving to be a very sensitive method of detecting viable MAP in cows' milk. The optimised PMS-phage assay was recently reported to have a limit of detection 50% (LOD_{50}) of ~1 MAP cell per 50 ml milk, making it a more sensitive detection method than existing MAP qPCR and conventional culture methods (Foddai and Grant, 2017).

As time passes, and the novel optimised 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 4–678 PFU/50 mL reconstituted PIF indicated by the plaque counts obtained. Since 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 prior to 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 decided to carry out a larger study. The objectives of the study were: (1) to test commercial CMR products sourced from within the USA using standard culture methods, two PMS-based methods (PMS-phage assay and PMS plus liquid culture) and IS*900* 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, in order to determine if the presence of any hygiene indicator microorganism might correlate with detection of viable MAP. An optimised 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 two CMR testing laboratories to maximise chances of detecting low numbers of viable MAP, if present, in the CMR samples.

MATERIALS AND METHODS

Acquisition of CMR Samples

CMR samples were acquired in two 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 collected with a sterile plastic scoop into sterile transport bags; with a separate sterile scoop being used for each sample. If a sealed bag of CMR was available on the farm that bag was used for sampling; the majority of the 50 Wisconsin sourced CMR samples were obtained from unopened bags of CMR (James Tarrant, University of Wisconsin-Madison, personal communication). Subsequently, during the spring of 2015, an additional 35 CMR samples were acquired from across the USA. For CMR collection outside Wisconsin, sample collection kits, including sterile scoops and bags, were mailed to veterinarians and veterinary students with detailed instructions about hygienic sampling technique and a request to use a sealed CMR bag if possible; all except two of the 35 CMR samples obtained in other US states were recorded as being from unopened bags. The 83 CMR samples ultimately tested originated from 15 US states and included 35 different brands of CMR representing six unique manufacturers.

CMR Sample Reconstitution

Unless otherwise stated, 6-9 g of each CMR sample (as indicated by the manufacturer's label) was aseptically weighed into a sterile 50 ml centrifuge tube, reconstituted to 50 ml with pre-warmed (37^{o}C) sterile distilled water, shaken thoroughly for several minutes to ensure resuspension of all powder, and then placed at 4^{o}C overnight to fully rehydrate. Next day, the reconstituted CMR samples were removed from the fridge and allowed to equilibrate to room temperature for at least 1 h before being centrifuged at 3000 x g for 15 min. Each CMR pellet was then resuspended in 1-5 ml Phosphate buffered saline (pH 7.4) containing 0.05% Tween 20 (**PBS-T**), as appropriate depending on the size of pellet obtained, in order to obtain what was considered to be a suitable consistency for PMS. The volume of PBS-T added was recorded so that a correction factor could be applied to plaque counts obtained when 1 ml of each CMR pellet sample was subjected to the PMS-phage assay. Finally, the reconstituted CMR pellet samples were ultrasonicated (pulse mode 37 kHz for 4 min in ice-water) in an Ultrasonic bath (FB-11201, Fisher Scientific Ltd, Loughborough, UK) to break up any MAP clumps in the sample before PMS was performed. Only one sample of each CMR was tested by each of the methods described below, so intra- or inter-laboratory variation for the methods employed was not evaluated during this study.

PMS of MAP from reconstituted CMR

PMS was performed on 1 ml of each sample (prepared as indicated above) using 5 µl biotinylated-aMp3 peptide- and 5 µl biotinylated-aMptD peptide- coated MyOne^{TM} Tosylactivated Dynabeads (Life Technologies, Paisley, Scotland, UK), prepared in-house as previously described (Foddai et al. 2010). A positive (1 ml of a 10^{-1} dilution of a broth culture of MAP B2) and negative (1 ml of 7H9 broth) control sample was included with each batch of 15-20 CMR samples processed. Magnetic separation was carried out using a Dynal BeadRetriever (Life Technologies). MAP cell capture was carried out for 30 min at room temperature under continuous mixing, followed by two washes in 1 ml PBS-T, and final resuspension of the beads in 1 ml Middlebrook 7H9 broth containing 10% (v/v) OADC

supplement (**7H9/OADC broth**) and **NOA** antibiotic supplement (Abtek Biologicals Ltd., Liverpool, UK; final concentrations per litre: Nystatin 50,000 IU, Oxacillin 2 mg, Aztreonam 30 mg). This final 1 ml bead sample was split equally between the phage assay and culture, carried out as described below. The positive and negative PMS controls were also processed through the phage assay (PMS-phage assay) and culture (PMS-culture) along with each batch of test samples.

PMS-Phage Assay

The PMS-phage assay and confirmatory plaque-PCR were carried out as follows: after PMS, 500 µl of each bead sample was transferred to a 11 ml flip-top vial (Capitol Vials, Auburn, AL, USA) containing 500 µl 7H9/OADC/4 mM $CaCl_2$/NOA antibiotics (final $CaCl_2$ concentration in sample, 2 mM) before being incubated overnight at 37°C. The phage assay was carried out as described by Foddai et al. (2009). Briefly, 100 µl D29 mycobacteriophage suspension (10^9 PFU/ml) was added to each bead sample, before incubation for 2 h at 37ºC. Then, in order to inactivate exogenous/non-adsorbed seed phage, 100 µl freshly prepared 100 mM ferrous ammonium sulphate (**FAS**, Sigma-Aldrich, Poole, Dorset, UK) was added to each sample and allowed to incubate for 10 min at room temperature, with thorough vortexing of the sample after 5 min, before the addition of 5 ml 7H9/OADC/2 mM $CaCl_2$/NOA broth. Samples were returned to the incubator at 37°C for a further 90 min before being plated with tempered (55°C) Middlebrook 7H9 agar containing 10% OADC (both Difco) and 1 ml *Mycobacterium smegmatis* mc^2 155 (10^8 CFU/ml). Plaques were counted following overnight incubation of plates at 37°C. Plaque counts obtained were expressed as plaque-forming units (PFU)/50 ml reconstituted CMR; direct plaque counts were multiplied by a factor of 2 to take account of the fact that only half the bead sample (equivalent of 25 ml reconstituted CMR) was processed through the phage assay, and by a factor of 1, 3 or 5 to take account of the differing volumes of PBS-T originally added to resuspend the CMR pellets.

Plaque-IS*900* PCR, essentially as described by Swift et al. (2013), was carried out on DNA extracted from plaques in order to confirm that the DNA present was MAP DNA and not from some other *Mycobacterium* sp. Up to a maximum of 10 plaques were randomly selected from each PMS-phage assay positive sample plate to be excised for DNA extraction. The centre of each plaque was excised using a sterile loop and transferred to an Eppendorf tube. The DNA was extracted from the plaques using the Zymoclean™ Gel DNA Recovery kit (Cambridge Bioscience Ltd., Cambridge, UK), according to the manufacturer's instructions. DNA was eluted from the Zymoclean columns using 20 µl elution buffer (supplied with kit). DNA was stored at -20°C until required for plaque-PCR. A protocol modified from Whittington et al. (1998) was used to target the IS*900* insertion element. To 40 µl of master mix containing 1 X DreamTaq Green Buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 1 U Fermentas DreamTaq DNA polymerase (Thermo Fisher Scientific, UK) and 250 ng of P90 5'GAAGGGTGTTCGGGGCCGTCGCTTAGG'3 and P91 5'GGCGTTGAGGTCGATCGCCC ACGTGAC'3 primers, 10 µl of plaque DNA was added. The PCR cycling conditions were: 94°C for 5 min, 37 cycles of 94°C for 30 sec, 62°C for 30 sec and 72°C for 1 min, final extension at 72°C for 4 min, and then sample cooled to 4°C. PCR products were visualised by 2% agarose gel electrophoresis. The expected IS*900* PCR product size was 400 bp.

PMS-Culture at Queen's University Belfast (QUB)

PMS-culture and confirmation of suspect positive cultures was carried out as follows: 500 µl of each bead suspension after PMS was inoculated into screw-capped glass test tubes containing 5 ml modified Middlebrook 7H9 liquid medium (Pozzato et al. 2011), consisting of (per 900 ml) 4.7 g Middlebrook 7H9 powder, 1.0 g casitone, 5 ml glycerol supplemented with 10% v/v OADC supplement and PANTA plus antibiotic supplement (all Becton Dickinson) and mycobactin J (2 mg/l; Synbiotics Europe SAS, Lyon, France), but without the addition of 16% egg yolk. The broth cultures were incubated at 37°C and absorbance at OD_{600nm} was measured at time 0 and then from four weeks onwards every two weeks up to 16 weeks

using a Biowave CO8000 Density meter (Biochrom Ltd., Cambridge, UK). When an increase in OD_{600nm} was observed for any broth culture, Ziehl-Neelsen (ZN) staining was employed to determine if acid-fast bacteria were present and, if so, IS*900* PCR (Moss et al. 1992) was applied to confirm presence of MAP cells. Any primary PMS-culture suspected of containing viable MAP was sub-cultured to Dubos broth medium, prepared as described by Hammer et al. (2002) including the addition of 20% Newborn Calf Serum (Life Technologies), **PACT** antibiotics (Polymyxin B 50 IU/ml, Amphotericin B 5 µg/ml, Carbenicillin 25 µg/ml and Trimethoprim 2.5 µg/ml, all Sigma) and mycobactin J (2 mg/L, Synbiotics Europe SAS), and onto Herrold's egg yolk medium (**HEYM**) containing 2 mg/L mycobactin J (prepared in-house). Once again, OD_{600nm} of broth cultures was measured periodically until an increase was observed, at which point IS*900* PCR was applied to confirm the presence of MAP DNA. IS*900* PCR was also applied to suspect colonies growing on HEYM plates to confirm their identity.

PMS-Culture at University of Wisconsin-Madison (UW)

Reconstituted CMR was processed by PMS using the KingFisher Duo Prime Purification System (ThermoFisher Scientific, Fitchburg, WI) to concentrate MAP and then inoculated into MGIT ParaTB medium with 1.0 mL MGIT ParaTB supplement (which contains mycobactin J), 500 µL egg yolk emulsion and 200 µL MGIT PANTA added, and also onto HEY slants (all culture media and supplements from BD Diagnostic Systems, Sparks, Maryland). For one set of MGIT ParaTB medium cultures the CMR was reconstituted as previously described (as per manufacturer label instructions; 6-9 gm/mL). For the other cultures, all CMR samples were reconstituted at a standard 5g/50mL and allowed to rehydrate before testing commenced.

If suspicious colonies were seen on HEY or if the MGIT 960 instrument was signal-positive, a five-target multiplex PCR was performed to establish if MAP was present (Shin, 2010). The nature of the CMR material inoculated onto solid media tended to resemble

bacterial growth causing many cultures to be unnecessarily tested. In total 149 cultures (including both PMS-cultures and conventional cultures) were tested by multiplex PCR.

Conventional Culture for MAP at UW

The sample processing method described by Botsaris et al. (2016) in a study of powdered infant formula was adapted and used with three different culture media. CMR was first reconstituted in phosphate buffered saline pH 7.2 (**PBS**) at 1.0 g/5 mL, allowed to sit at 20°C for 1 h, centrifuged at 2,500 x *g* for 15 min. The pellet was resuspended in 0.75% hexadecylpyridinium chloride (**HPC**) and allowed to sit for 4 h at 20°C. After centrifugation at 2,500 x *g* for 15 min the pellet was resuspended in 1.0 mL sterile water. Three media were inoculated: 200 µL onto HEY slants (BD Diagnostic Systems), 100 µL into MGIT ParaTB medium (BD Diagnostic Systems) supplemented with mycobactin J, egg yolk and PANTA, and 100 µL onto modified 7H10 agar plates supplemented with mycobactin-J, ADC and VAN. Suspicious colonies on solid culture media (HEY or 7H10) and signal-positive MGIT cultures were tested by multiplex PCR, as described above for PMS-cultures, to establish if MAP had been isolated. The nature of the CMR material inoculated onto solid media tended to resemble bacterial growth causing many cultures to be unnecessarily tested.

IS900 qPCR at UW

Three method variations were used to test CMR samples for presence of MAP by IS*900* qPCR:

Method 1 - For the first qPCR method, the CMR was reconstituted at 5 g/50 mL in sterile water, then held at 4°C for 18-24 h before centrifugation at 2,500 x *g* for 15 min. The pellet was resuspended in 3.0 mL sterile PBS containing 0.05% Tween 20. DNA was extracted from 1.0 mL of this suspension using the Tetracore Extraction System (Tetracore, Rockville, MD) using the 1.0 g protocol after agitation in the Benchmark BeadBlaster 24 (Benchmark

Scientific, Inc., Edison, NJ). From the 25 µL extraction product, 5 µL was used as template DNA for IS*900* qPCR.

Method 2 – For the second qPCR method, CMR was first reconstituted in PBS at 1.0 g/5 mL, allowed to sit at 20°C for 1 h, centrifuged at 2,500 x *g* for 15 min. The pellet was resuspended in 0.75% HPC and allowed to sit for 4 h at 20°C. After centrifugation at 2,500 x *g* for 15 min the pellet was resuspended in 1.0 mL sterile water. DNA was extracted from this suspension using the Tetracore Extraction System (Tetracore) using the 1.0 g protocol after agitation in the Benchmark BeadBlaster 24 (Benchmark Scientific). From the 25 µL extraction product, 5 µL was used as template DNA for IS*900* qPCR.

Method 3 - For the third qPCR method, the CMR was reconstituted at 1.0 g/9 mL, then held at 20°C for 1 h, then overnight at 4°C for 18-24 h. After centrifugation at 2,500 x *g* for 15 min, the pellet was reconstituted in 1.0 mL sterile Tris-EDTA (**TE**) buffer pH 8.0. DNA was extracted from this suspension using the Tetracore Extraction System (Tetracore) using the 1.0 g protocol after agitation in the Benchmark BeadBlaster 24 (Benchmark Scientific). From the 25 µL extraction product, 5 µL was used as template DNA for IS*900* qPCR.

Conventional Microbiological Analyses at UW

All CMR samples were reconstituted as per manufacturer label instructions; 6-9 gm/50 mL. The resulting milk sample was then tested by the same methods used to characterize the microbiological quality of bulk tank milk. Freshly reconstituted CMR was tested at the following four final dilutions: 1:50, 1:500, 1:5000, and 1:50000. Heat-stressed reconstituted milk replacer (37°C for 6 h) was tested at the same dilutions with the addition of two higher dilutions to anticipate the higher microbial counts: 1:100000 and 1:500000. Final dilutions were achieved by inoculating 0.2 mL of each primary dilution onto Trypticase soy agar (BAP) supplemented with 5% sheep blood (Remel Inc., Lenexa, KS) for determination of microorganism growth and total bacterial counts and eosin methylene blue (EMB) agar (Remel Inc., Lenexa, KS) to quantify lactose-fermenters (coliforms) and non-coliform, gram-

328 negative bacteria. Culture plates were incubated at 36°C in 5% CO_2 and examined for
329 growth after 24, 48, and 36 hours of incubation. Each dilution was also inoculated onto XLT4
330 agar (Remel Inc., Lenexa, KS), incubated in ambient air at 35°C and examined for growth of
331 *Salmonella* after 24, 48, and 36 hours of incubation. In addition, enrichment culture for
332 *Salmonella* was performed to increase sensitivity of *Salmonella* detection. Undiluted
333 reconstituted CMR (0.2 mL) was inoculated into selenite and Rappaport-Vassiliadis
334 enrichment broths (Remel Inc., Lenexa, KS). Enrichment broths were incubated for 18 hours
335 in ambient air at 35°C and then subcultured onto XLT4 agar (Remel Inc., Lenexa, KS).
336 Subcultures were incubated in ambient air at 35°C and examined for colonies resembling
337 *Salmonella* after 24 and 48 hours of incubation.
338 Colony counts were recorded on the last day of incubation. All colony types were
339 identified and classified into the following groups: coliform or non-coliform, gram-negative
340 rods (lactose-positive or negative on EMB, respectively), streptococci (*agalactiae* or non-
341 *agalactiae*), staphylococci, (*Staph. aureus* or coagulase-negative staphylococci, CNS),
342 *Corynebacterium* spp., *Bacillus* spp. or other microorganism using standard microbiological
343 procedures (Hogan et al. 1999). Other microorganisms or species level identifications were
344 performed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass
345 spectrometry (Bruker Daltonics, Bremen, Germany).
346
347 ***Genomic Analysis of MAP Isolates***
348
349 When MAP isolates were successfully cultured at QUB, genomic DNA was extracted
350 from cells grown in Dubos broth according to a method supplied by Dr Adel Talaat, University
351 of Wisconsin-Madison (Hsu et al. 2011). Briefly, this involved heating at 80°C for 20 min to
352 kill the mycobacterial cells, lysozyme and and proteinase K treatments to weaken the cell
353 wall, and addition of 5M NaCl and CTAB/NaCl to lyse the MAP cells. This was followed by:
354 (1) phenol/chloroform/isoamyl alcohol (24:24:1) extraction, (2) chloroform/isoamyl alcohol
355 (24:1) extraction, (3) precipitation of DNA with isopropanol overnight, (4) washing with 70%

ethanol, and (5) resuspension of DNA pellet in 30 µl sterile molecular grade water. The purity and yield of DNA from each suspect MAP isolate was provisionally checked using a Biophotometer (Eppendorf, Germany) before the DNA samples were sent to the DNA Sequencing Facility, University of Wisconsin Biotechnology Center, Madison, WI. All DNA samples were sequenced using Illumina Miseq platform and were run in paired-end with 250 bp for each read. Raw sequencing files were imported to CLCBio Genomic workbench version 8 for reference assembly using MAP K10 (NC_002944). Single nucleotide polymorphisms (**SNPs**), multiple nucleotide variant (MNV) and whole genome insertions/deletions were also analyzed using CLCBio software. The criteria for variant calling included that a variant has ≥ 20 times sequence coverage where this variant is present in ≥ 50% of the sequence reads. The consensus sequence of each sample was then extracted from CLCBio software and used to build a phylogenetic tree using HarvestTools (Treangen et al. 2014). Finally, to distinguish MAP genotypes (Type I vs II or III), *hsp65, gyrA* and *gyrB* PCR followed by enzyme digestion were performed as detailed previously by Castellanos et al. (2007) and Ghosh et al. (2012).

Statistical Analysis

The microbiological quality of CMR was compared for CMR samples that tested positive for viable MAP by phage assay or culture and those that did not, using the Mann-Whitney test with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA); a difference with P-value ≤ 0.05 was considered significant at the 95% confidence level.

RESULTS

PMS-Phage Assay (QUB)

Thirty-three (39.8%) of the 83 CMR samples tested by the PMS-phage assay yielded plaques, in which the presence of MAP DNA was detected by Plaque-PCR in 17 cases (Table 2); thus the presence of viable MAP was confirmed in 17 (20.5%) of the 83 CMR samples tested. Viable MAP numbers, indicated by plaque numbers in confirmed PMS-phage assay positive samples, ranged from 6-1212 PFU/50 mL of reconstituted CMR (mean 248.5 PFU/50 mL).

PMS-Culture (QUB) or Conventional MAP Culture (UW)

At QUB, primary liquid cultures of 29 of the 83 CMR samples in modified Middlebrook 7H9 medium (Pozzato et al. 2011) without egg yolk demonstrated an increase in OD_{600nm} of 0.1-0.7 units within the 16 week incubation period. When ZN stained, 18 of these cultures were observed to have acid-fast cells present, so were deemed suspect positive cultures. When the 18 suspect positive primary broth cultures were sub-cultured to Dubos medium and HEYM, 12 continued to show evidence of growth (an increase in OD_{600nm}) in liquid culture or appearance of suspect colonies typical of MAP after 4-6 weeks on HEYM. All 12 suspect cultures tested *IS900* PCR so were provisionally identified as MAP. Ultimately, pure Dubos broth cultures of viable MAP were obtained from 12 (14.5%) of the 83 CMR samples originally tested. DNA was subsequently extracted from these pure broth cultures and sent to UW for whole genome sequencing.

At UW, CMR samples were cultured after PMS or conventional MAP culture protocols involving HPC decontamination in MGIT ParaTB medium supplemented with egg yolk. None of the UW cultures were positive for viable MAP.

IS900 qPCR for MAP (UW)

Seven (8.4%) of the 83 CMR samples were IS*900* qPCR-positive with Ct values of 28.3 to 35.6, which translates to roughly 10-100 CFU of viable and non-viable MAP/g of CMR.

411 Five of the seven qPCR-positive samples (71%) were also positive by a test for viable MAP
412 (PMS-phage assay or PMS-culture) at QUB (Figure 1).
413
414 ***Inter-relationships between test results***
415
416 Table 1 provides details of the PMS-phage assay, PMS-culture and qPCR results for 26
417 (31.3%) of the 83 CMR samples which yielded a positive result by any of the three tests
418 applied. Twenty-four (92.3%) of these CMR samples tested positive for the presence of
419 viable MAP by either PMS-phage assay or PMS-culture, or both tests. MAP isolates were
420 successfully cultured from 12 CMR samples overall. Only one CMR sample (KY-12),
421 originating from a dairy farm in Kentucky, tested positive for MAP by all three tests applied
422 (Table 1).
423
424 ***Limited and Whole Genome Sequencing of Suspect MAP Isolates from CMR***
425
426 Limited genotyping using *hsp65, gyrA* and *gyrB* indicated that the 12 suspected MAP
427 isolates are from cattle source (Type II) and do not belong to types I or III. These isolates
428 were from three different US states, but the majority of isolates (N=10/12) were from
429 Wisconsin. We also performed whole genome sequence (WGS) analysis to further assay
430 genomic diversity among isolates and begin to track the prevalence of MAP collected from
431 CMR. The WGS analysis confirmed that the 12 CMR isolates were MAP strains; as
432 expected from the limited genotyping. In fact, more than 99% of the sequencing reads were
433 mapped to reference cattle strain, MAP-K10 (Table 3). Identification of single nucleotide
434 polymorphism (SNPs) among isolates and the standard laboratory strain (MAP-K10)
435 revealed large numbers of SNPs that ranged between 151 and 267 SNPs per genome; a
436 majority of which were shared among isolates from Wisconsin source CMR (Figure 2A).
437 Interestingly, the percentages of non-synonymous SNPs were much higher than
438 synonymous SNPs, and only a few SNPs were present in the intergenic regions (Figure 2B).

As expected, a phylogenetic tree of SNPs predicted from each genome (Figure 2C) showed a clear distinction from the MAP_S397 strain originally isolated from a sheep (Bannantine et al. 2012), but were closely related to K10 isolate from a cow (Wynne et al. 2010). In addition, genomes from all CMR isolates clustered separately from genomes recently isolated from Elk circulating in California, USA (personal communications). The sheer number of SNPs and their cluster pattern, different from traditional bovine strains used in most Johne's testing laboratories, suggests a common source for these MAP isolates from CMRs collected in four different states.

Conventional microbiological analyses (UW)

Standard measures of microbiological quality of CMR are summarized in Table 2. Total bacterial counts for the reconstituted 83 CMR samples ranged from 0-5.65 Log_{10} CFU/mL of reconstituted CMR (mean 2.66 ± 1.28 Log_{10} CFU/mL). *Bacillus* spp. represented the largest proportion (61%) of bacteria in most CMR (mean 2.33 ± 1.23 Log_{10} CFU/mL), followed by *Streptococcus* spp. (not *agalactiae,* 18%, mean 1.01 ± 1.44 Log_{10} CFU/mL) and coagulase-negative *Staphylococcus* spp. (4%, mean 0.27 ± 0.91 Log_{10} CFU/mL) (Table 2). However, most striking was the large variation in microbiological quality among CMR samples tested. Eight samples had no bacterial growth detected at all. One CMR had a Log_{10} total count of 3.18 per mL after reconstitution, all of which was due to *Streptococcus* spp. Another CMR sample had a Log_{10} total count of 5.2 per mL after reconstitution, all of which was due to *Bacillus* spp. No *Salmonella* spp. were detected in any CMR sampled, even after heat stressing the reconstituted CMR and using enrichment culture for *Salmonella*. Furthermore, no *Streptococcus agalactiae* or coagulase-positive *Staphylococcus aureus* were detected in any of the tested CMR.

When comparing microbiological results for viable MAP-positive CMR (PMS-phage assay or PMS-culture positive) to MAP-negative CMR, only total plate counts were significantly higher for MAP-positive CMR (p=0.024; Mann-Whitney test).

DISCUSSION

To our knowledge, this is only the second report of testing of commercial CMR products for the presence of viable MAP. The first CMR study (Khol et al. 2017) reported negative results for 18 commercial CMR samples obtained from 15 different CMR manufacturers tested by qPCR and culture preceded by chemical decontamination. In the present study three MAP detection approaches were taken – culture/PMS-culture, PMS-phage assay and IS*900* qPCR – and viable MAP was detected in 24 (26%) of the 83 commercial CMR products tested, by either the PMS-phage assay or PMS-culture, or by both tests. As this CMR testing was not as a result of randomized sampling this figure may not reflect the true viable MAP contamination rate for CMR more generally. Also, the CMR were collected on-farm, rather than directly from CMR manufacturers, so the possibility of cross-contamination of some samples with MAP on potentially MAP-infected farms cannot completely be ruled out. Numbers of viable MAP indicated by the Plaque-PCR confirmed PMS-phage assay results ranged from 6-1,212 PFU/50 mL of reconstituted CMR (mean 248.5 PFU/50 mL). Plaques harvested from 16 of the 33 CMR samples yielding plaques were not confirmed to contain MAP DNA by Plaque-PCR; this may reflect insensitivity of the PCR when low levels of MAP DNA are present (e.g. DNA from a single MAP cell per plaque rather than a clump of MAP cells per plaque) and hence false negative Plaque-PCR results may have been obtained. However, the more likely explanation is that the virucide step did not achieve complete inactivation of the D29 phages added to the PMS sample at the start of the phage assay, and so some plaques observed were due to residual D29 phages and not mycobacterial cells bursting to release progeny D29 phages. This latter scenario is the main reason why the confirmatory Plaque-PCR step must be included. MAP DNA was directly detected in just seven CMR samples by IS*900* qPCR; five of which also tested PMS-culture or PMS-phage assay positive. It is unknown whether the mean number of viable MAP indicated by the PMS-phage assay for the MAP culture-positive CMR samples (248.5

PFU/50 mL reconstituted CMR), would represent sufficient inoculum to infect a calf, but the cumulative MAP dose from birth to weaning would not be insignificant, given the volume of CMR consumed by a calf over this period.

Suspected viable MAP isolates were cultured from 12 different CMR samples overall by liquid culture, with no chemical decontamination applied before culture. From the outset of the study, we were very cognisant that the suggestion may be made that laboratory cross-contamination could have contributed to any MAP positive cultures obtained, so the following practical measures were taken to avoid cross-contamination with viable MAP within the laboratory at QUB: (1) CMR samples were weighed out and/or reconstituted in a lab where MAP had never been worked with, and not in the CL2 laboratory where viable MAP may have been present; (2) Sterile, single-use disposable plasticware was routinely used for all manipulations and transfers in the CL2 laboratory; (3) Brand new screw-cap glass culture tubes were used for the CMR PMS-cultures and Dubos sub-cultures, and not recycled glassware that may have been previously used to culture MAP; and (4) CMR cultures were not opened until there was evidence of an increase in OD_{600nm}, at which point a ZN stain was carried out to check for presence of acid-fast cells. If acid-fast positive, the culture was aseptically sub-cultured into Dubos broth, which, likewise, wasn't opened until an increase in OD_{600nm} was observed. All 12 of the suspect MAP isolates were subsequently confirmed to be MAP by limited and whole genome sequencing; all were found to be of the cattle-type and all different from one another. The isolation of multiple different MAP strain types provides reassurance that the isolates obtained are not laboratory contaminants, but potentially have a common source that impacted herds in four different states out of the 15 where farms were visited and CMR collected. The findings of this study suggest that the common source could be a MAP-contaminated CMR ingredient originating from MAP-infected dairy cattle.

Culture of CMR was carried out using several different published methods. Conventional culture methods normally used for bovine fecal samples at UW or the method used to detect MAP in powdered infant formula by Botsaris et al. (2016), in the hands of very experienced

technicians in UW, failed to recover any viable MAP; these methods involved chemical decontamination prior to culture, which was similar to the approach taken by Khol et al. (2017) in their recent CMR study. Only a milk sample preparation and culture approach, optimised over recent years by researchers at QUB, was successful in isolating viable MAP from 12 CMR samples during this study. This approach included: (1) allowing time (overnight at 4^{o}C following reconstitution of CMR) for complete rehydration of MAP cells present in CMR before testing commenced, (2) PMS to selectively capture MAP cells from CMR rather than exposing potentially injured MAP cells to a chemical decontamination treatment, (3) primary culture for up to 16 weeks in a modified Middlebrook 7H9 liquid medium (first described by Pozzato et al. 2011), which had casitone added but no egg yolk, to permit resuscitation of sub-lethally injured MAP cells, and (4) sub-culture of any primary cultures once evidence of MAP growth was observed into richer Dubos liquid medium (without egg yolk) and Herrold's egg yolk medium to stimulate more copious growth of MAP. It is difficult to explain the discrepant culture results at UW and QUB; we can only speculate on possible reasons. There were several distinct differences in terms of CMR sample preparation and culture media employed. Firstly, at QUB, once the CMR pellet was resuspended, the samples were subjected to an ultrasonication treatment to break up clumps of MAP cells. This may have had the effect of releasing viable or sub-lethally injured MAP cells from amongst predominantly dead cells (particularly after heat treatment) in clumps, giving them (greater) access to nutrients during primary culture in Pozzato medium. Secondly, no egg yolk was added to either the primary (Pozzato modified Middlebrook medium) or secondary (Dubos broth) culture media at QUB, whereas UW adds egg yolk routinely to liquid MGIT medium. The purpose of adding egg yolk to culture media used for MAP is still unclear, but neutralisation of the chemical decontaminant HPC seems to be a common reason (Whittington 2010). Whittington et al. (2013) subsequently suggested that egg yolk provides major carbon and energy sources as well as the surfactant lecithin. The question arises, are sub-lethally injured MAP cells likely to benefit from, or be adversely affected by, such a rich source of nutrients when trying to repair heat or dessication damage? Thirdly, the primary

551 culture medium adopted by QUB contained added casitone (0.1%, 1 g/L) and a higher than
552 normal amount of glycerol (0.5%, as per Pozzato et al. (2011) recipe, rather than 0.2%,
553 which is the amount indicated by the manufacturer (Difco) of Middlebrook 7H9 broth). The
554 additional ingredient, casitone, is a pancreatic enzymatic digest of casein (milk protein) that
555 contains a particularly high content of amino acids and peptides of varying sizes making it a
556 nutritious hydrolysate (BD Biosciences 2017). Glycerol, or alternatively Tween 80, both of
557 which are surfactants, can be added to Middlebrook 7H9 broth to reduce clumping of
558 mycobacterial cells during culture; although, as stated above, 0.2% is the recommended
559 glycerol concentration for this purpose. Glycerol would also represent a carbon source; a
560 very old publication (Sattler and Youmans 1948) relating to the effects of glycerol on tubercle
561 bacilli (*M. tuberculosis*) reported that glucose and glycerol increased their total amount of
562 growth but not their initial rate of multiplication. In light of the MAP isolation success at QUB
563 during this study, we would like to suggest that the optimal culture approach for isolation of
564 MAP from powdered milk products could be liquid culture in the modified 7H9 medium of
565 Pozzato et al. (2011), with PANTA but without egg yolk, following PMS to capture MAP cells
566 from reconstituted CMR that has had time to completely rehydrate before testing
567 commences. Chemical decontamination with HPC should definitely be avoided because of
568 its known deleterious effects on the viability of MAP cells (Dundee et al. 2001, Gao et al.
569 2005), and, therefore, the distinct possibility that false negative results will be obtained when
570 testing dairy products containing low numbers of MAP. The latter, plus the fact that solid
571 Herrold's egg yolk medium rather than a liquid culture medium was used by Khol et al.
572 (2017), could explain their negative culture findings for CMR. Liquid culture is likely to be
573 more conducive to recovery of stressed MAP cells than culture on solid agar media simply
574 because the bacterial cells are able to access water and nutrients more readily.

575 When CMR samples were being sourced for this study, every effort was made to
576 hygienically collect CMR on the farms visited and to collect CMR samples from unopened
577 bags. The vast majority of the 26 CMR samples that tested MAP positive (Table 1) were
578 sampled from unopened bags; just one of the positive samples (OH-013) is known to be

579 from an already opened bag of CMR. However, the possibility of contamination of CMR
580 samples by manure on the farm during collection cannot be totally ruled out because of the
581 difficulties in maintaining hygienic practices in a dirty sampling environment, despite the best
582 efforts of sampling personnel involved. Generally, all of the conventional measures of
583 microbiological quality applied to the CMR samples were within normal limits for whole milk
584 powders as set down in United States Department of Agriculture-Agricultural Marketing
585 Service Standards for milk and dairy products. These Standards stipulate total bacterial
586 counts <500,000 CFU/g and coliform counts ≤ 10 CFU/g for dry whole milk powders (USDA-
587 AMS 2001). The only statistically significant association between the conventional
588 microbiological analyses and viable MAP presence in CMR was in relation to total plate
589 counts, which were found to be significantly higher for viable MAP-positive CMR than for
590 MAP-negative CMR ($p=0.024$; Mann-Whitney test). In our opinion, this association does not
591 necessarily indicate greater contamination of the CMR with MAP on-farm, but rather could
592 reflect the possibility that the raw milk used to produce CMR ingredients contained higher
593 numbers of MAP that have been able to survive heat treatments applied during milk powder
594 production.
595
596 **CONCLUSIONS**
597
598 Viable MAP were detected in 24 (28.9%) of 83 CMR samples collected at US dairy
599 farms; 12 were positive by PMS-culture, 17 by PMS-phage assay, and 5 samples were
600 positive by both of these tests. A further two CMR samples tested positive for MAP DNA by
601 IS*900* qPCR. The presence of viable MAP in CMR was significantly associated with higher
602 total bacterial counts, but with none of the other microbiological parameters. This is the first
603 report of viable MAP in CMR, but not the first report of viable MAP in powdered milk
604 products. The source of the viable MAP detected cannot be verified; whether pre- or post-
605 processing contamination. It is unknown if the quantity of MAP detected in CMR would be
606 sufficient to cause infection of a calf. However, the prospect that MAP has survived the

manufacture of dried milk and whey-based products which are destined for consumption by food animals could have far reaching potential consequences; further testing of CMR collected directly at manufacturing sites using the PMS and liquid culture approach described above is warranted to verify our findings. The broader food safety implications of detecting viable MAP in this type of dried dairy product are not insignificant given that powdered infant formulae is consumed by young babies with immature immune systems.

ACKNOWLEDGMENTS

Funding for this study was obtained from the Wisconsin Agriculture Experiment Station (Award number MSN169060), the American Association of Bovine Practitioners, and the Walter and Martha Renk Endowed Laboratory for Food Safety. James Tarrant's involvement in the study was funded by the Merial-NIH Summer Scholars Program. Whole genome sequencing of the suspect MAP isolates obtained from CMR was partially supported by grant NIFA 2013-67015 from the USDA to Dr Adel Talaat.

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Table 1. Detailed PMS-phage assay, PMS-culture and direct IS900 qPCR results, and US state of origin information, for the 26 calf milk replacer (CMR) samples that tested positive by one or more of the MAP detection tests.

CMR code (US state of origin-sequence no.)	PMS-phage assay		PMS-culture	Direct IS*900* qPCR
	No. of plaques per 50 ml reconstituted CMR	Plaque IS*900* PCR result		
WI-001	696	+	-	-
WI-002	822	+	-	-
WI-003	150	+	-	-
WI-004	38	+	+	-
WI-006	0	-	+	-
WI-007	0	-	+	-
WI-012	1720	-	+	+
WI-013	1212	+ (weak)	+	-
WI-014	0	-	+	-
WI-025	0	-	+	-
WI-038	30	+	-	-
WI-048	0	-	+	-
WI-050	80	+ (weak)	+	-
TX-004	12	-	-	+
KY-006	12	+	-	-
UT-010	30	+	-	-
KY-012	40	+	+	+
OH-013[1]	1020	+	-	+
NY-015	6	+	-	+
KY-016	0	-	+	-
GA-017	10	+	-	+
KY-020	0	-	-	+
IA-026	6	+	-	-
NY-027	6	+	-	-
WA-031	30	+ (weak)	+	-
NY-032	36	+	-	-
Total no. CMR samples positive		17	12[3]	7
Average no. MAP (PFU/50 ml reconstituted CMR) in test positive samples [2]		248.5	116.7	153.7

[1] Only CMR sample known to have been collected from an already opened bag of CMR.

[2] The average number of plaque-forming units (PFU)/50mL reconstituted CMR for PMS-culture and qPCR positive samples were calculated based on the number of PFU indicated by the corresponding PMS-phage assay result for those samples; a negative PMS-phage assay result equated to 0 PFU/50 ml reconstituted CMR.

[3] All 12 isolates from culture-positive CMR samples were subjected to whole genome sequencing with the results shown in Fig. 2.

767 **Table 2.** Results of microbiological analyses performed on the 83 calf milk replacer (CMR)
768 products that were tested for the presence of viable MAP in order to elucidate numbers and
769 types of bacteria present. Data presented are minimum, maximum and mean counts (Log_{10}
770 CFU/mL) obtained for each analysis following reconstitution of the CMR samples according
771 to manufacturer's instructions in sterile water.
772

	Total Plate Count	Gram negative bacteria		Gram positive bacteria*			
		Coliforms	Non-coliforms	Strep	CNS	Coryne	Bac
Min. count	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Max. count	5.65	2.19	2.74	5.23	5.29	2.65	5.65
Mean count	2.66	0.03	0.03	1.01	0.27	0.15	2.33
SD	1.28	0.24	0.30	1.44	0.91	0.54	1.23

773 *Strep, *Streptococcus* spp.; CNS, coagulase-negative staphylococci; Coryne,
774 *Corynebacterium* spp.; Bac, *Bacillus* spp.
775

776 **Table 3.** Reference assembly summaries for 12 MAP isolates cultured from CMR during
777 this study, and for four unrelated Elk MAP isolates. First two letters of MAP isolate no. relate
778 to US state where CMR sample was collected.

MAP Isolate No.	Total reads	% Mapped Reads	Average coverage	No. of Single Nucleotide Polymorphisms (SNPs)
WI-004	2596628	99.35	125.66	204
WI-006	2632574	99.44	127.26	251
WI-007	3050230	93.39	139.58	261
WI-012	2617894	99.57	127.73	213
WI-013	2990666	96.31	148.78	256
WI-014	2407414	99.28	123.36	259
WI-025	2535896	99.42	130.18	151
WI-048	2288424	99.47	117.59	260
WI-050	2612622	99.04	133.55	257
KY-012	2966692	99.41	152.25	267
KY-016	2594488	98.87	132.39	257
WA-031	2493940	99.2	127.79	252
Elk8B-1	2121874	99.38	100.83	142
Elk8B-2	2683464	99.41	130.28	150
Elk10A	2628938	99.61	128.71	146
Elk10B	2746440	99.59	134.07	148

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Figure captions

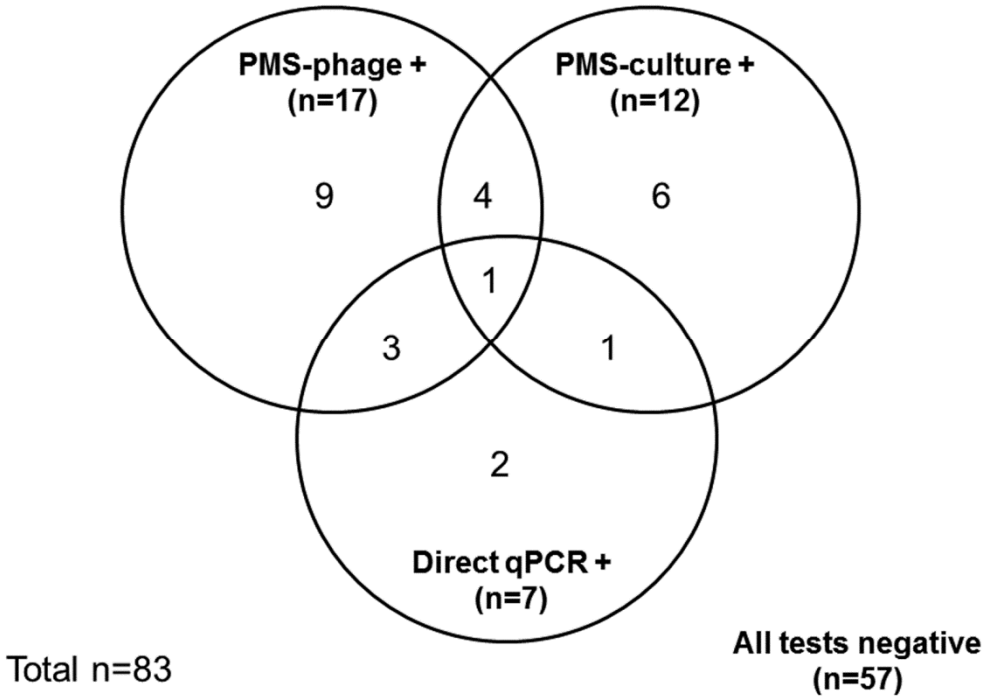
Figure 1

Venn diagram showing the interrelationships between results obtained by the PMS-phage assay, PMS-culture and direct IS*900* qPCR for 83 calf milk replacer (CMR) samples. Overlapping areas indicate numbers of CMR samples positive by more than one of the MAP detection tests.

Figure 2

Phylogenetic analysis of 12 MAP isolates obtained from different calf milk replacer (CMR) products and the MAP K10 reference strain. The first two letters of each strain ID indicate the US state of origin of the CMR sample from which the isolate was obtained. A) Venn diagrams displaying the number of common and different single nucleotide polymorphisms (SNPs) found among representative CMR isolates. B) The percentages of synonymous (SSNPs), non-synonymous (nSNPs) and intergenic SNPs found in the genomes of the 12 CMR isolates. C) A phylogenetic tree of the genomes of 12 CMR isolates with the presence of other genomes including the standard bovine type (K-10), an ovine type (S397), and four Elk isolates of US origin. Concatenated SNPs were used from all isolates to build a rooted Neighbor-joining tree using MAP-S397 genome as an out group. The percentages of a 1000 bootstrap replicates are shown on the dendogram.

803 Figure 1



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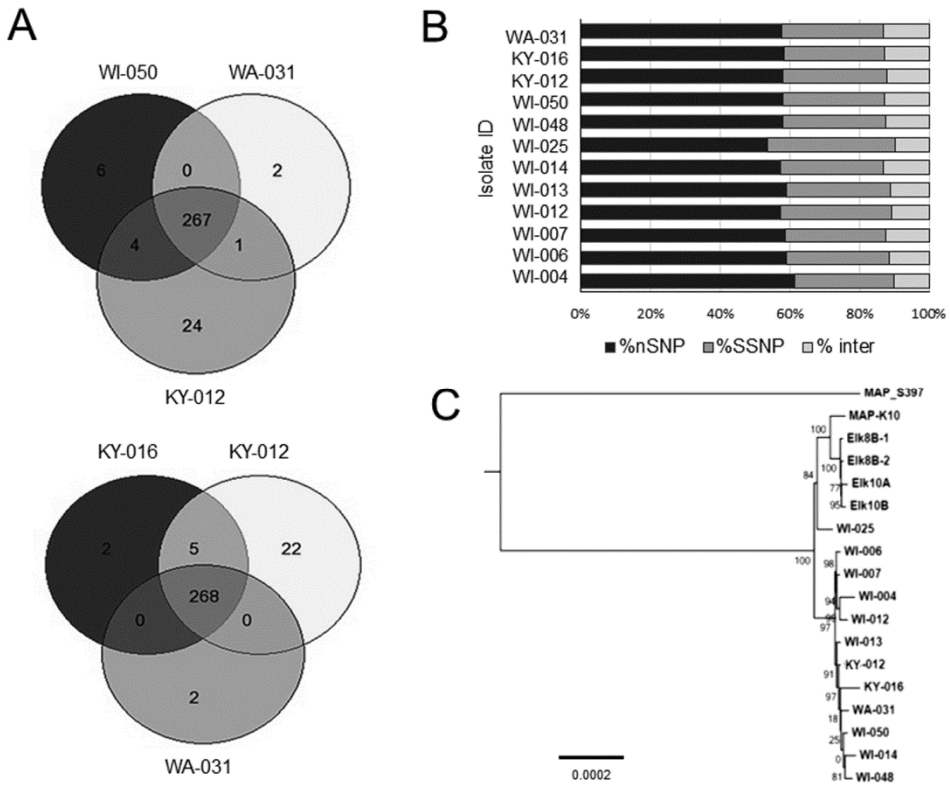
805 Figure 2



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