



Detection of viable *Mycobacterium avium* subspecies *paratuberculosis* in powdered infant formula by phage-PCR and confirmed by culture



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ABSTRACT

Surveys from different parts of the world have reported that viable *Mycobacterium avium* subsp. *paratuberculosis* (MAP) can be cultured from approximately 2% of samples of retail pasteurised milk samples. Pasteurised milk is used for the production of powdered infant formula (PIF) and therefore there is a concern that MAP may also be present in these products. Several studies have previously reported the detection of MAP in PIF using PCR-based assays. However, culture-based surveys of PIF have not detected viable MAP. Here we describe a phage amplification assay coupled with PCR (phage-PCR) that can rapidly detect viable MAP in PIF. The results of a small survey showed that the phage-PCR assay detected viable MAP in 13% (4/32) of PIF samples. Culture detected viable MAP in 9% (3/32) PIF samples, all of which were also phage-PCR positive. Direct IS900 PCR detected MAP DNA in 22% (7/32) of PIF samples. The presence of viable MAP in PIF indicates that MAP either survived PIF manufacturing or that post-production contamination occurred. Irrespective of the route of MAP contamination, the presence of viable MAP in PIF is a potential public health concern.

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1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of Johne's disease in cattle. Crohn's disease is a chronic inflammatory bowel disease (IBD) in humans and the first description of similarities between Crohn's disease and paratuberculosis in cattle was reported in 1913 by Dalziel, and an argument has been made that MAP may also be the causative agent of Crohn's. Although a causal link has not been proven, there are both individual studies and meta-analyses that have found that there is an association between exposure of humans to MAP and the occurrence of Crohn's disease (Atreya et al., 2014; Feller et al., 2007; Liverani et al., 2014; Naser et al., 2000; Waddell et al., 2008). More recently it has been suggested that this may be due to indirect immuno-modulatory effects on the host following exposure to the organism rather than infection per se (Abendano et al., 2013; Atreya et al., 2014; Nabatov, 2015). Hence the presence of viable MAP in the human food chain remains to be a concern for human health.

Johne's disease is a progressive inflammatory bowel disease of cattle, and in the latter stages of infection the organism spreads systemically and is then excreted in the milk of infected animals (Ayele et al., 2001), even when they are asymptomatic (Slana et al., 2008). Since the prevalence of paratuberculosis in dairy ruminants is high worldwide (Khol and Baumgartner, 2012), the detection of MAP DNA in raw milk is not surprising, and this has been demonstrated in many studies (see Eltholth et al., 2009; Nielsen and Toft, 2009). However, viable MAP has also been detected in surveys of retail pasteurised milk (Ayele et al., 2005; Carvalho et al., 2012; Ellingson et al., 2005; Grant et al., 2002; Paolicchi et al., 2012). Despite the concern for human health, contamination of milk with MAP is not legally restricted in any dairy products, including powdered infant formula (PIF).

Pasteurised milk is used for the production of PIF, and several studies have reported detection of MAP in PIF by PCR-based methods. For instance MAP was detected in 35% of PIF samples tested and the levels present were estimated to be equivalent to between 48 and 32 500 cells g⁻¹ (Hruska et al., 2005, 2011). More recently two small studies have also reported detection of MAP DNA in PIF by PCR in a high percentage of samples tested (6/6; El-Malek and Mohamed, 2011, 4/7; Hassan and Ali, 2012). In contrast a study in Indonesia did not detect MAP in any of 50 PIF samples tested (Nugroho et al., 2009). PCR methods can quantitatively detect MAP DNA with high specificity and sensitivity but cannot assess MAP

Abbreviations: ANV, amphotericin B, nalidixic acid and vancomycin; HEYM, Herold's Egg Yolk Medium; JD, Johne's disease; MAP, *Mycobacterium avium* subsp. *paratuberculosis*; PIF, powdered infant formula.

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viability and ideally the presence of MAP in processed food products should be confirmed by culture. In previous studies viable MAP has not been detected in PIF by culture, even from samples with high levels of MAP DNA (Hruska et al., 2011).

Unfortunately culture of MAP from any sample is laborious, time consuming, requires long periods of incubation and is often insensitive and therefore is not a method that is easily applied for surveys of foods (Rees and Botsaris, 2012). In contrast bacteriophage amplification assays have been used to detect MAP in a wide variety of matrices including milk, cheese and blood (Botsaris et al., 2013, 2010; Foddai et al., 2011; Stanley et al., 2007; Swift et al., 2013). Compared to culture, the phage-based assays are rapid (total time to detection and identification is 48 h), only detect viable MAP cells and also allows enumeration of the number of cells detected (Rees and Botsaris, 2012). The phage-PCR combines the ability to quantify viable MAP with the specificity of PCR (Stanley et al., 2007). Hence the aim of this study was to develop a method that could be used to detect viable MAP cells in PIF. Using this method, a small survey of commercial PIF was carried out and results compared with previously published methods for detection of MAP in PIF.

2. Materials and methods

2.1. Optimisation of the phage assay

Strain K10 was used for optimising the PIF sampling method. This was grown in Media Plus (MP; Middlebrook 7H9; Becton Dickinson supplemented with 10 mM CaCl₂) containing 2 mg ml⁻¹ Mycobactin J; Synbiotics, France). Cultures were grown with aeration at 37°C and the concentration of MAP cells determined using both culture onto MP and the phage amplification enumeration method (Rees and Botsaris, 2012). For optimisation experiments MAP cultures were diluted 10-fold from approximately 1 to 10³ cfu ml⁻¹ and 1 ml of each dilution added to 1 g of laboratory grade skim milk powder (Merck, Darmstadt, Germany). Three independent samples were prepared for each dilution of MAP and the sample was fully reconstituted using a further 8 ml MP to give a total volume of 10 ml. Samples were then centrifuged (2500 ×g, 15 min) and the pellet washed with 2 ml of MP to remove traces of milk protein. Finally pellets were reconstituted in 1 ml MP and the phage amplification assay performed as previously described by Botsaris et al. (2010). As a negative control 1 g of SMP was reconstituted in 9 ml Media Plus.

2.2. Sampling of milk-based infant formulae

A total of 32 samples of PIF were collected from the retailers in Cyprus from a total of eight different outlets. The samples selected all included skim-milk powder as a listed ingredient. The 32 different samples were from a total of ten different producers operating in seven different countries of the European Union. A sample of 3 g was taken aseptically from each PIF and this was separated into three 1 g aliquots which were tested independently using either phage-PCR, culture or direct IS900 PCR.

2.3. Phage-PCR

For the phage-PCR assay, the sample was prepared as described in Section 2.1. After the phage assay had been performed (Botsaris et al., 2010), the number of plaques was counted to record the number of mycobacterial cells detected. Then DNA was extracted from individual plaques (Rees and Botsaris, 2012) and the presence of MAP genomic DNA confirmed using PCR amplification of IS900 sequences (Botsaris et al., 2010).

2.4. Culture

For culture, PIF samples were reconstituted (1 g in 5 ml; Hruska et al., 2005) in sterile distilled water (SDW) and the mixture was centrifuged (2500 ×g, 15 min). The pellet was resuspended in 5 ml 0.75% HPC, incubated for 4 h at room temperature and recentrifuged (2500 ×g, 15 min). The pellet was resuspended in 1 ml SDW and an aliquot (200 µl) inoculated onto two Herrold's egg yolk medium (HEYM) slopes supplemented with Mycobactin J and antibiotics (ANV; Becton Dickinson, USA). Slopes were incubated for a period of 6 months at 37°C and visible growth detected as a thin layer of bacterial bloom containing some discrete colonies. The growth of MAP was confirmed by IS900 PCR as previously described (Botsaris et al., 2010). Tubes with no visible growth were also tested for MAP growth by washing the surface of the slopes with 200 µl SDW (Williams and Monif, 2009) before DNA was extracted and IS900 detected by PCR.

2.5. Direct IS900 PCR

For the direct detection of MAP in PIF by IS900 PCR, 1 g of milk powder was mixed with 9 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and then the sample was centrifuged (2500 ×g, 15 min). The pellet was resuspended in 1 ml TE and cells lysed by heating at 100°C for 20 min (Hruska et al., 2005). Cell debris was removed by centrifugation (13,000 ×g, 5 min), and the DNA was purified from the supernatant using a High Pure PCR Template Preparation kit (Roche Diagnostics, Germany; 25 µl elution volume); 5 µl of this was used for PCR amplification of the IS900 element using primers P90 and P91 (Whittington et al., 1998).

2.6. IS1311 PCR with Restriction Endonuclease Analysis (REA)

Cattle and sheep strains of MAP were distinguished by REA analysis of IS1311 using primers M56 and M94 and restriction of the PCR product with *Hinf*I (Whittington et al., 1998; Marsh et al., 1999).

3. Results and discussion

3.1. Development of a method to detect MAP in PIF

When testing milk sample using the phage amplification assay, it has been shown that the presence of milk protein can inhibit phage binding (Stanley et al., 2007) and therefore centrifugation is used to separate out MAP cells from the milk matrix (Botsaris et al., 2010). Hence when developing this protocol, the same processing procedures (centrifugation and rinsing of the pellet to remove residual milk protein; Section 2.1) were used to separate out MAP cells from the reconstituted PIF product.

To determine the ability of phage to infect cells in reconstituted PIF, tenfold dilutions of MAP cultures were used to inoculate samples of laboratory grade skim milk powder. Three independent samples were prepared for each dilution of MAP and then the sample processed (Section 2.1) before the phage assay was performed (Table 1). It was noted that the pellets formed were much smaller than when processing raw milk samples and therefore care is needed when washing the pellet. The control samples, to which MAP cells were not added, did not produce any plaques indicating that material from the sample matrix did not interfere with the inactivation of the phage during the phage amplification assay and that this processing method was appropriate for preparing PIF samples. The results indicated that the phage assay was able to detect viable MAP cells after recovery from PIF with a detection limit of approximately 10 cfu ml⁻¹ (Table 1). When plaques were detected, IS900 PCR was used to confirm that the cells detected were MAP. This demonstrated that any contaminants remaining following the PIF sample preparation did not inhibit the PCR reaction. While not sensitive enough to detect single cells, the detection limit is lower than the estimated of levels of MAP in naturally contaminated product reported by

Table 1
Determining detection limit of the phage-PCR method in PIF.

Sample	Approx. MAP CFU 1 g ⁻¹	MAP PFU 1 g ⁻¹ detected				MAP IS900 PCR ¹
		1	2	3	Average ± SD	
A	1.0 ²	ND [†]	ND	ND	–	NP*
B	1.0 × 10 ¹	3	2	2	2.3 ± 0.6	+
C	1.0 × 10 ²	54	48	66	56 ± 9.2	+
D ³	1.0 × 10 ³	740	820	850	803.3 ± 56.9	+
F	Control ⁶	ND	ND	ND	–	NP

*Not performed.

†Not detected.

¹ IS900 PCR was performed according to Botsaris et al. (2013). For sample B, all plaques were tested for the presence of MAP by PCR. For samples C and D a representative number of plaques (23) from each sample were tested.

² Original culture contained 1.3 × 10⁹ cfu ml⁻¹ and this was diluted in MP to prepare the inoculum used for each sample.

³ When testing 1 ml samples the number of plaques per plate was too numerous to count; the number of plaques recorded represents the number of countable plaques in a 0.1 ml sample, adjusted to a value per ml.

Hruska et al. (2011; 48 to 32 500 cells g⁻¹ based on the quantification of DNA), and therefore this simple procedure was deemed to be sufficient for the purposes of testing naturally contaminated products. If MAP cells are able to survive in the dried powder product, it is likely that they may be in a stressed or dormant state. Hence although it was possible to detect freshly cultured MAP cells that were spiked into PIF, it was possible that cells that have been in the product for some time may not be detectable using this method.

3.2. Survey of PIF products

To test whether the new protocol could detect MAP cells present in naturally contaminated product, a small survey of retail PIF products was performed. For comparison, samples were also tested using conventional culture and direct IS900 PCR both of which have been used previously to try and detect MAP in milk-based infant formulas (El-Malek and Mohamed, 2011; Hruska et al., 2011). A total of 32 samples of PIF were tested independently using the phage-based method, culture and direct IS900 PCR. Using culture, MAP was detected in 3 (9.4%) of the samples; these were produced by different companies and therefore did not represent one contaminated batch of product (see Table 2). No additional samples were found to contain evidence of MAP growth when samples from the surface of slopes that did not show visible growth were tested by PCR.

Using the direct IS900 PCR assay, MAP DNA was detected in seven (21.9%) samples including the three samples that were also MAP-positive by culture on HEYM (#12, #17 and #19). Genetic material from the three cultured isolates was subjected to IS1311 PCR REA and all three isolates were characterised as cattle strains. Two isolates (#12 and #19) were successfully sub-cultured, producing visible colonies on HEYM supplemented with mycobactin J and ANV after 8 weeks incubation, however one sample (# 17) could not be sub-cultured.

Four PIF samples were MAP-positive by phage-PCR and this group included the three culture-positive samples (#12, #17 and #19). Sample #3 was positive by phage-PCR but negative by culture and direct PCR. For the three culture-positive PIF samples the average plaque count recorded was 34.5. Five samples produced plaques that were not due to MAP (i.e. they were IS900 PCR-negative) and the average plaque number for these samples was 3.6. These may have been due to other mycobacterial in the sample or breakthrough in the assay as previously reported (Botsaris et al., 2013; Swift et al., 2013). In a study of raw milk samples, it was found that plaque number is a good predictor of whether or not a sample is likely to contain MAP (Botsaris et al., 2013). Hence the fact that the average plaque number for the MAP-

Table 2
MAP detection in PIF.

Producer	Sample no.	Culture	IS900 PCR	Phage combined with IS900 PCR	Plaque number ¹ pfu g ⁻¹
1	1	–	–	–	1
	2	–	+	–	(5)
3	3	–	–	+	30
	4	–	–	–	0
4	5	–	–	–	0
	6	–	–	–	0
5	7	–	–	–	(2)
	8	–	–	–	0
6	9	–	–	–	0
	10	–	–	–	0
7	11	–	–	–	0
	12	+	+	+	95
8	13	–	–	–	0
	14	–	–	–	0
9	15	–	+	–	0
	16	–	–	–	0
10	17	+	+	+	9
	18	–	–	–	0
11	19	+	+	+	13
	20	–	+	–	(5)
12	21	–	–	–	(2)
	22	–	–	–	0
13	23	–	–	–	0
	24	–	–	–	0
14	25	–	–	–	0
	26	–	–	–	(4)
15	27	–	–	–	0
	28	–	–	–	0
16	29	–	–	–	0
	30	–	–	–	0
17	31	–	+	–	0
	32	–	–	–	0
Total		3	7	4	
%	100	9.4	21.9	12.5	

+; MAP detected.

–; MAP not detected.

¹ samples that produced plaques but did not give a positive result with the IS900 PCR assay following DNA extraction from plaques are shown in brackets as these scored as MAP-negative.

positive samples was 10-fold higher than that for the MAP-negative samples is consistent with our previous findings.

Overall MAP was detected in eight (25%) of the PIF samples by at least one of the methods used. As expected, the highest number of MAP-positive samples (seven) was found using the direct IS900 PCR method and this is consistent with other surveys of pasteurised milk which compared both culture and direct PCR, where the number of PCR-positive samples was always higher than the number of culture-positive samples (Grant et al., 2002; O'Reilly et al., 2004). The phage-PCR assay detected viable MAP in more samples than by direct culture and is consistent with previous reports that the phage-PCR method is more sensitive than culture when detecting viable MAP in milk and other dairy products (Stanley et al., 2007; Foddai et al., 2009; Botsaris et al., 2010). This is not surprising since no chemical decontamination is required before carrying out the phage-PCR assay.

This is the first report of the use of the phage-PCR method to detect viable MAP cells in powder milk products. Not only is the method more sensitive, it is much faster than culture (results gained in 48 h). In addition the method includes the specificity of PCR but has the advantage that only viable cells are detected. The cells present in such dried milk products will experience stress during the skim-milk manufacturing process and it is possible that the cell surface is affected, reducing the ability of the phage to adsorb to the cell which will reduce the number of cells detected. Recently we have shown that stress conditions may force MAP cells into a dormant-non-stationary phase when they are no longer sensitive to bacteriophage D29 infection (Swift et al., 2014). Foddai et al. (2009) have also shown that the detection of MAP cells present in milk can be improved by a period of culture. We are currently

investigating whether introducing a recovery stage will increase the proportion of cells detected in the PIF and increase the sensitivity of the phage-PCR method.

This is also the first study to conclusively show that viable MAP can be present in PIF products, with this result being confirmed by two independent methods. The fact that these bacteria not only survive pasteurisation, but also further processing steps during the production of PIF is unexpected. Grant et al. (2002) found that the probability of the organism surviving pasteurisation may be related to variation in the number of MAP cells present in different batches of raw milk. Hence presence of MAP in PIF maybe the result of very high contamination levels of the milk used, or due to some other, as yet unidentified, underlying physiological factor or mechanism of survival, such as the possibility of the production of spore-like structures (Lamont et al., 2012).

The overall finding from the results was that 12.5% of the products tested were found to contain viable MAP cells. Previous authors have suggested that PIF may be a significant route of exposure of human infants to this organism (Hruska et al., 2011) and there is a concern that large numbers of mycobacterial cells could act as a pro-inflammatory trigger in premature babies and bottle-fed newborns even if no infection is established (see Atreya et al. (2014)). The development of a simple, rapid method that can be used to monitor the survival of MAP during the production of PIF will facilitate further investigations and allow improved control measures to be developed.

4. Conclusions

The phage-PCR assay provides a simple, rapid and quantitative method to detect viable MAP in PIF. Results from a small retail PIF survey indicated that the risk of exposure of infants to MAP is high (12.5% of 32 products harbored viable MAP). The zoonotic potential of MAP remains unproven, but this method provides a new tool to achieve the eradication of MAP from this very sensitive category of products.

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