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Detection of *Mycobacterium avium* subspecies *paratuberculosis* in powdered infant formula using IS*900* quantitative PCR and liquid culture media

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ABSTRACT

Mycobacterium avium subspecies *paratuberculosis* (MAP) has been implicated in Crohn's disease in humans resulting in public concern over the presence of MAP in powdered infant formula, which could contribute towards early human exposure to MAP or MAP components. Testing of representative powdered infant formula samples using effective tests is required to provide information on contamination of infant formula with MAP, so that consumers can make informed decisions. This study aimed to test representative powdered infant formula samples for the presence of MAP using a quantitative PCR and liquid culture method. For this purpose, an efficient DNA extraction method was developed and an optimum decontamination protocol for culture method was identified. A total of 122 powdered infant formula samples were tested, comprising 72 brands produced by 12 manufacturers from 9 countries. Powdered infant formula samples were reconstituted and centrifuged to separate the casein pellet, cream layer and whey fraction. A sensitive qPCR test was performed on DNA extracted from the casein pellet. In addition, the cream layer and casein pellet were cultured in liquid media, following decontamination with the optimum protocol. Of the 122 samples tested, 6 were positive for MAP DNA but none were positive for growth in culture at 12 and 20 weeks. The limit of detection of the quantitative PCR was less than 5 MAP organisms per 1.5g milk powder. The methods developed in the study could be used for quality assurance testing for infant formula and calf milk replacers. The low contamination level of MAP and absence of viable forms in our study suggests a relatively low risk of exposure of infants to MAP components.

1. Introduction

Johne's disease (JD) is a chronic gastroenteritis of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). The disease is endemic among dairy animals in many parts of the world and has been subject to voluntary or compulsory government-led control programs in many countries, while in other countries the JD control programs have been led by the producers. The major motivators for JD control are its economic impact on farm (Cho et al., 2012; Shephard et al., 2016), animal welfare concerns and recently, public health concerns (Atreya et al., 2014; Behr and Kapur, 2008; Waddell et al., 2016a, b). The economic losses to the dairy industry are due to disease management costs, mortality (Johnson-Ifearulundu et al., 1999), and reduced milk production (Raizman et al., 2009) chiefly among faecal shedders (Donat et al., 2014), increased culling rates (Raizman et al., 2009; Whitlock and Buergelt, 1996), decreased mean weight of the culled animals (Johnson-Ifearulundu et al., 1999) and in utero transmission (Whittington and Windsor, 2009) leading to reduced live weight of calves (Elzo et al., 2009), and reduced average weaning weight of calves (Bhattarai et al., 2013). The increase in consumer concern over compromised animal welfare due to the disease and the presence of MAP in milk and milk products intended for human consumption could contribute towards the future economic burden of JD.

Milk from animals in a JD infected farm could be contaminated with MAP. The bacteria, in free form or possibly intracellular within macrophages, can enter milk from direct secretion into milk (Sweeney et al., 1992) and/or via faecal contamination (Vissers et al., 2007), as faeces has been reported to contain a high load of MAP per gram (Fecteau and Whitlock, 2010; Whittington et al., 2000). MAP has been detected in milk derived from both clinically affected and sub–clinically infected animals (Logar et al., 2012; Streeter et al., 1995; Taylor et al., 1981).

Pasteurisation and spray drying processes used in the manufacture of powdered infant formula might not be sufficient to ensure freedom from viable MAP. Pasteurisation is achieved by heating milk to 72 °C for a holding time of at least 15 s (Food Standards Australia New Zealand, 2015). However, it has been noted that pasteurisation holding times would not be sufficient to inactivate MAP (Grant et al., 2005; Mullan, 2015; Sung and Collins, 1998), particularly when there is clumping of

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the organisms and/or the MAP load in milk is high (Grant et al., 2005; Hammer et al., 2006). Aside from the inherent ability of MAP to survive a number of stressors, including high temperature (Hammer et al., 2006; Sung and Collins, 1998) and desiccation (Larsen et al., 1956), milk has been shown to protect bacteria during spray drying and possibly against heat during pasteurisation (Ananta et al., 2005; Mañas et al., 2001). Therefore, it is possible that MAP could survive during pasteurisation and spray drying of milk to prepare powdered milk as evident from a study reporting the presence of viable MAP in powdered infant formula (Botsaris et al., 2016). No such information on powdered infant formula is available in Australia.

Human infant populations receive a significant portion of their nutritional needs from non-human milk and powdered infant formula (Australian Institute of Health and Welfare, 2010; NHMRC, 2011). Consequently, if powdered infant formula contains viable MAP or MAP components, it could result in exposure of human infants to MAP and MAP components, as at present there is no legal requirement to regulate MAP contamination in infant formula. However, there is increasing international concern over the potential for powdered infant formula to be contaminated with MAP, in association with increasing concerns over the role of MAP in Crohn's disease (CD), particularly in paediatric cases (Kirkwood et al., 2009). Although a causal link has not as yet been established, there is reported consensus among experts over the role of milk as a likely route of exposure of humans to MAP (Waddell et al., 2016a, b). In addition to the potential for viable MAP to be infective to susceptible humans (Kirkwood et al., 2009), MAP exposure may trigger detrimental inflammatory responses in the infant gut (Atreya et al., 2014). MAP antigens alone were able to exert a necrotising effect in the intestine of mice (Momotani et al., 2012). Furthermore, dysbiosis in the gastrointestinal microbial community of the infant gut is an additional concern, as has been noted in children with CD (Kaakoush et al., 2012). There is evidence of dysbiosis in the gastrointestinal microbial community of cattle following MAP infection (Fecteau et al., 2016). Dysbiosis in the infant gut is thus a possibility following early exposure to MAP through powdered infant formula, which could be facilitated by the underdeveloped mucosal immune system of infants.

Theoretically, many methods can be used to detect the presence of viable MAP and MAP DNA in powdered infant formula. However few methods have been used in studies to assess powdered infant formula for this purpose. There is a single study that used culture on solid media and phage PCR to confirm the presence of viable MAP in powdered infant formula (Botsaris et al., 2016). Culture can detect and isolate viable organisms in milk, with liquid media preferred over solid media owing to its higher sensitivity (Bradner et al., 2013; Whittington, 2009). However, the long incubation period (2–3 months) necessary for MAP growth and the requirement for a decontamination step are constraints for the culture methods. Decontamination of a clinical sample is essential to remove other competing bacteria present in powdered infant formula (Anonymous, 2011; Thompson, 2010); however, it could impact the limit of detection of culture (Reddacliff, Vadali, et al., 2003). Furthermore, there is little information regarding the optimal decontamination protocol for powdered milk or the interaction of different milk fractions (cream, whey and casein pellet) on the detrimental effects of decontaminants on MAP. Recently, more reliance is being placed upon molecular methods to detect MAP DNA owing to the rapidity of detection and the increased sensitivity of such methods. There have been attempts made to detect MAP DNA in powdered infant formula using conventional IS*900* PCR (Botsaris et al., 2016; El-Malek and Mohamed, 2011; Hruska et al., 2005; Nugroho et al., 2008) and f57 quantitative (q) PCR (Hruska et al., 2011); qPCR based assays targeting the IS*900* fragment of MAP are more sensitive than those detecting f57 (Donaghy et al., 2011; Hruska et al., 2011). The absence of a reported limit of detection (LOD) (Botsaris et al., 2016; Hruska et al., 2005), a high LOD (Donaghy et al., 2011; Hruska et al., 2011), and different approaches utilised to estimate LOD (Donaghy et al., 2011) are constraints to the adoption of these methods and interpretation of the results. The inclusion of a mechanical lysis step in the DNA extraction has been found not only to improve the sensitivity of qPCR methods (Bull et al., 2003; Madiraju et al., 2000; Odumeru et al., 2001; Plain et al., 2015) but also to facilitate DNA isolation from MAP present in unicellular, clumped form or within leucocytes. Furthermore, despite evidence that qPCR-based tests for MAP are prone to PCR inhibition resulting in false negative test results (Acharya et al., 2017), only one of the reported studies on powdered infant formula included inhibition control (Hruska et al., 2011).

Thus, the objectives of this study were to test representative samples of powdered infant formula in Australia for the detection of MAP contamination using a liquid culture method and a qPCR method to detect MAP DNA, which targeted the multi-copy insertion element IS*900*. This was achieved by the identification of a suitable decontamination protocol for the culture of viable MAP using liquid culture media, and the development of a DNA isolation technique for qPCR. Inhibition control for the qPCR method was achieved by a test and relief approach, through dilution of the extract.

2. Material and methods

2.1. Sample size and sample details

A sample size of 119 was determined to achieve 95% population sensitivity to detect a prevalence of 5% MAP contamination in powdered infant formula (Sergeant, 2016), assuming that the qPCR test was 50% sensitive and 100% specific. In total, 122 powdered infant formula samples were purchased both in-store and online from various commercial outlets in Australia for testing. The samples comprised 72 different brands produced by 12 manufacturers from 9 countries. Samples were purchased between September 2014 and July 2015, and stored at room temperature prior to sub-sampling.

2.2. Sub-sampling of powdered milk

All milk sampling was performed in a biosafety cabinet in a physical containment level 2 laboratory. The outer surface of each individual powdered infant formula container was disinfected using 1% Trigene advance (Medichem International) followed by 70% ethyl alcohol, with the same procedure used to sterilise the protective seal after removing the lid. Following disinfection, the protective seal was cut with a sterile scalpel blade.

A modified 50 ml sterile serological plastic pipette (Greiner bio-one), with the tip removed under aseptic conditions, was used to sample a column of milk powder from the full depth of the container. Samples were stored at − 20 °C in 50 ml falcon tubes until further use.

2.3. Reconstitution of powdered infant formula

Powdered infant formula samples (1.5 g) were reconstituted in 10 ml ultra-pure water heated in a water bath at 50 °C. Creation of a homogeneous solution was aided by shaking manually followed by vortexing for 20–30 s. The reconstituted milk formula was centrifuged at 2851 × *g* for 30 min resulting in three fractions: the cream layer, whey fraction and casein pellet.

2.4. Preparation of the bacterial suspension

A MAP reference strain (CM00/416) isolated from cattle tissue was used. This was a cattle (C) strain, determined using IS*1311* PCR-restriction endonuclease analysis (Marsh et al., 1999).

MAP suspensions were prepared as previously described (Plain et al., 2014). Briefly, bacteria were harvested from slopes (Middlebrook 7H10 agar supplemented with mycobactin J) and suspended in phosphate-buffered saline (PBS) supplemented with 0.1% (w/v) Tween 20. Single–cell suspension of MAP was prepared by passing the suspension