Occurrence of *Mycobacterium avium* subsp. *paratuberculosis* in foods and the impact of milk processing on its survival

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INTRODUCTION

Whilst the role of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in human disease (whether Crohn's or other disease) has remained the subject of continuing debate over recent years, the possibility that MAP could be transferred to humans via foods of animal origin has been the focus of considerable research effort. Published studies relating to MAP in food over the past five years (2000-2005) fall into two broad categories, those which investigated the natural occurrence of MAP in milk, other dairy products and beef (surveillance studies) and those which described the impact of various dairy processes on MAP in milk (survival studies). This Review and Perspectives paper summarises the information arising from both types of study.

SURVEILLANCE STUDIES

MAP in raw and pasteurised cows' milk

Several studies reporting the presence of MAP, by PCR and/or culture, in raw cows' milk from individual animals sub-clinically or clinically affected by Johne's disease (Giese and Ahrens 2000; Pillai and Jayarao 2002; Maher et al. 2004; Ayele et al. 2005), from bulk tanks at farm level (Corti and Stephan 2002; Sevilla et al. 2002; Stabel et al. 2002; Jayarao et al. 2004), and from bulk silos at processing level (Grant et al. 2002a; O'Doherty et al. 2002; O'Reilly et al. 2004) have been published over the last five years. Due to limitations in culture methods, accurate enumeration of MAP in milk is still not possible and, therefore, MAP counts reported in two of these studies (4-20 CFU/50 ml (Ayele et al. 2005) and <100 CFU/ml (Giese and Ahrens 2000)) are underestimates and need to be cited by others with due acknowledgement of this fact. It should also be noted that non-recovery or low recovery of viable MAP may have occurred in some studies (despite very high levels of IS*900* PCR positivity) because the decontamination methods employed would have damaged or killed the MAP present (Pillai and Jayarao 2002; Stabel et al. 2002; Jayarao et al. 2004). Whilst it is likely that MAP numbers in raw milk will decrease as contaminated milk from individual animals is mixed in the bulk tank at farm level and then milk from herds of differing Johne's disease status is mixed prior to processing, current evidence indicates that detectable levels of MAP may still be present in raw cows' milk prior to processing in various parts of the world.

The first survey to confirm the presence of low levels of viable MAP in commercially pasteurised cows' milk was carried out in the United Kingdom; 1.8% pasteurised milk samples tested MAP culture positive (Grant et al. 2002a; United Kingdom Food Standards Agency 2003). Whilst it was acknowledged that these culture positives could have arisen as a consequence of post-process contamination of milk by MAP, all the indications were that the MAP isolates obtained from pasteurised milk were genuine survivors (Grant et al. 2002a). Viable MAP has since been cultured from commercially pasteurised milk during surveys in California, Minnesota and Wisconsin, USA (Ellingson et al. 2005), Czech Republic (Ayele et al. 2005) and Argentina (Paolicchi et al. 2005), but not during surveys in Republic of Ireland (O'Doherty et al. 2002; O'Reilly et al. 2004) and Ontario, Canada (Gao et al. 2002). Table 1 summarises the key features of the published milk surveys. The decontamination method adopted for many of the surveys - 0.75% hexadecylpyridinium chloride for 4-5 h - has been shown to be the optimal method for recovery of MAP from milk when compared to other decontamination protocols (Dundee et al. 2001; Gao et al. 2005).

Table 1. Overview of surveillance studies to determine the presence of MAP in commercially pasteurised cows' milk.

Study / Country	Volume of pasteurised milk tested	No. (%) IS*900* PCR positive	No. (%) culture positive	Chemical decontamination before culture
Grant et al. (2002a) / UK	50 ml	67 / 567 (11.8%)	10 / 567 (1.8%)	0.75% HPC[a]/5h
Gao et al. (2002) / CAN	1 or 5 ml	110 / 710 (15%)	0 / 244[b]	None
O'Doherty et al. (2002) / IRE	50 ml	-[c]	0 / 77	0.75% HPC/5h
O'Reilly *et al*. (2004) / IRE	50 ml	35 / 357 (9.8%)	0 / 357[b]	0.75% HPC/5h
Ellingson et al. (2005) / USA	40 ml	452 / 702 (64%)	20 / 702 (2.8%)	None
Ayele et al. (2005) / CZ	50 ml	-	4 / 244 (1.6%)	0.75% HPC/5h
Paolicchi et al. (2005) / ARG	50 ml	2 / 70 (2.9%)	2 / 70 (2.9%)	0.75% HPC

[a] HPC, Hexadecylpyridinium chloride; [b] Suspect MAP positive cultures containing acid-fast cells that tested IS*900* PCR positive were encountered but sub-culture was unsuccessful; [c] IS*900* PCR was only used to confirm identity of acid-fast colonies, not to test milk samples directly.

MAP in raw sheep and goats' milk

DNA evidence of MAP in raw goats' milk in the UK (1.1% positive, Grant et al. 2001), Norway (7.1% positive, Djonne et al. 2003) and Switzerland (23.0% positive, Muehlherr et al. 2003) has been reported, but either culture was not attempted or the presence of viable MAP was not confirmed in any of these studies. In the UK study (Grant et al. 2001) raw sheep's milk was also tested but there was no cultural or PCR evidence of MAP, whereas in the Swiss study a similar percentage of raw sheep's milk samples (23.8%) as goats' milk samples (23.0%) tested MAP positive by PCR (Muehlherr et al. 2003). Detection rates observed probably reflect Johne's disease prevalence in the goat and sheep populations in the three countries concerned.

MAP in dairy products

The presence of viable MAP in dairy products other than liquid milk has not been extensively studied to date. The first reports of viable MAP being isolated from retail cheese and powdered infant formula were published recently. Gazouli et al. (2003) tested 42 samples of retail Feta cheese (made from a mixture of sheep and goats' milk) from Greece and reported the detection of MAP by PCR in 50% of samples and isolation of viable MAP from one sample. Hruska et al. (2005) tested 51 samples of powdered infant formula marketed by 10 manufacturers from seven European countries and detected MAP DNA in 49% (n=25) samples and cultivated viable MAP from one sample. More extensive surveillance of these and other dairy products is warranted in order to establish potential risk posed to consumers.

MAP in ground beef

In the advanced stages of Johne's disease in cattle, MAP infection is likely to be widely disseminated throughout the animal including muscle, lymph nodes and blood. It has, therefore, been suggested that meat from old dairy cows, used to make ground beef for human consumption in some parts of the world, may represent a source of MAP infection for consumers (Rossiter and Henning 2001). It is hypothesised that when ground (minced) beef is prepared, localised infection (for example in a lymph node) could be spread throughout a whole batch of ground beef. To date, there is no scientific evidence to substantiate this theoretical risk of MAP in beef. The only reported survey of 113 minced beef samples collected from a single meat processing plant in the Republic of Ireland over a 4-month period found no evidence of viable MAP (Maher et al. 2004). It should be noted, however, that details of the culture methodology adopted in this survey are not published and it is possible that a sub-optimal recovery method may have been employed for testing the beef. More extensive surveillance is needed to determine if beef is a significant potential vehicle of transmission of MAP to humans.

SURVIVAL STUDIES

HTST pasteurisation

Over the past five years there have been at least 10 pasteurisation studies published (Table 2). It is interesting to note that most researchers have moved away from laboratory studies (which have in the past been viewed as not accurately simulating commercial pasteurisation conditions), choosing instead to investigate pasteurisation of spiked or naturally infected milk in pilot- or commercial-scale plant. In recent studies, HTST pasteurisation has generally achieved a substantial $\log_{10}$ kill (4-7 $\log_{10}$) of MAP in milk. However, survival of low numbers of MAP after pasteurisation has been observed in the majority of studies involving both artificially spiked (7 of 8 studies) and naturally infected (2 of 2 studies) milk (Table 2) under a variety of time/temperature conditions.

Estimates of the number of MAP surviving HTST pasteurisation were 10-20 CFU/150 ml (Grant et al. 2005a) and 0.002-0.004 CFU/ml (McDonald et al. 2005). Factors such as the volume of milk tested, whether or not chemical decontamination was applied, and time lapse between pasteurisation and testing (immediate testing or after refrigerated storage) are now known to be important for the successful recovery of low numbers of viable MAP from pasteurised milk (Grant 2004; Grant and Rowe 2004; Gao et al. 2005).

The mechanism that enables MAP to survive pasteurisation remains to be fully elucidated. Protection of cells within clumps from the lethal effects of heat is widely discounted since heat penetration into clumps should be instantaneous. However, given that greater inactivation of MAP cells was reported when clumps were disrupted by homogenisation prior to pasteurisation (Grant et al. 2005a), clumping appears to have some influence on survival. A further hypothesis put forward to explain the presence of low numbers of MAP in pasteurised milk is heat activation of MAP cells above certain temperatures and extended holding times (Hammer et al. 2002; Herman et al. 2005).

Table 2. Overview of studies assessing the efficacy of HTST pasteurisation with respect to inactivation of MAP, 2000-2005.

Study / Country	Volume of pasteurised milk cultured	Chemical decontamination before culture	Time between pasteurisation and testing	MAP survival observed
(A) Laboratory-simulated pasteurisation of MAP spiked milk				
Gao et al. (2002) / CAN	600 µl	None	-[a]	Yes
Stabel and Lambertz (2004) / USA	50 ml	None	-	Yes
(B) Pilot/commercial-scale pasteurisation of MAP spiked milk				
Pearce et al. (2001) / NZ	50 ml	1% HPC/50 min	≤ 2 h	No
Hammer et al. (2002) / GER	50 or 100 ml	None	≤ 1 h	Yes
McDonald et al. (2005) / AUS	1500 ml	0.75% HPC/4h	-	Yes[b]
Grant et al. (2005a) / UK	150 ml	None	48 h	Yes[c]
(C) Commercial-scale pasteurisation of naturally infected milk				
Grant et al. (2002b) / UK	50 ml	0.75% HPC/5h	24 h	Yes
Ayele et al. (2005) / CZ	50 ml	0.75% HPC/5h	-	Yes

[a] Time lapse not stated in published paper but milk samples were apparently processed for culture within same working day; [b] Number of MAP surviving pasteurisation reported to be 0.002-0.004 CFU/ml (note: decontamination applied before culture); [c] Number of MAP surviving pasteurisation estimated to be 10-20 CFU/150ml (note: no decontamination applied before culture).

Homogenisation

Homogenisation is a process applied to milk to break up the milk fat globules by forcing them through a fine aperture under pressure. This prevents formation of a cream layer during distribution and storage. An additional effect of homogenisation on MAP cells was demonstrated recently (Grant et al. 2005a). Using a Mastersizer X spectrometer (Malvern Instruments Limited, Malvern, England) the cell size distribution in homogenised (2500 lb/in^2) and non-homogenised (control) MAP suspension was assessed. Homogenisation caused large MAP clumps to be disrupted into single cells or mini-clumps. However, clumps were shown to reform within a few minutes, particularly if the suspension was mixed. McDonald et al. (2005) reported a one $\log_{10}$ increase in numbers of MAP present when spiked milk was homogenised at 27,000 kg/cm^2, which provides further evidence of clump disruption by homogenisation.

Homogenisation is commonly applied to liquid milk prior to pasteurisation in many countries. Grant et al. (2005a) investigated the combined effects of homogenisation and pasteurisation on MAP inactivation. They

reported that heat treatments incorporating homogenisation (2,500 lb/in^2), applied upstream (as a separate process) or in-hold (at the start of the hold section), in conjunction with a 25 s hold time resulted in significantly fewer culture positive milk samples than pasteurisation treatments without homogenisation ($p<0.001$ for in-hold treatments and $p<0.05$ for upstream treatments). Given that homogenisation breaks up clumps, MAP cells would have been present as predominantly declumped cells when HTST pasteurisation was subsequently applied and this may explain the greater MAP inactivation achieved by the combination of homogenisation and pasteurisation. However, it should be noted that 100% inactivation of MAP is not guaranteed by combining homogenisation and pasteurisation since in that and previous studies low numbers of viable MAP have been isolated from pasteurised homogenised milk (Grant et al. 2002b; Grant et al. 2005a; McDonald et al. 2005).

Centrifugation

Centrifugation (or bactofugation) is used by the dairy industry for the pre-treatment of cheesemilk to remove clostridial spores that can otherwise cause problems during ripening. Essentially, bactofugation involves the application of centrifugal force (typically 7000 x g for a dwell time of 7s in commercial practice) to milk, which forces heavier particles (such as spores) out of suspension. Centrifugation is generally applied to milk preheated to 50-65^{o}C to aid the separation process. Clarification and separation are two other processes used by the dairy industry for liquid milk processing which also rely on centrifugal force. The application of centrifugation could have the potential to physically remove clumps of MAP from milk.
The impact of laboratory-simulated commercial bactofugation conditions on MAP in spiked milk samples was assessed recently (Grant et al. 2005b). Centrifugation at 7,000g for 10s (after pre-heating the spiked milk to 60^{o}C) resulted in 85-93% removal of MAP cells when de-clumped broth suspensions of two strains (NCTC 8578 and B4) were used to spike whole milk, and 74-79% removal when untreated (clumped) MAP suspensions were employed. The differences between numbers of clumped and de-clumped NCTC 8578 cells (0.26 $\log_{10}$), and clumped and de-clumped B4 cells (0.42 $\log_{10}$), removed approached statistical significance in both cases (paired t-test, P=0.0532 and P=0.0829, respectively), indicating that single MAP cells may be more readily sedimented during centrifugation than clumps of MAP cells, possibly as a consequence of the effects of entrapped air in intercellular spaces impacting on the buoyant density of clumps. However, as the data were acquired by laboratory methods simulating commercial centrifugation conditions, the efficacy of centrifugation for removal of MAP still needs to be verified by experiments employing appropriate pilot-scale plant and spiked or naturally infected milk.

Microfiltration

Membrane technologies were introduced into the dairy industry around 1970. Initially fouling problems hindered commercial application within the dairy industry but with the advent of ceramic membranes microfiltration of skim milk became a viable commercial process. Filtered milk is available commercially in a number of countries under the PurFiltre™ brand name. Typically for skim milk a pore size of 1.4 µm is used commercially. In theory, clumps of MAP should be efficiently removed from skim milk by microfiltration using a pore size of 1.4 µm. In a recent study simulated microfiltration using a 1.2 µm porosity filter achieved a reduction of between 1.3 and 3.4 $\log_{10}$ in numbers of MAP present in broth suspensions depending on strain, corresponding to the removal of 94.6-99.9% of cells present (Grant et al. 2005b). Although attempts were made to filter spiked skim milk, the 1.2 µm filter clogged up instantly preventing the filtration of no more than a few drops of spiked milk. In commercial microfiltration equipment this fat clogging problem would be overcome by cross-flow plant configuration and a pressure differential across the membrane, which it was not possible to simulate in the laboratory. The efficacy of microfiltration for removal of MAP from skim milk would need to be verified by experiments employing appropriate pilot-scale plant and spiked or naturally infected milk. However, it is important to note that microfiltration can only be applied to skim milk so MAP in the cream fraction would still need to be inactivated by heat treatment.

Cheesemaking

Cheese manufacture may be considered as a dehydration process in which the casein (protein) and fat of milk are concentrated, approximately tenfold. Conversion of milk into cheese involves several distinct but interrelated operations including coagulation, acidification, syneresis (separation of whey), dehydration, moulding, pressing and salting. A 10-fold concentration in MAP numbers occurs upon curd formation such that a level of 10 MAP/ml in milk ends up as 100 MAP/g of cheese (Donaghy et al. 2004). Donaghy et al. (2004) also reported that 1-4% of MAP cells present were lost in whey fraction. Survival of MAP in four varieties of laboratory-produced cheese has been studied: Queso Fresco (Sung and Collins 2000), Swiss

Tilsiter and Emmentaler (Spahr and Schafroth 2001), and Cheddar (Donaghy et al. 2004). All three studies reported that MAP counts decreased slowly but steadily over the ripening period in each of the cheese varieties, the rate of which was influenced by factors such as temperatures applied during cheesemaking, pH, water activity (a_w) and NaCl concentration. Survival D_{10} values (time required for MAP numbers to decrease by 1 $\log_{10}$) reported for MAP during ripening were: 36.5-59.9 d for Queso Fresco (Sung and Collins 2000), 45.5 d for Swiss Tilsiter (Spahr and Schafroth 2001), 27.8 d for Swiss Emmentaler (Spahr and Schafroth 2001) and 90-107 d for Cheddar (Donaghy et al. 2004).

Other potential processes for liquid milk

The impact of three processes that may have future commercial application for liquid milk processing on MAP inactivation has been investigated: gamma irradiation (Stabel et al. 2001), pulsed electric fields (Rowan et al. 2001) and high hydrostatic pressure (Sevilla et al. 2005). Stabel et al. (2001) reported that 10^6 MAP in raw milk were destroyed by 5, 10 or 15 kGy doses of gamma radiation. Rowan et al. (2001) reported that a 5.9 $\log_{10}$ kill of MAP in milk was achieved by 2500 pulses at 30 kV/cm in a 25 min period. A pressure treatment of 500 MPa for 10 min at 5 or 20^{o}C was shown by Sevilla et al. (2005) to effect a 4.8 $\log_{10}$ kill of MAP in milk. Whilst in theory all three processes could be possible alternatives to pasteurisation for milk, the impact of the processing conditions stated above on the organoleptic properties, and hence consumer acceptability, of milk would need to be assessed before commercialisation. Processing costs, relative to HTST pasteurisation, would also be an important consideration for the dairy industry.

DISCUSSION

To my knowledge, the above represents all the information on the occurrence of MAP in foods and the impact of milk processing on its survival currently in the public domain. As liquid milk has been the main focus of surveillance and research over recent years we know most about this food commodity. The results of recent studies indicate that MAP is present in raw cows' milk at detectable levels in various parts of the world and that HTST pasteurisation may not achieve 100% inactivation of MAP cells present on every processing occasion. Unfortunately, as current cultural methods do not permit the accurate enumeration of MAP in milk, we are unable to state with any confidence that if milk contains less than 'x' CFU/ml then HTST pasteurisation, or HTST pasteurisation in combination with some other process (such as homogenisation, centrifugation, microfiltration, cheesemaking), will completely inactivate this potential human pathogen. Information about the occurrence of viable MAP in other dairy products is not very comprehensive at present. Whilst the first reports of viable MAP in retail Feta cheese and powdered infant formula have recently been published, many more dairy products exist which have not been looked at, for example spray-dried milk, yoghurt, other cheeses (particularly those manufactured from raw milk) and UHT milk. Future surveillance/research efforts should, in my opinion, focus on dairy products and also on beef, rather than on liquid milk.

Clearly, if a potential food safety issue exists with a particular foodstuff the food industry needs to know about it to be able to take action to resolve the situation. It is vitally important that methods used to recover MAP from foods other than liquid milk are thoroughly evaluated prior to use to ensure maximal recovery of MAP from a different food matrix and sufficient detection sensitivity. When methods used to culture MAP from animal faeces were originally used to recover MAP from milk many false negative results were obtained because decontamination was too harsh and recovery of MAP from milk was not optimal. As decontamination methods for milk were evaluated and optimised the real picture about MAP in milk has emerged. Methods adopted in future studies of dairy products and beef must be capable of detecting MAP if present, otherwise false negative results could lull the dairy or beef industries into a false sense of security about the safety of their products.

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