Detection methods for *Mycobacterium avium* subsp. *paratuberculosis* in milk and milk products: a review

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ABSTRACT: *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) is the etiologic agent of paratuberculosis, a disease with considerable economic impact, principally on dairy cattle herds. Animals with paratuberculosis shed viable *MAP* especially in their milk, faeces and semen. *MAP* may have a role in the development of Crohn's disease in humans via the consumption of contaminated milk and milk products. The current methods of milk pasteurization are not sufficient to kill all *MAP* cells present in milk and *MAP* has been cultured from raw or pasteurized milk and isolated from cheese. The purpose of the present study was to review the different methods used for detection of *MAP* in milk and milk products. We analyze the current methods for direct or non direct identification of *MAP* and culture and molecular biology methods that can be applied to milk and milk products.

Keywords: PCR; cultivation; IS*900*; ELISA; MELISA; food safety; potential zoonosis; Johne's disease

List of abbreviations

ATP = adenosine triphosphate, **BACTEC** = fluorescence-quenching-based oxygen sensor, **BTM** = bulk tank milk, **CFU** = colony forming units, **CFT** = complement fixation test, **DNA** = deoxyribonucleic acid, **ELISA** = enzyme linked immunosorbent assay, **HEYM** = Herrold eggs yolk medium, **HPC** = hexadecyl pyridinium chloride, **HTST** = high-temperature short-time pasteurization, **IMS** = immunomagnetic separation, **IMS-PCR** = immunomagnetic separation and PCR, **IS** = insertion sequence, **LAM** = lipoarabinomannan, ***MAP*** = *Mycobacterium avium* subsp. *paratuberculosis*, **MGIT** = Mycobacteria Growth Indicator Tube (Becton Dickinson, USA), **PCR** = polymerase chain reaction, **SPC** = solid phase cytometry, **UHT** = ultra heat treated

Content

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1. Introduction

Mycobacterium avium subsp. paratuberculosis (*MAP*) is the causative agent of paratuberculosis or Johne's disease which primarily infects domestic and wild ruminants but has also been detected in other animals (e.g., wild rabbits). The digestive (intestinal) tract is considered the most common entrance route of *MAP* into the organism. Further development and multiplication of *MAP* in the intestinal mucosa depend on the natural immunity of animals and are determined by the ability of macrophages to reduce the intracellular multiplication of mycobacteria (Bendixen et al., 1981). Some animals can recover spontaneously. In other animals, *MAP* survives and multiplies in the intestinal tract mucosa. Having no clinical symptoms, these animals (often highly productive) become dangerous vectors of infection (sources of infection for other susceptible animals), shedding *MAP* into the external environment in their milk and faeces. Other animals may become carriers of *MAP* which survives in the intestinal tract. After phagocytosis by aggregated macrophages they are transferred from the mucosa of the intestinal tract (in a viable state and capable of multiplication) via lymph channels and then infiltrate regional lymph nodes. *MAP* can also occasionally penetrate other organs (Hole, 1958; Gilmour, 1976, 1985). In the later (clinical) stage of infection, owing to a thickening of the intestinal mucosa, *MAP* causes both a decrease in the absorption of nutrients and chronic diarrhoea with consequent wasting of the animals.

Pathogenesis of the disease and *MAP* shedding in milk and ejaculates has been studied most thoroughly in cattle (Ayele et al., 2004). *MAP* was detected in milk for the first time in 1935, when it was isolated from three out of four milk samples from clinically ill cows (Alexejeff-Goloff, 1935; Taylor et al., 1981). This finding was confirmed by a number of further studies (Smith, 1960; Taylor et al., 1981). The shedding of *MAP* through ejaculates has also been documented in bulls (Tunkle and Aleraj, 1965). Furthermore, *MAP* has been isolated/detected from udder tissue, supramammary lymph nodes and milk originating from cows with clinical signs of paratuberculosis. From asymptomatic cows, *MAP* has been isolated from supramammary lymph nodes, milk and from colostrum (Sweeney et al., 1992; Streeter et al., 1995). It has been suggested that *MAP*-infected macrophages are present in lipid droplets on the cream layer of milk (Koenig et al., 1993). The mechanism of the shedding of *MAP* organisms into milk has not yet been well investigated. Presumably, the shedding of *MAP* organisms into milk occurs by hematogenous or lymphatic spread.

Due to the deficiencies in current methodology (non-standardized methodology) for the isolation of *MAP* from milk, it is not possible to accurately determine the amount of *MAP* cells present in naturally infected milk. Sweeney et al. (1992) determined the level of milk contamination in asymptomatic cows as 2 to 8 CFU/50 ml. The level of contamination in clinical animals remains to be determined.

Recently, an increasing number of studies have been focused on the association of Crohn's disease with *MAP* (Hermon-Taylor and Bull, 2002). Publications dealing with the culture detection of *MAP* in milk and milk products have also been increasing in number over the last decade (Rademaker et al., 2007; Stephan et al., 2007). From Table 1 it can be seen that *MAP* has been detected by culture in milk in countries with advanced dairy breeding systems on all continents; the positivity has ranged between 0.3 and 35.0%. *MAP* has also been detected by culture and PCR methods in retail cheeses in the USA, the Czech Republic and Greece (Table 2). The culture method for *MAP* detection holds the advantage of specificity. However, the disadvan-

Table 1. Culture detection of *Mycobacterium avium* subsp. *paratuberculosis* in cows' milk

Country	Examined samples			Reference
	No.	positive	%	
Argentina	25	2	8.3	Paolicchi et al. (2003)
Australia	26	9	35.0	Taylor et al. (1981)
Czech Republic	244	4	1.6	Ayele et al. (2005)
	100	2	2.0	
Ireland	389	1	0.3	O'Reilly et al. (2004)
	357	0	0	
UK	312	15	4.8	Millar et al. (1996)
	60	4	6.7	Grant et al. (2002b)
	144	10	6.9	
	816	27	3.3	Grant et al. (2005)
USA	77	9	11.6	Sweeney et al. (1992)
	126	3	2.4	Streeter et al. (1995)
	7	2	28.6	Naser et al. (2000)
	8	0	0	Stabel (2001)
	156	0	0	Stabel et al. (2002)
	20	1	5.0	Pillai and Jayarao (2002)
	211	9	4.0	
	1 493	43	2.8	Jayarao et al. (2004)
	29	6	20.6	
	702	20	2.8	Ellingson et al. (2005)

tages of this method are the long time necessary for culture (six weeks or more) and the insufficient effectiveness of decontaminating methods (Harris and Barletta, 2001; Ayele et al., 2005). Hence, various methods for the rapid detection of *MAP* in milk and milk products are under development at present. These are in most cases molecular techniques based on the detection of *MAP* DNA.

Accordingly, the aim of the present review is to analyse the available methods of *MAP* detection (indirect and direct) with regard to their specificity and sensitivity, and the principles on which they are based.

2. Indirect *MAP* detection based on antibody detection in milk

The pathogenesis of paratuberculosis is characterized by two main stages: the first stage is the tuberculoid stage and is marked by a strong cell-mediated immune response. The second stage is the leproid stage, in which humoral immunity is important (Chiodini et al., 1984; Chiodini and Vankruiningen, 1986). Serological tests are not suitable for newly infected animals, because anti-*MAP* antibodies are not usually produced in the early stage of infection. These tests for *MAP* detection are more revealing when used to carry out a preliminary investigation of the disease prevalence in a herd and for confirmation of the diagnosis in clinically ill animals (Gumber et al., 2006).

The ELISA method was first used for detection of serum antibodies against *MAP* in cattle in the late 1980s (Jorgensen and Jensen, 1978). Larsen et al. (1963) detected high levels of antibodies in calves after the intake of colostrum from *MAP*-infected cows by means of a complement fixation test (CFT). Subsequently, the antibodies gradually disappeared. The indirect detection of antibodies in the blood serum of calves was later confirmed by the direct detec-

Table 2. *Mycobacterium avium* subsp. *paratuberculosis* detection in commercially available cheese by culture and PCR

Examined cheese					Detection method	Samples			Reference
Type	milk	country	NaCl[1] (%)	pH[2]		No.	positive	%	
Hard		Czech Republic	1.0	5.4	PCR[3]	23	4	17	Ikonomopoulos et al. (2005)
			1.0	5.4	culture		0	0	
Semi hard	bovine	Czech Republic	1.9	5.2	PCR[3]	5	1	20	
			1.9	5.2	culture		0	0	
		Switzerland	nk	5.6	PCR[4]	133	6	4	Stephan et al. (2007)
			nk	5.6	culture		0	0	
	goat and sheep	Greece	2.5	4.1	culture	42	2	5	Ikonomopoulos et al. (2005)
			2.5	4.1	PCR[3]		21	50	
Soft	bovine	Czech Republic	1.0	4.1	PCR[3]	14	0	0	
			1.0	4.1	culture		0	0	
		Switzerland	nk	nk	PCR[4]	9	0	0	Stephan et al. (2007)
			nk	nk	culture		0	0	
Cheese curds	retail	USA	nk	nk	culture	98	0	0	Clark Jr. et al. (2006)
			nk	nk	PCR[3,5]		23	23	
			nk	nk	PCR[6]		9	9	

nk = not known; [1]concentration of sodium chloride (NaCl) in the tested cheese; [2]pH value of the tested cheese
[3]IS*900* PCR; [4]*f57* Real-Time PCR; [5]single and quantitative PCR used; [6]heat shock protein X (*hspX)* gene

tion of anti-*MAP* antibodies in colostrum and milk. Comparable results were obtained in the colostrum of sheep from a *MAP*-infected herd (Muhammed and Eliasson, 1979). Richards (1990) investigated the occurrence of antibodies in cow's milk by a modified ELISA method designated as MELISA by the author. He detected levels of antibodies in serum and milk from cows with clinical manifestations of paratuberculosis comparable to those obtained by the ELISA method in the same cows. However, this finding was not confirmed by a later study which found that ELISA for antibodies to *MAP* in milk samples did not agree with ELISA results on serum samples from the same animals (Hardin and Thorne, 1996).

2.1. ELISA in individual and bulk tank milk samples

The ELISA method has been the only adopted procedure for *MAP* detection in milk with the use of specific antigens for *MAP* antibodies. Lipoarabinomannan (LAM) was the first antigen used in this method. Sweeney et al. (1994) were the first authors to use the ELISA method with the application of a home made antigen. By means of their home made method, they detected the presence of *MAP* in 15% of individual milk samples (Sweeney et al., 1994; Table 3).

A commercially available antigen and a home made procedure have been more often applied in the ELISA method for the analysis of individual milk samples. The highest sensitivity achieved by both the above mentioned approaches to *MAP* detection in individual samples of milk was 30%. However, their specificity was quite high. The literature also contains instances of the combining of a commercial antigen and a home made ELISA method for bulk tank milk samples. Moreover, in these cases, the reported sensitivity and specificity were 97% and 83%, respectively (Table 3).

It is common practice to have milk samples commercially examined and evaluated for anti-*MAP* antibodies by means of an ELISA method in specialized laboratories. It is also possible to buy a kit from a specialized laboratory and to perform the ELISA test in a normal laboratory. However, the evaluation of the obtained results may not be the same as if the analysis were performed in a specialized laboratory. A high specificity level (99%) of commercial examinations is a considerable advantage but these examinations have a very low sensitivity level (Table 3).

Table 3. The detection of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in cows milk by the ELISA method

Milk sample	Method		Examined samples			Parameters (%)		Reference
	antigen	procedure	No.	positive	%	sensitivity	specificity	
Individual	home made[1]	home	764	115	15.1	60.0	83.0	Sweeney et al. (1994)
	home made	home	26	23	88.5	28.0	91.5	Singh et al. (2007)
	commercial[2]	home	812	125	15.4	97.1	83.3	Nielsen et al. (2002)
			580	62	10.7	49.0–64.0	80.0–96.0	Klausen et al. (2003)
			602	203	33.7	97.1	83.3	Nielsen and Ersboll (2006)
	commercial examination[3]		2 122	36	1.7	40.0	99.0	Hendrick et al. (2005a)
			689	77	11.2	40.0	99.0	Hendrick et al. (2005b)
			1 921	64	3.3	40.0	99.0	Lombard et al. (2006a)
			34	28	82.4	40.0	99.0	Lombard et al. (2006b)
			1 576	124	7.9	67.0	99.7	Wells et al. (2006)
			4 901	123	2.5	21.0	99.8	Hendrick et al. (2006)
	commercial examination[4]		394	37	9.4	60.0	99.3	Salgado et al. (2007)
Bulk tank	commercial[2]	home	900	630	70.0	97.1	83.3	Nielsen et al. (2000)

[1]lipoarabinomanan antigen, LAM; [2]Allied Monitors, Fayette, Missouri, USA; [3]ELISA E, Antel Biosystems, Inc., Lansing, Michigan, USA; [4]IDEXX Laboratories, Inc., Westbrook, Maine, USA

Collins et al. (2005) analyzed the commercially available ELISA kits for *MAP* detection in milk. The tested kits showed high specificity levels in three independent laboratories (> 99%) but very low sensitivity levels (28%). Based on the results of the study the authors recommended the use of ELISA kits in support of paratuberculosis control programmes in dairy herds, only if data from ELISA kits are used judiciously and interpreted quantitatively.

2.2. Advantages and disadvantages of *MAP* antibody detection in milk

An explanation of results obtained by the ELISA method is always based on a compromise between sensitivity and specificity (Geue et al., 2007). Therefore, the evaluation of infected and non-infected herds always depends on the authors of the developed method and on its interpretation (Table 3). The problem of mutual cross-reactivity between antibodies (false positive reactions), which occurs when animals encounter atypical mycobacteria, remains to be solved. The commercially available examination of milk samples is one of the possible ways of standardizing this method, as specialized laboratories guarantee the level of sensitivity and specificity. Considerable advantages of the ELISA method are its high trough-put capability, its relatively low price and rapidity. A major disadvantage is the fact that although animals may shed *MAP* in milk, it does not necessarily mean that they will produce antibodies.

3. Direct detection of infectious agents of paratuberculosis

3.1. Visual detection of *MAP*

Direct visual detection of *MAP* by means of optical microscopy is theoretically possible after Ziehl-Neelsen staining of milk samples. However, no references concerning the use of direct optical microscopy for detection of *MAP* in milk are available in the literature.

3.1.1. Fluorescence microscopy

The use of fluorescence microscopy for the detection of *MAP* in milk is also very uncommon. Only the application of solid phase cytometry (SPC) can

be found in the literature. SPC is a recently developed laser scanning technique for the quantitative detection of fluorescently labelled bacteria on a membrane filter that eliminates the need for a growth phase. The resulting fluorescent cells are automatically detected and the results can be visualized using an epifluorescence microscope. SPC has mainly been used for the microbiological control of pharmaceutical waters and the detection of specific microorganisms in drinking water. D'Haese et al. (2005) investigated SCP as a rapid technique of detecting viable *MAP* cells in 50 ml of artificially contaminated pasteurized milk. To make SPC effective at detecting low amounts of *MAP* in unfilterable milk, a sample pre-treatment should focus on the removal of fat and proteins and the elimination of background flora. The authors reported a 73% recovery rate for this *MAP* detection method in milk (when 50 ml milk were spiked with 10^2 *MAP* cells).

3.1.2. Bioluminiscence

Bioluminiscence is generated by an enzyme-catalyzed chemoluminescence reaction, wherein the pigment luciferin is oxidised by the enzyme luciferase. The chemical reaction can occur either within or outside of the cell. The use of bioluminiscence for the detection of *MAP* in a variety of sources (water, milk, blood etc.) and organisms such as *M. leprae*, *M. tuberculosis* (Gupta et al., 1997; Martin-Casabona et al., 1997), *Saccharomyces cerevisiae*, *Citrobacter rodentium* and *Escherichia coli* (Jawhara and Mordon, 2004; Cho and Yoon, 2007) using firefly luciferase has been described in the available literature. Recently, studies dealing with the use of bioluminiscence in association with *MAP* have been published (Williams et al., 1999; Rosseels et al., 2006).

One of the detection strategies based on bioluminiscence is the luciferase system. Many luciferase reporter systems have been developed and are successfully used for specific detection (*M. tuberculosis).* The application of the luciferase system for *MAP* detection in milk is very rare and non-conventional. Nevertheless, Sasahara et al. (2004) used a bioluminescent construct (plasmid- and phage-based firefly luciferase constructs; gene *FFlux*) for the detection of viable *MAP* cells from milk artificially contaminated with *MAP* containing the bioluminescent construct. For the artificial milk contaminations, different isolates infected by plasmids and phages were prepared. The advantage of the use of *FFlux* as a reporter gene is that luciferase requires ATP in order to produce bioluminescence, and therefore only viable bacteria (who can produce ATP), including injured cells, will be detected. The lowest *MAP* population that could produce detectable luminescence was determined to be 10^2 cells/ml in skimmed milk and 10^3 cells/ml in whole milk. The detection of *MAP* using the luciferase system is much more rapid than cultivation and has comparable sensitivity to PCR methods.

3.2. *MAP* detection by culture examination

The culture detection of *MAP* in any matrix is considered the gold standard (reference method) for *MAP* detection. Despite widespread use, cultivation techniques are not standardized and the ability of different laboratories to cultivate varies considerably (Tables 4 and 5). A conventional culture of *MAP* requires special media enriched with Mycobactin J (an iron-chelating factor). Incubation is carried out at 37°C and colonies are in some cases visible after four weeks, but more commonly after 10 to 16 weeks. It is also possible to use a liquid medium to detect growing *MAP*. The radiometric system is known as BACTEC and the fluorescent one is known as MGIT (Becton Dickinson UK Ltd., Cowley, Oxford, United Kingdom).

MAP is usually detected in milk by culture. However, it has gradually been demonstrated that the sensitivity of this method largely depends on the way that an inoculum is prepared for application onto culture medium and the avoidance of contamination of the medium. Due to the fact that milk contains a number of other bacteria and milk elements, which can spoil samples if they are stored inadequately, an effort has been made to improve this method, or even to use this method concurrently with other methods of *MAP* detection.

Strategies traditionally employed for the detection of other mycobacteria, especially *M. tuberculosis,* have increasingly been adopted for *MAP* detection in recent years. The first adopted detection method was BACTEC. Subsequently, the FASTPlaqueTB assay (Biotec Laboratories Limited, Ipswich, United Kingdom) based on the exclusive detection of viable cells by means of plaque formation on culture medium within 24 h was modified for use on *MAP*.

Table 4. Culture methods used for the isolation of *Mycobacterium avium* subsp. *paratuberculosis* from fresh raw cows' milk

Milk sample	Centrifugation conditions			Decontamination			Media		Antibiotics	Reference
	volume (ml)	time (min)	speed (× 1 000 g)	chemicals HPC (%)	chemicals others	time (h)	HEYM	others		
Quarter	50	30	0.90	0.75		4.0	+		nk	Sweeney et al. (1992)
	50	30	1.95	0.75		own	+		without	Pillai and Jayarao (2002)
	50	30	1.95	0.75		own	+		without	Jayarao et al. (2004)
	50–100	nk	nk		5.00% OA, NaOH	nk	+		without	Taylor et al. (1981)
	10–12	30	4.00 rpm	0.90		own	+		without	Singh et al. (2007)
Tank	40	15	2.50	0.75		5.0	+		without	Dundee et al. (2001)
	40	15	2.50	0.75		own	+		VAN	
	40	15	2.50	0.90		own	+		VAN	
	40	15	2.50		1.25N NaOH	0.5	+		without	
	10	30	2.00 rpm	0.90		own	+		VN	Stabel et al. (2002)
	50	15	2.50	0.75		5.0	+		without	Grant et al. (2002a)
	50	15	2.50	0.75		5.0	+	BACTEC[1]	PANTA	
	50	30	1.95	0.75		own	+		without	Pillai and Jayarao (2002)
	40	30	1.95	0.75		own	+		without	Jayarao et al. (2004)
	50	15	2.50	0.75		5.0	+		without	O'Reilly et al. (2004)
	50	15	2.50	0.75		5.0	+		without	
	50	15	2.50	0.75		5.0	+	BACTEC[1]	PANTA	
	50	30	3.10	0.75		2.0–5.0	+		VAN,BHI	Gao et al. (2005)
	50	30	3.10	0.75		2.0–5.0	–	LB	VAN,BHI	
	50	15	4.00	without	without	without	+	BACTEC[1]	PANTA (+)	Grant et al. (2005)
	50	15	4.00	without	without	without	+[2]		VAN	
nk	250	30	1.95	0.75		own	+		nk	Streeter et al. (1995)
	5	nk	nk	nk	nk	nk	+		nk	Stabel et al. (1997)
	1–50	15	2.50	0.75		4.0	+		nk	Grant et al. (1998)
	18	10	10.00	without	without	without		Db+calf serum	VAN	Giese and Ahrens (2000)
	150	30	2.40	0.75		own	+[3]		VANN	Paolicchi et al. (2003)
	nk	30	1.20	0.90		own	+		VNF	Stabel et al. (2004)
	50	15	2.50	0.75		5.0	+		without	Ayele et al. (2005)

HPC = hexadecyl pyridinium chloride; HEYM = Herrold's egg yolk medium; OA = oxalic acid; nk = not known; own = over night; Db = Dubos broth; LB = Luria Bernati medium; PANTA = polymyxin B, amphoterin B, nalidixic acid, trimethoprim, azocillin; PANTA (+) = antibiotic supplement (Becton Dickinson UK Ltd., Cowley, Oxford, United Kingdom); VAN = vancomycin, amphoterin B, nalidixic acid; VNF = vancomycin, nalidixic acid, fungizone; VANN = vancomycin, amphoterin B nalidixic acid, nistatin; BHI = brain heart infusion; rpm = revolutions per minute

[1]Becton Dickinson UK Ltd., Cowley, Oxford, United Kingdom; [2]with 0.41% sodium pyruvate; [3]with no preciously described pyruvate

Table 5. Culture methods used for the isolation of *Mycobacterium avium* subsp. *paratuberculosis* from pasteurised and/or retailed cows' milk

Milk sample	Centrifugation conditions			Decontamination		Media		Antibiotics	Reference
	volume (ml)	time (min)	speed (× 1 000 g)	HPC (%)	Time (h)	HEYM	others		
Pasteurised	50	10	7.00	1.00	without	+		without	Pearce et al. (2001)
	50	10	7.00	1.00	without	+	BACTEC[1]	PANTA	
	50	15	2.50	0.75	5.0	+		without	Grant et al. (2002b)
	50	15	2.50	0.75	5.0	+	BACTEC[1]	PANTA	
	5	15	2.50	0.75	5.0	+	BACTEC[1]	PANTA	Grant and Rowe (2004)
	50	30	1.14	without	without		Db + calf serum	PACT	Stabel and Lambertz (2004)
	50	30	1.17[3]	without	without	+	BACTEC[1]	PANTA	
	50	30	1.17[3]	without	without	+		without	
	50	15	2.50	0.75	5.0	+		without	O'Reilly et al. (2004)
	50	15	2.50	0.75	5.0	–	BACTEC[1]	PANTA	
	50	20	1.50	0.75	4.0	+	BACTEC[1]	without	McDonald et al. (2005)
	50	20	1.50	0.75	4.0	+	BACTEC[1]	PANTA (+)	
	50	20	1.50	0.75	own	+		VAN	
	50	15	4.00	without	without	+	BACTEC[1]	PANTA (+)	Grant et al. (2005)
	50	15	4.00	without	without	+		VAN	
Retail	15	60	41.00	0.75[2]	own	–	Db	nk	Millar et al. (1996)
	8	15	2.50	0.75	5.0	+	BACTEC[1]	PANTA	Grant et al. (2003)
	8	15	2.50	0.75	5.0	+	MGIT[1]	PANTA	
	40	30	14.00	without	without	–	Db	PACT	Ellingson et al. (2005)
	50	15	2.50	0.75	5.0	+		without	Ayele et al. (2005)
Waste	25	30	2.00	0.90	own	+		VNF	Stabel (2001)

HPC = hexadecyl hyridinium hhloride; HEYM = Herrold's egg yolk medium (+ used, – not used); own = over night; nk = not known; Db = Dubos broth; PANTA = polymyxin B, amphoterin B, nalidixic acid, trimethoprim, azocillin; PANTA (+) = antibiotic supplement (Becton Dickinson UK Ltd., Cowley, Oxford, United Kingdom); VAN = vancomycin, amphoterin B, nalidixic acid; VNF = vancomycin, nalidixic acid, fungizone; PACT = polymyxin-B, carbenicillin, trimethoprim lactate, amphotericin B

[1]Becton Dickinson UK Ltd., Cowley, Oxford, United Kingdom

[2]HPC or 0.10% benzalkonikum chloride

[3]exactly 1 172 g

However, this method requires additional confirmation of *MAP* by means of specific PCR (Stanley et al., 2007). The reported sensitivity of the method is lower by 1 to 2 $\log_{10}$ than the sensitivity of culture on HEYM (Altic et al., 2007).

3.2.1. Volume and centrifugation conditions of analyzed samples

If the method of *MAP* detection in milk is to be standardized for routine purposes, it is necessary

to establish the sample volume for analysis which can vary between 1 l and several litres. Centrifugation is used for the processing and concentrating of samples for analysis. After the centrifugation of milk, up to 69.4% of *MAP* cells can be found in the pellet (Grant et al., 1998). The conditions of centrifugation of fresh cow's milk (volume, time and relative centrifugal force) have varied across different publications. The volume of centrifuged milk usually ranges between 40 and 50 l except for some publications, which report lower (1 to 18 ml) or higher (100 to 250 ml) volumes. The centrifugation time is usually between 15 and 30 min although some have reported times of 10, 20 or even 60 min. The rate of centrifugation specified by the relative centrifugal force has usually been lower than 9 000 g; 14 000 and 41 000 g have on rare occasion been used, however (Tables 4 and 5).

3.2.2. Decontamination of milk samples

This step is necessary when raw milk is used for the isolation of *MAP* but theoretically is not required when pasteurized milk is used, as the possibility that other microorganisms will grow in pasteurized milk products is very low. Methods to selectively kill non-mycobacterial flora in milk (of course in all samples) and decontamination are critical factors to ensure a high sensitivity of *MAP* detection. Raw milk usually contains a high level of contaminants, and the selection of the chemical decontamination step is an important consideration for the successful recovery of *MAP* (Dundee et al., 2001).

Hexadecylpyridinium chloride (HPC) is the most widely used and recognised decontaminating substance as it is the least harmful to *MAP*, and most efficient at killing other microorganisms. The method of decontamination is the same for fresh and pasteurized milk. Decontamination usually takes 4 to 5 h, or is performed overnight. A review of 25 publications reveals the use of the following chemical substances for milk decontamination: NaOH and HPC in two (8%) and 20 publications (80%), respectively; milk samples were not decontaminated in three (12%) publications (Tables 4 and 5).

3.2.3. Culture media and antibacterial and antimycotic compounds

HEYM is currently the most popular medium used for culture. In a few cases, the culture of milk was concurrently performed in the BACTEC system with the addition of various combinations and concentrations of antibacterial and antimycotic compounds. A mixture of antibiotic supplement substances named PANTA (+) is most often used for the culture of pasteurized retail milk. It is a commercially available combination of polymyxin B, amphoterin B, nalidixic acid, trimethoprim and azocillin. Antibiotic substances are usually not used for the culture of fresh milk. Nevertheless, VAN (vancomycin, amphoterin B, nalidixic acid; Tables 4 and 5) is used in some cases.

3.2.4. Culture examination on solid and in liquid media

The detection of *MAP* by means of culture on solid and in liquid media was first reported in 2001. Liquid media are used to accelerate *MAP* detection. The potential growth of viable *MAP* cells is first detected and then the culture of resuscitated cells is inoculated onto solid agar where the typical morphology of *MAP* colonies and their typical slow growth are confirmed (Grant and Rowe, 2004). In other studies, both types of culture media were used for the detection of *MAP* and the obtained results were compared (Tables 6 and 7).

Grant et al. (2003) compared the detection capabilities of the non-radiometric MGIT System and radiometric BACTEC Culture System with UHT milk samples spiked with different levels of *MAP* (from 10^1 to 10^7 cells/ml) which were inoculated into MGIT and BACTEC media with or without chemical decontamination before culture. The MGIT system was found to be very effective and could be used as a replacement for the radiometric system to detect *MAP* in pasteurized milk. The chemical decontamination caused a significant reduction in numbers of viable *MAP* in all spiked milk samples resulting in a decreased capability to culture. For obtaining *MAP* from heat-treated samples, liquid culture media are more suitable than agar media on which chemical decontamination cannot be applied (Grant et al., 2005; Ruzante et al., 2006).

3.3. *MAP* detection based on molecular techniques

The use of molecular biological methods for the detection of *MAP* in milk and other matrices was

Table 6. Culture methods used for the isolation of *Mycobacterium avium* subsp. *paratuberculosis* from raw cows's milk

Source of milk	Centrifugation conditions			Decontamination		Media	Antibiotics	Reference
	volume (ml)	time (min)	speed (× 1 000 g)	chemicals (%)	time (h)			
nk	50-100	nk	nk	5.00 OA, NaOH	nk	HEYM	without	Taylor et al. (1981)
	250	30	2.0[1]	0.75 HPC	own	HEYM + 0.41% sodium pyruvate	nk	Streeter et al. (1995)
	5	nk	nk	nk	nk	HEYM	nk	Stabel et al. (1997)
	1–50	15	2.5	0.75 HPC	4.0	HEYM	nk	Grant et al. (1998)
	18	10	10.0	no	without	Db + calf serum	VAN	Giesse and Ahrens (2000)
	150	30	2.4	0.75 HPC	own	HEYM + pyruvate	VANN	Paolicchi et al. (2003)
	nk	30	1.2	0.90 HPC	own	HEYM	VNF	Stabel et al. (2004)
	50	15	2.5	0.75 HPC	5.0	HEYM	without	Ayele et al. (2005)
Tank	40	15	2.5	0.75 HPC	5.0	HEYM	without	Dundee et al. (2001)
	40	15	2.5	0.75 HPC	own	HEYM	VAN	
	40	15	2.5	0.90 HPC	own	HEYM	VAN	
	40	15	2.5	1.25 N NaOH	0.5	HEYM	without	
	10	30	2.0 rpm	0.90 HPC	own	HEYM	VN	Stabel et al. (2002)
	50	15	2.5	0.75 HPC	5.0	HEYM	without	Grant et al. (2002a)
	50	15	2.5	0.75 HPC	5.0	HEYM – BACTEC[3]	PANTA	
	50	30	2.0[1]	0.75 HPC	own	HEYM	without	Pillai and Jayarao (2002)
	40	30	2.0[1]	0.75 HPC	own	HEYM	without	Jayarao et al. (2004)
	50	15	2.5	0.75 HPC	5.0	HEYM	without	O'Reilly et al. (2004)
	50	15	2.5	0.75 HPC	5.0	HEYM	without	
	50	15	2.5	0.75 HPC	5.0	BACTEC[3]	PANTA	
	50	30	3.1	0.75 HPC	2.0–5.0	HEYM	VAN,BHI	Gao et al. (2005)
	50	30	3.1	0.75 HPC	2.0–5.0	LB	VAN,BHI	
	50	15	4.0	without	without	HEYM – BACTEC[3]	PANTA (+)	Grant et al. (2005)
	50	15	4.0	without	without	HEYM	VAN	
Quarter[2]	50	30	0.9	0.75 HPC	4.0	HEYM	nk	Sweeney et al. (1992)
	50	30	2.0[1]	0.75 HPC	own	HEYM	without	Pillai and Jayarao (2002)
	50	30	2.0[1]	0.75 HPC	own	HEYM	without	Jayarao et al. (2004)

nk = not known; OA = oxalic acid; own = over night; Db = Dubos broth; HEYM = Herrold's egg yolk medium; HPC = hexadecyl pyridinium chloride; PANTA = polymyxin B, amphoterin B, nalidixic acid, trimethoprim, azocillin; PANTA + = antibiotic supplement (Becton Dickinson UK Ltd., Cowley, Oxford, United Kingdom); VAN = vancomycin, amphoterin B, nalidixic acid; VNF = vancomycin, nalidixic acid, fungizone; VN = vancomycin, nalidixic acid; VANN = vancomycin, amphoterin B, nalidixic acid, nistatin; BHI = brain heart infusion; LB = Luria Bernati medium; rpm = revolutions per minute

[1]exactly 1 950 g

[2]milk originated from four quarters

[3]Becton Dickinson UK Ltd., Cowley, Oxford, United Kingdom

Table 7. Culture methods used for the isolation of *Mycobacterium avium* subsp. *paratuberculosis* from pasteurised cows' and humans' milk

Source of milk	Centrifugation conditions			Decontamination		Media	Antibiotics	Reference
	volume (ml)	time (min)	speed (× 1 000 g)	chemicals (%)	time (h)			
Pasteurised	50	10	7.0	1.00 HPC	without	HEYM	without	Pearce et al. (2001)
	50	10	7.0	1.00 HPC	without	HEYM – BACTEC[3]	PANTA	
	50	15	2.5	0.75 HPC	5.0	HEYM	without	Grant et al. (2002b)
	50	15	2.5	0.75 HPC	5.0	HEYM – BACTEC[3]	PANTA	
	5	15	2.5	0.75 HPC	5.0	HEYM – BACTEC[3]	PANTA	Grant and Rowe (2004)
	50	30	1.1	without	without	Db + calf serum	PACT	Stabel and Lambertz (2004)
	50	30	1.2[1]	without	without	HEYM – BACTEC[3]	PANTA	
	50	30	1.2[1]	without	without	HEYM	without	
	50	15	2.5	0.75 HPC	5.0	HEYM	without	O'Reilly et al. (2004)
	50	15	2.5	0.75 HPC	5.0	BACTEC	PANTA	
	50	20	1.5	0.75 HPC	4.0	HEYM – BACTEC[3]	without	McDonald et al. (2005)
	50	20	1.5	0.75 HPC	4.0	HEYM – BACTEC[3]	PANTA +	
	50	20	1.5	0.75 HPC	own	HEYJ	VAN	
	50	15	4.0	without	without	HEYM – BACTEC[3]	PANTA +	Grant et al. (2005)
	50	15	4.0	without	without	HEYM	VAN	
Retail	15	60	41.0	0.10 BCh or 0.75 HPC	own	Db	nk	Millar et al. (1996)
	8	15	2.5	0.75 HPC	5.0	HEYM – BACTEC[3]	PANTA	Grant et al. (2003)
	8	15	2.5	0.75 HPC	5.0	HEYM – MGIT[3]	PANTA	
	40	30	14.0	without	without	Db	PACT	Ellingson et al. (2005)
	50	15	2.5	0.75 HPC	5.0	HEYM	without	Ayele et al. (2005)
Waste	25	30	2.0	0.90 HPC	own	HEYM	VNF	Stabel (2001)
Breast[2]	nk	nk	nk	without	nk	12B* + OADC	PANTA	Naser et al. (2000)

HPC = hexadecyl pyridinium chloride; HEYM = Herrold's egg yolk medium; OA = oxalic acid; BCh = benzalkonikum chloride; own = overnight; Db = Dubos broth; PANTA = polymyxin B, amphoterin B, nalidixic acid, trimethoprim, azocillin; PANTA (+) = antibiotic supplement (Becton Dickinson UK Ltd., Cowley, Oxford, United Kingdom); VAN = vancomycin, amphoterin B, nalidixic acid; VNF = vancomycin, nalidixic acid, fungizone; VANN = vancomycin, amphoterin B, nalidixic acid, nistatin; PACT = polymyxin-B, carbenicillin, trimethoprim lactate, amphotericin B; OADC = enrichment (DIFCO, Livonia, Michigan, USA)

[1]exactly 1 172 g; [2]milk originated from a human; [3]Becton Dickinson UK Ltd., Cowley, Oxford, United Kingdom

made possible by the discovery of specific DNA sequences, particularly IS*900* (Green et al., 1989) and *f57* (Poupart et al., 1993). The sequencing of the entire *MAP* genome (strain K-10) also significantly contributed to the precise detection and identification of *MAP* specific sequences (Li et al., 2005). With regard to the fact that various modifications of the PCR method are usually employed for *MAP* detection in milk, all possible "critical" steps will be analysed in detail in the following section.

Table 8. Direct PCR detection of *Mycobacterium avium* subsp. *paratuberculosis* in cows' milk by centrifugation

Milk source	Samples			PCR		Sensitivity or DL (CFU/ml)	Centrifugation			Reference
	No.	positive	%	type	target		volume (ml)	speed (×.1 000 g)	time (min)	
Quarter	11	2	18.0	single	IS*900*	10^3	18.0	20.0	5	Giese and Ahrens (2000)
	1 493	201	13.4	single	IS*900*	0.21[a]	50.0	2.0[d]	30	Jayarao et al. (2004)
	211	69	33.0	single	IS*900*	10^1–10^2	50.0	2.0[d]	30	Pillai and Jayarao (2002)
	26	6	23.0	single	IS*900*	nk	15.0-20.0	3.0 rpm	45	Singh et al. (2007)
Raw	24	0	0	single	IS*900*	nk	1.0	3.0	15	Paolicchi et al. (2003)
	16	2	13.0	Q	*f57*	10^1	10.0	5.0[e]	30	Tasara and Stephan (2005)
Udder[b]	84	3	3.6	Q	*f57*	10^2	10.0	5.0[e]	30	Bosshard et al. (2006)
	9	7	77.8	MS[c]	IS*Mav2*	10^2	1.0	MS		Stratmann et al. (2002)
Bulk tank	29	9	27.5	single	IS*900*	0.87[1]	50.0	2.0[d]	30	Jayarao et al. (2004)
	1 384	273	20.0	nested	IS*900*	nk	20.0	4.5	30	Corti and Stephan (2002)
	20	10	50.0	single	IS*900*	10^1–10^2	50.0	2.0[d]	30	Pillai and Jayarao (2002)
	52	35	68.0	nested	IS*900*	10^2	0.5	150 rpm	5	Stabel et al. (2002)
	2	2	100.0	Q	*f57*	10^1	10.0	5.0[e]	30	Tasara and Stephan (2005)
	100	3	3.0	Q	*f57*	10^2	10.0	5.0[e]	30	Bosshard et al. (2006)
	7	5	71.4	MS[c]	IS*Mav2*	10^2	1.0	MS		Stratmann et al. (2002)
	423	23	5.5	MS[c]	IS*Mav2*	5×10^2	1.0	MS		Stratmann et al. (2006)
	328	13	3.9	nested	IS*900*	nk	30.0	1.0	15	Pinedo et al. (2008)
Retail	312	22	7.0	single	IS*900*	2×10^2 –3×10^2	20.0	41.0	60	Millar et al. (1996)
	710	110	15.5	nested	IS*900*	DL 10^2	1.0	18.0	90	Gao et al. (2002)
Dried	51	25	49.0	single	IS*900*	nk	20.0 mg	nk	nk	Hruska et al. (2005)
	51	18	35.0	Q	*f57*	nk	20.0 mg	nk	nk	

DL = detection limit; Q = quantitative Real-Time PCR; MS = magnetic separation; nk = not known; CFU = colony forming unit; S*900* = insertion sequence 900 specific to *Mycobacterium avium* subsp. *paratuberculosis* (Green et al., 1989); *f57* = fragment 57 specific to *Mycobacterium avium* subsp. *paratuberculosis* (Poupart et al., 1993); IS*Mav2* = insertion sequence specific to *Mycobacterium avium* subsp. *paratuberculosis* (Strommenger et al., 2001)

[a]KELA test; [b]milk from cows from the slaughterhouse; [c]peptide-mediated capture; [d]exactly 1 950 g; [e]exactly 4 500 revolutions per minute (rpm)

3.3.1. *MAP* concentration by centrifugation

The isolation of DNA by means of PCR is a critical step in its detection in any matrix. Accordingly, it is necessary to pay due attention to this step. The procedures used for the centrifugation of samples with the aim of concentrating *MAP* cells present in milk before PCR examination is performed are the same as those procedures carried out before culture examination (Chapter 3.2.1.; Tables 8 and 9).

3.3.2. Isolation of DNA from *MAP*

Commercially available kits, developed for DNA isolation from different matrices, are commonly used for this purpose. There are three commercial kits for the rapid isolation and detection of *MAP* in milk:

(1) Aureon Biosystems (ParaStatus^{TM} isolation kit for milk, Vienna, Austria) – at present for research use only.

(2) Labor Diagnostik Leipzig (Bactotype Detection Kit *MAP* milk test, Leipzig, Germany) – at present for research use only.

(3) ADIAPURE (Adiagene, Saint Brieuc, France) is specialized for mycobacterial DNA extraction from milk and is marketed for diagnostic use.

Other kits are also available on the market. However, these can only be used for the PCR reaction itself. The manufacturer's instructions only state that high quality DNA is required for the test and give no details concerning the principle of DNA isolation.

3.3.2.1. Immunomagnetic separation of *MAP*

In addition to centrifugation (Tables 8 and 9), the isolation of DNA (and *MAP* cells) from samples may be achieved through immunomagnetic separation (IMS; Table 10).

The immunomagnetic separation (IMS) technique is useful for the specific isolation of mycobacteria from foodstuffs, clinical samples or excrement. The big advantage of this method is its ability to strongly concentrate mycobacteria in samples with low bacterial cell counts. This method involves a reversible interaction between target cells and antibodies settled on magnetic particles. These complexes (cell-antibody-particle) are easy to separate from the suspension by the application of a strong magnetic field (Grant et al., 1998).

The IMS procedure has proved to be very effective in separating a desired organism from a heterogeneous suspension of microorganisms, such as are found in milk samples and from substances in milk which could inhibit the PCR reaction. Additionally, it also concurrently concentrates bacteria from larger sample volumes (Grant et al., 1998; Metzger-Boddien et al., 2006).

An IMS protocol was developed to isolate *MAP* cells from milk by Grant et al. (1998) using commercial Dynabeads coated with polyclonal rabbit anti-*MAP* Imunoglobin G antibodies. The potential value of this method is the rapid detection of *MAP* in milk when used in conjunction with an "end point" detection method. Table 10 shows that 90% of strategies that aim to detect *MAP* after IMS are PCR assays, and that they all target the IS*900* specific element. This method is relatively new in comparison with culture and PCR techniques. The

Table 9. PCR detection of *Mycobacterium avium* subsp. *paratuberculosis* in artificially contaminated cows' milk

Type of milk	*MAP* (CFU/ml)	Samples			PCR		Sensitivity or DL (CFU/ml)	Centrifugation			Reference
		No.	positive	%	type	target		volume (ml)	speed (× 1 000 g)	time (min)	
Raw	5×10^1–2×10^3	6	4	66.7	single	IS*900*	2×10^2–3×10^2	20	41	60	Millar et al. (1996)
	10^6–10	nk	nk	nk	single	IS*900*	10^1 CFU/2 ml	10	8	10	Khare et al. (2004)
	10^6–10	nk	nk	nk	Q	IS*900*	10^1 CFU/2 ml	10	8	10	
Bulk tank	10^0–10^8	24	10	50.0	single	IS*900*	10–10^2	50	2[c]	30	Pillai and Jayarao (2002)
	3×10^1	nk	nk	nk	single	IS*900*	1	50	3	30	Gao et al. (2007)
Pasteurized	10^1–10^5	nk	nk	nk	single	IS*900*	10–10^2	1	42	90	Odumeru et al. (2001)
	10^7–10^5	11	2	18.2	nested	IS*900*	DL 10^2	1	18	90	Gao et al. (2002)
	1×10^6–5×10^1	1	1	1.0	Q	IS*900*	4×10^1	25	6 rpm	5	O´Mahony and Hill (2004)
Skimmed[a]	10^6–10^0	1	1	1.0	Q[b]	*dnaA*	10^4	20	2	30	Rodriguez-Lazaro et al. (2004)
	10^6–10^0	1	1	1.0	Q	IS*900*	10^2	20	2	30	Rodriguez-Lazaro et al. (2005)

DL = detection limit; Q = quantitative Real-Time PCR; nk = not know; rpm = revolutions per minute; *MAP* = *Mycobacterium avium* subspecies *paratuberculosis*; CFU = colony forming unit

[a]semi-skimmed

[b]nucleic acid sequence-based amplification (NASBA)

Table 10. *Mycobacterium avium* subsp. *paratuberculosis* detection in cows' and goat milk by the PCR method after immunomagnetic separation

Examined milk		Examined samples			Methods		Detection limit	Reference
Animal	Type	No.	positive	%	isolation	detection		
Cow	raw[1]	244	19	7.8	nk	IS*900* PCR	10^1 CFU/50 ml	Grant et al. (2002b)
		389	50	12.9	nk	IS*900* PCR	10^0 CFU/50 ml	O'Reilly et al. (2004)
		423	23	5.5	home made	PMC, MS (IS*Mav2*)	5×10^2 CFU/ml	Stratmann et al. (2006)
	raw[2]	60	11	6.7	Dynabeads	IS*900* PCR	nk	Grant et al. (2002a)
		243	25	10.3	home made	Q IS*900* PCR	5–10^1 CFU/ml	Metzger-Boddien et al. (2006)
	pasteurised[1]	567	67	11.8	nk	IS*900* PCR	10^1 CFU/50 ml	Grant et al. (2002b)
		357	35	9.8	nk	IS*900* PCR	10^0 CFU/50 ml	O'Reilly et al. (2004)
	pasteurised[2]	144	29	20.8	Dynabeads[3]	IS*900* PCR	nk	Grant et al. (2002a)
Goat	raw[2]	340	5	1.5	Dynabeads[3]	IS*900* PCR	10^0 CFU/ml	Djonne et al. (2003)
		340	24	7.1	Dynabeads[3]	IS*900* PCR	10^{-1} CFU/ml[4]	

CFU = colony forming unit; PMC = peptide mediated capture; MS = magnetic separation; IS = insertion sequence; nk = not known; Q = quantitative Real-Time PCR

[1]bulk milk

[2]individual milk samples

[3]Dynal UK Ltd., Wirral, Merseyside, United Kingdom

[4]after the dot blot hybridisation

particles which are used for magnetic separation are commercially available. Home made particles were used in two cases only.

Mutual cross-reactivity between antibodies can cause problems in IMS. The production of polyclonal antibodies may also be an issue (unpublished data). On the other hand, the fact that *MAP* cells are not exposed to potentially damaging chemicals, as they are during traditional decontamination procedures, resulting in no effect on the physiological state of the cells, may be an advantage of IMS. Another indisputable advantage of the method is the fact that it incorporates dual protection of specificity which is guaranteed by the bound *MAP*-specific antibody and the use of *MAP*-specific primers (Grant et al., 2000; Metzger-Boddien et al., 2006).

3.3.3. PCR

PCR is a rapid and sensitive method for the detection of *MAP* in milk and other types of samples. At this time a number of different variants of the PCR assay have been described for the detection *MAP* in milk. Almost all PCR protocols target the IS*900* insertion sequence, which has been accepted as a standard marker for *MAP*. However, analysis of the genomes of other environmental *Mycobacterium* species has revealed sequences that are highly homologous to *MAP* IS*900* (Englund et al., 2002). Therefore, other *MAP*-specific genetic elements have to be evaluated to improve the reliability of PCR detection of *MAP*. To date alternative target elements such as single copy *f57* and *HspX* elements and the IS*Mav2* element present in three copies in the *MAP* genome have been found (Poupart et al., 1993; Ellingson et al., 1998; Strommenger et al., 2001). The detection of these elements may not provide for as high a sensitivity as the multicopy IS*900* element, but they are less prone to generating false-positive results and are much more accurate for purposes of quantification.

From Table 8 it can be seen that considerable variations exist among the used PCR methods and that the same is true for culture examination. The analyzed volume of milk samples, conditions of centrifugation, the number of examined samples, and also the sensitivity and detection limit of the used PCR method differ from method to method.

A disadvantage of the PCR methods currently used for the detection of *MAP* in milk is the fact that they cannot distinguish between viable and nonviable bacteria in the analyzed samples.

3.3.3.1. Single PCR

Single PCR exclusively targets the IS*900* element. For the assessment of the sensitivity of a developed technique, the artificial contamination of milk with a known amount of *MAP* cells detected by culture ("gold standard" of *MAP* detection) is carried out. After the obtaining of an acceptably high sensitivity, the developed PCR methods are applied (including the method of isolation) for the examination of real milk samples. It must be borne in mind that the analyzed volume of milk varies considerably (from 1 ml to 50 ml). Accordingly, the correct explanation of the obtained results, including the dilution effect in bulk tank milk (BTM) or market milk, remains a challenge (Tables 8 and 9).

3.3.3.2. Nested PCR

The specific DNA sequence IS*900* is also used as the target for *MAP* detection in this modification of PCR. In 2002, IS*900*-nested PCR was used to determine the specificity and sensitivity of a commercial ELISA test (Stabel et al., 2002). This type of PCR was also used to establish *MAP* prevalence in BTM in Switzerland (Corti and Stephan, 2002). However, the nested PCR method is now being replaced by Real-Time PCR.

3.3.3.3 Real-Time PCR

The Real-Time PCR method is a novel modification of single PCR. It differs from single PCR by its use of fluorescently labelled probes. This PCR assay monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (i.e., in Real-Time as opposed to endpoint detection). The signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescent emission at each cycle, it is possible to monitor the PCR reaction during the exponential phase when the first significant increase in the amount of PCR product correlates to the initial amount of target template.

This method has been used for *MAP* detection in milk since 2002 (Khare et al., 2004; O'Mahony and Hill, 2002, 2004). The "classic" detection target IS*900* is also used as a template for Real-Time PCR, but it is evident that this detection target is not suitable for accurate quantification. On the other hand, this detection target is particularly suited to and sensitive for the detection of very low numbers of *MAP* cells; this is possible because of the multiple copies of the gene present in the *MAP* genome. For the accurate quantification of *MAP* cells in samples, single copy genes (e.g., *f57*; Table 8) are used in Real-Time PCR.

4. Presence of *MAP* in milk and milk products

Several studies provide evidence that *MAP* is secreted into the milk of a proportion of cattle clinically and sub-clinically infected with *MAP*. Detectable quantities of *MAP* have previously been reported in the milk of clinically infected (Taylor et al., 1981; Giese and Ahrens, 2000) and sub-clinically infected (Sweeney et al., 1992; Streeter et al., 1995) cattle.

Colostrum and milk are good sampling materials for the isolation of *MAP* from infected dairy cattle, but some practical matters should be considered before the collecting of samples. Firstly, the udder must be washed and dried in order to decrease the risk of environmental contamination. It is necessary to collect milk from each teat or from bulk tank milk and to immediately chill to 4°C. The samples should be transported to the laboratory without delay and for best results should be analyzed immediately. If a milk sample is not analyzed immediately after it is taken, it can be frozen at –70°C, but this is not preferable.

4.1. Colostrum

Colostrum is an important source of nutrients and an immediate source of passively absorbed maternal antibodies, which are critical to the protection of the newborn calf against infectious diseases in the first weeks and months of life. However, colostrum can also represent one of the earliest potential exposures of dairy calves to infectious agents like *MAP* (Chiodini et al., 1984). For example, *MAP* has been recovered from the colostrum of 22.2% of clinically normal but *MAP* infected cows (36% of

heavy fecal shedders; 16% of light fecal shedders; Streeter et al., 1995).

MAP has also been detected in milk and supramammary lymph nodes from asymptomatic *MAP* infected cows at the time of slaughter (Sweeney et al., 1992). *MAP* was found in 27% of lymph nodes in marked contrast to examined milk, which was positive by culture only in 11.6% of animals. The authors concluded that the serological ELISA status was not useful for predicting infection and that the shedding of *MAP* is lower in the milk of asymptomatic infected cows than that of clinically ill cows. These results were confirmed in the colostrum and milk of cows subclinically infected with *MAP* (Streeter et al., 1995).

4.2. Unpasteurized milk

Nowadays, consumers can buy unpasteurized milk and dairy products made from this milk only from private small-scale producers and from grassland farmers. The examination of unpasteurized milk for the presence of *MAP* is now only performed for experimental purposes. Legislation related to milk quality does not require that milk be completely free of *MAP*.

4.3. Pasteurized milk

The first commercial pasteuriser was built in Germany in 1882. To begin with the commercial pasteurization of milk was not generally accepted, but many companies adopted the practice in secret. The first legislation on pasteurized milk was passed in 1924 and defined pasteurization as a heating process of not less than 61.1°C for 30 min in approved equipment ("holder method"). Although the "holder method" has previously been the most widely used, this technique has now in large part been superseded by high-temperature short-time (HSTS) pasteurization methods. The standard of 71.1°C for 15 s was agreed upon after the evaluation of HSTS treatment on the creaming ability of milk, practical experience and numerous other considerations (Holsinger et al., 1997).

Studies published in the scientific literature concerning the ability of *MAP* to survive heat treatment report varied findings. McDonald et al. (2005) found that pasteurization performed at different temperatures and for different times was very effective in *MAP* devitalisation, resulting in a greater than 6 $\log_{10}$ and 4 $\log_{10}$ reduction in all 85% and 14% of pasteurized samples, respectively. Whilst in Ireland, O'Doherty et al. (2002) found that all 396 samples of pasteurized milk examined were negative; the occasional occurrence of viable cells has been reported in other studies (Grant et al., 2001). Millar et al. (1996) noted the presence of *MAP* DNA in pasteurized milk from a small-scale production unit in England and Wales, detected by the PCR method. The survival of *MAP* was also demonstrated in a study of 827 pasteurized milk samples from 241 dairies in England, Wales, Northern Ireland and Scotland over a 17-month period (Grant et al., 2002a).

In the study of Grant et al. (2005) *MAP* was isolated from milk samples (12 out of 27) after heat treatment at 72.5°C, i.e., a temperature close to the minimum pasteurization temperature specified by the legislation or from non-homogenized milk (13 out of 27). It is therefore clear that standard pasteurization temperature and time fail to unequivocally guarantee the full inactivation of *MAP*. The number of positive findings decreased with increasing temperature, but *MAP* was also detected in milk treated by temperature of 82.5°C with a longer dwell time and upstream homogenisation.

MAP survival is likewise associated with the initial number of cells in raw milk. *MAP* has also been detected after HTST, if more than 10^5 CFU/ml/milk were present in milk (Gao et al., 2002; Grant et al., 2002b). With regard to the fact that *MAP* cells may clump together in milk, pasteurization combined with homogenization is much more efficient than pasteurisation alone as documented by Grant et al. (2005). The survival of cells in clusters after pasteurization has been confirmed by the studies of Keswani and Frank (1998), Sung and Collins (1998) and Hammer et al. (2002). Heat treatment with concurrent homogenization applied either as a separate process (upstream – usually performed after the second regeneration, after centrifugation and standardization unit) or in the dwell zone (entry to the dwell zone) resulted in a significantly lower number of positive samples than pasteurization without homogenization ($P < 0.001$ for homogenization in the dwell zone, $P < 0.05$ for upstream homogenization) (Grant et al., 2005).

It has been shown that viable *MAP* can be detected even after application of different levels of pressure in conjunction with pasteurization (Donaghy et al., 2007).

4.4. Powdered milk

Powdered milk (coffee creamer, whole milk powder, half-fat milk, skimmed milk, baby food – sunar, etc.) can also become a source of *MAP* infection. The only study dealing with this potential source of infection was performed in 2004, a year in which an increasing incidence of Crohn's disease in children was observed prompting the authors to investigate the phenomenon. Samples of dried milk baby food products, originating from ten European countries, available on the Czech market, were tested. The occurrence of *MAP* in baby food products from all ten European countries was confirmed (Hruska et al., 2005).

4.5. *MAP* in cheese

Two methods are used to ensure the safe production of cheeses: either pasteurization of milk or holding the finished cheese for at least 60 days at a temperature of 2°C (curing). The two most important factors contributing to the inactivation of bacterial pathogens during the 60-day curing process are low pH and high salt concentration (Spahr and Schafroth, 2001).

The culture method is used for *MAP* detection in cheese. Cheese produced in a laboratory is artificially contaminated and the relationship between *MAP* survival and time is detected by culture. In such studies, heat treated or non-heat treated milk is used and three standard types of cheese are produced: soft, semi hard and hard. In 2005 and 2006, some studies appeared which also used PCR for detection, besides the above mentioned culture method. The IS*900* and *hsp*X genetic elements were used as the detection targets (Table 2).

In the above mentioned cases *MAP* was detected in cheese available on the market (Ikonomopoulos et al., 2005; Clark, Jr. et al., 2006). Both studies documented that higher detection rates of *MAP* can be obtained by PCR than by culture. Nevertheless, PCR does not provide information about the viability of *MAP* cells. The screening of cheese found detectable quantities of *MAP* DNA. *MAP* detection rates obtained by means of PCR in Greece, CZ and USA were 50%, 12% and 5%, respectively.

4.6. *MAP* in milk – legislative regulations

According to the legislation of the Czech Republic (Act No. 166/1999 Coll. on veterinary care and amendments of some related acts), milk and products made from this milk can be released onto the market after treatment performed in dairy plants. Non-heat treated raw milk and milk products can be sold with the consent of the Regional Veterinary Administration directly to consumers in the place of production, provided that the milk originates from healthy animals and herds free of dangerous infections, to which the animals may be susceptible. The sellers of raw milk or products made from this milk are obliged to have it tested in a laboratory at least once every 6 months. However, legislation currently in force in the Czech Republic does not require that milk be free from *MAP*. Consequently, milk from herds with a certified outbreak of paratuberculosis can be delivered to dairy processing plants.

According to European Union legislation (Anonymous, 2004b) as amended by Anonymous (2006), the managers of food processing plants, when considering whether to subject raw milk and colostrum to heat treatment, must take into account procedures developed in accordance with the principles of Hazard Analysis and Critical Control Points (HACCP) according to Anonymous (2004a). Heat treatment of raw milk or colostrum must meet the following specifications: pasteurization must be performed using high temperature treatment for a short time (at least 72°C for 15 s) or low temperature for a long time (at least 63°C for a period of 30 min) or any other combination of time and temperature leading to an equivalent effect. The ultra high temperature (UHT) treatment is, according to Anonymous (2006), a continuous flow of heating medium for a short time (at least 135°C in combination with an adequate dwell time). Accordingly, the EU legislation does not ban the sale of milk from herds with the occurrence of paratuberculosis; the testing of milk for the presence of the causative agent of paratuberculosis is not required by law although recently, some states have adopted national control and certification programmes.

5. Conclusions

The presented tables show that the detection of *MAP* in milk has been reported from countries on the European continent, Argentina, the UK and Australia. Due to the fact that detection methods are still under development and that the movement of infected animals between farms and different

countries is not controlled, *MAP* has been detected in growing numbers of cattle herds and countries worldwide.

In the past decades several methods for the detection, isolation and identification of *MAP* in milk have been described. The preparation of samples for analysis is a basic and critical step in *MAP* detection. A basic procedure for the detection of *MAP* in milk includes centrifugation to collect the pellet fraction, chemical decontamination, and subsequent processing of samples by a detection method (cultivation, PCR etc.). The most often used method of *MAP* detection is culture. The second most frequent method of *MAP* detection in milk is PCR (single, nested, Real-Time PCR). Methods of visual detection of *MAP* such as bioluminescence and indirect *MAP* detection method – ELISA/MELISA have been used in a few cases.

Recent studies suggest that *MAP* may survive the pasteurization of milk and have led to an increase in the testing of milk and milk products for the presence of this organism (Grant, 2006). Milk constitutes the primary means of transmission of paratuberculosis infection from cow to calf (mother to offspring). On the other hand milk forms a significant element of the diet of many children and adults. As most milk is consumed after pasteurization, the focus has been on the identification of *MAP* in raw milk and pasteurized milk and in milk products (Grant et al., 2005).

Published studies document the detection of *MAP* in colostrum, non-pasteurized and pasteurized milk, baby milk powder and all types of cheese. Nevertheless, other dairy products which are not mentioned in the present study such as sour milk products or creamers have not yet been tested. However, the occurrence of some life forms of *MAP* can also be expected in these matrices. Generally, the detection of any organism from these matrices depends upon a good isolation method. The sensitivity and detection rate will increase with the further development of the detection and isolation methods of *MAP* in these matrices.

All the above mentioned products have cow's milk as their major constituent. However, potential *MAP* contamination of milk from other ruminant species remains to be elucidated. In sheep and goats, *MAP* was detected by means of IS*900* PCR in 23% of bulk tank milk samples from 403 different farms throughout Switzerland in the year 2003 (Muehlherr et al., 2003). The first report of isolation of cultivable *MAP* from goat's milk originated from India (Singh and Vihan, 2004). Hence, it is evident that paratuberculosis also occurs in herds of these animals.

According to the experience from some infected herds, bringing a disease under control takes several years and is very expensive (Pavlik et al., 2000; Hasonova and Pavlik, 2006). Dairy cattle breeders should therefore go to great lengths to protect their herds from infection with paratuberculosis. Certification programs may be a reasonable solution to the problem. They may decrease the distribution of infected animals within a state and among states worldwide. Regarding food safety, it should also be guaranteed that milk and dairy products are free of *MAP*. These measures could significantly decrease the potential occurrence of Crohn's disease in groups of people at risk. At high risk are above all children, the next of kin of patients suffering from Crohn's disease and immunocompromised patients. Certification programmes will not eradicate paratuberculosis. However, they may considerably contribute to a reduction in the presence of the infectious agent in foodstuffs of animal origin from certified herds.

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