Detection of *Mycobacterium Avium Subsp. Paratuberculosis* in Milk from Cclinically Affected Sheep and Goats

Zoi Dimareli-Malli, DVM, MSc, PhD

Veterinary Research Institute of Thessaloniki,
National Agriculture Research Foundation,
Thermi 57001, P.B.O : 60272, Thessaloniki, Greece
Tel:+302310365372
e-mail: dimareliz@hotmail.com

KEY WORDS: Culture, *Mycobacterium avium subsp.paratuberculosis*, milk, goat, sheep

ABSTRACT

Milk and faecal samples from sheep and goats with clinical symptoms of paratuberculosis were examined for the presence of *Mycobacterium avium subsp.paratuberculosis* by culture in M7H11 and Herrold's, as well as in MGIT. The determination was based on two groups of clinically suspected animals in Northern Greece: group I consisted of 65 goats and group II consisted of 35 sheep, belonging to 8 goats' flocks and 5 sheep flocks respectively. All animals had known paratuberculosis infection. In total, MAP was isolated from the faeces of 16 goats (24.6 %) and from the milk of 5 goats (7.7 %). However, MAP strains were detected from the faeces of 3 sheep (8.6 %) and from the milk of 2 sheep (5.7 %). Out of the 16 faecal-culture-positive goats, 10 (62.5 %) had normal stools and 6 (37.5 %) had intermittent and severe diarrhea. The 3 faecal-culture-positive sheep had softened, pasty stools. The faecal culture status was compared with milk culture results. In the group of goats with intermittent and severe diarrhea, 4 of 6 (66.7 %) were milk-culture-positive and 1 of 10 (10 %) goats with normal stools was milk culture positive. This difference was statistically significant ($p < 0.05$).

INTRODUCTION

Paratuberculosis disease, or Johne's disease, is a chronic granulomatus enteritis of ruminants caused by *Mycobacterium avium subsp. paratuberculosis* (MAP).[1,2]. MAP infection is widespread in domestic livestock, including cattle, sheep, and goats. Rabbits, deer, and other animals have been identified as wildlife hosts of MAP.[3] Paratuberculosis in animals is usually acquired at an early age from feces-contaminated environments and from milk, younger animals being more susceptible than adults. Successful control of the disease in a herd is dependent on minimizing the exposure of young animals to the organism.[4]

In small ruminants, the only consistent, though nonspecific, clinical sign of the disease is progressive weight loss. Diarrhea is not a constant feature and is often intermittent, although it can be severe in some goats in individual herds.[5] The prevalence of the disease in goats and sheep in Greece is high, and infection is the cause of significant economic losses. The first reported case of paratuberculosis in sheep was diagnosed in 1967-1968 and in goats in 1968 in the Zaanen breed. Since then the number of infected herds and animals has grown rapidly and the disease is now widespread in small ruminants in Greece, it presents a serious problem, causing significant economic losses.

Generally, goats are more susceptible to the disease than sheep.[6,7,8] Despite their frequent contact with goats and sheep, the disease was not reported in cattle until 1988, when it appeared in at least 10 cattle herds during 1988-2003.[9] In ruminants, the gastrointestinal tract is the primary site of infection. Adult animals shed the causative agent in the milk even if they are not manifesting clinical signs of paratuberculosis.[10] When contaminated milk is ingested by young animals, they acquire the infection.

MAP causes Johne's disease, which is similar to Crohn's disease in humans. Chiodini and co-workers described the discovery of evidence of mycobacterial infection in Crohn's disease,[1] followed by a review of Crohn's disease and the Mycobactrerioses.[3,11,12] Several studies support the hypothesis that MAP causes or contributes to Crohn's disease,[13] but there are other studies that dispute these findings.[14,15] If indeed Crohn's disease is initiated by MAP infection, the question of how the infection is contracted remains.

It is known that cows infected with MAP, both clinically and sub-clinically, shed the bacterium in their milk.[16,4,17] A number of studies have been and are being carried out to determine if MAP can be transmitted to humans via milk and the ability of MAP to survive industrial or laboratory pasteurization has been assessed by several research groups.[18,19] There are two reports [20,21] on the incidence of MAP in raw sheep and goats' milk samples by Immunomagnetic PCR (IMSPCR). The primary purpose of the study reported here was to determine the proportion of infected animals that excrete MAP in their milk. A second objective was to determine whether an association exists between stool status and presence of organism in the milk. Solid and liquid culture systems were attempted for the detection of MAP in the raw sheep and goats' milk.

MATERIALS AND METHODS

Milk–sampling

Before collecting samples, the udder was washed and dried with a clean towel. The first two samples of milk (25 mL) were collected separately from each animal and stored in sterile, centrifuge-sized tubes. Samples were transported to the laboratory at 4º C in an insulated box.

A total of 100 raw milk samples - goats (n = 65) and sheep (n = 35) - were tested for the presence of MAP by culture in M7H11 and Herrold's solid medium, and in MGIT liquid culture system. The milk samples were obtained from 100 clinically suspected animals, selected on the basis of chronic weight loss and diarrhea, from herds with established paratuberculosis problems. The diagnosis was confirmed by microscopical examination, culture, or histopathology and PCR.

Fecal sampling

Fecal samples were taken from the rectum using disposable gloves, then faeces was transferred to a re-sealable plastic container. The faecal samples were obtained from all animals described above.

Decontamination and culture

Fifty mL of each milk sample was centrifuged (15 min at 2,500 rpm), resulting in a pellet suspended in 10 mL, 0.75 % (w/v), HPC. Following incubation at room temperature (21º C) for 4 h, and further centrifugation, as above, the pellet was re-suspended in 2 mL sterile distilled water. Two slopes of M 7H11 agar - supplemented with Mycobacteria selectatabs (Amphotericin B, Polymixin B, Carbenicillin and Trimethoprim: code MS24: MAST Laboratories, Ltb; Merseyside, United Kingdom), 10 % OADC enrichment medium (Difco), and 2 mg/mL mycobactin j (Merieux) - were inoculated with 200 µl of the re-suspended pellet and two slopes of Herrold's egg yolk medium with mycobactin j and Mycobacteria selectatabs (Greig et al.1999). Two vials of MGIT medium (Becton Dickinson, UK) - supplemented with 0.5 mL of MGIT OADC, 0.1 mL of MGIT PANTA antibiotic mixture (Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azboullin), and 2 µg/ml mycobactin j (Merieux) - was inoculated with 500 µl of the re-suspended pellet.

Slopes were examined periodically for the presence of colonies.

MGIT tubes were read daily via UV light source beginning on day 2. When growth was observed in either medium, an acid-fast stain was carried out by the Ziehl-Neelsen method to confirm the presence of acid-fast organisms. When acid-fast bacilli were observed, further confirmatory tests (as detailed below) were carried out on each suspect isolate to determine whether it was MAP.

Confirmatory tests

When acid–fast cells were observed in a culture, subculture was performed to confirm slow growth rate, typical colony morphology, and mycobactin-dependence. A portion of the MGIT culture, or a colony from a M7H11 slope and Herrold's, were sub-cultured to fresh M7H11, both with and without mycobactin j and incubated at 37º C for up to 18 weeks. Colony morphology and time of appearance of colonies was noted, as well as the mycobactin dependence.

Analysis by PCR

PCR was performed on isolated colonies to detect the *M.a.paratuberculosis* insertion sequence IS900. DNA extraction and PCR was carried out using the protocol described by Challans et al. [22] Briefly, the process consisted of extracting the mycobacteria with xylene, pelleting them, and washing to remove traces of xylene. Then, samples were lysed by shaking them with zirconium beads, on a mini-bead beater, three times at high speed for 90 seconds each time. DNA was extracted with chloroform/octanol and precipitated with ice-cold isopropanol. Oligonucleotide primers 90 and 9123 were selected to amplify a 388 base-pair product from the 5' region of IS900 from M.a.paratuberculosis, and a 252 base-pair product from the 5' region of IS90124 for Mycobacterium avium subsp. silvaticum. PCR amplifications were performed with 30 cycles of denaturation at 94ºC for 30 seconds, annealing at 55º C for 30 seconds and extension at 72ºC for one minute. The final extension occurred at 72ºC for two minutes in a Techne PHC-3 thermocycler. PCR products were analysed by agarose gel electrophoresis and the DNA fragments were visualised by ethidium bromide.

Statistical analysis

The Student-Fisher test was used to compare the faecal culture status with milk culture results. Significant differences were considered at $p < 0.05$.

RESULTS AND DISCUSSION

Out of the 65 caprine faecal cultures, 10.8 %, 18.5 %, and 23% were positive in Herrold's, M7H11, and MGIT respectively,

Table 1: *Detection of MAP in caprine and ovine milk samples by culture in Middlebrook 7H11, Herrold's and MGIT liquid culture system*

Milk samples	Milk culture		
	Middlebrook 7H11 +	Herrold's	MGIT +
Caprine 65	4 (6,15 %)	1(2,85%)	5 (7,69 %)
Ovine 35	2 (5,71 %)	0	2 (5,71 %)

Table 2*: Detection of MAP in caprine and ovine fecal samples by culture in Middlebrook 7H11, Herrold's and MGIT liquid culture system*

Fecal samples	Fecal culture		
	Middlebrook 7H11 +	Herrold's	MGIT +
Caprine 65	12 (18,46 %)	7 (10,76%)	15 (23 %)
Ovine 35	3 (8,57 %)	1 (2,85%)	3 (8,57 %)

***Table 3**: Analysis of caprine – ovine fecal and milk samples MAP culture positive in MGIT, Herrold's and M7H11*

Fecal culture in liquid culture system MGIT and solid media M7H11, Herrold's						
Caprine samples	65	16 +	→	6 (ISD)	→	4 MCP
			↘	10 (NS)	→	1 MCP
Ovine samples	35	3 +	→	(SPS)	→	1 MCP

ISD: Intermittent severe diarrhea
NS: Normal stools
SPS: Softened pasty stools
MCP : Milk culture positive

***Table 4**: Detection of MAP in caprine, ovine fecal and milk samples by culture in Middlebrook 7H11, Herrold's and MGIT*

Samples	No	M7H11	Herr-old's	MGIT detection by		
				Fluoresence	ZN Microscopy	Subculture in M7H11
Caprine fecal	65	12	7	22	19	15
Ovine fecal	35	3	1	5	3	3
Caprine milk	65	4	1	9	6	5
Ovine milk	35	2	0	4	3	2

whereas 2.85%, 6.2 %, and 7.7 % of the caprine milk samples were positive in Herrold's, M7H11, and MGIT respectively. Three (8.6 %) ovine faecal samples were positive in M7H11 and MGIT, while 2.9 % was positive in Herrold's. Under examination, 5.7 % of 35 ovine milk samples were found to be positive in M7H11and MGIT, whereas all of them showed negative results regarding the Herrold's (Table, 1.2).

After liquid cultivation, a second step is necessary to confirm positive results. Since confirmation of positive signals has to be done by subcultivation on solid media, the method does not markedly reduce overall detection time in comparison to cultivation on solid medium. The 3 techniques (fluorescence, ZN, and subculture in M7H11) revealed 15 fecal samples to be positive for MGIT. Three more strains were isolated in MGIT than in M7H11. In this case, MGIT was an enrichment for the isolation of the MAP (Table 4).

A comparison of the 3 media reveals a significant difference between Herrold's and M7H11-MGIT, while the difference between M7H11 and MGIT was not significant ($p < 0.05$). A sample was considered positive when MAP was isolated on any single medium. In total, MAP was isolated from the feces of 16 goats (24.6 %) and from the milk of 5 goats (7.7 %). However, MAP strains were mycobactin-dependent detected from the feces of 3 sheep (8.6 %) and from the milk of 2 sheep (5.7 %) (Figure1.2). All the above were positive in IS900 PCR. Of the 16 fecal-culture-positive goats, 4 were positive in all 3 culture methods, M7H11, MGIT, and Herrold's. Seven samples were positive in MGIT and M7H11. Two were positive in MGIT and Herrold's. Two were positive only in MGIT. Finally, only 1 was positive in M7H11 and Herrold's.

In sheep, 2 of the fecal samples were positive in M7H11 and MGIT, and 1 was positive in all three media. Of the 16 fecal-culture-positive goats, 5 (31.2 %) were milk culture positive for MAP. However, of the

3 fecal-culture-positive sheep, only 1 (33.3 %) was milk culture positive for MAP. Out of the 16 fecal-culture-positive goats, 10 (62.5 %) had normal stools and 6 (37.5 %) had intermittent and severe diarrhea. The 3 fecal-culture-positive sheep had softened, pasty stools.

The fecal culture status was compared with milk culture results. In the group of goats with intermittent and severe diarrhea, 4 of 6 (66.6 %) were milk-culture-positive. One of 10 goats (10 %) with normal stools was milk culture positive. This difference was statistically significant ($p < 0.05$). Among sheep, 1 of 3 (33.3 %) was milk culture positive. MAP was detected from 5 of 65 (7.7 %) clinically suspected goats and from 2 of 35 (5.7 %) clinically suspected sheep by cultivation (Table 3).

DNA evidence of MAP in raw goat's milk was reported as follows: 1.1 % positive in the UK,20 7.1 % positive in Norway,[21] and 23 % positive in Switzerland.[25] According to our results, an association was observed between stool status of the sheep and goats and the presence of the organism in milk.

In cows, association with faecal shedding has been reported in the following situations: presence of the organism in the colostrum and milk,[17] in the milk and supramammary lymph nodes,[10] and in fetuses in subclinically infected cows. Giese and Ahrens [26] detected MAP in milk samples from 6 of 11 animals infected. Taylor et al.[16] found MAP in the milk of 35 % of the ani-

Figure 1: *Proportion of goats shedding MAP in milk and faeces*

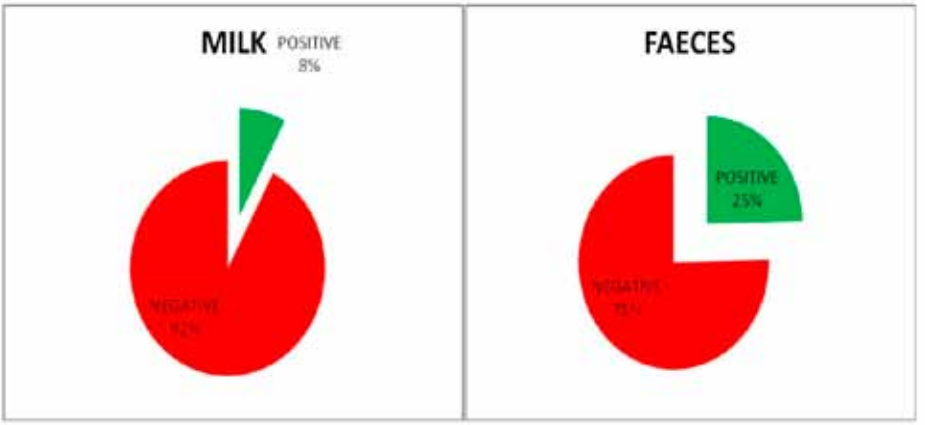
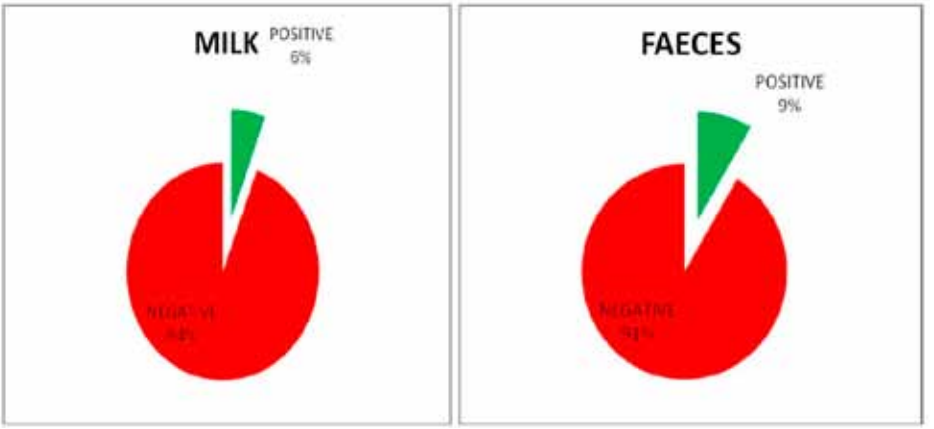


Figure 2: *Proportion of sheep shedding MAP in milk and faeces*



mals investigated.

Since MAP is excreted in the milk of infected animal, kids and lambs can become infected by nursing from, or being bottle-fed milk from, an infected doe and ewe. According to our results, the risk that the organism is being shed into the milk is believed to be higher in goats and sheep that have intermittent and severe diarrhea. This symptom indicates that animals moved into later stages of the infection. Thus, clinically affected goats and sheep (as shown by weight loss) are more likely to infect their offspring than goats and sheep still in good condition.

Milk and dairy products are important components of human nutrition. MAP has been linked to Crohn's disease in humans. During the last 10 years, there has been progress in the research of the relationship between this microorganism and Crohn's disease. The focus of much of the research is food products as the transmission pathway.[27] The way in which MAP is transmitted is not yet fully understood, but some lines of evidence suggest that humans can become infected through contaminated milk. Additionally, some authors showed that when MAP is present in milk, the bacteria are able to resist pasteurization conditions, typically heat treatment. Confirmed MAP isolates were cultured from 1.8 % of the commercially pasteurized milk samples in the UK.[28] Similar data were published from the USA.[18]

However, the autoimmune character of Crohn's disease does not exclude a risk for genetically susceptible people when linked with bacterial triggers. Crohn's may occur even though live MAP cells are not present in food. The possibility of a health risk for consumers resulting from not only viable MAP, but also from inactive or dead cells, and even from their structural components renders the presence of MAP cells important. Introducing MAP-free milk and dairy products would provide the least risk for consumers.[19]

REFERENSES

1. Chiodini RJ, Van Kruiningen HI, Merkal RS: Ruminant paratuberculosis Johne's disease: current status and future prospects. *Cornell Vet* 1984; 74: 218-262.
2. Harris NB, Barletta RG: Mycobacterium avium subsp. Paratuberculosis in veterinary medicine. *Clin Microbiol Rev* 2001; 14: 489-512.
3. Chiodini RJ: Crohn's disease and the mycobacterioses a review and comparison of two disease entities. *Clin Microbiol Rev* 1989; 2: 90-117.
4. Streeter RN ,Hoftsis GF , Bech-Nielsen S, Shulaw WR, Rings M: Isolation of Mycobacterium paratuberculosis from colostrum and milk of subclinically infected cows. *Am J Vet Res* 1995; 56: 1322-1331.
5. Eamens G :Johne's disease in goats. In : Milner a., Wood p.(eds), Current trends in research, diagnosis and management. CSIRO Australia , 1989;105-112.
6. Xenos G, Christopoulos Chr, Psychas V, Leontides S, Mitliangas P, Magoutas P: Paratuberculosis in sheep and goats in certain regions of Northern and Central Greece. *Workshop in Paratuberculosis*, CEC, Denmark 1989; 113-121
7. Dimareli Z, Xenos G, Argyroudis S, Papadopoulos O: A survey of ovine and caprine paratuberculosis in the Thessaloniki area, Greece.*The Paratuberculosis Newsletter*1991; Vol 3, 2, 8-9.
8. Dimareli-Malli Z , Sarris K: Comparison of DNA probe test and cultivation methods for detection of Mycobacterium avium subsp. Paratuberculosis in caprine and ovine faeces. *Aust Vet J* 2001; 79: 47-50.
9. Dimareli-Malli Z, Sarris K, Papadopoulos G: Paratuberculosis in cattle in Greece. *Geotechnical scientific issues* 1998; Vol 9, 4: 20-25.
10. Sweeney RW, Whitlock RH, Rosenberger AE: Mycobacterium paratuberculosis cultured from milk and supramammary lymph nodes of infected asymptomatic cows. *J Clin Microbiol* 1992; 30: 166-171.
11. Hermon-Taylor J, Bull T: Crohn's disease caused by Mycobacterium avium subspecies paratuberculosis; a public health tragedy whose resolution is long overdue.*J Med Microbiol* 2002; 51: 3-6.
12. Autschbach F, Fisold S, Hinz U, Zinser S, Linnebatcher M, Giese T, Loffler T, Buchler MW, Schmidt J : High prevalence of Mycobacterium avium subspecies paratuberculosis IS900 DNA in gut tissues from individuals with Crohn's disease. *Gut* 2005 ; 54: 944-949.
13. Hermon-Taylor J, Bull TJ, Seridan JM, Cheng J, Stellakis ML, Sumar N: The causation of Crohn's disease by Mycobacterium avium subspecies paratuberculosis.*Can J Gastroenterol* 2000;
14: 521-531.
14. Kanazawa K, Haga Y, Funakoshi O, Nakajima H, Munakata A, Yoshida Y: Absence of Mycobacterium paratuberculosis DNA in intestinal tissue from Crohn's disease by nested polymerase chain reaction. *J Gastroenterol* 1999; 34: 200-206.
15. Van Kruiningen HJ: Lack of support for a common etiology in Johne's disease of animals and Crohn's disease in humans. *Inflamm Bowel Dis* 1999 ;5: 183-191.

16. Taylor TK,Wilks GR, Mcqeen DS: Isolation of Mycobacterium paratuberculosis from the milk of a cow with Johne's disease. *Vet Rec* 1981;109: 532-533.

17. Stephan R, Buhler K, Corti S: Incidence of Mycobacterium avium subsp. Paratuberculosis in bulk-tank milk samples from different regions in Switzerland. *Vet Rec* 2002; 150: 214-215.

18.Ellingson JLE , Anderson JL, Koziczkavski JJ : Detection of viable Mycobacterium avium subsp. paratuberculosis in retail pasteurized whole milk by two culture methods and PCR. *J Food Prot* 2005; 68: 966-972.

19. Hruska K, Bartos M, Kralik P, Pavlik I: Mycobacterium avium subsp. Paratuberculosis in powdered infant formula. . Proceedings of the 8th International Colloquium on Paratuberculosis,Copenhagen, Denmark. *International Association for Paratuberculosis* 2005; 311-319.

20.Grant JR, Oriordan LM,Ball HJ, Rowe MT: Incidence of Mycobacterium paratuberculosis in raw sheep and goat's milk in England, Wales and Northern Ireland. *Vet Microbiol* 2001; 79:123-131.

21.Djonne B, Jensen MR, Grant IR: Detection by immunogenetic PCR of Mycobacterium avium subsp. paratuberculosis in milk from dairy goats in Norway. *Vet Microbiol* 2003; 92:135-143

22.Challans J A, Stevenson K, Reid H W, Sharp M : A rapid method for the extraction and amplification of Mycobacterium paratuberculosis DNA from clinical samples. *Vet Rec* 1994 ;134: 95-96.

23. Sanderson JD, Moss MT, , Tizard ML, Hermon-Taylor J, : Mycobacterium paratuberculosis DNA in Crohn's disease tissue. *Gut* 1992; 33: 890-896.

24.Kunze ZM, Portaels F, Mcfadde J J : Biologically distinct Subtypes of Mycobacterium avium differ in possession of insertion sequence 15901. *J Clin Microbiol* 1992 ;30: 2366-2372.

25. Muehlherr JE, Zweifel C, Corti S: Microbiological quality of raw goat's and ewe's bull-tank milk in Switzerland. *J Dairy Sci* 2003; 86: 3849-3856.

26.Giese SB, Ahrens P: Detection of Mycobacterium avium subsp. Paratuberculosis in milk from clinically affected cows by PCR and culture. *Vet Microbiol* 2000; 77: 291-297.

27.Grant IR: Occurrence of Mycobacterium avium subsp. Paratuberculosis in foods and the impact of milk processing on its survival. Proceedings of the 8th International Colloquium on Paratuberculosis,Copenhagen, Denmark. *International Association for Paratuberculosis*2005 ;271-277.

28.Grant JR, Ball HJ, Rowe MT : Incidence of Mycobacterium paratuberculosis in bulk raw andcommercially pasteurized cow's milk from approved dairy processing establishments in theUnited Kingdom. *Appl Environ Microbiol* 2002; 68: 2428-2435.