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Detection of *Mycobacterium avium* subsp. *paratuberculosis* in Retail Cheeses from Greece and the Czech Republic

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We investigated the presence of *Mycobacterium avium* subsp. *paratuberculosis* in retail cheeses from Greece and the Czech Republic. We found that 31.7% and 3.6% of our samples reacted positive by PCR and culture, respectively. Consumption of these cheeses is likely to result in human exposure to *M. avium* subsp. *paratuberculosis*, albeit at a low level for viable cells.

Paratuberculosis, which is caused by *Mycobacterium avium* subsp. *paratuberculosis*, affects domestic and wild ruminants (1). *M. avium* subsp. *paratuberculosis* has also been implicated in the pathogenesis of Crohn's disease in humans (4, 5, 19). Animals may shed *M. avium* subsp. *paratuberculosis* in their milk, even during subclinical infection (12, 25), and human exposure to *M. avium* subsp. *paratuberculosis* through dairy products has become an issue of concern, despite the fact that there is no conclusive evidence to support association of this pathogen with Crohn's disease (9, 10). The ability of *M. avium* subsp. *paratuberculosis* to survive industrial or laboratory pasteurization has been assessed by several research groups (8, 13, 17, 22, 23). Recently, Ayele et al. (2) demonstrated the presence of viable *M. avium* subsp. *paratuberculosis* in retail milk. The viability of this pathogen in cheese has been investigated, to our knowledge, by only three groups. These studies referred to a soft Hispanic-style (queso fresco) cheese (21), to hard (Swiss Emmentaler) and semihard (Swiss Tisliter) cheeses (24), and to cheddar cheese (7). In the present study we used cultivation and PCR to investigate the presence of *M. avium* subsp. *paratuberculosis* in retail cheese from Greece and the Czech Republic.

Collection of samples was performed from February to June 2003. The sampling plan was designed to include the maximum possible number of the most popular brands (commercial labels) and/or types of cheese, based on data published annually by the cheese producers in both countries. Hence, we sampled five brands of feta cheese from Greece and a total of three brands of cheese from the Czech Republic, one for each type commonly available, i.e., soft, hard, and semihard. Selection of the outlets used for sampling was done by random ballot draw of the major outlets (supermarkets) in both countries. Each outlet was visited once, when a single sample was obtained from each brand to the maximum number of brands available. The minimum number of retail points to be visited, hence the minimum number of samples collected for each brand, was five. Each brand was assumed homogenous across sites. At 5% prevalence of *M. avium* subsp. *paratuberculosis*, this sample size represents 20% "precision" (or deviation from precision) at a 95% confidence level. However, due to low availability, the number of samples that were examined for one feta cheese (brand A) was three (25% "precision"). In this manner, a total of 84 samples of eight different brands of cheese was collected from Greece (A, B, C, D, and E) and the Czech Republic (F, G, and H) (Table 1).

Each sample consisted of 50 g of cheese and was stored at 4°C for less than 48 h before examination. After disposal of the external layer, the remaining cheese was homogenized by stomacher blender (Kleinfeld Labortechnik, Germany). Thirty milliliters of homogenate was used for cultivation as previously described (2). In brief, decontamination was performed by incubation with 0.75% hexadecyl-pyridinium chloride (Sigma Chemical Co., St. Louis, Mo.) in a dark room, at room temperature, for 5 h. A 200-μl aliquot of the suspension was inoculated onto three slants of Herrold's egg yolk medium (Sigma Chemical Co., St. Louis, Mo.) with 2 μg/ml mycobactin J (Allied Monitor Inc., Fayette, Mo.). To rule out fast-growing mycobacteria and early contamination of cultures, the vials were monitored during the first week of incubation. Further observation was at biweekly intervals until ample colonies appeared for diagnosis. Incubation was carried out for no less than 8 months. DNA was isolated from 200 mg homogenate, using the QIAamp DNA tissue kit (QIAGEN GmbH, Düsseldorf, Germany). PCR amplification was performed as previously described (10). In brief, the primers P1N, 5′ GCATGG CCCACAGGACGTTGAG 3′, P2N, 5′ CTACAACAAGAG CCGTGCCG 3′, P3N, 5′ GGGTGTGGCGTTTTCCTTCG 3′, and P4N, 5′ TCCTGGGCGCTGAGTTCCTC 3′ (international patent application PCT/GR2004/000051) were incorporated in a one-tube nested PCR, amplifying a 257-bp fragment within the *M. avium* subsp. *paratuberculosis*-specific IS*900* element (11). The PCR products were analyzed by electrophoresis on 2% agarose gel, followed by UV transillumination. For confirmation of the PCR results, DNA isolated from 50% of the samples collected in both countries was marked with coded

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TABLE 1. Characteristics of cheeses tested and the results of testing by culture and PCR

Cheese type (country of origin)	Prepared with:	Brand's code	No. of samples	PCR positive (%)	Culture positive (%)	Physicochemical characteristics of cheese[a]					
						pH	% NaCl	Temp (°C) during:			
								Pasteurization (°C/s)	Manufacturing	Ripening	Storage
Feta (Greece)	Mixture of sheep and goat milk[b]	A	3	0/3 (0)	0/3 (0)	4.1–4.5	2.5–3.5	72–74/15	45–55	15–16	4
		B	12	2/12 (16.6)	0/12 (0)	4.1–4.5	2.5–3	72–74/15	45–55	15–16	4
		C	10	7/10 (70.0)	1/10 (10)	4.1–4.5	2.5–4	72–74/15	45–55	15–16	4
		D	7	6/7 (85.7)	1/7 (14.3)	4.1–4.5	1.8–2	72–74/15	45–55	15–16	4
		E	10	6/10 (60.0)	0/10 (0)	4.1–4.5	2.5–4	72–74/15	45–55	15–16	4
Subtotal		5	42	21/42 (50)	2/42 (4.7)						
Hard (Czech Republic)	Bovine milk	F	23	4/23 (17.4)	1/23 (4.3)	5.4–5.9	1–1.2	72–74/15	53–55	10–22	3–8
Semihard (Czech Republic)	Bovine milk	G	5	1/5 (20.0)	0/5 (0)	5.2–5.7	1.9–2.4	74–78/15	38–41	8–14	3–8
Soft (Czech Republic)	Bovine milk	H	14	0/14 (0)	0/14 (0)	4.1–4.3	1–1.2	85/15–25	28–32	20–25	3–8
Subtotal		3	42	5/42 (11.9)	1/42 (2.4)						
Total		8	84	26/84 (30.9)	3/84 (3.6)						

[a] As reported by the manufacturers.
[b] The ratio was not specified by the manufacturers.

numbers and cross-examined by the collaborating laboratories. Results were then communicated and compared.

PCR allowed amplification of the 257-bp, *M. avium* subsp. *paratuberculosis*-specific DNA fragment of the IS*900* element in 21 (50.0%) and 5 (12.0%) of the 42 samples collected from Greece and the Czech Republic (Table 1; Fig. 1). Positive results varied, depending on the brand and type of cheese, from 0% to 85.7%, the highest being recorded for brand D feta cheese (Table 1). The reproducibility of the PCR results in each laboratory was 100%. From the 84 samples that were tested by cultivation, 3 (3.6%) were *M. avium* subsp. *paratuberculosis* positive (Table 1). These samples also produced positive results by PCR. Our isolates were identified as *M. avium* subsp. *paratuberculosis* only by PCR, since after 8 months of incubation, they produced very few visible colonies and they failed to grow on secondary cultures.

Studies on the survival of *M. avium* subsp. *paratuberculosis* in model cheeses have shown that certain physicochemical characteristics, mainly pH, salt concentration, and temperature during manufacture and ripening, prevent multiplication of the pathogen, which is by nature very fastidious (20). These factors, which varied in the cheeses that we sampled (Table 1), have been shown to cause the death of most pathogenic bacteria found in cheese within 7 days of ripening (3). Despite indications that this adverse environment may be less inhibitory to *M. avium* subsp. *paratuberculosis* survival (21), it may be responsible for decreased viability or even death of *M. avium* subsp. *paratuberculosis* in our cheese samples, which probably accounts for the culture-negative, PCR-positive results that we recorded. Indeed, *M. avium* subsp. *paratuberculosis* cells of low viability have been associated with false-negative culture results generated by the neutralization of these cells during decontamination (20). For this reason, incubation of our samples with hexadecyl-pyridinium chloride was carried out for only 5 h, which is less than half the time of that which is recommended (18). However, even under these circumstances decontamination could still be a source of false-negative culture results, especially for samples containing small numbers of low-viability cells of *M. avium* subsp. *paratuberculosis* (15). Therefore, it can be stated that the results that we recorded by culture may be understating the true prevalence of this pathogen in our cheese samples. The latter could also be associated with the fact that sheep and goat strains of *M. avium* subsp. *paratuberculosis* seem to be more likely to fail to grow on culture compared to those isolated from bovines (6, 15, 16).

Detection of *M. avium* subsp. *paratuberculosis* by PCR varied

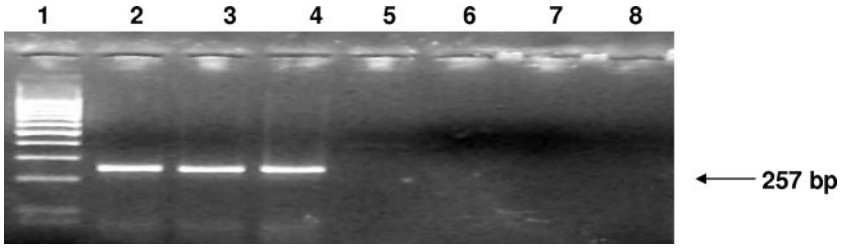

FIG. 1. Agarose gel electrophoresis of PCR products obtained by IS*900* one-tube nested PCR performed on representative cheese samples. Lane 1, 100-bp molecular weight ladder (New England BioLabs Ltd., United Kingdom); lane 2, positive control sample; lanes 3 and 4, positive samples showing the 257-bp amplification product specific for *M. avium* subsp. *paratuberculosis*; lanes 5 and 6, negative samples; lane 7, negative control containing only the PCR mixture and water; lane 8, negative control with DNA isolated from a confirmed PCR- and culture-negative cheese sample.

considerably among different types and brands of cheese, especially feta (0 to 85.7% positive samples). Although identification of the source of this variation is very difficult (21), it seems probable that our results reflected the impact of the specific physicochemical characteristics of each type of cheese on the elimination of *M. avium* subsp. *paratuberculosis* and/or the prevalence of this pathogen in the relevant animal populations. Indeed, the highest level of positivity (85.7%) was recorded for the brand D feta, which is produced on a Greek island with milk derived from a rather isolated population of animals that is enzootic for paratuberculosis (14). Interestingly, this brand is advertised for its low (<2%) NaCl concentration (Table 1).

In conclusion, DNA sequences specific for *M. avium* subsp. *paratuberculosis* can be amplified by PCR from a considerable percentage of retail cheeses in Greece and the Czech Republic. This finding and the isolation of viable *M. avium* subsp. *paratuberculosis* from the types of cheese mentioned above, albeit at a low frequency, indicate that they are a means of human exposure to *M. avium* subsp. *paratuberculosis*.

REFERENCES

1. **Ayele, W. Y., M. Machackova, and I. Pavlik.** 2001. The transmission and impact of paratuberculosis infection in domestic and wild ruminants. Vet. Med. Czech. **46:**205–224.
2. **Ayele, W. Y., P. Svastova, P. Roubal, M. Bartos, and I. Pavlik.** 2005. *Mycobacterium avium* subspecies *paratuberculosis* cultured from locally and commercially pasteurized cow's milk in the Czech Republic. Appl. Environ. Microbiol. **71:**1210–1214.
3. **Bechmann, H. P., and U. Spahr.** 1995. The fate of potentially pathogenic bacteria in Swiss hard and semihard cheeses made from raw milk. J. Dairy Sci. **78:**476–483.
4. **Chamberlin, W., D. Y Graham, K. Hulten, H. M. T. El-Zimaity, M. R. Schwartz, S. Naser, I. Shafran, and F. A. K. El-Zaatari.** 2001. *Mycobacterium avium* subsp. *paratuberculosis* as one cause of Crohn's disease. Alim. Pharmacol. Ther. **15:**337–346.
5. **Chiodini, R. J.** 1989. Crohn's disease and mycobacterioses: a review and comparison of two disease entities. Clin. Microbiol. Rev. **2:**90–117.
6. **Dimarelli, Z., G. Xenos, S. Argyroudis, and O. Papadopoulos.** 1991. A survey of ovine and caprine paratuberculosis in the Thessaloniki area, Greece. Paratuberculosis Newsletter **3:**8–9.
7. **Donaghy, J. A., N. L. Totton, and M. T. Rowe.** 2004. Persistence of *Mycobacterium paratuberculosis* during manufacture and ripening of cheddar cheese. Appl. Environ. Microbiol. **70:**4899–4905.
8. **Grant, I. R., H. J. Ball, S. D. Neill, and M. T. Rowe.** 1996. Inactivation of *Mycobacterium paratuberculosis* in cows' milk at pasteurization temperatures Appl. Environ. Microbiol. **62:**631–636.
9. **Hermon-Taylor, J., and T. Bull.** 2002. Crohn's disease caused by *Mycobacterium avium* subspecies *paratuberculosis*: a public health tragedy whose resolution is long overdue. J. Med. Microbiol. **51:**3–6.
10. **Hermon-Taylor, J.** 2001. Protagonist *Mycobacterium avium* subspecies *paratuberculosis* is a cause of Crohn's disease. Gut **49:**755–756
11. **Ikonomopoulos, I., M. Gazouli, I. Pavlik, M. Bartos, P. Zacharatos, E. Xylouri, E. Papalambros, and V. Gorgoulis.** 2004. Comparative evaluation of PCR assays for the robust molecular detection of *Mycobacterium avium* subsp. *paratuberculosis*. J. Microbiol. Methods **56:**315–321.
12. **Johnson-Ifearulundu, Y., and J. B. Kaneene.** 1999. Distribution and environmental risk factor for paratuberculosis in dairy cattle herds in Michigan. Am. J. Vet. Res. **60:**589–596.
13. **Keswani, J., and J. F. Frank.** 1998. Thermal inactivation of *Mycobacterium paratuberculosis* in milk. J. Food Prot. **61:**974–978.
14. **Leontides, S., D. Tomopoulos, C. Christopoulos, T. Tsangaris, and G. Exarchopoulos.** 1975. Paratuberculosis in goats in Greece, p. 1426–1428. *In* Proceedings of the 20th World Veterinary Congress, Thessaloniki, Greece.
15. **Machackova, M., J. Lamka, W. Y. Ayele, I. Parmova, P. Svastova, T. Amemori, and I. Pavlik.** 2002. Infection of ruminants by uncultivable strains of *Mycobacterium avium* subsp. *paratuberculosis* in the Czech Republic, p. 191–196. *In* R.A Juste (ed.), Proceedings of the Seventh International Colloquium on Paratuberculosis. International Association for Paratuberculosis, Inc., Madison, Wis.
16. **Merkal, R. S.** 1984. Paratuberculosis: advances in cultural, serologic and vaccination methods. J. Am. Vet. Med. Assoc. **184:**939–943.
17. **Meylan, M., D. M. Rings, W. P Shulaw, J. J. Kowalski, S. Bech-Nielsen, and G. F. Hoffsis.** 1996. Survival of *Mycobacterium paratuberculosis* and preservation of immunoglobulin G in bovine colostrums under experimental conditions simulating pasteurization. Am. J. Vet. Res. **57:**1580–1585.
18. **OIE.** 2000. Manual of standards for diagnostic tests and vaccines for terrestrial animals, p. 1–19. OIE, Paris, France.
19. **Pavlik, I., L. Bejckova, B. Fixa, O. Komarkova, and J. Bedrna.** 1994. DNA fingerprinting as a tool for epidemiological studies of paratuberculosis in ruminants and Crohn's disease, p. 278–289. *In* R. J. Chiodini, M. T. Collins, and E. O. E Bassey (ed.), Proceedings of the Fourth International Colloquium on Paratuberculosis. International Association for Paratuberculosis, Inc., Madison, Wis.
20. **Reddacliff, L. A., A. Vadali, and R. J. Whittington.** 2003. The effect of decontamination protocols on the numbers of sheep strain *Mycobacterium avium* subsp. *paratuberculosis* isolated from tissues and faeces. Vet. Microbiol. **95:**271–282.
21. **Spahr, U., and K. Schafroth.** 2001. Fate of *Mycobacterium avium* subsp. *paratuberculosis* in Swiss hard and semihard cheese manufactured from raw milk. Appl. Environ. Microbiol. **67:**4199–4205.
22. **Stabel, J. R., E. M. Steadham, and C. A. Bolin.** 1997. Heat inactivation of *Mycobacterium paratuberculosis* in raw milk: are current pasteurization conditions effective? Appl. Environ. Microbiol. **63:**4975–4977.
23. **Sung, N., and M. T. Collins.** 1998. Thermal tolerance of *Mycobacterium paratuberculosis*. Appl. Environ. Microbiol. **64:**999–1005.
24. **Sung, N., and M. T. Collins.** 2000. Effect of three factors in cheese production (pH, salt, and heat) on *Mycobacterium avium* subsp. *paratuberculosis* viability. Appl. Environ. Microbiol. **66:**1334–1339.
25. **Van Kruiningen, H. J., R. J. Chiodini, W. R. Thayer, J. A. Coutu, R. S. Merkal, and P. L. Runnels.** 1986. Experimental disease in infant goats induced by a mycobacterium isolated from a patient with Crohn's disease. A preliminary report. Dig. Dis. Sci. **31:**1351–1360.