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Short communication

Detection of mycobacterium avium subsp. paratuberculosis in cheeses from small ruminants in Tuscany

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ABSTRACT

Paratuberculosis is an infectious disease which affects mainly domestic and wild ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map). Map has been associated with human diseases like Crohn disease, type-1 diabetes, sarcoidosis, multiple sclerosis and Hashimoto's thyroiditis. The aim of this study was to determine the level of Map positivity of cheeses produced in Tuscany (Italy) as an indication of human exposure to the specific pathogen. Sampling was focused on artisanal cheeses produced without commercial starter culture from raw sheep or goat milk, on small-scale farms.

Samples were tested by quantitative PCR (qPCR) and culture. Map DNA was detected in 4/7 (57.14%) goat, and in 14/25 (56%) sheep cheeses by qPCR, whereas cultivation produced a positive result in only one case. This corresponded to a goat cheese that had also reacted positively by qPCR and yielded a viable Type S (sheep) strain of Map. The Map load of the tested samples based on qPCR ranged from 6×10 to 1.8×10^4 Map cells/g of cheese. The results indicate on average 56.57% and 66.6% positivity of cheese samples and farms, respectively. Hence, the type of cheeses that were analyzed within the context of this study seem to constitute a considerable source of human exposure to Map; although the question remains of whether the Map cells were present in a viable form, since positive results were almost exclusively recorded by qPCR.

1. Introduction

Paratuberculosis is an infectious disease which affects mainly domestic and wild ruminants and is caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map). Human exposure to Map has been identified as a potential risk factor for genetically susceptible individuals in connection to the development of Crohn's disease, type-1 diabetes, sarcoidosis, multiple sclerosis and Hashimoto's thyroiditis (Sechi and Dow, 2015). The reputed association of Map with the specific human diseases has generated concern about its presence in food of animal origin and its ability to survive pasteurization (Collins, 2011; Grant et al., 1996). Interestingly, positivity of raw sheep and goat milk to Map has been investigated, to the best of our knowledge, only within the context of ten studies. These were performed in connection to milk produced in England, Wales and Northern Ireland (Grant et al., 2001), Norway (Djønne et al., 2003), Switzerland (Muehlherr et al., 2003), India (Ronald et al., 2009; Singh and Vihan, 2004), Italy (Galiero et al., 2015; Nebbia et al., 2006), Greece (Dimareli-Malli, 2008), Cyprus (Botsaris et al., 2010) and Mexico (Favila-Humara et al., 2010). Map positivity of cheese produced from sheep and goat milk to Map has been investigated in five studies, in connection to products available in Greece (Ikonomopoulos et al., 2005; Liandris et al., 2014), Scotland (Williams and Withers, 2010), Cyprus (Botsaris et al., 2010) and Italy (Galiero et al., 2015). Isolation of Map in culture was substantiated only in five of the studies mentioned above, two of which referred to cheese (Ikonomopoulos et al., 2005; Williams and Withers, 2010) and the rest to raw milk (Dimareli-Malli, 2008; Galiero et al., 2015; Ronald et al., 2009; Singh and Vihan, 2004). Isolation of Map by culture has not been performed before in Italy in connection to cheese produced from sheep and goat milk. However, the prevalence of paratuberculosis has been examined with molecular and cultural methods by two teams in connection to sheep (Attili et al., 2011; Galiero et al., 2015), and to goats (Cerri et al., 2002; Nebbia et al., 2006). Notably, the investigation carried out by Cerri et al. (2002) and Galiero et al. (2015) referred respectively to a specific goat flock and a sheep farm in Tuscany, which is the geographic region targeted by the present study.

Considering public concern about human exposure to Map, the significance of traditional cheeses in the agricultural and local touristic sectors, and the fact that Map positivity of cheeses produced by small ruminant milk has not been investigated before in Italy, this study was focused on the assessment of Map positivity of cheese produced from raw small ruminant milk in traditional, small-scale farms, in Tuscany, Italy.

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2. Materials and methods

2.1. Sample collection

Sample collection was restricted to artisanal cheeses produced locally in the traditional manner from sheep or goat milk, particularly in the districts of Pisa, Lucca, Livorno (Tuscany, Italy). Within this context all types of cheese included in this study were produced without commercial starter culture from bulk unpasteurized milk, collected from animals bred locally. Ripening period of cheeses ranged between three days and two months (Tables 1 and 2).

Sample collection was performed on a voluntary basis from 9 of the 21 (42.85%) registered cheese producers by Veterinary doctors who visited each of the farms between September 2013 and April 2014, and interviewed farmers in relation to the method of milk collection and cheese production. Based on this information, the cheeses to be tested were classified as soft (water activity — aw 0.97–0.99), semi-hard (aw 0.96–0.93) and hard (aw 0.92–0.86), which was correlated to the duration of their ripening period. One sample (200 g) of cheese was selected randomly (ballot draw) among those available in the farm at the time of sampling (Tables 1 and 2), taking every possible precaution to avoid cross-contamination. The number of samples finally collected was thirty-two (n = 32), of which 25 (78.12%) were produced from sheep, and the rest (n = 7, 21.87%) from goat milk; this sampling secures 95% level of confidence (z value = 1.96; margin of error 20) for an expected prevalence of 50% (Ikonomopoulos et al., 2005).

After collection, all samples were stored in ice-containing isothermic containers for transportation, which was completed within the same working day. Upon arrival to the laboratory, samples were stored at 4–6 °C for no more than 48 h. At the beginning of processing, samples were divided using aseptic technique in four portions each of 10 g. After having discarded the external layer of cheese, the first portion was used for DNA extraction, the second for cultivation, the third for assessment of pH and aw, and the last as back-up.

Table 1
Results recorded by qPCR and culture on the samples of cheese produced from sheep milk.

Sample code number	Farm code	Type of cheese	aw	pH	Result recorded by	
					qPCR (Map cells/g)	Culture
1	B	Soft cheese	0.972	5.085	3×10^2	–
2	H	Soft cheese	0.976	5.46	3×10^2	–
3	F	Soft cheese	0.975	4.75	3×10^2	–
4	F	Soft cheese	0.978	4.86	–	–
5	B	Semi-hard cheese	0.946	5.28	–	–
6	B	Semi-hard cheese	0.95	4.98	6×10^2	–
7	B	Semi-hard cheese	0.948	5.01	1.5×10^2	–
8	B	Semi-hard cheese	0.934	5.73	1.5×10^2	–
9	C	Semi-hard cheese	0.966	5.39	6×10^2	–
10	D	Semi-hard cheese	0.953	4.865	–	–
11	F	Semi-hard cheese	0.953	5.14	3×10^2	–
12	F	Semi-hard cheese	0.954	4.825	–	–
13	G	Semi-hard cheese	0.945	5.03	1.8×10^4	–
14	G	Semi-hard cheese	0.949	5.19	–	–
15	I	Semi-hard cheese	0.963	5.135	–	–
16	I	Semi-hard cheese	0.957	5.275	–	–
17	B	Hard cheese	0.925	5.5	6×10^2	–
18	B	Hard cheese	0.923	5.6	–	–
19	F	Hard cheese	0.867	5.44	1.5×10^2	–
20	F	Hard cheese	0.926	4,94	–	–
21	I	Hard cheese	0.91	5.66	–	–
22	G	Hard cheese	0.898	4.96	1.8×10^4	–
23	G	Hard cheese	0.864	5.29	6×10^2	–
24	G	Hard cheese	0.797	4.97	1.8×10^4	–
25	H	Hard cheese	0.883	4.94	–	–

Table 2
Results recorded by qPCR and culture on the samples of cheese produced from goat milk.

Sample code number	Farm code	Type of cheese	aw	pH	Result recorded by	
					qPCR (Map cells/g)	Culture
26	A	Soft cheese	0.978	5.47	–	–
27	A	Soft cheese	0.975	5.44	1.5×10^2	–
28	A	Soft cheese	0.979	5.53	6×10	+
29	C	Semi-hard cheese	0.956	4.92	6×10^2	–
30	E	Semi-hard cheese	0.955	4.685	–	–
31	A	Hard cheese	0.926	5.48	–	–
32	A	Hard cheese	0.92	5.3	4.8×10^3	–

2.2. Culture

A 10 g portion of each cheese sample was transferred aseptically into a sterile Stomacher bag containing 90 ml of 0.9% NaCl and was homogenized using a Stomacher blender (Stomacher 400 circulator, PBI Int. USA) at 230 rev/min for 3 min. The homogenate (50 mL) was transferred into a 50 mL sterile tube and was centrifuged at 2500 g for 15 min. After having discarded the supernatant, the pellet was suspended in 25 mL of 0.75% HPC (hexa-decyl-pyridinium chloride, Sigma Chemical Co. St. Louis Mo, USA), and was incubated in the dark at room temperature for 2 h. Centrifugation was repeated (2500 $\times g$ for 15 min) and the pellet was resuspended in 1 mL of PBS-Tween. A volume of 200 μL of the latter solution was inoculated onto each of the following media: (a) Herrold's egg yolk medium (HEYM), (b) HEYM supplemented with mycobactin j (Mj) (ID vet, Grabels, France) and (c) Middlebrook 7H11 containing Mj (Ikonomopoulos et al., 2005). Incubation was carried out at 37 °C for up to ten months, with the growth media being examined weekly for bacterial growth. Identification of Map in culture was performed by Ziehl–Neelsen (ZN) stain and the qPCR assay described below (Liandris et al., 2014).

2.3. DNA isolation

DNA was isolated from cheese and ZN-positive bacterial colonies grown on culture. In the latter case, the procedure was performed as previously described by dispersing 1–2 colonies into 50 μL of distilled water and heating at 100 °C for 20 min (Whittington et al., 1999).

Before DNA isolation, 10 g of each cheese sample were homogenized as mentioned above (Stomacher blender, 230 rev/min for 3 min) into a sterile Stomacher bag containing 90 mL of pre-warmed (37 °C) sodium citrate (2% *w*/*v*, Sigma-Aldrich, St. Louis, MO, USA). The homogenate was incubated for 1 h at 37 °C (modified Donaghy et al., 2011) and was processed (10 mL) for DNA isolation using a commercially available kit according to the instructions provided by the manufacturer (Adiapure ™, bioMérieux SA, France); DNA products were stored at −20 °C.

The quality of the extracted DNA was evaluated with regard to purity and integrity by submerged gel electrophoresis followed by image analysis using a Bio-Rad ChemiDoc XRS + Molecular Imager (Bio-Rad Laboratories Inc., U.S.A.), and by OD 260/280 nm ratio, using a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific Inc., U.S.A.).

2.4. qPCR

qPCR was applied to confirm the identity of the isolates positive to Ziehl–Neelsen and for the quantitative detection of Map DNA in the cheese samples that were collected (Liandris et al., 2014). In brief, the primers F 5′-AATGACGGTTACGGAGGTGGT-3′, R 5′-GCAGTAATGGTC GGCCTTACC-3′, and the probe, 5'FAM-TCCACGCCCGCCCAGACAGG-TAMRA 3′ were incorporated in the qPCR assay, amplifying a 89 base pairs (bp) fragment within the Map IS900 element. Each reaction consisted of 1× master mix (LightCycler TaqMan master mix, Roche,