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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 6x10 to $1.8x10^4$ 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 analysed 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.

Keywords: small ruminants; *Mycobacterium avium* subsp. *paratuberculosis*; cheese; culture; 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.

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 unpasteurised 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.

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 homogenised 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 (2,500 × 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'-GCAGTAATGGTCGGCCTTACC-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, Germany), 10 pmole of each primer, 5 pmole of the probe, 5 µL of target DNA and PCR grade water to a final volume of 20 µL. Amplification was performed in a LightCycler 2.0 (Roche, Germany) using the following thermal profile: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 60 s and 72 °C for 2 s, followed by a final step of 43 °C for 30 s. QPCR for the detection of DNA belonging to Map was applied in full compliance with ISO17025 requirements.

Positive and negative controls incorporated in each assay corresponded approximately to 10% of the samples tested per batch and consisted respectively of the reaction mixture containing Map DNA (positive control) and total DNA of confirmed PCR-negative fecal samples or water (negative control). Quantification of Map DNA detected in cheese samples was performed with reference to serial dilutions of DNA extracted from Map isolated in pure culture after determining DNA quantity by Picogreen (Quant-iT TM PicoGreen dsDNA Assay Kit) according to the manufacturer's instructions (Life Technologies Benicia, USA). The results were confirmed using a plasmid recombinant constructed to contain the IS900 of Map, kindly provided by Tim Bull of St George's University of London.

2.5 Molecular typing of Map isolated in culture

Molecular typing of Map isolated in culture was performed as previously described using the following set of oligonucleotide primers designed to bind specifically to IS900 of Map strains that affect sheep (DMC529), bovines (DMC531) or both (DMC533) (Collins et al., 2002):

DMC529: 5'-TTGACAACGTCATTGAGAATCC-3'

DMC531: 5'-TCTTATCGGACTTCTTCTGGC-3'

DMC533: 5'-CGGATTGACCTGCGTTTCAC-3'

The multiplex PCR reactions consisted of 5 µL of Map DNA, 12.5 µL of EconoTaq master mix (Lucigen, Lucigen Inc., USA), primers DMC 529 and DMC 531 at a final concentration of 0.6 µM (1.5 µL), and DMC 533 at 1.2 µM (3µL). The reaction mixture was completed with the addition of PCR grade water to a total volume of 25µL. Amplification was performed using the following thermal profile: 3 min at 95 °C and 40 cycles of 30 s at 94 °C, 62 °C, and 72 °C, followed by a final extension at 72 °C for 7 min.

DNA products were submitted to submerged gel electrophoresis and its result to image analysis, using a Bio-Rad ChemiDoc XRS+ Molecular Imager (Bio-Rad Laboratories Inc., U.S.A.). The isolate was identified as belonging to Map Type C (cattle) or S (sheep), depending on whether the size of the PCR product was 310 or 162 base pairs, respectively.

3. RESULTS

3.1 Bacterial isolation and typing

Cultivation of cheese produced a positive result on M7H11 after 7 months of incubation, in connection to one (1/32, 3.12%) of the samples that were tested. The specific sample which corresponded to a cheese produced from goat milk, had also reacted positively by qPCR. The isolate was identified by PCR as a sheep strain (Type S).

3.2 QPCR

The analysis performed by qPCR indicated the presence of Map DNA in 18 (56.25%) of the 32 samples that were tested. Four (4) and 14 of the samples that reacted positively corresponded to cheese produced from goat (4/7, 57.14%) and sheep (14/25, 56%) milk, respectively. Positivity at farm level reached 66.6% (6/9) (Fig. 1). The Map load of the tested samples indicated by qPCR, ranged from 6x10 to $1.8x10^4$ Map cells/g of cheese (Table 1 and 2). The respective assessment performed in connection to the culture-positive cheese sample indicated a microbial load of 6x10 Map cells/g of cheese.

Investigation for correlation at a statistically significant level between Map positivity by qPCR and animal species (sheep/goat), using Mann Whitney U test, did not provide a positive result. The same was concluded in connection to Map positivity by qPCR and aw or pH of the tested cheese samples, using Pearson's coefficient.

In relation to cheeses produced from sheep milk the percentage of Map positivity by cheese type was (Table 1): Soft cheeses 3/4 (75%), semi-hard cheeses 6/12 (50%), hard cheeses 5/9 (55.5%); likewise in relation to cheeses produced from goats milk the percentage of Map positivity by cheese type was (Table 2): Soft cheeses 2/3 (66.7%), semi-hard cheeses 1/2 (50%), hard cheeses 1/2 (50%).

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)	Result recorded by Culture
1	B	Soft cheese	0.972	5.085	$3x10^2$	-
2	H	Soft cheese	0.976	5.46	$3x10^2$	-
3	F	Soft cheese	0.975	4.75	$3x10^2$	-
4	F	Soft cheese	0.978	4.86	-	-
5	B	Semihard cheese	0.946	5.28	-	-
6	B	Semihard cheese	0.95	4.98	$6x10^2$	-
7	B	Semihard cheese	0.948	5.01	$1.5x10^2$	-
8	B	Semihard cheese	0.934	5.73	$1.5x10^2$	-
9	C	Semihard cheese	0.966	5.39	$6 x10^2$	-
10	D	Semihard cheese	0.953	4.865	-	-
11	F	Semihard cheese	0.953	5.14	$3x10^2$	-
12	F	Semihard cheese	0.954	4.825	-	-
13	G	Semihard cheese	0.945	5.03	$1.8x10^4$	-
14	G	Semihard cheese	0.949	5.19	-	-
15	I	Semihard cheese	0.963	5.135	-	-
16	I	Semihard cheese	0.957	5.275	-	-
17	B	Hard cheese	0.925	5.5	$6x10^2$	-
18	B	Hard cheese	0.923	5.6	-	-
19	F	Hard cheese	0.867	5.44	$1.5x10^2$	-
20	F	Hard cheese	0.926	4,94	-	-
21	I	Hard cheese	0.91	5.66	-	-
22	G	Hard cheese	0.898	4.96	$1.8x10^4$	-
23	G	Hard cheese	0.864	5.29	$6x10^2$	-
24	G	Hard cheese	0.797	4.97	$1.8x10^4$	-
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)	Result recorded by Culture
26	A	Soft cheese	0.978	5.47	-	-
27	A	Soft cheese	0.975	5.44	$1.5x10^2$	-
28	A	Soft cheese	0.979	5.53	6x10	+
29	C	Semihard cheese	0.956	4.92	$6 x10^2$	-
30	E	Semihard cheese	0.955	4.685	-	-
31	A	Hard cheese	0.926	5.48	-	-
32	A	Hard cheese	0.92	5.3	$4.8x10^3$	-

4. DISCUSSION

Based on the results presented above, DNA belonging to Map is present in more than half of the cheese samples that were tested, at amounts that indicate a considerable level of contamination. Unfortunately, the latter cannot be correlated to results recorded by others, since to the best of our knowledge quantitative detection of Map in small ruminant cheese has not been performed before (Botsaris et al., 2010; Galiero et al., 2015; Ikonomopoulos et al., 2005; Liandris et al., 2014; Williams and Withers, 2010). The same can be stated with regard to the prevalence of small ruminant paratuberculosis in Italy, given that, with the exception of the studies targeted to isolated farms in Tuscany (Cerri et al., 2002; Galiero et al., 2015), the only published report refers to a neighboring geographic region (Marche), in which Map infection was detected by fecal culture and end-point PCR in 12.6% of the tested farms (Attili et al., 2011).

High Map load in the tested cheeses does not indicate a proportionally high level of raw milk contamination, since the specific pathogen is most probably concentrated in the final product. This may occur at syneresis of the curd or when the curds and whey are separated during cheese-manufacturing (Hanifian, 2014). In this regard, the recorded level of Map positivity obviously implies contamination of raw small ruminant milk in the districts of Pisa, Livorno and Lucca in the Tuscany area, but it does not prove that the specific animal product constitutes a considerable source of human exposure to Map. The latter, however, may be affirmed in connection to artisanal cheeses produced from this milk, according to traditional cheese-making procedures on small-scale farms, although this was observed almost exclusively by PCR and not culture, which does not substantiate exposure to live pathogens (Klein, 2002).

The discrepant results recorded here between culture and PCR are usually attributed to the negative impact of cheese ripening on Map viability (Botsaris et al., 2010; Ikonomopoulos et al., 2005), though its survival is clearly not always inhibited. The latter, i.e. Map survival, has been demonstrated during the ripening phase of cheeses made from bovine milk, even in connection to low-moisture, low pH and long curing period cheeses produced with commercial starters (Hanifian, 2014). A similar finding was also recorded in connection to small ruminant cheeses (Cirone et al., 2006). According to this, Map (2.5 mL of suspension containing $1.6x10^{13}$ Map cfu/mL) inoculated into 5 L of goat milk used for cheese production was still viable as indicated by cultivation at the end of a 60-day ripening period, though its population

was considerably decreased from 98 to 8 cfu/g. In support of Map being able to survive cheese ripening as well as pasteurization, Ikonomopoulos et al. (2005) reported isolation of Map from commercially available, low pH and high salt concentration Feta cheese samples, made from a mixture of sheep and goat pasteurized milk (72–74 °C for 15 s). In agreement with the above, Williams et al. (2010) have reported that sheep cheeses made from unpasteurized milk ripened for more than three months were positive to culture.

In addition to Map inactivation during cheese processing, low positivity of culture compared to PCR has been linked to several technical details of the former. Effectively, it has been reported that cultivation results in a considerable under-estimation of true prevalence of Map in cheese (Botsaris et al., 2010; Ikonomopoulos et al., 2005). In order to increase recovery of viable Map by culture, the decontamination stage was reduced in this study to only 2 h. Furthermore, Herrold Egg Yolk medium was combined with M7H11, which is recommended specifically for the isolation of Map from small ruminant specimens (Dimareli-Malli et al., 2013). Given that, even though under these conditions the level of Map positivity recorded here by culture was very low, one is obliged to consider that the discrepancy between the latter and PCR is not an artifact, but should be attributed to the inactivation of Map during cheese ripening due to the prevailing physic-chemical (aw, pH and [NaCl]) conditions. This seems to be supported by the fact that the only culture positive result that was recorded, corresponded to a high aw (0.979) and pH (5.53) cheese ripened for three days. This is notably in agreement with Donaghy et al. (2004), who reported that the highest survival rate of Map during Cheddar cheese curing was recorded in 1-day cheeses, although the organism was culturable after the 27-week ripening period.

A comparison of the level of cheese PCR-positivity reported here, to that of similar studies performed in other countries, would require the application of the same or similar PCR assays, since significant variations of their sensitivity or minimum detection limit would definitely influence the final results. Excluding the bias that is introduced to the comparison mentioned above by the specific parameter, it can be noted that traditional small ruminant cheeses and particularly semi-hard cheeses in Tuscany are PCR positive to Map at a percentage which is higher (7/14, 50%) than that reported by Botsaris et al. (2010) (1/10, 10%) at a statistically significant level ($p=0.04036$, $p<0.05$).

Whilst numbers of each type of cheese tested are small and statistical comparison is not feasible, the above findings do suggest higher Map positivity rates for soft cheeses [75% (sheep milk), 66.7% (goat milk)] compared to semi-hard [50% (both sheep and goat milk)] and hard [55.5% (sheep milk), 50% (goat milk)]. This indicates that ripening has a considerable impact on Map inactivation, which is probably associated with its effect on aw, pH and salt concentration. This is not necessary in conflict with the fact that the highest number of Map cells/g were detected in semi-hard and hard cheese ($4.8x10^3$ and $1.8x10^4$ respectively), since given that PCR is not influenced directly by microbial viability, the specific finding could be attributed to the greater physical concentration of Map in the latter types of cheese compared to those characterized as soft.

Based on the evidences that were recorded, the fact that the specific category of cheeses under study here is made from raw milk, does not seem to influence its safety significantly, though the latter would most definitely benefit by the application of pasteurization. The efficiency of pasteurization in connection to Map inactivation was shown to be impaired when applied to raw milk containing the specific pathogen at a high concentration (Grant et al., 2005). However, 1 log of Map Type C has been inactivated in bovine milk after heat treatment at 70 °C for 15 s (Whittington et al., 2010) or in connection to goat milk and pattern A (RC17 European type), at 65 °C for 20 min (Cirone et al., 2006). Furthermore, the efficacy of thermal processes can be affected by the strain: S strain of Map appeared to be less thermotolerant than the C strain (Whittington, 2010).

In conclusion the type of cheeses that were analysed 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.

5. CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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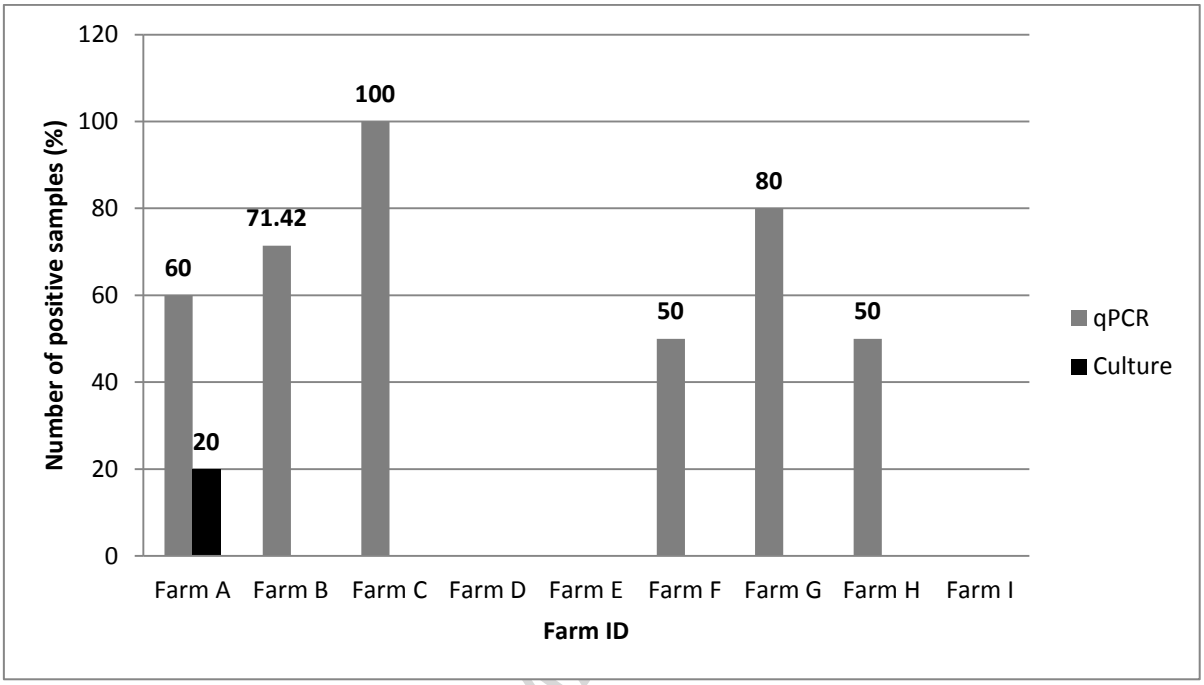

Figure 1. Percentage of positivity at farm level by qPCR and culture.

HIGHLIGHTS

- *Mycobacterium avium* subsp. *paratuberculosis* (Map) positivity was assessed by quantitative PCR (qPCR) and culture in sheep and goat artisanal cheeses produced traditionally in Tuscany, Italy.
- Positive result were recorded by qPCR in 4/7 goat (57.14%), and 14/25 sheep (56%) cheeses. Positivity of farms by qPCR reached 66.6% (6/9).
- The only culture-positive result (1/32, 3.12%) was recorded in connection to a qPCR-positive goat cheese sample. The isolate was identified as a Type S (sheep) strain.
- Results indicate that this specific category of cheeses may constitute a considerable source of human exposure to Map, although the questions remain about the viability of Map cells detected.