Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* in milk and dairy cattle in Southern Italy: preliminary results

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Abstract

Paratuberculosis affects all ruminants worldwide. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) could have a role in human diseases like Crohn's. Some extra European Union (EU) countries request importation of MAP-free products. Italy has not yet actualised a control programme and the diffusion of the infection is still unknown in Southern Italy. The aim of this study was to evaluate the prevalence of the infection in five regions of Southern Italy. Bulk tank milk and in-line milk filters were sampled in 780 dairy cattle herds and respectively analysed by ELISA and real time-polymerase chain reaction (PCR). One hundred and 55 out of 780 herds (19.9%) were found positive by ELISA and/or real time PCR. Individual milk samples were then collected from all the producing animals of positive herds and from a selection of negative herds. The estimated prevalence varies from region to region between 2.8 and 5.5%. Our results indicate that the disease is widespread in the five regions. The observed prevalence could be underestimated.

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the etiological agent of paratuberculosis, also known as Johne's disease (JD), a chronic and progressive granulomatous enteritis affecting ruminants, especially dairy cattle, and a variety of domestic and wildlife species (Beard *et al.*, 2001; Chiodini *et al.*, 1984; Kennedy and Benedictus, 2001). In cattle the disease is characterized by chronic diarrhea, weight loss, decreased milk production, and often leads to death (Ayele *et al.*, 2001; Chacon

et al., 2004), causing important economic losses in dairy and beef industry.

The zoonotic potential of MAP has been debated for almost a century due to clinical and pathological similarities between ruminants JD and humans Crohn's disease (CD) and in last years the role of MAP as a cause of human type I diabetes, Hashimoto's thyroiditis and multiple sclerosis has been hypothesized (Cossu *et al.*, 2013; Shanahan and O'Mahony, 2005; Sisto *et al.*, 2010). Patients with CD have similar symptoms to those of JD and evidence of MAP in the gut has been found in 52-92% of Crohn's patients using polymerase chain reaction (PCR) (Autschbach *et al.*, 2005; Bull *et al.*, 2003). The transmission of MAP to humans most likely occurs via contaminated milk and milk products (Gill *et al.*, 2011).

MAP can survive low-temperature holding (63°C for 30 min) and high temperature-short time (HTST) (72°C for 15 sec) pasteurization (Grant *et al.*, 1996; Grant, 2006; Millar *et al.*, 1996; Sung and Collins, 1998; Van Brandt *et al.*, 2011): some surveys showed the presence of MAP in commercially pasteurized milk purchased at retail (Carvalho *et al.*, 2012; Ellingson *et al.*, 2005; Grant *et al.*, 2002; Shankar *et al.*, 2010).

Available evidences of the involvement of MAP in human diseases have been inconclusive, but still some extra EU countries, like China and India, are beginning to request certifications to ensure importation of MAP-free products.

The prevalence of MAP infection is still unknown in many regions of the world and there is the need to study the spread of the disease for many reasons: the economic losses; the likelihood of the zoonotic potential of MAP; its capability of surviving the pasteurization; the increase of the request of MAP-free animals and products.

Many countries have developed voluntary or mandatory strategies aimed to eradicate or control the disease. In Italy, during the year 2012, national institutions (Arrigoni, 2012) have developed a voluntary national plan of probable forthcoming emission with the purposes of supporting exportations, decreasing the diffusion of the infection, creating positive conditions for a conscious commerce of animals and their products. Actually, the national plan is still in process and the knowledge on the diffusion of MAP infection in Italian herds is fragmented and incomplete; several studies have investigated MAP diffusion in Northern and Central Italy (Cenci-Goga *et al.*, 2010; Lillini *et al.*, 2005; Pozzato *et al.*, 2011), but the only information on the presence of MAP in Southern Italy date back to 1989 (Vesco *et al.*, 1990) and no data on the prevalence of the disease in dairy cattle herds are available.

In this study was conducted a survey on 780 dairy cattle herds in five different regions of

Southern Italy. The aim of the study was to evaluate the prevalence of paratuberculosis at herd and within-herd level in order to estimate the prevalence of the disease in these regions where the situation was still unknown.

Materials and Methods

Collection of samples

In this study, 780 farms located in Southern Italy were considered. Bulk tank milk (BTM) and in-line milk filter (ILMF) samples were requested to farmers. Samples were collected in sterile Falcon tubes and immediately frozen at -20°C and shipped to the laboratory within 15 days from sampling. BTM samples were obtained from 778 herds and ILMF were received from 496 herds. Many little herds did not require the use of in line milk filters, therefore only BTM were analysed in the study (Table 1). Overall the number of dairy cattle herds sampled for BTM and/or ILMF was 780. To ensure a collection of samples representing all the producing animals of each herd, a second sampling of BTM and ILMF was requested to farmers after six months from the first sampling. Details on the number of BTM and ILMF collected in the first and in the second sampling are reported in Table 1. The complete sampling was performed in 410 out of 780 farms (52.5%).

In order to estimate the within-herd prevalence of MAP infection, individual milk samples from all the lactating cows were collected from 115 of the 155 (74.2%) positive herds and from 35 negative herds Individual milk samples were collected six months to one year later

than BTM and ILFM previously collected samples. In total 12189 individual milk samples were analysed.

ELISA method

BTM and individual milk samples were tested for antibodies against MAP using a commercially available ELISA (ID Screen® *Paratuberculosis* indirect confirmation test; IDVET, Montpellier, France), according to the manufacturer's instruction.

DNA extraction

ILMF were cut into small pieces (about 15 cm²), put in filtered Stomacher bags with 100 mL of Phosphate Buffered Saline added with Tween 20 0.05% (PBS Tween 20) and homogenized at 300 rpm for 2 min in a Stomacher 400 Circulator (International PBI, Milan, Italy). Fifty mL of homogenate were collected into a falcon tube and centrifuged at 2500 x g for 15 min. The obtained pellet was resuspended in 1 mL of PBS Tween 20, vortexed and transferred into a 1.5 mL eppendorf tube.

Five µL of MyOne Tosylactivated Dynabeads (Life Technologies, Milan, Italy) coated with aMp3 peptide (NYVIHDVPRHPA) and 5 µL of MyOne Tosylactivated Dynabeads coated with aMptD peptide (GHNHHHQHHRPQ) (Research Biochemicals, Cambridge, UK) were added to the suspensions obtained by ILMF. The samples were subjected to continuous rotation on a Stuart Rotator SRT6 (Sigma-Aldrich, St. Louis, MO, USA) mixer for 30 min at 30 rpm at room temperature. The samples were then subjected to magnetic separation for 10 minutes and washed two times with 1 mL PBS Tween 20. The beads were resuspended in 300 µL of PBS Tween 20, 200 µL of sterile water, added of 300 mg of SIGMA glass beads (150-212 µm diameter) and subjected to bead beating in Tissue Lyser for 10 min at 30 Hertz.

Two-hundred µL of surnatant were collected in a new 1.5 mL Eppendorf tube and samples were then subjected to DNA extraction with a commercially available DNA extraction kit following the manufacturer's instructions (DNeasy Blood and Tissue Kit; QIAGEN, Milan, Italy).

Real-time polymerase chain reaction

Real-time PCR was performed targeting IS900 sequence with primers and probe already described (Ricchi *et al.* 2009). The amplification reactions were performed in duplicate for each sample using a StepOne Plus System (Life Technologies) in 20 µL of master mix containing 300 nM of each primer, and 6 nM of the probe. An internal positive control (Life Technologies) was also used. PCR reactions were performed at the following conditions: 40 cycles of denaturation for 15 sec at 95°C and annealing/elongation for 60 sec at 60°C. The limit of detection of this method was estimated to be 10-20 CFU/filter. The limit of detection was estimated by spiking MAP-free ILMF with ten-fold diluitions of one ATCC 19698 strain and one field strain. ILMF were then tested with to the previously described DNA extraction and real time PCR, comparing the results with culture on Herrold Egg Yolk Medium (data not shown). The efficiencies of the reactions were evaluated according to Pfaffl (2001).

Results

A total of 155 of 780 herds (19.9%) were found positive on BTM and/or on ILMF. In particular, 104/778 herds (13.4%) were positive by ELISA on BTM, 92/496 herds (18.5%) were positive by RT-PCR on ILMF and 41/494 (8.3%) were positive on both BTM and ILMF. In four of the five tested regions the percentage of positive herds varied from 20.5% to 23.4% and in one region we found positive 12.8% of dairy herds. The within-herd estimated prevalence

(EP) varied from region to region from a minimum of 2.8% to a maximum of 5.5%. In total 649 (5.3%) out of 12189 individual milk samples resulted positive. Details on the within-herd prevalence of the 115 herds resulted positive to BTM and/or ILMF analysis and of the 35 negative farms tested for individual samples which are reported in Table 2.

Discussion

In this study we analyzed BTM and/or ILMF from 780 dairy herds in five regions of Southern Italy. Real time PCR on ILMF allowed to detect 18.5% positive dairy herds versus 13.4% detected by ELISA BTM analysis.

The herd level EP showed similar distributions between four regions (20.5-23.4%). Only one region showed a remarkable lower herd level EP value (12.8%); this can be due to the lower number of ILMF samples collected in this region (only from 26.2% of herds, in contrast with 49.5-88.5% of herds from other regions). Two main factors may be the cause of the lower prevalence: i) the lower capability demonstrated by BTM analysis in detecting positive herds and ii) the fact that the absence ILMF is usually associated to herds with low numbers of milking cows, which are reported to be at lower risk for paratuberculosis infection (Collins *et al.,* 1994; Wells and Wagner, 2000).

In the surveyed five regions, the EP of paratuberculosis at herd level seems to be remarkably lower than in other surveyed italian regions. In the region of Latium the prevalence was estimated to be 42% (Lillini *et al.,* 2005). In the regions of Lombardy and Veneto the EP resulted, respectively, 48 and 65% (Pozzato *et al.*, 2011). The European (Nielsen and Toft, 2009) and US (APHIS, 2008) prevalence estimations are >50%. The difference can be due to the use of different tests and to the procedure of selection of herds. For exam-

Table 1. Number of bulk tank milk, in-line milk filters samples, and sampled farms.

	First sampling	Second sampling	Total samples	Sampled farms (n)	Farms where the complete sampling plan was performed (n)
BTM	730	687	1417	778	410
ILMF	463	448	911	496	410

BTM, bulk tank milk; ILMF, in-line milk filter.

Table 2. Within-herd estimated prevalence in farms resulted positive or negative to bulk tank milk and in-line milk filters.

Sampled farms (n)		Within-herd EP (%)			Total sampled animals (n)	Positive) animals (n
		Minimum	Mean	Maximum		
BTM and ILMF negative	35	0	1.94±0.02*	12.7	3184	77
BTM or ILMF positive	115	0	6.10±0.02*	26.1	9362	572

EP, estimated prevalence; BTM, bulk tank milk; ILMF, in-line milk filter. *Values are presented as ±standard deviation.

ple, the prevalence of MAP infection in Lombardy and Veneto regions (Pozzato *et al.*, 2011) was estimated by sampling the serum of all the animals older than 12 months of age in a group of randomly selected herds. In our study were sampled individual milk of 115 dairy farms resulted positive to BTM or ILMF screening test. Testing the 35 farms which resulted negative to the screening tests showed that 19 herds resulted with at least one infected animal and thus the number of infected herds and the EP was underestimated. One additional cause for the lower prevalence detected could be the largeness of the herds which is usually lower in southern Italy than in Northern Italy.

On the contrary, the mean observed within-herd EP in four regions (4.45%) is comparable to the one observed in Veneto region (4.1-5.9%) (Pozzato *et al.*, 2011) and higher to the ones observed in Lombardy (2.6%) (Pozzato *et al.*, 2011) and in Latium (2%) (Lillini *et al.*, 2005). However in the comparison of these data two main aspects must be taken in account: first of all, the majority of animals tested in our study were sampled from herds tested positive to the analysis on BTM and/or ILMF. In fact, we tested all the lactating animals of 115 positive herds and only of 35 negative herds. This can lead to overestimate the mean within-herd EP. The other aspect is that we analyzed individual milk samples using a commercial ELISA kit (IDVet), which has a sensitivity of 60% (Lavers *et al.*, 2012). In the other studies, ELISA was performed on serum with two different commercial kits (Pourquier and IDEXX), which sensitivities varied from study to study (Nielsen and Toft, 2008) and in the study by Pozzato *et al.* (2011) were assumed to be, respectively, 15 and 9%. Additionally, the prevalence of infected animals detected in our study was evaluated in lactating cows, thus in animals that are at least 22 month-old.

The screening sampling plan tested may be a useful tool to detect farms with higher intra herd prevalence and with higher risk to produce milk contaminated by MAP in comparison to sampling serum of animals of all the animals older than 12 months as performed in previous studies (Pozzato *et al.*, 2011). Sampling the serum requires time, money and employees. Analyzing BTM and ILMF samples is faster and cheaper. Although it probably leads to underestimate the true prevalence of MAP, the screening sampling plan performed detected as positive dairy herds with higher within-herd prevalence (6.10±0.04%), while herds with lower within-herd prevalence resulted negative (1.94±0.02%).

By the way, the lack of sensitivity of the screening sampling plan can be overcome by repeating the tests. In fact, 55 of the 115 positive herds (47.8%) resulted negative to the first sampling and positive after six month. This trend is probably due to the fluctuation of the concentration of antibodies (Gilardoni *et al.,* 2012; Sweeney *et al.,* 2006) and the intermittent shedding of MAP with milk and faeces (Gill *et al.,* 2011), as well as the turnover of milking cows.

Conclusions

Our study demonstrates that MAP is widespread in dairy cattle herds of Southern Italy. Our approach could lead to underestimate the true prevalence, but repetitions of the sampling in time can overcome the lack of sensitivity and the method is a fast and cheap alternative to screen a great number of dairy cattle herds in a short time. The study is still in progress and our data should be analysed with a Bayesian model (Branscum *et al*., 2004), in order to obtain the true prevalence of infection taking into account the EP, the variability in the accuracy of diagnostic tests and the uncertainty about the prevalence.

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