Serological survey of paratuberculosis in dairy cattle in Garfagnana district (Tuscany)

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To cite this article:

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ABSTRACT. *Mycobacterium avium* subsp. *paratuberculosis* (Map) is the agent of a chronic, progressive granulomatous enteritis in ruminants known as Johne's disease or paratuberculosis. Nowadays, the interest regarding this pathogen is increasing not only because Map causes economic losses, but it has also been suggested as a potential risk factor for the development of human diseases such as Crohn disease, autoimmune diseases like type-1 diabetes, sarcoidosis, multiple sclerosis and Hashimoto's thyroiditis.

The aim of our study was to determine the presence of paratuberculosis in Garfagnana (Tuscany, Italy) where raw milk is not submitted to pasteurization, but it is directly sold to consumers or it is destined for cheese making without the employment of heat treatment.
The survey was conducted in Garfagnana district where there are 17 herds which produce and market bovine milk for direct human consumption. Serum samples (n=162) were obtained form 16 herds and were analyzed performing ELISA ID screen® Paratuberculosis Indirect screening test (ID.VET, Montpellier, France) and positive samples were tested with ELISA ID screen® Paratuberculosis Indirect confirmation test (ID.VET, Montpellier, France), according to the instructions provided by the manufacturer.
The analysis performed by ELISA revealed that the true seroprevalence was 29.1% at the herd level and 4.6% at the animal level.
In our opinion, although ELISA is a very useful tool to screen herds for paratuberculosis, more studies should be carried out in our territory combining serological data with cultural and PCR analysis.

Keywords: paratuberculosis; cattle; ELISA; Tuscany; Italy.

INTRODUCTION

M*ycobacterium avium subsp. paratuberculosis* (Map) is a bacterium which causes a chronic, progressive granulomatous enteritis, known as Johne's disease or paratuberculosis in ruminants (Stevenson, 2015). Nowadays, the interest regarding this pathogen is increasing not only because Map causes economic losses due to decrease in milk production and poor body condition followed by death or culling (Hasonova and Pavlik, 2006), but it has also been suggested as a potential risk factor for the development of human diseases such as Crohn disease, autoimmune diseases like type-1 diabetes, sarcoidosis, multiple sclerosis and Hashimoto's thyroiditis (Sechi and Dow, 2015; Koriem, 2016). With reference to this, ruminants are the main source of infection, both directly, through the contamination of the food chain and particularly of milk and meat (Savi et al., 2015; Ricchi et al., 2016), and indirectly, through the fecal contamination of water (Whan et al., 2005).

Concerning the level of contamination of bovine bulk tank milk, it has been proven that it can contain a median load of 32.4 Map cells mL^{-1} and maximum load of 1424 Map cells mL^{-1} (Ricchi et al., 2016). With reference to this, in Italy, as suggested by previous studies, not only bovine raw milk, but also pasteurized milk is a significant source of Map exposure for consumers (Giacometti et al., 2012; Serraino et al., 2014a; Serraino et al., 2014b). Unfortunately, while the disease has been highlighted in the Tuscan caprine and ovine population (Galiero et al., 2015; Galiero et al., 2016), no official data have been published on the occurrence of paratuberculosis in cattle in Tuscany.

The aim of our study was to determine the presence of paratuberculosis in Garfagnana, which is a district of Lucca province located in northern Tuscany (Italy) where raw milk is not submitted to pasteurization, but it is directly sold to consumers or it is destined for cheese making without the employment of heat treatment.

MATERIALS AND METHODS

The survey was conducted in Garfagnana district which is a large area situated in Lucca province located in northern Tuscany (Italy) (www.ucgarfag-nana.lu.it); in this territory there are 17 closed herds which produce and market bovine milk for direct human consumption and the main domestic breeds farmed are "Pezzata Rossa", "Bruna Alpina", "Frisona" and crossbreeds. In this territory milk production relies upon small-scale family farms and extensive cattle farming based on the traditional system of pasturing in which animals graze in unfenced pastures. With reference to this, it should be born in mind that this kind of breeding promotes the potential spread of paratuberculosis between infected wildlife and livestock.

In addition, it should be noted that no animals were vaccinated against Map because in Italy cattle immunisation against paratuberculosis is forbidden.

During the period between July 2014 and December 2015 samples were collected from 16 of the 17 (94.11%) herds registered for milk production. Within each dairy herd, the animals were monitored on the basis of the current Italian regulation concerning paratuberculosis (Italian Ministry of Health, 2013); according to this, which establishes to test all lactating cows older than 36 months, all breeding males over 24 months of age and all cattle over 24 months of age introduced in the last 12 months with serological tests, 162 animals were examined.

From each cow, 5 mL of blood were collected into tubes (Vacutainer, BD Biosciences) without anticoagulant. Then, the samples were transported, under refrigeration conditions, to the laboratory where sera were separated from the clot by centrifugation at 200 g for 10 min and frozen at -20 °C until performing enzyme-linked immunosorbent assay (ELISA) tests.

Serum samples (n=162) were analyzed performing ELISA ID screen® Paratuberculosis Indirect screening test (ID.VET, Montpellier, France) following the manufacturer's instructions; according to kit manufacturer interpretation, animals with serum results of S/P ≥ 70 % were classified as positive, while those with values of S/P > 60% and < 70% were considered dubious. Then, positive and dubious samples were tested using the kit ELISA ID screen® Paratuberculosis Indirect confirmation test (ID.VET, Montpellier, France) according to the instructions provided by the manufacturer; with reference to this, samples with S/P ≥ 70% values were considered positive according to kit manufacturer interpretation. These tests included a pre-ab-

sorption step of bovine serum with a suspension of *M. phlei* which allows to reduce false positive reactions increasing test's specificity.

Apparent prevalences were calculated by dividing the number of test positive results by the corresponding total number of samples tested and the 95% CI for apparent prevalences were estimated with the Wilson method using free online software available at: http://epitools.ausvet.com.au (Epitools, Sergeant, ESG, 2017). With regard to calculation of true prevalence, true prevalences at herd, animal and farm level were calculated with the Rogan-Gladen method using the free online software Epitools taking into account that ELISA ID screen® Paratuberculosis Indirect screening test (ID.VET, Montpellier, France) has a reported

sensitivity (Se) of 41.5 % and specificity (Sp) of 99.42 (Fry et al., 2008).

RESULTS

Regarding to the screening ELISA test, three animals were positive and two animals were dubious; subsequently, all these sera were analyzed by the confirmatory ELISA test and, with reference to this, all the three positive samples were confirmed, while regarding the dubious, one resulted positive and the other one negative (Tables 1). The analysis performed by ELISA revealed that the apparent seroprevalence was 12.5% (2/16) at the herd level and 2.5% (4/162) at the animal level. The true prevalence was 29.1% at herd level and 4.6% at animal level (Table 2).

Table 1. Results recorded by ELISA tests carried out on the serum samples

Herd code	N° tested animals	Results recorded by		
		ELISA screening test (values of S/P and age of dubious and positive animals)		ELISA confirmation test (values of S/P of positive animals)
		Positive	Dubious	Positive
A	6	0	0	0
B	4	0	0	0
C	10	0	0	0
D	13	0	1 (61.87%, 8 years and 10 months)	0 (22.49%)
E	2	0	0	0
F	4	0	0	0
G	14	0	0	0
H	22	1 (71.04%, 3 years and 5 months)	1 (67.65%, 5 years and 8 months)	2 (82.84%) (73.59%)
I	1	0	0	0
L	27	0	0	0
M	5	0	0	0
N	7	0	0	0
O	8	0	0	0
P	19	2 (154.47%, 8 years and 2 months) (123.11%, 6 years and 3 months)	0	2 (171.32%) (138.03%)
Q	12	0	0	0
R	8	0	0	0

Table 2. Apparent and true herd, animal and farm level prevalence estimates.

Prevalence type	Number positive for Map	Number tested	Apparent prevalence (Wilson CL)			True prevalence (Rogan-Gladen CL)		
			estimate	Lower 95% CL	Upper 95% CL	estimate	Lower 95% CL	Upper 95% CL
herd level	2	16	0.125	0.0350	0.36	0.291	-0.105	0.687
animal level	4	162	0.025	0.01	0.062	0.046	-0.012	0.105
farm H level	2	22	0.091	0.025	0.278	0.208	-0.086	0.502
farm P level	2	19	0.105	0.029	0.314	0.243	-0.094	0.58

DISCUSSION

The current study is the first conducted on the prevalence of paratuberculosis in Tuscany cattle herds carried out using commercial ELISA; unfortunately, our results cannot be correlated to those recorded in our territory by others, since, to the best of our knowledge, detection of paratuberculosis in cattle has not been previously performed by ELISA test. In fact, the only published study reported that 3.4% of bovine population tested positive by AGID in Tuscany (Lillini et al., 2005). The discrepant results could be attributed to the fact that AGID is less sensitive than ELISA (OIE, 2104). On the contrary, paratuberculosis has been already assessed in this region not only in goats (Cerri et al., 2002) and in sheep (Galiero et al., 2015), but Map has been identified in small ruminant dairy products (Galiero et al., 2016). Based on the evidences recorded by previous studies concerning the presence of Map in food chain (Savi et al., 2015; Ricchi et al., 2016) and its potential role in the development of human diseases (Sechi and Dow, 2015; Koriem, 2016), it is advisable to pasteurize bovine milk before sale or cheese making. Similarly, nowadays no official epidemiological data have been published regarding beef cattle and wild ruminants. With reference to the latter, it could be useful to know the prevalence of the disease in these species because they could constitute an important source of infection grazing on unfenced pastures where also livestock species could access (Carta et al., 2013).

Our study indicates that the true herd seroprevalence of paratuberculosis in Garfagnana district was 29.1%; in this regard, comparing these data with those collected by Pozzato et al. (2011) who highlighted that the true herd prevalence was 70% for Lombardy and 71% for Veneto, we can say that in Garfagnana there is a lower seroprevalence. With reference to this, the variation in the herd prevalence rates could be attributed to the higher sample size and to the higher number of herds tested in the previous study. In fact, unlike Veneto and Lombardia, in Garfagnana district there are few and small sized family farms which could be tested. This is in accordance with the fact that prevalence varies by numbers of animals in the herds; in the study of Wells and Wagner (2000) dairies with < 50 cows, 18.6 ± 3.3% of herds were positive for paratuberculosis, while for dairies with 50 to 99, 100 to 299, and ≥ 300 cows, 20.5 ± 2.5%, 25.7 ± 3.4%, and 39.7 ± 4.5% of herds, respectively, were positive for paratuberculosis. Furthermore, with reference to the study of Pozzato et al. (2011), unlike our research, it should be noted that, because it was carried out on subjects older than 12 months, the prevalence could has been underestimated considering the chronic nature of the infection (Nielsen and Toft, 2008).

In addition, the low prevalence obtained in our study may be a consequence of the low sensitive of the ELISA test as well as the management practice. With reference to the former, the sensitivity of ELISA test is highest for the animals which are in the later stages or in the clinical phase of the disease; on the contrary, animals in the early stages of infection cannot necessarily be detected by this test because seroconversion may take months or years (Whitlock et al., 2000). Furthermore, concerning management practice, the fact that famers maintain closed herds avoid introduction of infected animals (Wells and Wagner, 2000).

Comparing our true herd prevalence rate with that calculated by Cenci-Goga et al. (2010) (10.1%) who

analyzed subjects ≥ 24 months, it should be noted that there is a higher prevalence of paratuberculosis in Gafagnana district than in Umbria. Furthermore, our result is higher also in comparison with that recorded by Sechi et al. (2013) who reported a true herd prevalence of 9.7%, although the age of the animal tested was not been specified.

In addition, as shown in the Table 1, in our study the highest values of S/P% were recorded by the older animals; this could be due to the fact that the sensitivity of ELISA test increases with the age of the animal (Nielsen et al., 2103).

CONCLUSIONS

Data obtained by ELISA test revealed the presence of paratuberculosis in cattle herds in Garfagnana district, contributing to improve the knowledge of the diffusion of paratuberculosis in Tuscany.

In addition, our survey draw attention to the lack of studies which analyze the presence of this disease in small sized family farms which sell, although in a small area, raw milk and cheeses produced from unpasteurized milk which are more harmful than non-handicraft products which are submitted to pasteurization, process capable of inactivating the pathogen.

In our opinion more researches should be carried out in our territory on paratuberculosis analyzing samples collected not only from livestock, but also from wildlife. In addition, it is advisable to cull or to isolate positive animals to reduce the disease's transmission to susceptible subjects and to decrease the human exposure to Map through the ingestion of contaminated food.

Furthermore, in our opinion, although ELISA is a very useful tool to screen herds for paratuberculosis, further researches should be carried out in our territory combining serological data with cultural and PCR analysis to genotype the circulating strains.

ACKNOWLEDGEMENTS

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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