PAPER

Seroepidemiological study of Johne's-disease in dairy cattle in Umbria, Italy

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Abstract

A total of 788 serum samples from dairy cattle in Umbria, Italy, were tested for the presence of antibodies to *Mycobacterium avium* subspecies paratuberculosis (*Map*) using a commercial enzyme-linked immunosorbent assay (ELISA) kit. The sampled animals came from 19 herds representative of the central area of the Umbria county (Perugia and Assisi districts). Using the manufacturer suggested cut-off for a positive test, 44 animals (5.6%) were positive. Using the sensitivity and specificity claimed by the manufacturer of the ELISA kit, the true prevalence in Umbria dairy cattle overall was calculated as 9.7% (99% CI, 7.0%, 12.4%).

Introduction

Johne's disease is a chronic debilitating disease of ruminants caused by the bacterium *Mycobacterium avium* subsp. *paratuberculosis* (*Map*). This disease has gained increased notoriety in recent years because of concerns about a possible relationship to gastrointestinal diseases of humans. The increased awareness of Johne's disease in the dairy community also has been fostered by reports of the negative impact of Johne's disease on milk production and the overall economic health of dairies. The disease is an important issue also in meat cattle since in the final and terminal aspects of the disease, animals become emaciated with fluid diarrhea and develop bottle jaw and the carcass may not pass meat inspection for human consumption. Moreover any prophylaxis measures are not effective until thoroughly applied in all farms in a given area. From this perspective paratuberculosis is not a dairies-only problem. There have been also discussions in Europe of disease control programs on a state-by-state or even national level and on the possible links between Crohn's disease and paratuberculosis (European Commission, 2000). A possible association between *Mycobacterium avium* subsp. *paratuberculosis* and Crohn's disease was first suggested in 1913, when similarities between the gross pathology and symptoms of Johne's disease in cattle and those of Crohn's disease in humans were first noted. Crohn's disease is now generally believed to have a multifactorial aetiology with genetic predisposition, environmental factors (infectious agent, diet or smoking), and abnormal inflammatory response all playing a part. Evidence supporting a link between *M. avium* subsp. *paratuberculosis* and Crohn's disease includes: higher detection rates of *M. avium* subsp. *paratuberculosis* by PCR and culture in gut samples from Crohn's patients compared to controls; demonstration of a serological response to *M. avium* subsp. *paratuberculosis* antigens in Crohn's patients; and anti- *M. avium* subsp. *paratuberculosis* antibiotic therapy resulting in remission, or substantial improvement in disease condition, in many patients (Clancy *et al.*, 2007). The available scientific evidence has been reviewed by a number of expert groups in recent years. The consensus opinion, at present, is that the available information is insufficient to prove or disprove that *M. avium* subsp. *paratuberculosis* is the cause of Crohn's disease, but the hypothesis is still plausible. The recent discovery of a susceptibility gene in Crohn's patients, NOD2/CARD15, does not preclude a role for *M. avium* subsp. *paratuberculosis* in the pathogenesis of at least some cases of Crohn's disease, since the function of this gene is bacterial sensing in the gut. If *M. avium* subsp. *paratuberculosis* does contribute to the causation of Crohn's disease then it may not be acting as a conventional infectious agent (European Commission, 2000; Clancy *et al.*, 2007). To establish program effectiveness, baseline prevalence data are needed (especially for different geographic areas and management schemes). Surveys for *Map* are difficult to accomplish because of the chronic nature of the infection and the lack of good testing methods for animals in the pre-clinical stages. Surveys done in USA in the past typically have examined limited geographical regions and reported seroprevalences up to 7.29% of cows and of 50% of herds and fecal-culture prevalences of 3.05%. These prevalences compare to herd-level prevalence of 18 and 16.7% in Belgium and the maritime provinces of Canada, respectively. In Austria, individual-animal prevalence in Holstein cattle was reported 3 or 8% depending on the enzyme-linked immunosorbent assay (ELISA) technique used and Belgium had a reported individual-animal prevalence of 0.87% (Adaska and Anderson, 2003). Management styles and facilities differ widely in the dairy industry. Smaller herds often use pasture grazing for an important percentage of the diet. The Assisi district features some of the earliest dry-lot dairies in the county. These dairies use total-mixed rations comprised of a variety of commodities as well as forage crops fed in bunks. These dairies often raise the calves in areas of the dairy that are entirely separate from the milking herd or (in about 50% of the herds) delegate raising and milk feeding of the calves to offsite calf raisers. The central part of the county (Perugia and Assisi districts) has the largest population of dairy cattle, with approximately 5000 dairy cows. The whole region has a population of approx. 10,000 dairy cows. The dairies in this area are typically newer free-stall dairies that have many management characteristics in common with the dairies in the

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northern part of Italy. The herds frequently purchase replacement cows and heifers as well as bulls. Therefore, these dairies have a high risk for bringing animals infected with Johne's disease into the herds. Dairies in these regions also tend to cull (less than 15% annually). With many cows >5 years old in these herds, the herds tend to have relatively high clinical Johne's disease. In an attempt to characterize the risk of the diffusion of paratuberculosis a study has been undertaken to determine Johne's disease-*Map* seroprevalence in dairy cattle in Umbria, Italy.

Materials and methods

Nineteen dairies in the Perugia and Assisi districts were visited and 788 blood samples collected. The 19 dairies were randomly selected using premise-identification numbers dairy database at the onset of the study (specifically all numbers for given region were printed, cut out and drawn from a *hat*). These dairies were selected to represent the central Umbria dairy industry spatially; therefore more dairies were selected from areas of higher dairy density. The selected area extends approx from the latitude 43.063611° to 42.92786° and the longitude from 12.200556° to 12.645067° (Figure 1). Samples size were calculated using the formula

$$n = Z^2 * p * (1-p)/C^2$$

where
Z is the Z-value (*e.g.*, 1.96 for a 95% confidence level);
p is the expected prevalence, expressed as decimal;
C is the confidence interval, expressed as decimal (Brixton Health, 2009).

With approximately 10,000 cows and 350 dairies and in Umbria, the samples size provide a 95% confidence level (CL) for county-wide cow-level prevalence with a confidence interval (CI) of 20 and a 95% CL for a county-wide herd-level prevalence with a CI of 3.4 All dairies were milking >50 cows. The samples were tested, within 3 hours from sampling (kept refrigerated at 4°C prior to testing), according to manufacturer's specifications with a commercial Johne's-disease ELISA kit (HerdChek M.pt, IDEXX Laboratories Italia S.r.l., Milano, Italy). Samples initially were tested in single wells and any samples with an S/P ratio between 0.15 and 0.30 were re-tested using the double-well format. In re-tested samples, the positive, negative or suspect classification was based on the results of the double-

well test. In the kit insert, the manufacturer states that: the test shows a sensitivity in excess of 50% and specificity above 99%. True prevalences were calculated using standard methods (Bottarelli, 2009).

Results and discussion

Out of the 788 cows sampled, 44 (5.6%) were positive using an S/P ratio of 0.30 as the cut-off point (as suggested by the manufacturer of the ELISA kit). Using a sensitivity value of 50% and a specificity of 99% (as claimed by the manufacturer) the true prevalence in Umbria dairy cattle overall was calculated as 9.7% (99% CI, 7.0%, 12.4%). Experience suggests that sensitivity and specificity claimed by the manufacturer are optimistic. If sensitivity and specificity for the ELISA kit of 27% and 90% are assumed, then the true prevalence in Umbria dairy cattle overall is instead 18% (99% CI, 16.3%. 19.7%) and the true prevalence values are calculated as 14.6 (99% CI, 18.1%, 21.7%). Using the manufacturers suggested cut-off point, and if an individual herd is classified as positive based on having one or more seropositive animals, then 9 (47.4%) of the herds in the area would be classified as positive. Using a test with a specificity of <100% and repeated testing results in increased chance of falsely identifying an animal and, by extension, a herd as positive. Therefore, it might be more appropriate to classify a herd as positive only if multiple animals are seropositive. Using this more-rigorous standard (≥2 seropositive ani-

mals) results in 6 (31.6%) of the herds being classified as positive.

The seroprevalence of Johne's disease we found is within range of apparent prevalence previously reported for Italy and other parts of the EU (EuropeanCommission, 2000; Dorshorst *et al.*, 2006; Khol *et al.*, 2007; Nielsen and Toft, 2009; Cenci-Goga *et al.*, 2010). Aggressive culling practices (like those used in northern Italy dairies), might mitigate some economic effects (Gonda *et al.*, 2007). Until in-depth studies of the epidemiology and economics of Johne's disease in dairies are performed, it remains to be determined whether *Map* has a large-enough economic impact to justify a mandatory state-wide control program (rather than the current voluntary strategy that relies on individual producers to decide if Johne's disease is a significant problem that justifies aggressive control strategies in their own herds) (Kudahl *et al.*, 2007).

It has long been known that *Map* can be cultured from raw milk of clinically infected cows with paratuberculosis. It is probable that the organisms are present within the monocyte population abundant in milk, but they also occur extracellularly (Woo *et al.*, 2009). More recent work has shown that *Map* can also be cultured from the milk of apparently healthy subclinically infected cows (European Commission, 2000). Clinically infected animals may shed up to 10^{12} *Map* per mL in their faeces. Subclinically infected animals also shed the organism though usually in lower amounts. Infected dairy cows and sheep shed *Map* in their milk (Mac-Johnston *et al.*, 2004). Given the high prevalence of *Map* in the dairy
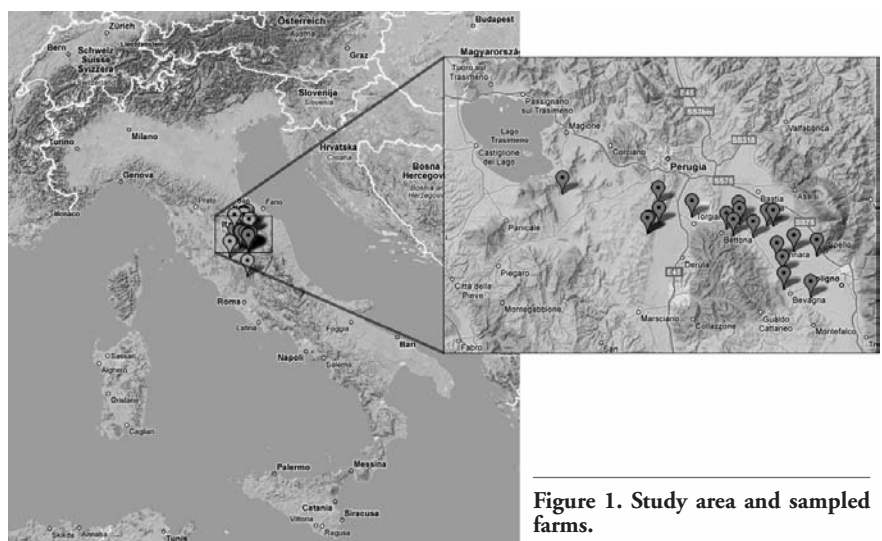

Figure 1. Study area and sampled farms.

herds and domestic livestock of western Europe and North America, it is inevitable that *Map* will from time to time be present in bulked tank milk being brought to pasteurization plants (Shankar *et al.*, 2009). This raw milk is normally subjected to treatment either by pasteurization, UHT treatment or sterilization. The proportion of milk consumed in each of these categories varies considerably throughout Europe. Because of difficulties in culturing the organism, it has proved problematic to experimentally determine the efficiency of treatments such as pasteurization. Several studies have shown that *Map* prepared in *in vitro* cultures, spiked into whole cows' milk at a range of microbial concentrations and then treated with experimental pasteurization, remained cultivable from some samples, after exposure to 65°C for 30 min (Standard Holder method) or 72°C for 15 sec (High Temperature Short Time method) (European Commission, 2000; Méndez *et al.*, 2006).

These studies have been criticized principally on the grounds that experimental pasteurization does not accurately reproduce the conditions such as turbulent flow that occur in commercial pasteurization units. In addition, because of the presence of the organism in monocytes, spiking studies may not be representative. A recent interim report of ongoing work in the UK has reported the finding of viable *Map* in around 5% of raw milk samples examined and in about 3% of pasteurized samples examined, although no information is yet available on the numbers of organisms present (Cerf *et al.*, 2007). Because of its presence in raw milk, *Map* may be initially present in cheeses made from raw milk from infected animals, or in those made from milk exposed to pasteurization at lower temperature prior to the cheese-making process (Eltholth *et al.*, 2009). *Mac* (*Mycobacterium avium* complex) are generally resistant to acid conditions and are known to be able to resist the acidic conditions created in intracellular phagolysosomes as part of their strategy for survival within the host cell. *Mac* may thus survive low pH conditions involved in cheese-making, although the recovery of *Mac* from cheese, by growth in conventional culture, may be unreliable. At the present time there are no publications from laboratory based research specifically addressing the question of whether *Map* is present in cheese, but epidemiological studies for environmental risk factors for *Mac* in patients with HIV disease, identified an increased risk associated with the consumption of hard cheeses (Cerf *et al.*, 2007). Macrophages containing *Map* are known to be found throughout the body of animals with the advanced pluribacil-

lary form of Johne's disease. Most tissues including lymph nodes, spleen, bone marrow, liver, kidney and lung are affected and, although titers of bacteria are low, *Map* can be successfully cultured.

In areas with high *Map* prevalence it has been postulated to apply to all farms a program to prevent and control this costly disease (Wells *et al.*, 2008). Basically, any program should be aimed at controlling Johne's bacteria in manure, colostrum and milk management, and management of infected animals. The reduction of exposure of newborn calves to manure that may contain the bacteria is a fundamental step, followed by providing clean feed for young stock and mature animals, along with clean water for both young and mature animals. It is therefore vital to keep manure from mature stock from contacting young stock. The control of Johne's bacteria by colostrum and milk management is obtained using low risk colostrum and low risk milk, *i.e.* using only colostrum from healthy cows that were negative on a recent Johne's test and taking into account that pooling increases the chances of infecting many calves from a single infected cow. Especially in meat cattle farming colostrum from "low risk" cows can be used by freezing or refrigerating. Another indication is the use of colostrum supplements particularly if the beef cattle herd is highly infected. The same suggestions apply to milk: it is better to use a milk replacer instead of unknown status milk and pasteurized milk or milk only from cows with recent negative Johne's test. Thoroughly clean the udder and teats before collecting milk for calf feeding is another good procedure. The management of infected cows is achieved by the identification and removal of clinical and late stage animals as soon as possible and by testing to spot subclinical infections and define the herd status.

Conclusions

There are very few valid Johne's disease prevalence estimates available in Europe, mainly due to problems with test characteristics and partly due to study design unsuitable for prevalence inference. This inconsistency is due to the different procedures, changes to methods, and use of different reagents and the choice of different cut-off levels. These conditions render the results almost random, and we would venture to say useless, if one thinks of doing large-scale epidemiological estimates. Observational statistics studies, such as the longitudinal designs or the cross-sectional

studies, which are of the utmost importance to draw inferences when it is impossible for the investigators to assign subjects into a treated group versus a control group, loose most of their power when applied to diseases such as Johne's disease. For these reasons we believe that basic and robust descriptive studies, such as this one, are ideal to determine whether *Map* has a large enough impact to justify or not a mandatory control program in a given area.

Since to establish program effectiveness data are needed (especially for different geographic areas and management schemes), this research represents the first attempt to collect baseline prevalence data in Umbria, Italy for further considering the implementation of Johne's disease in dairy cattle herds.

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