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RESEARCH ARTICLE

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Latent infections are the most frequent form of paratuberculosis in slaughtered Friesian cattle

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Abstract

Paratuberculosis is a chronic mycobacterial infection causing granulomatous enteritis in ruminants, whose pathogenesis and epidemiology poses numerous challenges, including latency and reactivation. The most recent and complete classification of paratuberculosis immunopathological types in cattle recognized five categories. In this study, 1031 slaughtered Friesian cattle were submitted to serological, microbiological and pathological examinations with the aim of maximizing the rate of case detection. In most cases, infected animals had minimal lesions and almost no other proof of infection (38.9%), while the more characteristic types with the whole constellation of microbiological and immunological evidences accounted for a lower proportion (7.7%). As these findings in cattle suggest similarities with the epidemiology of tuberculosis in humans, we propose to re-group the original immunopathological types into two broader paratuberculosis epidemio-pathogenic forms or states: latent and patent. The former term would define infections with focal lesions and might constitute an apparent resilience status representing a difficult to detect reservoir of infection whose role could become critical if later immune-compromising factors lead to re-activation. The latter would group those cases with multifocal and diffuse inflammatory lesions with higher mycobacterial load and viability suggestive of a more immediate epidemiological risk. Interestingly, the relative frequency of presentation of each profile varied with age. The proportion of latent forms remained relatively constant between 33.8% and 54.3% through adulthood from 3 years of age, while patent forms were more frequent during the first years of age and tended to decrease among the oldest individuals.

Additional key words: bovine paratuberculosis; immunopathology; microbiology; epidemiology; pathogenesis; latency.

Introduction

Paratuberculosis (PTB) or Johne's disease is a widespread granulomatous enteritis of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Chiodini *et al*., 1984). This chronic infection that could affect more than half of European dairy cattle herds (Nielsen & Toft, 2009) causes considerable economic losses (Lombard, 2011) and raises growing public health concerns due to the presence of viable MAP in the human food chain and its possible role in the pathologically similar human Crohn's disease (Collins, 2011; Chiodini *et al*., 2012).

It is generally accepted that most MAP infections occur within the first weeks of life by the fecal-oral route and that afterwards, new infections become less

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This work has one supplementary table that does not appear in the printed article but that accompanies the paper online.

Abbreviations used: AFB (acid fast bacilli); DI (distal ileum); EP (epidemio-pathogenic); GHPs (good hygiene practices); HE (hematoxylin and eosin); HEYM (Herrold Egg Yolk); HPC (hexa-decyl pyridinium chloride); ICV (ileocecal valve); I-LN (ileal lymph node); JC-LN (jejunal caudal lymph node), LJ (Löwenstein-Jensen); MAP (*Mycobacterium avium* subsp. *paratuberculosis*); PPA3 (protoplasmatic antigen 3); PTB (paratuberculosis); rtPCR (real time PCR); ZN (Ziehl-Neelsen).

frequent due to an age-related resistance increase (Windsor & Whittington, 2010). In any case, most infected animals do not develop clinical signs, nor become positive to laboratory tests until reaching the more advanced stages of the infection. This type of pathogenesis lends to PTB a spectral character that has been repeatedly pointed out since the first pathological studies of sheep cases series in the 1950s by Stamp & Watt (1954) and suggests a latency state similar to that recognized in human tuberculosis (Flynn & Chan, 2001).

For cattle, immunopathological types were summarized into five types: focal, multifocal, diffuse lymphocytic or lymphoplasmacytic, diffuse intermediate and diffuse multibacillary or histiocytic, based on an exhaustive histological characterization of lesions (González *et al*., 2005) and its nomenclature revision (Vazquez *et al*., 2013). This classification acknowledges the existence of one type, the focal type, that would represent an infectious state similar to that of latency that is considered the most frequent in human tuberculosis (Flynn & Chan, 2001; Corbett *et al*., 2003), but that has not been well defined in cattle mycobacteriosis (Cassidy, 2006; Van Rhijn *et al*., 2008). Indeed, other terms representing that concept but with a broader and more epidemiological perspective, have been defined by Nielsen & Toft (2008) as: free of infection, infected (non-shedders), infectious (MAP shedders) and affected (clinical PTB and MAP shedding). However, no pathological or microbiological evidence supports these concepts in the case of PTB, in spite of the pathogenetic and epidemiological relevance of such a state as discussed for tuberculosis (Jones *et al*., 2011). Actually, the pathological studies leading to the description of these PTB types and also inferring into some immunological and microbiological associations have not been done in such a large and broad representation of the susceptible adult cattle population (over 1,000 animals) but on two Spanish cattle populations. One consisting of animals from affected farms submitted to PTB control programs including culling follow up (González *et al*., 2005) and the other selected according to positive and negative reactions in the bovine tuberculosis intradermal test (Balseiro *et al*., 2003; Balseiro, 2004). This is so because histopathological studies are costly due to the destructive nature of the post-mortem procedures and to the need of a highly trained pathologist to carefully examine all the samples. However, in order to get a less biased picture of the frequency of the different immunopathological types in natural cases, a broader approach is necessary. In this sense, the most practical strategy without destroying whole healthy and affected herds is through a slaughterhouse study at the end of the productive life of the individual animals. Even though this kind of study suffers from several biases like farmer culling strategies, commercial differentials between slaughterhouses, transport costs, prevalence of different diseases, public health policies, etc., it is still the most economical way to have access to a large set of cattle biological samples that includes internal organs.

Since most current PTB control programs involve some form of testing for detection of infected individuals, the performance of these tests is a critical factor to early and efficiently detect infectious animals (Whitlock *et al*., 2000). Moreover, as there are evidences that age and immunopathological types of PTB are associated with different frequencies of positive results in both immune and microbiological tests, estimating their performance in natural cases is very important to design improved control strategies.

This report presents the findings of a large cross-sectional study on adult Friesian cattle processed in two Basque Country slaughterhouses regarding immunologic, microbiologic and histopathological variables in order to determine the prevalence and age distribution of the different immunopathological PTB forms and, more specifically, of the one that might correspond to a pathological and microbiological form of latency, and to assess its implications on PTB manifestation throughout the lifespan of dairy cattle. Although subject to local conditions, these associations are of interest because they may help to understand the pathogenesis and epidemiology of the different PTB infection conditions and the subsequent interpretation of standard diagnostic tests in order to increase the efficiency of control measures.

Material and methods

Animals and sample collection

A systematic weekly sampling was carried out at two local abattoirs in the Basque Country (Bilbao, Bizkaia, Basque Country, Spain and Donostia-San Sebastián, Gipuzkoa, Basque Country, Spain) from March 2007 to November 2010, at a rate of about 10 animals per week. Both abattoirs were operated by two muni-

cipally owned companies and complied with the pertinent Basque (Basque Government Decree 454/1994), Spanish (Spanish Government Law 32/2007 and Royal decree 731/2007) and European (Council Regulation (EC) No 1099/2009) legislation on animal welfare, under the supervision of official veterinarians. Permission to collect and use the samples was obtained from the managers of each slaughterhouse: Matadero Municipal de Bilbao and Matadero Frigorífico Donostiarra S.A.L (MAFRIDO), respectively.

The selection of animals was focused on Friesian cattle aged 30-60 months to maximize genetic homogeneity for pathogenesis and to increase the chances of picking up infected animals by maximizing exposition according to the standard model of early life infection on the lower side and clinical progression on the upper side. Usually the first 4 or 6 animals in the line satisfying breed and age requirements were selected. Neither body condition nor farm of origin was taken into account. This resulted in a total of 1,031 animals included in the study. All were born in herds located in the Basque Country (36.4%), other regions in North-East Spain (57.0%) and other European countries (6.6%).

Duplicate whole blood samples were collected from the jugular vein from each animal into sterile Vacutainer EDTA tubes (BD Vacutainer®, Franklin Lakes, NJ, USA) at the beginning of the slaughter line, right after stunning and before bleeding. According to good hygiene practices (GHPs), the esophagus was tied off ("rodding") and the rectum was sealed off ("bagging") previous to the evisceration of the gastrointestinal tract.

Once at the gut and tripe room, the intestinal tissue and associated mesenteric lymph nodes from each animal were introduced into a labeled plastic bag and individually transported in plastic baskets to NEIKER-Tecnalia necropsy room. There, fresh samples of about 5 g were aseptically taken from the jejunal caudal lymph node (JC-LN), distal ileum (DI) and ileocecal valve (ICV) within 24 hours. In order to prevent cross contaminations, all non-disposable instruments were washed and immersed in Virkon® (Bayer Healthcare LLC, Shawnee Mission, KS, USA) after each animal tissue collection. Scalpel blades were cleaned and disinfected after each tissue use and changed between animals. Gross examinations of tissues were focused on typical PTB changes locations: mucosal surface corrugation and wall thickening of the ileum, enlarged mesenteric lymph nodes and prominent lymph vessels. Samples from DI, ICV, JC-LN and ileal lymph node (I-LN) were collected and fixed in 10% neutral buffered formalin for histopathological examination.

Slaughterhouse veterinary inspectors provided the date of birth of each animal as recorded in the EU bovine identification documents (Council Regulation (EC) No 1760/2000).

Histopathological examination

The selection of tissue sections submitted to histological examination was based on the reported preferential entrance point of MAP (the intestinal Peyer's patches) (Sweeney *et al*., 2006) for ICV-DI samples, and on the more frequently reported affected lymph nodes (JC-LN and I-LN), respectively. Formalin-fixed tissues were routinely dehydrated, embedded in paraffin, cut at 4 µm and mounted in standard glass slides. All sections were stained with hematoxylin and eosin (HE). If PTB compatible lesions were identified, Ziehl-Neelsen (ZN) staining was applied to an additional section in order to identify acid-fast bacilli (AFB). Histopathological lesions were classified as focal, multifocal, diffuse lymphoplasmacytic, diffuse intermediate and diffuse histiocytic, according to the classification of PTB immunopathological types proposed for cattle by González *et al*. (2005) and modified by Vazquez *et al*. (2013). Briefly, focal lesions were small well-delimited granulomas containing mostly macrophages, some lymphocytes and Langhan's giant cells. The localization of these lesions was restricted to the interfollicular areas of ICV or, more frequently, to the cortical area of associated mesenteric lymph nodes. The cell-composition of multifocal granulomas was similar to that observed in the focal type, but they were larger in size and number. These lesions were usually located in the same areas, but tended to extend to the apex of some ileal villi. Normal architecture of both small intestinal and associated lymph nodes was strongly altered in diffuse lesions that were specifically named according to the predominant cell-type infiltrate: histiocytic, lymphoplasmacytic or intermediate.

Immune response: indirect ELISA

A two-step commercial ELISA kit (Pourquier® ELISA paratuberculosis, currently IDEXX Paratuberculosis Screening Ab Test and IDEXX Paratuberculo-

sis Verification Ab Test; IDEXX Laboratories, Inc., Westbrook, ME, USA), which includes an initial stage of plasma pre-absorption with *Mycobacterium phlei* and based on *M. avium* strain 18 protoplasmic antigen (PPA-3) (Mon *et al*., 2012), was used for detecting specific antibodies against MAP in plasma samples according to the manufacturer's instructions. In each assay, negative and positive controls included in the kits were tested in duplicate. For the screening ELISA, interpretation of positive (≥ 70%), doubtful (> 60% and < 70%) and negative results (≤ 60%) was done according to sample to positive (S/P) ratios, as indicated by the manufacturer. This criterion was also used for interpretation of verification ELISA results during the period 2007-2009. However, it is important to mention that a modification for defining the three categories was indicated by the manufacturer for samples analyzed during 2010. Hence, the new cut-off points were as follows: positive if ≥ 55%, doubtful if > 45% and < 55%, and negative if ≤ 45%. Serological response to the ELISA testing was defined as positive if positive or doubtful results were found at the verification ELISA and negative if negative interpretation resulted from the screening or the verification ELISA.

Microbiological findings: MAP isolation and IS*900* amplification

A pool consisting of mucosa from the ICV-DI area and JC-LN in the same proportions was used for microbiological testing. In order to avoid cross contaminations between pools, in addition to one-use scalpel blades and sterile Petri dishes, pincers, scissors and scalpels were cleaned with 10% chloramine, dipped into 70% ethanol and flame sterilized between samples.

A 2 g tissue sample was decontaminated overnight with 0.75% hexa-decyl pyridinium chloride (HPC), as previously described (Juste *et al*., 1991). Cultures were performed in homemade Herrold Egg Yolk (HEYM) (Becton Dickinson, Franklin Lakes, NJ, USA) and Löwenstein-Jensen (LJ) (Difco Laboratories, Inc., Detroit, MI, USA) media, both supplemented with Mycobactin J (Allied Monitor, Inc., Fayette, MO, USA), in duplicate. Both media are the most widely recommended for primary isolation of MAP cattle strains (Type C or II) (Whittington, 2010). After allowing drying-off the excess of water by incubation in a horizontal position at 37°C for one week, tubes were checked for contamination and, if negative, had their caps tightened. The first growth control was performed at 8 weeks under a stereoscopic microscope. Subsequent observations were performed every 4 weeks up to the 20th week of incubation. Cultures were considered positive if one or more characteristic MAP colonies were noticed in any of the four tubes. Confirmation of colony identity was based on positive polymerase chain reaction (PCR) amplification of MAP specific IS*900* insertion sequence as described before (Moss *et al*., 1992).

Another 2.5 g tissue pool was used for MAP DNA isolation and amplification by real time PCR (rtPCR). Previous to DNA extraction each pool was homogenized in 10 mL of sterile water for one minute in a Stomacher® 80 Biomaster (Seward, Worthing, UK). The whole homogenate was transferred into a 15 mL sterile tube. Then, an adapted protocol of Adiapure® MAP DNA extraction and purification kit (Adiagene, Saint Brieuc, France) for fecal samples was applied to tissue samples. Briefly, 300 µL of the homogenized sample were transferred to 2 mL vials containing 300 mg of glass beads (Adiagene, Saint Brieuc, France) and 300 µL of Lysis 1 buffer. These tubes were submitted to cell disruption in a Hybaid RiboLyser (Hybaid, Teddington, UK) at 3 cycles of 45 s at 4 ms^{-1} (4,000 rpm), followed by a centrifugation at 7,500 g for 5 min. Next, 300 µL of the supernatant were transferred to Eppendorf tubes with 20 µL of Lysis 2 buffer where they were incubated at 70 ± 2°C for 10 min and subsequently, at 95 ± 2°C for 15 min. After an additional pulse centrifugation, 300 µL of supernatant of each sample and the extraction control were transferred to a well of the filtration plate. After filtration on a vacuum manifold at –81 kPa for a maximum of 15 min, and a new filtration step at –68 kPa, two wash series of 100 µL with Wash buffer were carried out.

Purified DNA was mixed with the Eluent buffer, and approximately 100 µL of eluted DNA were collected into autoclaved Eppendorf vials where they were kept at 6 ± 2°C in case of immediate PCR processing, or at –20 ± 5°C if PCR was not performed in the following 24 hours. MAP DNA detection based on the amplification of the specific IS*900* insertion sequence was carried out using the ADIAVET® PARATB Real Time commercial kit (Adiagene, Saint Brieuc, France) and the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as previously described (Juste *et al*., 2009). Samples were scored positive if their threshold cycle (Ct) was less than 40.00.

The results from both microbiological tests were used to calculate a MAP viability percentage that would further support the infection latency form from a microbiological point of view. This parameter was defined as the quotient of the culture result (0: negative; 1: positive) by 1 counting only the positive PCR results. For each age group and/or PTB form it was the proportion of positive isolations out of the number of PCR positive results (%MAP viability = number of positive cultures/number of positive rtPCRs × 100).

Paratuberculosis latency definition

Ruminant PTB is an spectral disease that, for practical purposes, has been classified into five granulomatous inflammatory forms ranging from the diffuse types of González *et al*. (2005) that are the active or patent cases readily detected by clinical, pathologic, serologic and microbiologic methods, to the cases in which no evidence of infection can be found and that are, therefore, considered as healthy. Then it would remain a middle ground where clear but limited pathological evidence accompanied or not with immunologic and microbiological positive results could be comparable to the reported human tuberculosis latent forms. Therefore, considering that presence of a lesion was indicative of some degree of host response to infection, while MAP cells could be recently ingested passing through bacteria (Sweeney *et al*., 1992) and raised specific antibodies might represent contact without infection, three PTB epidemio-pathogenic (EP) forms were defined by regrouping the immunopathological types according to the frequency of positive immunological and microbiological results as follows:

— Apparently free of PTB: Absence of any pathological evidence of MAP infection in the examined sections (lesion free) and very low frequency of microbiological or immunological positive results.

— Latent PTB: Presence of focal lesions with low or moderate frequency of microbiological or immunological evidence of infection.

— Patent PTB: Presence of multifocal and diffuse lymphoplasmacytic, intermediate and histiocytic inflammatory lesions with a high frequency of microbiological or immunological evidence of infection.

Each individual animal in this study was allocated to one EP form according to its primary immunopathological type (González *et al*., 2005).

Statistical analysis

Significance of differences in the frequency of positive immunological and microbiological results for each lesion type (lesion *vs.* no lesion and focal, multifocal and diffuse) as well for latent and patent PTB EP forms were assessed by level means comparison with the Tukey-Kramer correction for multiple comparisons in the Proc GLM of the SAS 9.1 statistical software (SAS Institute, Cary, NC, USA). Estimations of 95% Wilson confidence intervals (CI) for the proportion of positive testing to ELISA, microbiology and histopathology of each age group and total data were done with Epi Tools (AusVet Animal Health Services; http://epitools.ausvet.com.au/).

Association of age with prevalence of latent and patent EP form was examined attending to age in years from 1 year to 11 or more years to cover the whole range of ages found in the present study. This classification yielded unequal size groups that had different amounts of variability. However, it was preferred over other statistically simpler alternatives, because it was considered to better match the natural seasonal and productive cycles of farming and animal growth and husbandry. Except for the youngest animals, all classes had at least 25 individuals. This was estimated as a sample size giving a resolution of less than 5% in proportion estimates and providing a small deviation from infinite degrees of freedom in normal distribution probability calculations. The youngest group was kept separate because it represented the different physiological status of heifer (versus cow in the rest of the sample) and actually showed an unexpectedly high prevalence in PTB indicators. This distribution provided a good resolution plotting for the age in years and the frequency of cases that was tested for correlation with the Pearson option of the SAS CORR procedure, to compare fitting for total PTB age-prevalence and for each of the two forms (latent and patent). Additional confirmation of the age effect was obtained by testing age in months and frequency of each of the forms with the Kendall rank correlation coefficient.

PTB EP forms were taken as the diagnostic reference for the ELISA test, over which the sensitivity, specificity, positive and negative predictive values of the ELISA results were estimated. Additionally, the agreement between serological testing and histopathology results was evaluated by Cohen's kappa (K) statistic and interpreted as follows: < 0.2, poor; 0.2-0.4,

fair; 0.4-0.6, moderate; 0.6-0.8, good; and > 0.8, excellent. Calculations of immunological diagnosis sensitivity and specificity error estimates were performed with the VassarStats (Statistical Computation Web Site by Richard Lowry; http://vassarstats.net). The level of significance for all statistic tests was set at the standard $p < 0.05$, although the specific values are given for each analysis.

Results

Histopathological lesions

PTB lesions were found in 46.7 % of the animals (Fig. 1). Most lesions were of a focal type (38.9%) whereas inflammatory lesions classified as multifocal and diffuse accounted for 3.4% and 4.3%, respectively. Among the diffuse-type, histiocytic enteritis represented 78.6% of these types (Table 1). MAP bacilli were found in 10.4%, 64.7% and 100% of ZN-stained tissue sections with focal, multifocal and diffuse lesions, respectively. Local autolysis or loss of tissue in the sections of an animal accounted for the loss of 45 cases which were not retested.

Immunological findings

Specific antibodies against MAP were detected in 76 animals (7.4%) (Table 1). Seropositivity rate significantly differed between the apparently free (1.7%) and PTB lesion groups (14.1%) ($p < 0.0001$). The likelihood of testing positive in the ELISA test was closely related to the development of multifocal (41.2%) ($p < 0.0001$) and diffuse types (92.9%) ($p < 0.0001$), but not with focal types ($p = 0.1832$), when compared with the apparently free group (without detectable lesions).

Microbiological findings

Similar patterns of MAP detection rates according to histology were found by both tissue culture and molecular testing, but the frequency of rtPCR positive results was always higher than that of culture (Table 1). The mycobacterial detection by tissue culture and rtPCR were respectively 3.7 and 1.8 folds higher for animals with PTB lesions related to those apparently free ($p < 0.0001$). Animals with focal lesions, presented a higher proportion of positive results in both microbiological tests (tissue culture: $p = 0.0005$; rtPCR: $p = 0.0247$) but the highest differences with the apparently free group were those of the other two main lesion types: multifocal (tissue culture and rtPCR: $p < 0.0001$) and diffuse (tissue culture and rtPCR: $p < 0.0001$).

Regarding MAP viability estimates, the proportion was more than twice in animals with lesions (66%) than in those without them (32%). Animals apparently free of PTB as well as those with focal lesions were associated with MAP viability estimates below 50% whereas multifocal and diffuse lesion groups had rates above 80% (Table 1).

Table 1. Percentage of paratuberculosis (PTB) immunopathological types, humoral responses, *Mycobacterium avium* subsp. *paratuberculosis* (MAP) detection rates and MAP viability estimates. Comparisons between each PTB immunopathological type and the no-lesions group. Grouping according to PTB forms. The number of animals appears in parentheses. Mean S/P: Mean optical density of individual serum divided by optical density of positive control

Immunopathological type	No. of animals (%)	Serology		Microbiology			PTB form
		% ELISA+	Mean S/P	% Isolation	% rtPCR+	% MAP viability	
No lesions	526 (53.3)	1.7 (9)	12.70	7.0 (37)	22.2 (117)	31.6	Apparently free
Focal	384 (38.9)	3.1 (12)	12.99	14.3 (55)***	28.9 (111)*	49.6	Latent
Multifocal	34 (3.4)	41.2 (14)***	71.87***	64.7 (22)***	79.4 (27)***	81.5***	Patent
Diffuse	42 (4.3)	92.9 (39)***	173.40***	100.0 (42)***	100.0 (42)***	100.0***	Patent
D. lymphoplasmacytic	3 (0.3)	100.0 (3)	157.78	100.0 (3)	100.0 (3)	100.0	Patent
D. intermediate	6 (0.6)	83.3 (5)	167.96	100.0 (6)	100.0 (6)	100.0	Patent
D. histiocytic	33 (3.3)	93.9 (31)	175.72	100.0 (33)	100.0 (33)	100.0	Patent
Total PTB lesions	460 (46.7)	14.1 (65)***	31.63***	25.9 (119)***	39.1 (180)***	66.1***	Latent/Patent

D.: diffuse immunopathological subtypes. rtPCR: real-time PCR. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Comparison to no-lesions group).

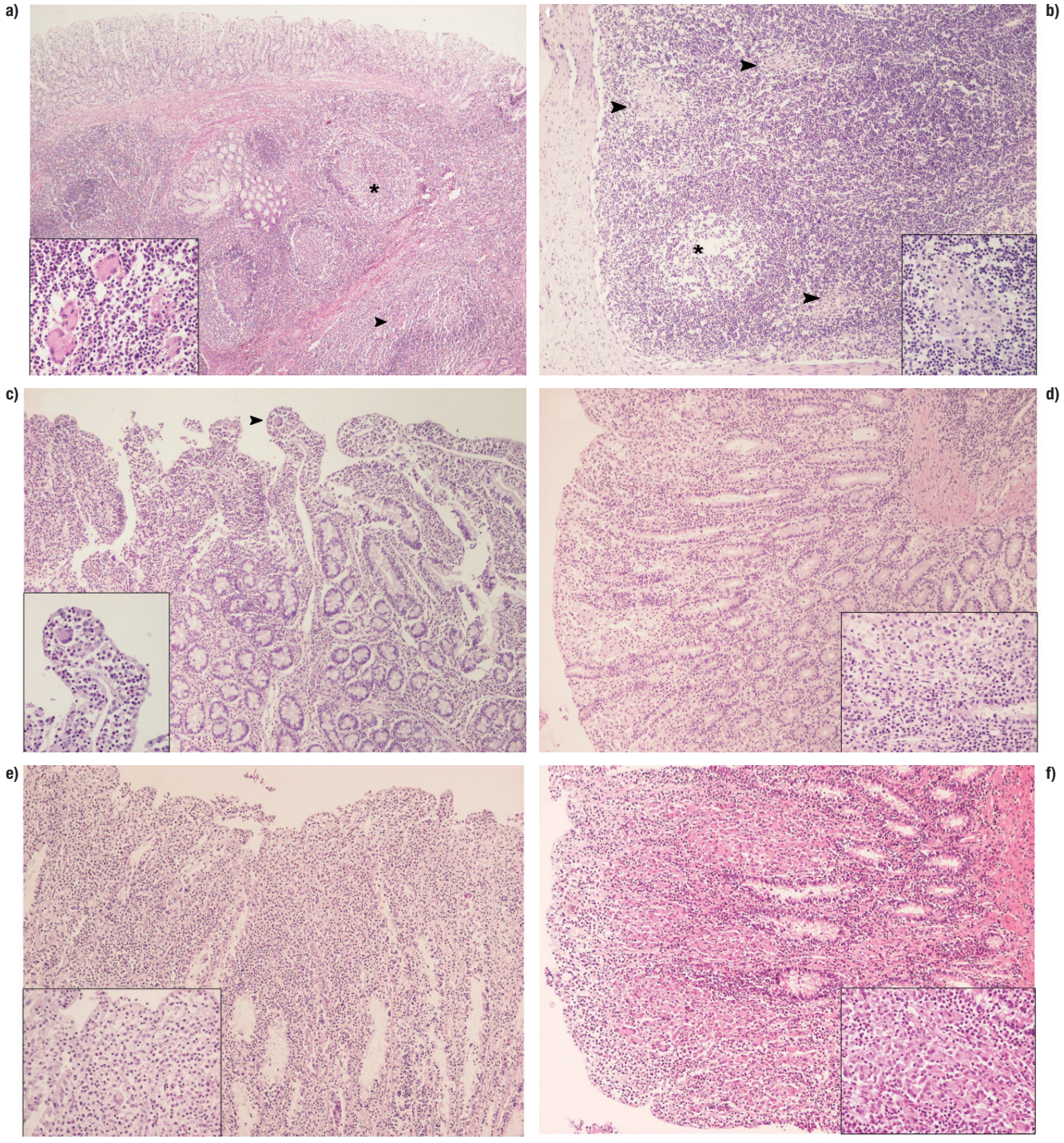

Figure 1. Paratuberculosis lesions: details of focal, multifocal and diffuse lymphoplasmacytic, diffuse intermediate and diffuse histiocytic lesions. a) Focal lesion. Distal ileum, ileo-cecal valve (DI-VIC). HE 40x. Detail of multinucleated cells (➤) located in the lymphoid tissue of DI-VIC (* lymphoid follicle). HE 400x. b) Focal lesion. Jejunal caudal lymph node. HE 100x. Detail of a granuloma (➤) formed by macrophages located in the cortical area near to a lymphoid follicle (*). HE 400x. c) Multifocal lesion. DI-VIC. HE 100x. Detail of a giant cell (➤) located in the apex of a villi. HE. 400x. d) Diffuse intermediate lesion. DI-VIC. HE 100x. Detail of the inflammatory infiltrate causing thickening of the mucosa. Notice the presence of macrophages and lymphocytes. HE 400x. e) Diffuse lymphoplasmacytic lesion. DI-VIC. HE 100x. Detail of the inflammatory infiltrate formed mainly by lymphocytes, causing thickening of the mucosa. HE 400x. f) Diffuse histiocytic lesion. DI-VIC. HE 100x. Detail of the inflammatory infiltrate formed mainly by macrophages causing thickening of the mucosa. HE 400x.

Table 2. Paratuberculosis (PTB) prevalence values and their corresponding 95% confidence intervals (CI), according to the diagnostic method, PTB form and age at slaughter[1]

Group	Age (years)	No. of animals	ELISA		Microbiology				Paratuberculosis form					
			% Pos	95% CI	%rtPCR+	95% CI	%MAP viab.	95% CI	% App. Free	95% CI	% Latent	95% CI	% Patent	95% CI
1	< 2	8 (8)	12.5	2.2-47.1	37.5	13.7-69.4	66.7	20.8-93.9	62.5	30.6-86.3	12.5	2.2-47.1	25.0	7.1-59.1
2	≥ 2, < 3	77 (74)	9.1	4.5-17.6	24.7	16.4-35.4	68.4	46.0-84.6	66.2	54.9-76.0	24.3	16.0-35.2	9.5	4.7-18.3
3	≥ 3, < 4	173 (167)	12.1	8.1-17.8	33.5	26.9-40.9	63.8	50.9-74.9	49.7	42.2-57.5	38.3	31.3-45.9	12.0	7.9-17.8
4	≥ 4, < 5	241 (227)	6.6	4.1-10.5	30.3	24.8-36.4	54.8	43.4-65.7	50.7	44.2-57.1	40.5	34.4-47.0	8.8	5.8-13.2
5	≥ 5, < 6	181 (175)	8.8	5.5-13.9	30.4	24.2-37.4	49.1	36.4-61.9	45.7	38.5-53.1	46.3	39.1-53.7	8.0	4.8-13.0
6	≥ 6, < 7	123 (118)	6.5	3.3-12.3	27.6	20.5-36.1	32.4	19.1-49.2	58.5	49.5-67.0	34.7	26.8-43.7	6.8	3.5-12.8
7	≥ 7, < 8	82 (78)	0.0	0.0-4.5	19.5	12.4-29.4	37.5	18.5-61.4	59.0	47.9-69.2	38.5	28.4-49.6	2.6	0.7-8.9
8	≥ 8, < 9	47 (44)	4.3	1.2-14.2	31.9	20.4-46.2	33.3	15.2-58.3	63.6	48.9-76.2	34.1	21.9-48.9	2.3	0.4-11.8
9	≥ 9, < 10	44 (43)	4.5	1.3-15.1	45.5	31.7-59.9	45.0	25.8-65.8	48.8	34.6-63.2	46.5	32.5-61.1	4.7	1.3-15.5
10	≥ 10, < 11	27 (26)	11.1	3.9-28.1	14.8	5.9-32.5	75.0	30.1-95.4	65.4	46.2-80.6	34.6	19.4-53.8	0.0	0.0-12.9
11	≥ 11	28 (26)	0.0	0.0-12.1	28.6	15.3-47.1	62.5	30.6-86.3	50.0	32.1-67.9	50.0	32.1-67.9	0.0	0.0-12.9
Global		1,031 (986)	7.4	5.9-9.1	29.6	26.9-32.4	51.8	46.2-57.4	53.3	50.2-56.4	38.9	36.0-42.0	7.7	6.2-9.5

[1] App. free: apparently free; Map viab.: *Mycobacterium avium* subsp. *paratuberculosis* viability; Pos.: Positive; rtPCR: real-time PCR. The number of animal with valid histopathological results appears in parentheses.

Paratuberculosis forms

Table 2 shows the frequency of positive results by serological and microbiological methods as well as the frequency of each EP form across ages at slaughter. MAP infection was detected by microbiological and pathological methods at all ages. With the exception of the older age groups, seropositive reactions were also found at all ages. Even though the total proportion of animals with lesions remained relatively constant between 33.8% and 54.3% through age (non-significant correlation with years of age; r = 0.11687; p = 0.7322), splitting into latent (r = 0.65716; p = 0.0280) and patent (r = –0.86344; p = 0.0006) EP forms showed clear opposite age-related trends (Fig. 2). Latent cases predominated over patent ones in older cattle, but the more extended lesion types were more frequent during the first years of life, decreasing again among the oldest animals. In fact, 93.4% of patent PTB cases corresponded to animals within their first 6 years of life. Kendall correlation for age in months and frequency of each form yielded a total of 124 levels ranging between 1 and 24 observations per level. This non-parametric correlation confirmed the yearly results association with tau values of 0.0054 (p = 0.9309) for total PTB lesion frequency and 0.12545 (p = 0.0451) for latent and –0.39243 (p < 0.0001) for patent EP form frequency.

Serologic testing showed a high specificity for both PTB forms (98.3%) but sensitivity was very low for latent cases, of which about 96.9% were not detected, and moderate for patent cases of which 30.3% were not picked up as positive by the ELISA test (Table 3). Poor agreement was found between ELISA and histopathological results for latent and total cases (latent and patent forms) (K_{Latent} = 0.016; K_{Total} = 0.131) but pa-

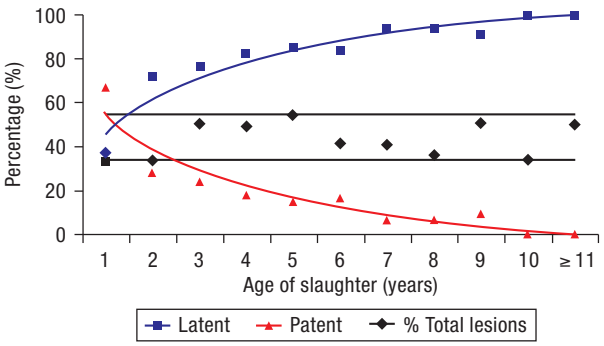

Figure 2. Proportion of total paratuberculosis (PTB) lesions and PTB epidemio-pathogenic (EP) form through age. Frequency of total cases with PTB lesions over total number of individuals as well as proportion of latent and patent PTB cases over number of cases with lesions are plotted versus age at slaughter in years except for the 11 years group which include also older animals. While the proportion of total cases remained relatively constant through age between 34% and 54% (r = 0.1170), splitting into patent and latent EP forms showed clear opposite trends (r = 0.9473) where the former predominated during the first years of age and the latter in the older animals. Solid black lines represent the minimum and maximum rates of overall lesions whereas blue and red lines depict the frequency of latent and patent EP forms as a logarithmic function of age in years, respectively.

Table 3. ELISA performance according to paratuberculosis epidemio-pathogenic forms. Estimations of sensitivity (Se), specificity (Sp), positive (PPV) and negative predictive values (NPV) with 95% confidence intervals (CI)

Paratuberculosis form	No. of animals	ELISA test			
		% Se	% Sp	% PPV	% NPV
Apparently free	526	—	98.3 (96.7-99.2)	—	56.7 (53.4-59.9)
Latent	384	3.1 (1.7-5.5)	—	57.1 (34.4-77.4)	—
Patent	76	69.7 (58.0-79.5)	—	85.5 (73.7-92.8)	—
Total (Latent + Patent)	460	14.1 (11.2-17.7)	—	87.8 (77.7-94.0)	—

tent ones showed a good agreement between seropositivity and presence of lesions ($K_{Patent} = 0.738$).

ELISA sensitivity did not change much through age for latent cases, but was clearly lower at the older ages in the patent form (Suppl. Table S1 [pdf online]).

Discussion

The results obtained in this study confirmed the broad range of immunopathological features that can match parts of the chronic pathologic entity known as bovine paratuberculosis (PTB) and fitted the immunopathological types proposed by González *et al*. (2005) that provide the most detailed picture of the range of pathological variability of PTB in cattle. However, its novelty as a large cross-sectional histopathological survey has led to further grouping them into only two forms, latent and patent, that simplify the assessment of these two pathogenetically and epidemiologically relevant states (Jones *et al*., 2011; Nielsen *et al*., 2013). Thus, the latent epidemio-pathogenic (EP) form, would be defined as a case with focal granulomatous lesions associated with scarce humoral responses and low mycobacterial detection rates and moderate MAP viability proportions indicative of a pathogenetically and microbiologically quiescent state. On the contrary, the patent EP form would represent the more active inflammatory PTB types or states (multifocal and diffuse lymphoplasmacytic, intermediate and histiocytic immunopathologic types) associated with high rates of seropositivity and mycobacteria detection suggestive of more pathologically and epidemiologically active infections.

In agreement with previous observations both in calves (Pérez *et al*., 2009) and adult cattle (Balseiro *et al*., 2003), our study showed that the majority of MAP infections are of the latent type (38.9%) and thus, practically silent in immunological and microbiological terms. Just like that, the tip of the iceberg model frequently used to more easily convey the relationship between clinical and subclinical MAP infections (Magombedze *et al*., 2013), would be also supported by the ratio of patent to total (1/6) infection cases found here.

Since the predominance of latent forms is something that is assumed to be frequent in human tuberculosis (Corbett *et al*., 2003; Barry *et al*., 2009), it could be hypothesized that MAP may follow the pathogenesis model of *Mycobacterium tuberculosis* infection, where most infections are latent and re-infections or reactivations of delimited foci may occur occasionally in immuno-compromised or senescent individuals (Parikka *et al*., 2012); although this has remained a controversial subject in bovine tuberculosis (Jones *et al*., 2011). Thus, the well-known focal types, located in both small intestine walls and associated lymph nodes, could be interpreted as a manifestation of latency in PTB, and approaching the definition of PTB epidemiological patterns based not just on the apparently histopathologically quiescent state of this form, but also on the low mycobacterial viability estimates for the latent form, which would explain previous observations on increased age-related resistance (Larsen *et al*., 1975). More speculatively, this form could be defined as one of resilience like a kind of natural vaccination or premunition phenomenon maintained by a sustained low level priming of the immune response that could help prevent further spread or re-infection while immunity is not compromised.

Indeed, the low number of AFB observed in focal granulomas by ZN staining was already pointed out by Balseiro *et al*. (2003) and González *et al*. (2005), and it seems to be more related to the difficulties to stain dormant MAP whose cell wall has lost acid-fastness than to absence of this agent in the macrophagic cells (Seiler *et al*., 2003).

In addition, our classification share great immunological and microbiological similarities with that pro-

posed by Nielsen & Toft (2008) since most latent courses would correspond to the denominated "infected" target condition, whereas patent infection may include the other two ones: "infectious" and "affected". Here, the degree in which animals showing latent and patent PTB forms could act as a source of infection for other animals was also indirectly but more sensitively estimated by tissue culture, instead of by its *in vivo* proxy, fecal culture or PCR. In that sense, to further precise the relevance of bacterial spreading by infected animals according to their PTB profile, and to speed up the interpretation of bacteriological testing, the proportion between classical bacterial isolation and molecular detection is being proposed as an indicator of comparative bacterial viability between profiles and age. Although this variable does not only represent true bacterial viability, but also bacterial burden determined by the differences in culture and PCR sensitivity, the fact is that it might roughly indicate the amounts of live bacteria that are available for transmission according to PTB form. In any case, the results presented in this study would provide a reference of what can be expected in terms of infectivity if the lengthy and labor-intensive traditional isolation methods are replaced by the faster molecular PCR tests (Pathak *et al*., 2012).

From a clinical and epidemiological perspective, the distribution of each of these two PTB forms through age seems to point out to different levels of production damage (Vazquez *et al*., 2012). The observation that persistent latent PTB occurred at any age during adulthood, and showed an increased relative frequency pattern that made the total PTB frequency to remain stable at nearly 50%, would be consistent with the standard paradigm of early life infection (Groenendaal *et al*., 2002; Balseiro *et al*., 2003; Bastida & Juste, 2011), where factors related to the immune system priming-maturation-boosting during the first years of life might influence in a greater measure progression towards clinical disease than the more classical hypotheses on physiological changes of parturition and aging. On the contrary, the observation of patent forms usually among animals under 6 years confirms the period of maximum clinical incidence according to the literature (Chiodini *et al*., 1984; Nielsen & Ersbøll, 2006) and our own direct field experience, suggesting that the length of productive life of dairy cows showing patent disease could be substantially shortened, as reported elsewhere (Alonso-Hearn *et al*., 2012). However, it was somewhat noteworthy that patent forms were present in 25% of animals less than 2 years old. In this sense, the small number (8) of studied animals within the younger age group would simply show that it is uncommon to kill these individuals, because they are just either too old to be killed for meat and too young to have paid their raising and exhausted their productive life. Thereby, it could be assumed that they are just the most PTB susceptible part of the population suffering readily visible health or production failures that lead the farmers to a culling decision. Following with this high susceptibility hypothesis, it would also explain the diffuse lymphoplasmacytic types as corresponding to an even more susceptible sub-group that shows the shortest and more homogeneous incubation period leading their victims to a life-span of 3 years (Vazquez *et al*., 2012) even with the lowest mycobacterial loads among diffuse types (González *et al*., 2005). All this suggests that the different forms do not represent a sequence, but that they would correspond to an intrinsically different character of PTB pathogenesis. While it is clear that a cross-sectional study like this one does not allow drawing firm conclusions on this point, it provides a hypothesis to be tested in further cohort-type studies.

This study shows a similar age-specific distribution between patent PTB form and reactors to *Mycobacterium bovis* with tuberculous lesions at post-mortem examinations (Brooks-Pollock *et al*., 2013), which strongly remarks the importance of both *Mycobacterium* spp. infections during the first years of life.

From a diagnostic point of view, difficulties for *in-vivo* detection of latent form cases compared to patent form cases have been repeatedly reported, and are the main cause of failure of some radical testing and culling eradication programs (Jungersen *et al*., 2012). As expected, the sensitivity of serological PTB diagnosis strongly varied between both profiles (Nielsen & Toft, 2008; Jungersen *et al*., 2012).

Regarding the antibody responses against MAP through age, most seropositive results were found among animals aged 3-6 years-old (80.3%), supporting previous findings of increasing seropositivity with age (Nielsen *et al*., 2013). Despite this, there was good agreement between ELISA testing and histology for patent PTB. In this study, 69.7% of all patent cases and more specifically, 92.9% of cases with diffuse enteritis were detected by ELISA, confirming that this immunological test is a useful indicator of severe tissue damage (Vazquez *et al*., 2013). However, sensitivity and agreement sharply decreased for the latent

PTB cases. Furthermore, since overall ELISA sensitivity barely reached 14% when referred to global histological lesions, those control strategies in which the decision is based on a positive serologic result testing might even enhance rather than control MAP spread within the herd due selection pressure potentially favoring antibody-silent infections. Further research to determine specific antigen reactivity patterns (Jones *et al*., 2011) should help confirm the latent or inactive nature of each case and could open the way to a more sensitive detection strategy of those otherwise silent infections.

In summary, we propose two EP PTB forms, as the outcome of a comprehensive post-mortem study of a large number of individual slaughtered Friesian cattle, taking into account histopathological, microbiological and serological variables. Thus, patent PTB would represent a less prevalent but more active pathological type of infection (multifocal and diffuse lymphoplasmacytic, intermediate and histiocytic immunopathologic types), that is readily detected by *in vivo* testing methods and that would progress to clinical disease and death. In turn, latent PTB would constitute the vast majority of PTB cases that would be nearly undetectable by current *in vivo* diagnostic methods, without usually progressing to substantial clinical nor subclinical losses, and which might be indicative of a pathogenetically and microbiologically quiescent state. Both forms appeared to follow divergent trends through the lifespan of Friesian cattle causing that the overall prevalence of PTB appeared relatively stable. Finally sensitivity discrepancies between the ELISA test and microbiological and histological methods further confirm that currently available serologic tests might fail to detect too many infected animals, and that this could have a negative effect on current culling control strategies according to their results.

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