Age structure of *Mycobacterium avium* subsp. *paratuberculosis* infection in culled Friesian cattle

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ABSTRACT

The aim of this study was to determine the age structure of paratuberculosis infections in culled dairy cattle as a mean to improve the design and use of diagnostic tests and control strategies.

581 adult Friesian cattle slaughtered in the Basque Country from March 2007 to December 2008 were included in the study. Samples of blood, intestinal tissue and mesenteric lymph nodes were taken from each animal. Blood was used in an ELISA test whereas intestinal tissue was used for bacterial isolation, DNA detection by a commercial real time PCR (RTi-PCR) and histopathological examination. All methods showed a peak at 3-4 years of age that ranged from 42.3% (histopathology) to 16.5% (ELISA). Minimum prevalence was found at 7-8 years. Overall prevalence ranged from 8.3% (ELISA) to 20.0% (RTi-PCR). Sampling within the 3-5 years of age group could improve the chances of herd infection detection with a minimum number of samples. Focusing testing on this age group could also save resources and maximize efficiency in testing and culling programmes.

INTRODUCTION

It is generally accepted that paratuberculosis (PTB) is a slow infection that begins during the first weeks of life in a contaminated environment, but that do not develop into visible lesions and disease until the early adult life of cows in some animals.

Sensitivity of PTB diagnosis varies with the stage of the infection and lack of sensitivity of diagnostic tests is the main hurdle for the successful use of test and cull strategies.

In this slaughterhouse study, the main goal was to determine the age structure of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection in Friesian cattle as well as to obtain new data on the efficacy of four diagnostic tests for detection of subclinically infected animals.

MATERIALS AND METHODS

Animal selection and sampling

Using a weekly systematic sampling, 581 Friesian animals were examined from March 2007 to December 2008, at two local slaughterhouses in the Basque Country. After stunning, blood samples from the jugular vein were collected into sterile tubes containing EDTA. Additionally, fresh and formalin-fixed samples of jejunal caudal lymph node (JCL) and distal ileon and ileocecal valve (ICV) were aseptically taken. Bovine identification documents (BID) were reviewed for age data collection.

ELISA

The Pourquier® ELISA Paratuberculosis Antibody Screening kit (Institut Pourquier, Montpellier, France) was used on plasma samples according to the manufacturer’s instructions. Samples with positive and doubtful results for the screening ELISA were retested using Pourquier® ELISA Paratuberculosis Antibody Verification kit (Institut Pourquier, Montpellier, France). Positive results corresponded to sample to positive (S/P) ratios above 70%.
Culture
Gut tissue samples, consisting in 1 g of JCL and 1 g of mucosa from the ICV area and distal ileon, were decontaminated with hexa-decyl pyridinium chloride (HPC) (0.75%) and inoculated in duplicate mycobactin J supplemented Herrold’s Egg Yolk (HEYM) and Löwstein-Jensen (L-J) media as previously described (Juste et al., 1991). Cultures were first examined after 8 weeks of inoculation and subsequently every 4 weeks up to 20 weeks. No evidence of bacterial growth after this incubation period was considered as a negative result. Isolated colonies were confirmed by conventional PCR amplification of MAP specific IS900 insertion sequence (Moss et al., 1992).

RTi-PCR
Sample preparation involved the homogenization of 2.5 g of JCL and mucosa from the ICV and distal ileum area (1:1) in 10 ml of sterile water for 1 min at medium speed in a Stomacher® 80 Biomaster (Seward, Worthing, UK). Afterwards, 300 µl of the homogenized sample were submitted to a modified protocol of Adiapure® MAP DNA extraction and purification kit (Adiagene, Saint Brieuc, France) for tissue samples. Purified DNA samples were eluted to a final volume of 100 µl. MAP DNA detection, based on the amplification of the specific IS900 insertion sequence, was carried out using the ADIAVET® PARATB Real Time commercial kit (Adiagene) and ABI Prism® 7000 Sequence Detection System instrument (Applied Biosystems, Foster City, CA). Samples were considered positive if the cycle threshold (Ct) value was <37.

Histopathological examination
Formalin-fixed tissue samples were processed using standard histological procedures. All sections were stained by haematoxylin and eosin (HE) and observed under the light microscope. In case of paratuberculosis compatible lesions Ziehl-Nielsen (ZN) staining was performed. Lesions were classified according to González et al., (2005).

Test combinations: Prevalence analysis and sensitivity and agreement study
PTB prevalence was estimated by serial and parallel analysis. Only animals with all tests positive were scored positive in serial evaluation while animals with at least one positive result in any of the tests were scored positive in the parallel analysis.

Complementary sensitivity was calculated in order to evaluate test combinations and maximize sensitivity in PTB diagnosis. This value was defined as the additional number of samples testing positive to an evaluated test in relation to the reference one.

Agreement between tests was measured by Kappa (κ) value. Sensitivity estimations and Kappa values were calculated with WinEpi software (www.winepi.net).

Statistical analysis
Fisher’s exact probability was calculated in order to detect significant differences in MAP diagnosis according to age. The level of significance was set at p-value <0.05.

RESULTS
Overall PTB prevalence ranged from 8.3% by ELISA (n=581) test to 20.0% by PCR (n=581). The prevalence of positive MAP tissue cultures was 18.7% (n=576). Characteristic paratuberculosis lesions were observed in 13.6% of the studied samples (n=213). Serial prevalence analysis estimated PTB prevalence at 9.5% whereas parallel analysis estimation was nearly 3 times higher (27.7%).

The average age at which MAP infection was detected by serology, culture, RTi-PCR and histopathology was 4.6, 5.3, 5.4 and 4.2 years; respectively. All tests evidenced a peak of paratuberculosis infection prevalence when animals were 3-5 years. At this age prevalence determined by a combination of tests read in parallel was 33.2%. Age distribution of PTB infection is shown in Fig. 1.
A statistically significant lower frequency in MAP infection was observed in animals older than 5 years compared to 3-5 years-old animals ($p_{ELISA}=0.0002; p_{CULTURE}=0.0087; p_{RTi-PCR}=0.0078; p_{HP}<0.0001$).

![Fig. 1](image_url)

**Fig. 1.** Age structure of MAP infection in relation to diagnostic tests: ELISA (A), culture (B), RTi-PCR (C) and histopathological examination (D). Line: MAP prevalence and bars: number of samples tested.

**Table 1.** Sensitivity, complementary sensitivity and agreement ($\kappa$) values of diagnostic test combinations. Interpretation of agreement: poor ($\kappa=0.00-0.20$), fair ($\kappa=0.21-0.40$), moderate ($\kappa=0.41-0.60$), good ($\kappa=0.61-0.80$) and excellent ($\kappa=0.81-1.00$).

<table>
<thead>
<tr>
<th>EVALUATED TEST</th>
<th>REFERENCE TEST</th>
<th>Sensitivity (%)</th>
<th>Complementary sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEST</td>
<td>ELISA</td>
<td>CULTURE</td>
<td>RTi-PCR</td>
</tr>
<tr>
<td>ELISA</td>
<td>-</td>
<td>37.0</td>
<td>37.1</td>
</tr>
<tr>
<td></td>
<td>($\kappa=0.454$)</td>
<td>($\kappa=0.461$)</td>
<td>($\kappa=0.696$)</td>
</tr>
<tr>
<td>CULTURE</td>
<td>85.1</td>
<td>-</td>
<td>63.4</td>
</tr>
<tr>
<td></td>
<td>($\kappa=0.562$)</td>
<td></td>
<td>($\kappa=0.536$)</td>
</tr>
<tr>
<td>RTi-PCR</td>
<td>89.6</td>
<td>65.7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>($\kappa=0.525$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HP</td>
<td>75.0</td>
<td>48.0</td>
<td>47.2</td>
</tr>
</tbody>
</table>
Broadly, diagnostic test combinations resulted in moderate agreement ($\kappa=0.41-0.60$) excepting for the combination ELISA and histopathological examination that showed a good agreement ($\kappa=0.696$). The highest relative and complementary sensitivity values were obtained with microbiological methods (RTi-PCR and Culture)(Table 1.).

**DISCUSSION**

Microbiological methods as well as histopathological examination corroborated that most PTB infected animals were between 3 and 5 years old as traditionally described. Our serological findings were in agreement with the assumption of the age of 2.5 to 4.5 years as the highest risk of detecting antibodies against MAP suggested by other authors (Nielsen Ersbøll, 2006). The fact of finding one seropositive 18 months-old animal with diffuse lesions and MAP isolation from gut tissue may support the implication of animals younger than 2 years in horizontal MAP spread suggested by other authors (Wells *et al.*, 2000).

With regard to diagnostic tests, RTi-PCR showed a similar sensitivity to MAP isolation. The good agreement between ELISA and HP indicates that ELISA could be a good predictor of intestinal lesion and therefore might anticipate clinical disease. However, the poor complementary sensitivity of ELISA and culture and RTi-PCR indicates that humoral immune response is a bad predictor of subclinical infection. Therefore, a combination of RTi-PCR and ELISA appears to give the best chances to maximize PTB diagnosis.

**CONCLUSION**

Based on the yearly infection rates obtained from this study it seems appropriate to intensify time testing at the age of 3-5 years in order to maximize the likelihood of detecting infected animals before they spent a too long time in the herd acting as a source of infection for the rest of the susceptible animals. On the other hand, we can conclude that no single test can detect all infected individuals and that, therefore, a combination of tests yields the maximum sensitivity.

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