Prevalence of bovine paratuberculosis in the Latium region (Italy)

Lillini E, Bitonti G, Gamberale F, Cersini A

Istituto Zooprofilattico Sperimentale Lazio e Toscana, Via Appia Nuova 1411 – 00178 Rome, Italy
Phone +390679099451 Fax +390679340724 elillini@rm.izs.it

ABSTRACT

In Italy there are few studies about the prevalence of bovine paratuberculosis. In the Veneto region a serosurvey on 416 dairy farms recorded a herd seroprevalence of 49% by ELISA and in Tuscany 3.4% of bovine population tested positive by AGID. In order to have a wider evaluation of the infection in cattle population of Latium region we carried out a serological survey on 369 herds (2% of regional herds) uniformly distributed in 5 province districts using an ELISA. A total of 19,627 animals were examined (6.5% of the whole population). ELISA-positives totaled 472 animals (2% ± 0.4% -CI 95%), ranging from one to a maximum of 65 cows per farm. 155 herds were seropositive with at least one positive cow (42% ± 5% -CI 95%). Fecal samples (77) were collected from ELISA-positive herds and from at least one of the seropositive subjects and than examined by Ziehl-Nieelsen (Z-N) stain, bacteriological culture and two PCR techniques. Only in one case was the molecular test negative when culture was positive; in the remaining fecal samples we found a concordance of 99% between the serological and molecular tests. The ELISA is useful for screening herds due to low cost and fast and easy execution. The results obtained confirm a widespread distribution of bovine paratuberculosis in our region, affecting up to at least 42% of cattle farms.

Key words: Latium region (Italy), seroprevalence, bovine, paratuberculosis.

INTRODUCTION

Bovine paratuberculosis is widespread in the world and has been diagnosed in Italy. It was first described in cattle in 1927, in sheep in 1968 and in buffalo in 1996 (Lillini et al., 1999). The prevalence of the infection differs among the countries but generally high. Epidemiological studies on advanced animal husbandry countries show that the average of positive percentage is about 50% and it could reach the 80% within 2020 if surveillance control programmes will be not implemented (Collins, 2002, personal communication). In the Veneto region of north eastern Italy, 49% of 416 herds examined by ELISA were test-positive (Robbi et al., 2002).

In the last 10 years in the Latium region (Central Italy) the infection was found in dairy and beef herds on the basis of a clinical suspicion or on a specific request of breeders.

The aim of the present study was to carry out a survey to determine the seroprevalence of paratuberculosis in Latium region (Central Italy) herds.

MATERIALS AND METHODS

A total number of 2,605 herds with 29 or more cattle are present in the whole Lazio region: Rome 27.5% (n=717), Frosinone 18.1% (N=470), Latina 28.7% (N=747), Viterbo 13% (N=339), Rieti 12.7% (N=332). A stratified sample size was calculated on the basis of herd number for each province and a total of 19,627 bovine sera from 369 dairy and beef herds of the 5 Latium provinces, Rome (100), Frosinone (72), Latina (100), Viterbo (53) and Rieti (44) were tested. This represented 6.5% of the Latium bovine population and 2% of the total herds. An ELISA (commercial IDEXX Antibody ELISA Kit, using the manufacturer’s recommended protocol) was used.
Seventy seven follow-up fecal samples (from selected ELISA-positive cows in various herds) were stained with the Z-N TB QUICK STAIN (B.D., USA). The isolation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) was carried out following the National Animal Disease Center protocol (NADC, Ames USA): a) decontamination of fecal specimens with 1% of HPC (w/v); b) growth in solid home-made HEYM additioned with mycobactin J (ALLIED MONITORLABS, USA) and sodium piruvate. The Qia amp DNA Mini® Kit (QIAGEN, USA) was employed for DNA extraction from fecal samples. The DNA amplification of MAP, carried out with the PCR kit ADIAVET PARA tub® (ADIAGEN, France), is based on amplification of a DNA fragment insertion sequence IS900 specific for MAP strains (Lillini et al., 2002). The nested PCR was employed by PCR1 and PCR2. The target of PCR1 primers (p90/p91) was 400bp fragment localized within the IS900 insertion element:

- primer p90: 5’-GAAGGGTGTTCGGGGCCGTCGTTAGG-3’
- primer p91: 5’-GGCGTTGAGGTCGATCGCCCACGTGAC-3’

To generate a specific internal probe sequence, we designed a second pair of oligonucleotide (p25/p26) for a Nested PCR application; these primers amplify a 229 bp fragment using the product of PCR1 as DNA template:

- primer p25: 5’-CCAGGGACGTCGGGTATGGC -3’
- primer p26: 5’-GGTCGGCCTTACCGGCGTCC –3’

PCR amplification was carried out in 50µl volume reaction (final concentration):

**Statistical analyses**
The sensitivity and the specificity of the ELISA kit test was required to calculate the sample size of dairy and beef herds to be tested for the bovine paratuberculosis seroprevalence estimation at a population level. Therefore, in order to assure the validity of the investigation and to improve the statistical significance of the seroprevalence estimates a performance evaluation of the ELISA serological test was carried out in a preliminary phase.

**ELISA performance**
Data related to sensitivity and specificity, plus predictive values of negative and positive sera were deduced from a study carried out in 2002 on 6 herds in Latium region. Five herds were considered infected by MAP and one free of infection on the basis of the following characteristics: during the last five years neither clinical signs of paratuberculosis were observed nor new animals were introduced. Half yearly two faecal culture controls and serological examinations were completed and all gave negative results. During this investigation 633 cows over 2 years old were examined. The faecal culture test was used as gold standard to verify animal infection. Diagnostic sensitivity (DSn) and diagnostic specificity (DSp) of a kit ELISA for antibodies were determined on the basis of results obtained with cultural examinations (Sockett et al., 1992).

The predictive value of negative (PV-) and positive (PV+) sera was calculated on the relative DSn, DSp and on the sero-prevalence value expected by the gold standard (Sweeney et al., 1995).

**1° ELISA paratuberculosis test IDEXX**
DSn and DSp evaluation were calculated by a contingency 2x2 table, which links the sanitary situation of reference animals with test results obtained. Assay was executed by investigation on 633 cows (Table 1)

<table>
<thead>
<tr>
<th>Reference animals</th>
<th>infected</th>
<th>non infected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TP</td>
<td>FP</td>
</tr>
<tr>
<td>positive</td>
<td>44</td>
<td>26</td>
</tr>
<tr>
<td>negative</td>
<td>13</td>
<td>550</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>FN</td>
<td>TN</td>
</tr>
</tbody>
</table>

Diagnostic sensitivity ELISA (DSn) = 0.772
Diagnostic specificity ELISA (DSn) = 0.955
Figs 1 and 2 show positive and negative predictive values in infected herds.

![Fig. 1. ELISA positive predictive value (PV+)](image1)

Predictive positive value of test calculated on population with 9.93% seroprevalence:
Positive ELISA test result: \( PV_+ = \frac{P \times SE}{P \times SE + (1 - P) \times (1 - SP)} \) = 0.654

![Fig. 2. ELISA negative predictive value (PV-)](image2)

Predictive negative value of test calculated on population with 9.93% seroprevalence:
Negative ELISA test result: \( PV_- = \frac{P \times SE}{P \times SE + (1 - P) \times (1 - SP)} \) = 0.974; with a confidence interval (CI) of 95%, ELISA sensitivity and specificity range is reported in the following Table 2.

<table>
<thead>
<tr>
<th>Estimate</th>
<th>CI 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>77.193 - 86.685</td>
</tr>
<tr>
<td>Specificity</td>
<td>95.486 - 96.967</td>
</tr>
</tbody>
</table>

Considering the low positive predictive value of ELISA test IDEXX and using a test-positive threshold value S/P of 0.15, herds were defined as infected if at least 2 results were test-positive; with a cut off of S/P of 0.30 the herd was considered infected if one result was test-positive.
Preliminary data showed that raising the cut-off from 0.15 to 0.30 improved specificity, and consequently the positive predictive value of the test, without decreasing the sensitivity and negative predictive value. So the number of culture tests could be reduced and used only on ELISA positive samples.

**Sampling method**

Calculation of the number of herds to sample: considering a total of 2,605 herds with herd size greater than 29 animals in the Lazio region, assuming an expected 10% prevalence of infected herds in this category, 3% accuracy and a 95% confidence level, a sample size of at least 335 herds was calculated using Win Episcope 2.0 software. The number of herds to be sampled was then stratified for each province of the Lazio region on the basis of the respective herd number.

Calculation of the number of animals to test: as diagnostic sensitivity of ELISA, on cattle aged >= 24 months, is more than 25% (this value represents the lower value verified in asymptomatic cattle), the probability of finding ELISA positives in infected animals is more than 95% in a herd with a real prevalence of >=2%. So any cattle aged more than 24 months were tested.

**RESULTS**

Table 3 shows the number of positive herds and cattle for a single Latium province and the respective test-positive percentage. The higher number of positive herds (52%) is in Rieti province while in Frosinone province the percentage is the lowest (28%).

Among 369 examined herds, 155 (42% ± 5% - CI 95%) tested positive. Of the 19,627 tested sera, 472 (2.4% ± 0.4% - CI 95%) ELISA-positives were found.

Fig. 3 represents the distribution of ELISA-positive cattle among the infected herds in Latium region. In Fig. 4 it is shown that in almost 50% of herds one ELISA-positive cow is present; 22% of herds have two positive animals and 16 herds (11%) more than 5 ELISA-positive cattle.

Fig. 5 represents the percentage of positive herds related to the size of the herd. At least one positive cow is found in almost 70% of controlled herds less than 100 head.

<table>
<thead>
<tr>
<th>Province</th>
<th>Examined herds</th>
<th>Positive herds</th>
<th>Examined heads</th>
<th>Positive heads</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N°</td>
<td>%</td>
<td>N°</td>
<td>%</td>
</tr>
<tr>
<td>Frosinone</td>
<td>72</td>
<td>20</td>
<td>28</td>
<td>2315</td>
</tr>
<tr>
<td>Latina</td>
<td>100</td>
<td>37</td>
<td>37</td>
<td>4465</td>
</tr>
<tr>
<td>Rieti</td>
<td>44</td>
<td>23</td>
<td>52</td>
<td>2516</td>
</tr>
<tr>
<td>Roma</td>
<td>100</td>
<td>40</td>
<td>40</td>
<td>6105</td>
</tr>
<tr>
<td>Viterbo</td>
<td>53</td>
<td>25</td>
<td>47</td>
<td>4226</td>
</tr>
<tr>
<td>Total</td>
<td>369</td>
<td>155</td>
<td>42</td>
<td>19627</td>
</tr>
</tbody>
</table>
To confirm the serological results, one or more fecal samples were collected from 77 ELISA-positive cattle in 40 positive herds in Rome province. Culture and slide examination were positive in 9 and 26 herds.
respectively; the results obtained by both PCR showed 31 infected herds of a total of 38 examined (81%). Only in one case was the molecular assay negative, even if the bovine was very low shedding, whereas in the remaining 8 herds there was full concordance between the two tests. Fecal shedding of MAP may be intermittent and of few organisms (1-5 colonies). We found one fecal sample positive to MAP culture and microscopic examination for acid fast bacilli but negative to PCR tests.

We found that 34 herds out of 40 sero-positive were confirmed as infected by at least one of the confirmatory tests performed on fecal samples (commercial PCR and/or PCR home-made or bacterial culture or microscopic examination for acid fast bacilli), giving an overall accordance of 84% of test-positive herds.

A more realistic vision on the true situation in Latium region may have been obtained if we had used a seroprevalence calculation with hypergeometric distribution or with other statistical formulas.

CONCLUSION

This epidemiological study could be used to improve control programmes for the eradication of the infection. In our study we found that the elevated number and percentage of infected herds correspond to those obtained in other seroepidemiological surveys carried out in other countries with advanced animal husbandry (Allworth et al., 2002a. Allworth et al., 2002b. Piaggio et al., 2002). In our experience 47% of the examined herds presented only one ELISA-positive animal.

In previous work it was found that in some cases animal trade was the source of infection (Lillini et al., 1986. Lillini et al., 1998).

In conclusion, the prevalence of the infection could rise in future if surveillance control programs are not implemented. Despite its limits, the ELISA remains a suitable method for the identification of the infected herds because it is inexpensive, fast and simple to perform.

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REFERENCES


