In this study, we characterized the humoral responses in cattle of Sardinia. The animals were divided into three groups:
1) 28 cattle infected with *Mycobacterium bovis*;
2) 48 cattle from herds in which foci of infection was notified;
3) 50 cattle from herds that were TB-free.
Levels of IgG antibody were measured against the following antigens of *M. tuberculosis*: Heparin-Binding-Haemagglutin (HBHA), Ag85B, PPE44, and PE_PGRS33 to investigate their potential to diagnose TB in animals. Our results indicated that HBHA is a potential candidate for the development of a serological assay for rapid diagnosis of cattle infected with *M. bovis*.

**KEY WORDS:** HBHA, *Mycobacterium bovis*, Humoral response, Bovine tuberculosis
able, and the humoral response can target several antigens, we assayed the sera for HBHA (both methylated and unmethylated) (Zanetti et al., 2005), and three other mycobacterial proteins, PE_PGRS33 (Rv1818c), PPE44, and Ag85B, identified in several studies as potentially important targets of the immune response to tubercular disease (Brennan and Delogu, 2002, Demangel et al., 2004, Fifis et al., 1992, Morris et al., 1994). These proteins are present in M. tuberculosis complex and in other mycobacterial species (Gey van Pittius et al., 2006) (Lilenbaum et al., 2001).

A total of 126 bovine sera collected in the Sardinian provinces of Oristano and Sassari were analyzed. The specimens were divided into three groups. Group 1 contained 28 sera collected from different cattle in Oristano Province that were PPD + and in which M. bovis was demonstrated by molecular analysis performed on biopsy specimens. All biopsies were tested according to standard protocols for IS6110 specific for M. tuberculosis complex (Thierry et al., 1990), and for the M. bovis-specific 500-bp fragment Rodriguez et al., 1995). All these samples were Polymerase Chain Reaction (PCR) positive. Group 2 included 48 sera collected from cattle belonging to herds of the Oristano province in which foci of tubercular infection were reported. These cattle were PPD negative also after multiple testing. Later these cattle were slaughtered and no anatomo-pathologic lesions were found. Group 3 contained 50 sera collected from TB-free herds (the negative control group). Proteins were purified by affinity chromatography, as previously described (Delogu and Brennan 2001). All the proteins were expressed in Escherichia coli, and the HBHA was also expressed in M. smegmatis to obtain the methylated protein (Delogu et al., 2004). The specific IgG humoral response against M. tuberculosis antigens was determined with an enzyme-linked immunosorbent assay (ELISA) as previously described (Zanetti et al., 2005). The arbitrary cut-off point (ODs405nm 0.5) was defined as the mean found in groups 1 and 2, increased
by 0.1 unit to reduce the false positive results. The statistical analyses were based on the chi-squared test.

Figure 1 shows the levels (expressed as OD$_{405\text{nm}}$) of IgG against both forms of HBHA, PE_PGRS33, PPE44, and Ag85B in the sera of animals belonging to three groups. In Group 1 assessment of reactivity to HBHAe revealed humoral responses (i.e., OD$_{405\text{nm}}$ equal or greater than 0.5) in 17 of out 28 sera (Table 1). Twelve sera of Group 1 displayed reactivity to HBHAe (Table 1). Eight of the sera that recognized the native protein also recognized HBHAe, but in most cases (5/8) the OD values for HBHAe were approximately twice as high as those for HBHAs. Reactivity to the Ag85B protein was documented in five of the 28 sera in Group 1 (Table 1). Three of the five Ag85B-positive sera recognized both the native and recombinant forms of HBHA, while the remaining two displayed no reactivity to either of the HBHA proteins.

There were significant differences between the humoral responses to HBHAe, HBHAs, and Ag85B in Group 1 and those in the control Group 3 (p=0 for HBHAe, p=0 for HBHAs, p=0.0159 with an odds ratio of 9.80 for Ag85B). In this group no significant differences were observed between the responses to HBHAe, HBHAs, and Ag85B (p=0.21, odds ratio=0.6 for HBHAe and p=0.063, odds ratio=0.46 for HBHAs). None of the 50 negative control sera displayed reactivity to either the HBHA proteins or to Ag85B, (all of the OD$_{405\text{nm}}$ values for these sera were between 0.1 and 0.3) (Table 1). Three sera from Group 1 and five from Group 2 displayed reactivity to the PPE44, and there were no humoral responses in any of the three groups to PE_PGRS33 (OD$_{405\text{nm}}$ values ranging from: 0.1-0.3 nm) (Table 1). There were

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Group 1 (N*=28)</th>
<th>Group 2 (N*=48)</th>
<th>Group 3 (N*=50)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>HBHAe</td>
<td>17 p=0</td>
<td>11 p=0</td>
<td>19 p=0</td>
</tr>
<tr>
<td>HBHAs</td>
<td>12 p=0</td>
<td>16 p=0</td>
<td>16 p=0</td>
</tr>
<tr>
<td>Ag85B</td>
<td>5 p=0.0159</td>
<td>23 p=0</td>
<td>25 p=0</td>
</tr>
<tr>
<td>PE_PGRS33</td>
<td>0</td>
<td>28 p=0</td>
<td>0</td>
</tr>
<tr>
<td>PPE44</td>
<td>3 p=0.087</td>
<td>25 p=0</td>
<td>5 p=0.0596</td>
</tr>
<tr>
<td>PPD</td>
<td>28</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

N*= number of cattle
no significant differences between the infected groups and controls regarding reactivity to these two antigens (for PPE44: p=0.0807 for Group 1 versus Group 3 and p=0.0596, for Group 2 versus Group 3). An important result of this study is the remarkable difference in the humoral response against the HBHA and Ag85B antigens measured in sera from cattle belonging to group 2 compared with those of group 3. Though Group 2 animals belonged to herds in which foci of M. bovis infection had developed, the 48 cattle were all PPD negative, and therefore should be considered healthy and not infected with M. bovis. Remarkably, 19 sera of Group 2 were reactive against HBHAe, 16 against HBHAs and 25 against Ag85B. Since no reactivity against these two antigens was observed in sera collected from animals belonging to Group 3, the antigenic reactivity observed against these antigens may be indicative of M. bovis infection. The serological assay based on the HBHA and Ag85B antigens may therefore detect infected cattle in a PPD-negative population of contacts. An interesting result may be observed when the antigenic reactivity of Group 1 and Group 2 was compared. Of the 28 sera belonging to Group 1, 17 reacted with HBHAe, 12 recognized HBHAs and 5 sera reacted with Ag85B (3 of these recognize also HBHA). Nineteen of the 48 sera belonging to Group 2 reacted against HBHAe, 16 against HBHAs and 25 displayed reactivity to Ag85B (13 of these also recognized the HBHA). The proteins that make up the Ag85 complex are highly immunogenic and capable of stimulating a strong antibody response in humans infected with M. tuberculosis and M. leprae and in M. bovis-infected cattle (Lilenbaum et al., 2001, O’Reilly et al., 1995). They are secreted by M. bovis, and are also present in the BCG vaccine strain. Although they are immunogenic, antibody reactions to these proteins have also been observed in healthy animals, and they are strongly cross-reactive. Therefore, the higher percentage of Ag85B-reactive sera in Group 2, considering the few numbers of sera reactive with Ag85B in Group 1, might reflect environmental contact with non-pathogenic mycobacteria. Hence, Ag85B may lack the specificity required for a serological assay although it could still be used as a general indicator of mycobacterial infection. Conversely, serological tests relying on HBHA might be able to differentiate between tuberculin positivity caused by M. bovis infection and that caused by contact with environmental mycobacteria. Sera reactivity against un-methylated and methylated HBHA were similar, suggesting that methylation does not affect the antigenic reactivity in cattle. This is different from what has been observed in TB patients (Temmerman et al., 2004, Zanetti et al., 2005) and may reflect differences in the immunopathogenetic process of the disease. The fact that HBHAs in Group 1 was better recognized with respect to HBHAs in terms of OD values might indicate that cattle with TB better recognized the unmethylated protein. This aspect might be evaluated in a further study. We did not observe any humoral responses to the PPE44 or PE-PGRS33. The results of the present study, which are concordant with those reported for human sera, indicate that these antigens are not the main targets of the humoral response to mycobacteria. In conclusion, considering the results obtained in humans and in cattle, HBHA is a potentially sensitive and specific marker for an antibody assay to M. bovis, and, in conjunction with other microbiological tests, it can be used for the diagnosis of bovine TB.

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REFERENCE

Lilenbaum W., Pessolani M.C., Fonseca L.S. (2001). The use of Ag85 complex as antigen in ELISA for...


