Heparin binding haemagglutin as potential diagnostic marker of *Mycobacterium bovis*

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SUMMARY

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

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Bovine tuberculosis (TB) is a serious infectious disease caused by *Mycobacterium bovis,* a member of the *Mycobacterium tuberculosis* (MTB) complex (O'Reilly *et al.,* 1995). The immune response to bovine TB is predominantly cell-mediated, however when the bacterial burden is high, the host mounts a significant humoral response to *M. bovis* antigens (O'Reilly *et al*., 1995, Hewinson *et al.,* 1996). Several attempts have been made to develop simple, low-cost serological assays that can identify those animals with advanced infection that are nonetheless skin-test negative. The earliest assays of this type, based on the use of *M. bovis* Purified Protein Derivative (PPD) and phosphatide antigens (Harboe *et al.,* 1995), lacked specificity, and subsequent efforts were thus aimed at identifying antigens expressed exclusively by *M. bovis* or at least by a limited number of mycobacterial species. Several antigens have proved to be specific for *M. bovis*, but assays based on their detection have displayed low sensitivity (Hanna *et al.,* 1989, Hewinson *et al.,* 1996, Lilenbaum *et al.,* 2001; Harboe *et al.,* 1990, Lightbody *et al.,* 1998).

Recently, our group demonstrated that the Heparin Binding Haemagglutin (HBHA) (Zanetti *et al.,* 2005), expressed by *M. tuberculosis* and *M. bovis* (Menozzi *et al.,* 1996), is a specific and sensitive marker that can discriminate between patients with active TB and those vaccinated with BCG who were PPD positive. The aim of this work was to investigate whether an antibody-based test could be useful to diagnose TB in animals. Since the immune response to TB is vari-

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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_PGRS*33* (Rv1818c), PPE*44*, 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 IS*6110* 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 (ODs_{405nm} 0.5) was defined as the mean found in groups 1 and 2, increased

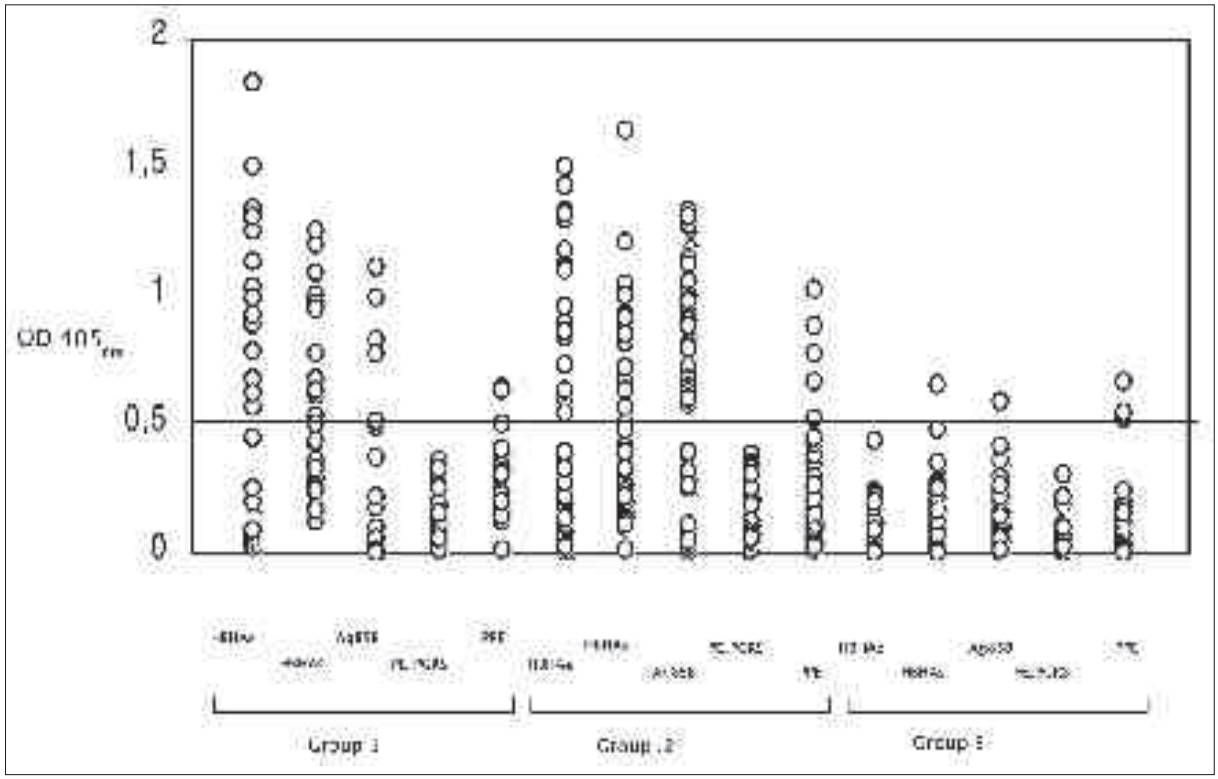

FIGURE 1 - *Humoral responses to the five mycobacterial antigens in the three groups of bovine sera. Results are expressed as optical density at 405 nm (y axis), and values of 0.5 nm or more are indicative of reactivity. The line in the graphic indicated the cut-off value.*

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_{405nm}) of IgG against both forms of HBHA, PE_PGRS*33*, PPE44, and Ag85B in the sera of animals belonging to three groups. In Group 1 assessment of reactivity to HBHA*e* revealed humoral responses (i.e., ODs_{405nm} equal or greater than 0.5) in 17 of out 28 sera (Table 1). Twelve sera of Group 1 displayed reactivity to HBHA*s* (Table 1). Eight of the sera that recognized the native protein also recognized HBHA*e,* but in most cases (5/8) the OD values for HBHA*e* were approximately twice as high as those for HBHA*s*. 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 HBHA*e*, HBHA*s*, and Ag85B in Group 1 and those in the control Group 3 (p=0 for HBHA*e*, p=0 for HBHA*s,* and p=0.0159 with an odds ratio of 9.80 for Ag85B). Moreover, while the difference between the responses to the two forms of HBHA was not significant (p=0.181, odds ratio=2.6), significant differences were observed between the responses to HBHA*e* and Ag85B (p=0.001, odds ratio=7.11) and between the responses to HBHA*s* and Ag85B (p=0.0419, odds ratio=3.45). In Group 2 (Table 1) nineteen of the 48 sera displayed reactivity to HBHA*e*, HBHA*s* elicited an antibody response in 16 sera and 11 of these also recognized HBHA*e*. In these cases, reactivity to the two proteins was similar. Twenty-five sera of Group 2 reacted to Ag85B (Table 1). Thirteen of the Ag85B-positive sera also recognized both forms of the HBHA. Humoral responses to HBHA*e*, HBHA*s*, and Ag85B in Group 2 were significantly different from those Group 3 (p=0 for HBHA*e*, p=0 for HBHA*s,* p=0 with an odds ratio of 53.26 for Ag85B). In this group no significant differences were observed between the responses to HBHA*e*, HBHA*s,* and Ag85B (p=0.21, odds ratio=0.6 for HBHA*e* and p=0.063, odds ratio=0.46 for HBHA*s*). None of the 50 negative control sera displayed reactivity to either the HBHA proteins or to Ag85B, (all of the $OD_{405\ nm}$ values for these sera were between 0.1 e 0.3) (Table 1). Three sera from Group 1 and five from Group 2 displayed reactivity to the PPE*44*, and there were no humoral responses in any of the three groups to PE_PGRS*33* ($OD_{405\ nm}$ values ranging from: 0.1-0.3 nm) (Table 1). There were

TABLE 1 - *Comparison of PPD test and the mycobacterial antigens performance for three group of animals samples analyzed in our study. In the table were indicated the p value for comparison between Group1 (TB) and Group 2 (Group with foci of tubercular infection) with Group 3(Control Group).*

Antigens	*Group 1 (N*=28)*		*Group 2 (N*=48)*		*Group 3 (N*=50)*	
	+	-	+	-	+	-
HBHAe	17 p=0	11	19 p=0	29	0	50
HBHAs	12 p=0	16	16 p=0	32	0	50
Ag85B	5 p=0,0159	23	25 p=0	23	0	50
PE_PGRS33	0	28	0	48	0	50
PPE44	3 p=0,087	25	5 p=0,0596	43	0	50
PPD	28	0	0	48	0	50

N*= number of cattle

no significant differences between the infected groups and controls regarding reactivity to these two antigens (for PPE*44*: 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 HBHA*e*, 16 against HBHA*s* 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 HBHA*e*, 12 recognized HBHA*s* and 5 sera reacted with Ag85B (3 of these recognize also HBHA). Nineteen of the 48 sera belonging to Group 2 reacted against HBHA*e*, 16 against HBHA*s* 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 HBHAe 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 PPE*44* or PE-PGRS*33*. 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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