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Use of recombinant proteins in antibody tests for bovine tuberculosis

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Abstract

Tuberculosis (TB) in cattle remains a major zoonotic and economic problem in many countries. Since the standard diagnostic assay, the intradermal test (IDT) with bovine PPD tuberculin, has less than optimal accuracy in all situations, other diagnostic methods such as serological assays have been investigated. Because of fundamental concerns for the low sensitivity and specificity of previous ELISA protocols, a profiling ELISA with nine purified, recombinant proteins of TB complex mycobacteria, was employed on samples from four groups of cattle: (a) naturally *Mycobacterium avium*-exposed and experimentally *Mycobacterium bovis*-infected, (b) officially-certified TB-free herds, (c) exposed to *M. bovis* in two field TB outbreaks and scored as bovine reactors in the γ -IFN assay for bovine TB, (d) paratuberculosis (para TB)-infected. The described ELISA proved to be highly specific. In fact, the antibody (Ab) response could be consistently detected in 3 out of 3 endotracheally-infected calves and in 1 out of 3 contact-infected calves. There was also a very low prevalence of low-titered, non-specific Ab responses in paraTB-infected animals. As for the animals exposed to field TB outbreaks, 16 out of 28 γ -IFN positive cattle were also Ab-positive; importantly, 7 out of 12 γ -IFN positive, IDT-negative cattle showed Ab responses to TB proteins. In general, the profile of the Ab response varied among animals; the reaction to single recombinant antigens was sometimes transient and fluctuating, whereas the panel of antigens on the whole was indeed more effective in Ab detection. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Cattle; Bacteria; Antibody; ELISA; *Mycobacterium bovis*; Recombinant proteins; Serodiagnosis

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1. Introduction

Bovine tuberculosis (TB) is a serious infectious disease caused by *Mycobacterium bovis* (O'Reilly and Daborn, 1995), which gives rise to both large economic losses and a major public health concern. The disease control programmes carried out in most countries (Caffrey, 1994) are based on a test and removal strategy utilizing the intradermal test (IDT) with purified protein derivative (PPD) tuberculin (Monaghan et al., 1994). In this respect, it is widely recognized that tests for cell-mediated immunity are far more sensitive than antibody (Ab) tests in bovine TB (Wood et al., 1992), in terms of both precociousness and prevalence of the positive reactions in TB-affected cattle. Disease control based on IDT can be facilitated by the gamma-interferon (γ -IFN) test for bovine TB (Wood and Rothel, 1994), which was reported as more sensitive (Wood et al., 1991; Archetti et al., 1996), but sometimes less specific (Lauzi et al., 2000) than IDT. An inverse relationship was also described between the courses of cell-mediated and humoral immunity, the latter being preferentially detected in cases of advanced disease (Ritacco et al., 1991). Despite these drawbacks, Ab tests for *M. bovis* have been extensively investigated for a number of reasons. First, Ab tests may help detect IDT-negative, anergic cattle in advanced phases of infection, thus complementing the results of the tests for cell-mediated immunity (Plackett et al., 1989). Second, useful results can be obtained in deer and cattle in the anamnestic ELISA assay carried out after injection of PPD tuberculin (Hanna et al., 1992; Chinn et al., 2000; Lightbody et al., 2000). Finally, Ab tests might elucidate the disease status of farms, characterized by dubious, inconclusive IDTs (Amadori et al., 1998). Several antigens and protocols of Ab assays were proposed and different rates of success were reported (Hanna et al., 1992; Cataldi et al., 1994; O'Loan et al., 1994; Costello et al., 1997; Ostyn et al., 1997; Sugden et al., 1997; Lightbody et al., 1998; Lightbody et al., 2000). TB serology is actually compounded by considerable technical difficulties related to the choice of antigens and immunoassay format, the phase of the infection and the Ab isotype involved in the response. In practice, no serological procedure for bovine TB has met the demands of both sensitivity and specificity for large-scale field usage. The often delayed seroconversion (Hanna et al., 1992; Lyashchenko et al., 1998), the cross-reactions induced by environmental mycobacteria (Amadori et al., 1998), the often conflicting requirements between specificity and sensitivity of the test antigens (Fifis et al., 1992; Wood et al., 1992), all accrue to the difficulties in establishing a satisfactory serological protocol for bovine TB. In general, Ab tests based upon PPD tuberculins and/or different crude extracts of *M. bovis* are characterized by a low discriminating power, the distribution of the Ab titers between infected and non-infected individuals being widely overlapping (Amadori et al., 1998). There is thus a case for use of TB complex-specific antigens; some of them have been successfully employed in tests on naive and *M. bovis*-infected cattle (Lyashchenko et al., 1998). Purified antigens can be obtained by electrophoretic separation and blotting on nitrocellulose (Abou-Zeid et al., 1987), chromatographic procedures (Fifis et al., 1991), electroelution (Weldingh et al., 2000) and genetic engineering (Lyashchenko et al., 1998). The rationale behind an ELISA protocol with multiple recombinant antigens of *M. bovis* can be summarised as follows.

- There is a fairly good knowledge of the main antigens involved in the Ab response of *M. bovis*-infected cattle, which can pave the way for a correct choice of the test antigens

(Cataldi et al., 1994; O’Loan et al., 1994; Lightbody et al., 1998; Lyashchenko et al., 1998; Lightbody et al., 2000).

- A combination of several antigens should cover a variation of the Ab responses of cattle to multiple antigens (Fifis et al., 1992; Lyashchenko et al., 1998).
- Recombinant proteins can be produced on a large scale; they are cost-effective and guarantee satisfactory conditions of both homogeneity and consistency of the test antigens.

In the present study we examined the serological specificity of nine recombinant antigens by testing sera from *M. avium*-exposed and experimentally or naturally *M. bovis*-infected cattle and from paratuberculosis (paraTB)-infected cattle, to evaluate the diagnostic potential in a range of situations.

2. Materials and methods

2.1. Animals

2.1.1. Naturally *M. avium*-exposed and experimentally *M. bovis*-infected calves (group 1)

This study was undertaken in a licensed, high-security animal isolation unit under the supervision of a responsible biosecurity officer and the Animal Health and Welfare Division of the Italian National Veterinary Service. Three, 6-month-old, cross-bred calves (numbers 39, 40, 43) from a bovine tuberculosis-free herd, scored as avian reactors in the γ -IFN assay for bovine tuberculosis (Wood and Rothel, 1994) (Bovigam test kit, CSL, Melbourne, Australia), were sedated and inoculated endotracheally with 50,000, 5000 and 500 colony forming units, respectively, of a virulent *M. bovis* strain. The strain used for the experimental infection had been isolated in Modena Province (northern Italy) in February 1998 from a bovine lymph node; after isolation on solid Löwenstein-Jensen medium, the identification was unambiguous in terms of microscopy (Ziehl-Neelsen staining), cultural characteristics (growth in Löwenstein-Jensen, Stonebrink with crystal violet, liquid Middlebrook 7H9, solid Middlebrook 7H10 media), biochemical reactions (urease+, nitrate reduction–, arylsulfatase–, tellurite reduction \pm , T2H-susceptible), temperature requirements (growth at 43 °C, negative) and animal pathogenicity (guinea-pig test positive). The strain, frozen in aliquots and given code number 503, was titrated on solid Middlebrook 7H10 medium. Fifty-seven days later, three other avian reactors of the same herd (numbers 71, 111, 117) were housed with the above calves (contact infection). Endotracheally-infected calves (EIC) were slaughtered at day 105 post infection (p.i.); contact-infected calves (CIC) were slaughtered at day 92 after exposure to EIC. Blood samples were collected at weekly intervals from each EIC and CIC. These animals were selected to facilitate investigation of the possible interference of *M. avium* infection on diagnosis of bovine TB (Amadori et al., 2002). No IDT was carried out before any blood sampling; in agreement with the definition of *avian reactors* in the γ -IFN assay *before the experimental infection*, both *M. bovis* and *M. avium* were isolated from the calves; the three EIC and one of the CIC had TB lesions at autopsy (Amadori et al., 2002).

2.1.2. Cattle from TB-free herds (group 2)

Twenty-one sera of cattle were collected in four officially-certified, TB-free herds; on the day of sampling, animals were submitted to a comparative IDT with bovine and avian PPD tuberculin, and to the γ -IFN test for bovine TB (Wood and Rothel, 1994). The four herds had never been affected by TB and paraTB in the last 10 years, nor had there been suspected lesions at the slaughterhouse, or dubious IDTs in the previous annual checks.

2.1.3. Cattle from two confirmed field outbreaks of bovine TB (group 3)

Twenty-eight sera were collected from bovine reactors (Wood and Rothel, 1994) to the γ -IFN test for bovine TB. Some of these cattle were also IDT-positive.

2.1.4. ParaTB-affected cattle (group 4)

Fifteen sera were collected from cows showing clinical signs of Johne's Disease. These animals had scored positive for Ab to MAP (*M. avium*, subsp. *paratuberculosis*) by using two commercially available kits; they were also shedding MAP in the feces at the time of blood collection.

2.2. Recombinant antigens

The genes encoding the 14 kDa protein, ESAT-6, MTSA-10, MPT51 and MPT63 of *M. tuberculosis* were expressed in *E. coli* as NH₂-terminally polyhistidine-tagged fusion proteins, as previously described (Manca et al., 1997a,b). Recombinant antigens were purified to near homogeneity by using a three-step chromatographic protocol (Colangeli et al., 1998). The genes encoding MPB59, MPB64, MPB70 and MPB83 of *M. bovis* were expressed as previously described (Lightbody et al., 1998). The antigens used in this study are listed in Table 1. Some have been already used in serological studies on experimentally-infected cattle (Lyashchenko et al., 1998). MTSA-10 is identical to the CFP10 protein described elsewhere (Skjot et al., 2000).

Table 1
Recombinant protein antigens of *M. bovis* used in this study

Antigen	Molecular mass (kDa)	Reference for sequence	Specificity for TB-complex
ESAT-6	6	(Sørensen et al., 1995)	±*
14 kDa protein	14	(Verbon et al., 1992)	–
MPT 63	16	(Manca et al., 1997a)	+
MPB 70	22	(Matsumoto et al., 1995)	+
MPB 64	23	(Yamaguchi et al., 1989)	+
MPT 51	27	(Ohara et al., 1995)	–
MPB 59	30	(Matsuo et al., 1988)	–
MTSA-10	10	(Colangeli et al., 2000)	±*
MPB 83	22	(Matsuo et al., 1996)	+

(+) protein expressed in the TB complex, only; (–) protein expressed in the TB complex and in other mycobacteria; (*) ESAT-6 and MTSA-10 are also present in *M. kansasii* and *M. marinum*.

2.3. ELISA

The recombinant proteins listed in Table 1 were stored at -20°C in 50% glycerol at a concentration of 20 $\mu\text{g}/\text{ml}$. They were used to coat Nunc Maxisorb ELISA plates at 1 $\mu\text{g}/\text{ml}$ at 4°C overnight in carbonate/bicarbonate buffer 0.1 M, pH 9.6. The indirect ELISA in use had been developed and validated in an earlier study (Amadori et al., 1998). Briefly, plates were washed three times with PBS plus 0.05% Tween 20 and overcoated with 0.5% skim milk in PBS for 1 h at 37°C . This was next discarded and sera were added at 1:16 final dilution in PBS/Tween/1% BSA. After 1 h at 37°C in a rotary shaker and three additional washings, a rabbit anti-bovine IgG (H + L), alkaline phosphatase (AP)-conjugated anti-serum in PBS/Tween/1% BSA was added and plates were incubated for 60 min at 37°C in a rotary shaker; after three additional washings, *para*-nitro-phenyl phosphate (1 mg/ml) was used as the substrate of the reaction; after 1 h at room temperature plates were read spectrophotometrically at 405 nm. A pool of Ab-positive sera, selected in our previous study (Amadori et al., 1998), was used to check the consistency of the results; these were validated if the above positive control read within a pre-defined OD range.

2.4. Data analysis

In the case of experimentally infected cattle (group 1), all results were scored on the basis of the day 0 control, in agreement with a previous study (Lyashchenko et al., 1998); results were expressed in terms of optical density index (ODI), i.e. the ratio between OD_{405} of a test sample and OD_{405} of day 0 (before infection) (Lyashchenko et al., 1998). An $\text{ODI} \geq 2$ was taken as indicative of a consistent Ab response; samples within ODI values between 1.5 and 1.9 were scored as weakly Ab-positive.

Cattle sera of group 2 were used to determine a suitable cut-off value for field sera. This was set on the basis of the mean net OD + 3 standard deviations. Net OD was determined as the OD difference between Ag-coated and blank wells, minus the same difference in control wells where no serum was reacted.

Repeated measure ANOVA was applied to the results of the Ab tests for each recombinant protein in endotracheally-infected calves (group 1). One-way ANOVA was used to check the differences observed with the nine recombinant proteins in cattle of group 4. The Newman-Keuls post test was adopted to perform multiple comparisons among groups.

3. Results

3.1. *M. avium*-exposed and experimentally *M. bovis*-infected calves (group 1)

All calves showed signs of infection in terms of post mortem pathological findings and/or results of the IDT and γ -IFN tests for bovine TB (Amadori et al., 2002). The three endotracheally-infected calves mounted an Ab response to different recombinant proteins (Table 2). Of the three contact-infected calves, only one (number 111) developed a transient Ab response to MTSA-10, MPT51 and MPT63; Ab reactions to these proteins

Table 2
Ab response to recombinant *M. bovis* proteins after endotracheal infection. Calves 39, 40, 43

Proteins days p.i.	MPB59	MPB64	MPB70	MPB83	14 kDa	ESAT-6	MTSA-10	MPT51	MPT63
7	+, -, -	±, -, -	±, -, -	±, -, -	-, -, ±	±, -, -	±, -, -	+, -, -	±, -, -
14	-, ±, -	-, -, -	-, -, -	-, -, ±	-, -, -	-, -, -	-, -, ±	-, -, ±	-, -, -
23	-, +, ++	-, ±, +	-, +, +	-, ±, ++	-, ±, ++	-, +, ++	-, +, +	-, +, +	-, +, +
28	-, +, ±	-, ±, -	-, ±, ++	+, +, ++	-, ±, ±	-, ±, -	-, +, -	±, +, ±	-, ±, ±
35	-, ++, -	-, ++, -	-, ++, ++	++, ++, ++	-, ++, -	-, ++, -	-, ++, -	-, ++, -	-, ++, -
42	-, ++, -	-, ++, -	-, ++, ++	++, ++, +	-, ++, -	-, ++, -	-, ++, -	-, ++, -	-, ++, -
52	-, +, -	-, +, ±	-, +, ++	±, ++, +	-, +, ±	-, +, ±	-, +, -	-, -, ±	±, +, ±
60	-, -, -	-, -, -	±, -, +	±, -, +	-, -, -	-, -, -	-, -, -	-, ±, ±	-, -, -
67	-, -, -	-, -, -	-, -, +	±, -, +	-, -, -	-, -, -	-, -, -	-, -, -	-, -, -
74	-, -, ±	-, -, ±	-, -, +	±, -, +	-, -, ±	-, -, ±	-, -, ±	-, -, ±	-, -, ±
81	-, ±, ±	-, +, -	±, -, +	-, ±, +	-, -, +	-, ±, ±	-, ±, ±	±, -, +	±, -, ±
88	-, -, ±	-, -, -	-, -, +	-, ±, +	-, -, ±	-, -, -	-, -, -	-, -, ±	-, -, ±
95	-, -, ±	-, -, ±	-, -, +	-, -, +	-, -, +	-, -, ±	-, -, ±	±, -, +	±, -, +

Results for calves 39, 40 and 43, respectively, are reported in a row, spaced by commas. (-) Ab negative; (±) weakly Ab positive (ODI 1.5–1.9); (+) Ab positive (ODI 2.0–2.9); (++) strongly Ab positive (ODI ≥ 3).

were detected at days 51 and 58 after contact infection, and then again at day 86. It is worth noting that these latter proteins were not preferentially recognized by endotracheally-infected calves; their Ab response was mainly directed in fact to MPB70 and MPB83 (calves 39 and 43), or to a larger array of proteins (calf 40), the peak of reactivity in the Ab tests being always observed between days 35 and 42 p.i. Interestingly, there was also evidence of an earlier Ab response to bovine PPD tuberculin in two endotracheally-infected calves, possibly due to a cross-reaction with antigens expressed by mycobacteria of the *avium/intracellulare* group (data not shown). By repeated measure ANOVA, the test results for MPB83 were shown to differ significantly as time elapsed after infection ($P < 0.01$). There was no correlation between infectious dose of *M. bovis* and level of the subsequent Ab response in endotracheally-infected animals.

3.2. Cattle from TB-free herds (group 2)

All animals were IDT-negative; 12 were scored NIL reactors and 9 were scored AVIAN reactors in the γ -interferon test. Results of Ab tests are shown in Table 3. On the basis of the net OD values corresponding to mean + 3 S.D.s, a common cut-off of 0.07 net OD was adopted for all the proteins under study. This common cut-off was chosen because it was representative of the results obtained with most TB complex-specific antigens. Also, there were no differences if the animals were separated into NIL and AVIAN types. The high background reactions for MBP59 and 14 kDa protein (non-specific for TB complex) were probably due to exposure to environmental mycobacteria.

3.3. Field outbreaks of bovine TB (group 3)

In two field outbreaks of bovine TB, 16 out of 28 γ -interferon positive cattle showed Ab responses to at least one protein antigen. Seven out of nine recombinant proteins used in this study were serologically recognized by those animals, showing variable reactivity patterns. Importantly, 7 of these Ab responders were found among 12 IDT-negative cattle. Further, 12 animals showed Ab responses against TB complex-specific proteins, and 5 of

Table 3
Ab tests in cattle from TB-free herds

	Mean	S.D.	Mean + 3 S.D.
Ab to recombinant, non-TB complex-specific proteins			
MPB59/net OD	0.0226	0.0233	0.0925
14 kDa protein/net OD	0.0253	0.0212	0.0889
MPT51/net OD	0.0075	0.0109	0.0402
Ab to recombinant, TB complex-specific proteins			
MPB64/net OD	0.0208	0.0239	0.0925
MPB70/net OD	0.0145	0.0158	0.0619
MPB83/net OD	0.0126	0.0208	0.0750
ESAT-6/net OD	0.0176	0.0196	0.0764
MTSA-10/net OD	0.0071	0.0132	0.0467
MPT63/net OD	0.0053	0.0082	0.0299

Table 4
Ab to *M. bovis* proteins in paraTB-affected cows

Animal	MPB59	MPB64	MPB70	MPB83	14 kDa	ESAT-6	MTSA-10	MPT51	MPT63
2	–	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	–	–
6	–	–	–	–	–	–	–	–	–
9	+	–	–	–	+	–	–	–	–
10	–	–	–	–	–	–	–	–	–
14	–	–	–	–	–	–	–	–	–
15	–	–	–	–	+	–	–	–	–
17	+	–	–	–	–	–	–	–	–
19	–	–	+	–	–	–	–	–	–
27	+	–	–	–	–	–	+	–	–
43	–	–	–	–	–	–	–	–	–
44	–	–	–	–	–	–	–	–	–
46	–	–	–	–	–	–	–	–	–
50	–	–	–	–	–	–	–	–	–
64	–	–	–	–	–	–	–	–	–

(–) Ab negative sample; (+) Ab positive (net OD ≥ 0.07).

those were IDT-negative. Among TB complex-specific proteins, the most reactive ones were ESAT-6, MTSA-10 and MPB64 (5, 4 and 3 responders, respectively); there was no detectable Ab response to MPT51 and MPT63.

3.4. ParaTB-affected cattle (group 4)

Four animals out of 15 showed an Ab response to MBP59 and/or 14 kDa protein. Two animals were Ab-positive for MBP70 and MTSA-10, respectively (see Table 4). There were significant differences in the reactivity of the sera to the nine proteins under study (one-way ANOVA, P : 0.001). The mean titres (net OD) to MPB70, 14 kDa protein and MPB59 showed the highest values of standard deviation. By performing the Newman-Keuls post test, the test results for MPB59 and 14 kDa proteins were shown to differ significantly from those of other proteins under study ($P < 0.05$).

4. Discussion

Our results confirm that a panel of recombinant antigens enables detecting the Ab response of *M. bovis*-infected cattle, while keeping a high specificity towards infections sustained by mycobacteria of the *avium/intracellulare* group. In fact, the previous sensitisation of group 1 calves by such mycobacteria did not prevent a successful recognition of the Ab response to *M. bovis*-specific antigens after a lag phase, characterized in some animals by a preferential recognition of cross-reacting antigens like MPB59. As for the endotracheally-infected animals, the peak of Ab response was observed between day 35 and 42 p.i.; this phase was set between a distinct increase of the body temperature at week 4 p.i. and a later phase of *M. bovis* excretion from the nasal cavities (Amadori et al., 2002).

The good specificity of the test system was further confirmed by the results obtained on paraTB infected cattle (group 4); it should be noted that an Ab response to MPB59 but not MPB70 had been already described in paraTB-affected cattle (Harboe et al., 1990). As expected (Harboe et al., 1990), the highest level of cross-reaction was observed for MPB59, a component of the Ag85 complex (Wiker and Harboe, 1992). An Ab response to such cross-reacting proteins could be effectively checked by a further ELISA for Ab to specific, purified antigens of *Mycobacterium avium* subsp. *paratuberculosis* (Olsen et al., 2001).

The need for a wide panel of antigens could be also inferred from the results on cattle from two field TB outbreaks. In fact, the profiles of Ab response did vary considerably; also, they were more restricted than those of experimentally infected calves (group 1). The transient course of the Ab response to single antigens in group 1 calves was also a prominent feature; nevertheless, the simultaneous usage of several recombinant antigens enabled recognizing the ongoing Ab response over time, thus highlighting the profiling ELISA described in this study. In the case of an experimental study with repeated samplings, the test conditions can be controlled by inserting on the same plate the day 0 serum (see results of group 1 calves); needless to say, this negative reference value will vary according to the background exposure to environmental mycobacteria. On the contrary, the situation would be ill-defined in the case of field samples of unknown origin. By using the net OD values as readout, a good specificity of our ELISA could be demonstrated.

Within a profiling assay with a panel of recombinant proteins, sensitivity and specificity might be improved by staging the Ag/Ab reaction in liquid rather than in solid phase, in the framework of a competition, inhibition or liquid phase blocking ELISA. In particular, this could possibly counteract the non-specific binding of IgM Abs onto the solid phase. In addition, isotype-specific ELISAs could be better suited to the purpose, as previously described for human and bovine TB (Alifano et al., 1996; Lightbody et al., 2000).

For the time being, the described ELISA protocol can be of use under the aforementioned conditions: first, this Ab test could detect *M. bovis*-infected, IDT-negative cattle, as shown in some group 3 animals. Secondly, it could be properly employed in IDT-negative cattle for confirmation of the data provided by the γ -IFN assay, which may show a rather low specificity in some areas (Lauzi et al., 2000). Finally, it may help elucidate the infection status of farms, characterized by dubious, inconclusive IDTs (Amadori et al., 1998).

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