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Further Evaluation of an Indirect Enzyme-Linked Immunosorbent Assay for the Diagnosis of Bovine Tuberculosis

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With 2 figures and 2 tables

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Summary

The sensitivity and specificity of an ELISA for the detection of bovine IgG anti-*Mycobacterium bovis* antibodies were 73.6 % and 94.1 %, respectively, as determined in 53 bacteriologically confirmed tuberculous cattle and 101 healthy cattle from a tuberculosis-free area.

In addition, the results of ELISA and tuberculin tests in 149 cattle were compared with those of subsequent necropsy studies. Both tests failed to detect 2 animals with tuberculous lesions and positive culture; 3/12 cattle with *M. bovis* isolation and no lesions, and 2/7 with atypical mycobacterial infection reacted to tuberculin, but none had antibodies; in 128 cattle with neither lesions nor mycobacterial isolation, 6 were tuberculin reactors and 7 others had antibodies.

Negative results were obtained by ELISA in 21/22 paratuberculous cattle. Antibodies were not detected in 88.9 % to 96.4 % of 697 cattle from two tuberculin negative herds of an endemic area. In a herd with proved *M. bovis* infection, distribution of seropositive animals in tuberculin and non-tuberculin reactors was similar.

Antibody responses to cutaneous tuberculin stimuli were observed in 4 experimentally infected cattle, but only in 2/10 healthy controls after repeated PPD stimuli. Nine controls which had either received a single tuberculin dose or none showed no increase in antibody levels.

The low sensitivity of this ELISA limits its usefulness as a diagnostic tool for bovine tuberculosis eradication campaigns. However, it could be helpful in epidemiological surveillance if its efficiency to identify infected herds is demonstrated.

Key words: Bovine tuberculosis, ELISA, mycobacterial antibodies

Introduction

In our previous assessment of an enzyme-linked immunosorbent assay (ELISA) for the detection of bovine circulating IgG mycobacterial antibodies, 90.0 % of bacteriologically confirmed tuberculous cattle were found positive (18/20), whereas negative reactions

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were observed in 89.8 % healthy cattle (44/49) from a tuberculosis free area (RITACCO et al., 1987). Such sensitivity and specificity, which are at least comparable to those attributed to the tuberculin skin test (FRANCIS et al., 1978), encouraged us to explore further the applicability of this method to the diagnosis of bovine tuberculosis. In our attempt to achieve this aim, we conducted a series of studies involving naturally and experimentally infected cattle, the results of which are reported here.

Material and Methods

The presence of antimycobacterial antibodies was examined in 2,568 serum samples obtained from naturally and experimentally infected cattle and their controls.

Cattle

Naturally infected

1. *Bacteriologically confirmed tuberculous cattle and controls.*

(i) Animals with macroscopic lesions compatible with tuberculosis were selected at the slaughterhouse. Serum was obtained from the intracardiac clot, and lesion samples were taken for bacteriological studies. Only the sera from 53 animals (n: 53) with *Mycobacterium bovis* isolation were examined by the ELISA.

(ii) Healthy animals from a tuberculosis-free area (Campo DILFA, Uruguay) were used as controls (n: 101).

2. *Blindly selected necropsied cattle.* Seventy-two hours prior to slaughter serum samples were collected from 149 animals and tuberculin skin tests were simultaneously performed. Samples of macroscopic lesions or, when not observed, of mediastinic and retropharyngeal lymph nodes were selected for mycobacterial isolation.

3. *Bacteriologically confirmed paratuberculous cattle.* Serum samples were collected from cattle with positive faecal culture for *M. paratuberculosis* (n: 22). The animals belonged to a tuberculosis free herd with proved paratuberculosis infection.

4. *Tuberculin-negative cattle from a tuberculosis endemic area.*

i) Cattle from a carefully controlled herd (n: 137) from which occasional tuberculin reactors were promptly eliminated, were included.

ii) Serum samples were obtained in two consecutive years from 523 and 560 cattle, respectively. The animals were part of a formerly infected herd in which all tuberculin reactors had been eliminated recently. Twelve animals were retested six months after the last bleeding.

5. *Cattle from a tuberculosis-infected herd.* (n: 594) Infection had been confirmed by the isolation of *M. bovis* from lesions of 2 necropsied animals.

Experimentally infected

1. Three steers aged 8 months were inoculated intravenously with 0.01 mg (wet weight) of *M. bovis* strain CPZ 7773 isolated from a cow. One tenth mg of bovine PPD was injected intradermally at weeks 6, 9, 11, 14, and 16 post inoculation (p. i.).

Another steer of the same age was inoculated intravenously with 0.001 mg (wet weight) of *M. bovis* AN5, a collection strain with a lower virulence than that of *M. bovis* strains recently isolated from lesions. PD at the above-mentioned dose was injected intradermally at weeks 26, 36, 72, 74, 77, 79, and 110 p. i.

Blood samples were collected periodically until week 47 p. i. from the first three animals, and until week 116 p. i. from the last. Lesion samples were obtained at necropsy for bacteriological studies.

2. Nineteen healthy steers aged 6 to 36 months, grouped into four lots of 4 or 5 animals each, served as controls: lots 1 and 2 received 0.1 mg of bovine PPD intradermally every 14 and 56 days, respectively; lot 3 received a single dose of PPD at the onset of the experiment; the last lot was not tuberculinized. Blood samples were obtained fortnightly up to week 36. All experimental animals came from tuberculosis-free farms.

Cattle selected at the slaughterhouse lacked a recorded history of previous tuberculinizations. Experimental animals have not been tuberculinized before starting the experiment. For the remaining cattle studied here, a period of at least six months elapsed between the last tuberculin test and the bleeding.

Bacteriological studies

Specimens were minced in a tissue grinder, decontaminated by adding 4 % sodium hydroxide, and cultured on LOWENSTEIN-JENSEN and STONEBRINK media. Species identification was based on previously described tests (LENNETTE, 1980).

Faecal samples were processed and cultured according to methods previously described for *M. paratuberculosis* isolation (MERKAL, 1973).

Enzyme-linked immunosorbent assay

The assay was performed as described previously (RITACCO et al., 1987), with the following modifications: *M. bovis* PPD antigen was employed at a concentration of 10 µg/ml in carbonate buffer; affinity purified rabbit anti-bovine IgG (H + L chain) coupled to horseradish peroxidase (Accurate Chemical Scient. Corp. Westbury, N. Y.) was used at an optimal dilution of 1 : 2,400; the working substrate-chromogen solution was prepared combining 200 µl of 2.2 azinobis (3-ethylbenzthiazoline sulphonic acid) (Sigma Chemical Co.) stock solution (32.9 mg/ml), 50 µl of H_2O_2 9 % (v/v) and 12 ml of citrate buffer, pH 4; volumes of 100 µl per well were used, except for the antigen, 120 µl of which were dispensed into each well.

Results

Sensitivity and specificity: O. D. values obtained in sera from cattle of the tuberculosis-free area ranged from 0.010 to 0.330. The cut-off value was determined by the addition of two standard deviations to the mean of this group (mean + 2 SD: 0.0556 + 2 [0.057] ≅ 0.170).

Antibody levels were above the cut-off point in 73.6 % (39/53) of the sera from bacteriologically confirmed tuberculous cattle, whereas values for 94.1 % of the sera from the tuberculosis-free cattle were below it (95/101) (Table 1).

Serological and tuberculin skin test results in blindly selected necropsied cattle: The results of the ELISA and of the tuberculin skin test in 149 cattle blindly selected at the slaughterhouse are summarized in Table 2.

Only two animals showed tuberculous lesions confirmed by culture. Neither had tuberculin skin reactivity nor detectable circulating antibodies. Of the remaining 147 animals with no visible lesions at necropsy, 12 had *M. bovis* positive culture of which three were tuberculin reactors and none showed detectable antibodies. Atypical mycobacteria were isolated from 7 and tuberculin skin reactivity was observed in 2; antibodies were not detected in any of them.

Table 1. Serological analysis by ELISA of naturally infected and control cattle populations

Group	Description	Positive/number tested	Percentage positive
1	— With active tuberculosis bacteriologically confirmed	39/53	73.6
	— Healthy controls from a tuberculosis-free area	6/101	5.9
2	— Blindly selected at the slaughterhouse	See Table 2	
3	— With confirmed paratuberculosis	1/22	4.5
4	— Long-dated tuberculin negative	5/137	3.6
	— Recently cleaned herd		
	1st sampling	22/523	4.2
	2nd sampling	62/560	11.1
5	— *M. bovis* infected herd		
	Tuberculin-positive cattle	46/139	33.1
	Tuberculin-negative cattle	126/455	27.7

Table 2. Serological and tuberculin skin test results in 149 blindly selected necropsied cattle

Necropsy Findings	Bacteriological Findings	Tuberculin Skin Reactors	Serological Reactors
Macroscopic lesions compatible with tuberculosis (n = 2)	2 *M. bovis*	0	0
No visible lesions (n = 147)	12 *M. bovis*	3	0
	7 Atypical Mycobacteria	2	0
	128 Culture Negative	6*	7*

* Tuberculin and serological reactors did not overlap.

Of 128 animals with neither lesions nor mycobacterial isolation, 6 were tuberculin reactors, while 7 others had circulating antibodies.

Antibody determination in paratuberculous cattle: A low positive ELISA result was found in the sera from only 1 out of 22 confirmed paratuberculous animals; the remaining 21 were negative.

Antibody determination in tuberculin-negative herds from a tuberculosis-endemic area: In a herd that had been tuberculin-negative for many years, the ELISA showed that 96.4 % of the cattle (132/137) lacked detectable anti *M. bovis* antibodies.

Antibody determinations in sera from a herd which had been recently freed of tuberculosis showed 95.6 % seronegative animals (501/523). A year later, this percentage dropped to 88.9 % (498/560). Different animals were found positive in the two testings. Nine out of 12 animals with strongly positive results in the second testing became negative six months later. The whole herd was negative to the tuberculin skin test during the period of the study (Table 1).

Antibody determination in a herd with proved M. bovis infection: Twenty-nine percent (172/594) of cattle from this group had detectable antibodies, and 23 % were tuberculin reactors. However, seropositive animals were evenly distributed between the tuberculin reactors and the non reactors (33.1 % and 27.7 %, respectively) (Table 1).

Anamnestic antibody response to cutaneous PPD in experimentally infected cattle: The 3 steers infected with a highly virulent wild strain of *M. bovis* developed detectable antibodies 10 weeks p. i. Antibody levels increased three or four weeks after the first PPD stimulus and persisted at high levels for eight weeks. After the stimulus was discontinued the descent occurred between weeks 23 and 38 (Fig. 1).

The reaction was similar in the steer inoculated with *M. bovis* strain AN5: no spontaneous increase of antibody levels and brief anamnestic responses to PPD stimuli were oberved (Fig. 1). *M. bovis* was isolated from all animals.

One of the controls from lot 1 had moderately high antibody levels at the onset of the experiment, which persisted during the whole period. After the third tuberculin injection, an increase in antibody levels was observed in one animal from lot 1 and in one from lot 2. The remaining 16 controls were seronegative throughout the experiment (Fig. 2).

Discussion

The results obtained in the present study show that: a) mycobacterial antibodies were present in a high percentage of animals with active tuberculosis, but absent in subclinically infected cattle; b) there was little coincidence between the results obtained with the tuberculin skin test and the ELISA; c) anamnestic antibody responses to tuberculin were observed in all the infected cattle. In a small number of the control animals these responses occurred after repeated PPD stimuli; d) the ELISA showed a fairly high specificity in the

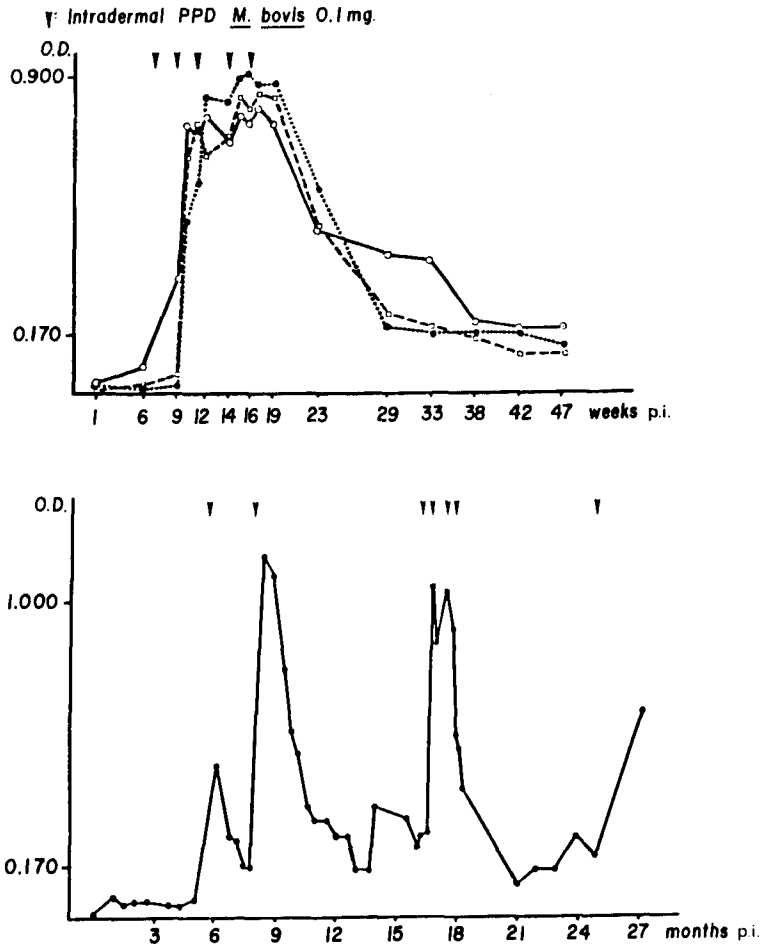

Fig. 1. Serum antimycobacterial antibodies detected by ELISA at different times after infection in 4 cattle experimentally infected with *Mycobacterium bovis* and periodically injected with tuberculin PPD

testing of animals both from tuberculosis-free and endemic areas, although fluctuations in antibody levels were seen in a small percentage.

Mycobacterial antibodies were found in almost 3 out of every 4 cattle with active tuberculosis. The discrepancy with the sensitivity levels of our previous assessment (90.0 %) could be due to the use of larger samples, to technical modifications which increased its specificity, and to the fact that a different criterion was adopted in the selection of the cut-off point. On the other hand, no IgG antibody response was detected in the early stages of bovine tuberculosis or in its latent form. This was shown by the seronegativity of all the infected animals with no visible lesions at necropsy and by the absence of spontaneous antibody production in the early stages of experimental infection. This low sensitivity represents a serious drawback for the ELISA under consideration. The use of monoclonal antibodies (MAbs) to specific *M. bovis* determinants for the purification of antigens and/or in competition assays would certainly result in maximum specificity (Wood et al., 1988). However, it is unlikely that sensitivity could be improved by this means. The reported work on the application of MAbs to human tuberculosis diagnosis is

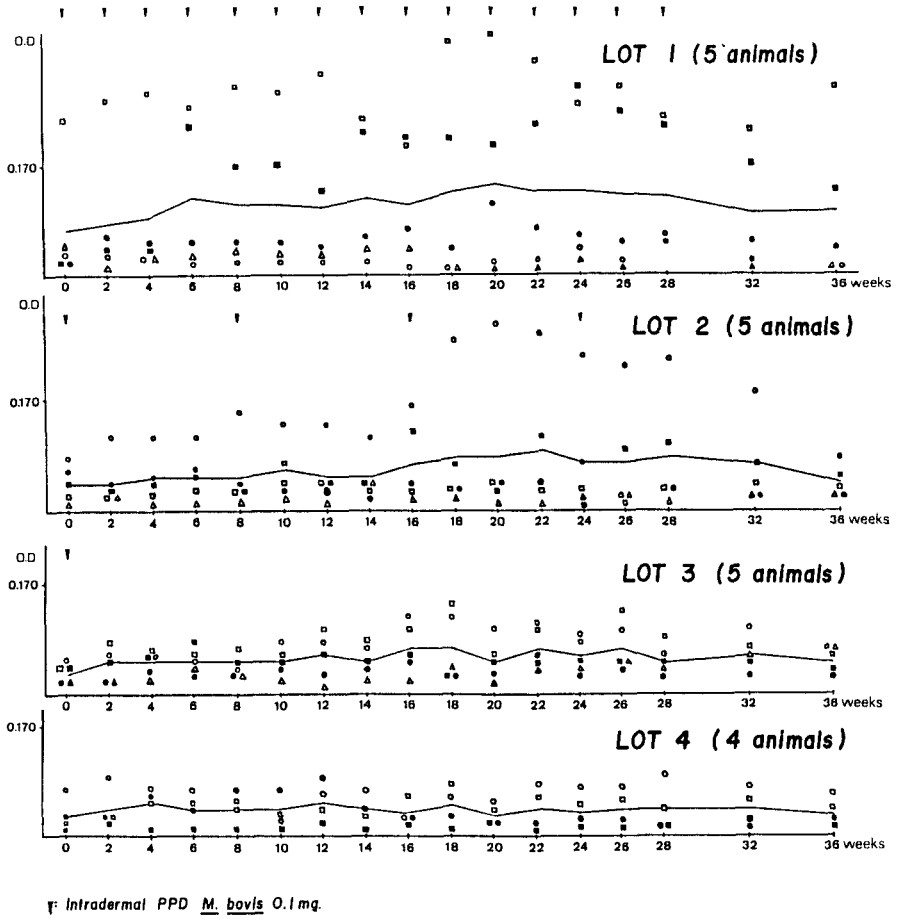

Fig. 2. Serum antimycobacterial antibody levels by ELISA in 19 uninfected control cattle. (—): Trend line of medium values

not very encouraging, probably because of the wide heterogeneity of individual responses to specific epitopes and the immunodominance of shared epitopes (STANFORD, 1983; BOTHAMLEY et al., 1988; JACKETT et al., 1988).

Positive results of the ELISA and of the tuberculin skin test in a *M. bovis* infected herd coincided in only one animal out of 3. It is interesting to note that coincidence was also lacking in the false positive results of the blind sampling performed at the slaughterhouse. A balance between cellular and humoral immune responses in human tuberculosis was postulated more than a decade ago (LENZINI et al., 1977) and is supported by recent investigations (HUYGEN et al., 1988). Cellular response tends to be strongest in the early stage of infection and decreases as the disease progresses. In fact, a complete cutaneous energy is commonly found in long-termed or acutely disseminated tuberculosis. Antibodies, on the contrary, are not present in healthy tuberculin-positive individuals (BENJAMIN and DANIEL, 1982; KARDJITO et al., 1982; KALISH et al., 1983), but tend to appear most frequently in advanced, long-dated or disseminated disease, when there is a heavy antigenic load (DANIEL and DEBANNE, 1987). Our results and those of PLACKETT et al. (1989) suggest that a similar balance would occur in bovine tuberculosis.

Anamnestic antibody responses elicited by tuberculin were described previously in tuberculous as well as healthy cattle (MALLMAN et al., 1964; RICHARDS et al., 1966; YUGI

and NOZAKI, 1972; THOEN et al., 1983). In the present study, transient anamnestic IgG antibody responses were detected by the ELISA in all the experimentally infected cattle. Although a single PPD stimulus did not elicit increase in antibody levels in healthy controls, 2 out of 10 uninfected animals which received repeated PPD doses did respond in an anamnestic-like manner. Admittedly, previous tuberculin test performed on the slaughtered animals included in our study could have biased our sensitivity figures. However, this is not highly probable because systematic tuberculin skin testing in Argentina is carried out only in a few dairy herds on a voluntary basis.

In contrast with recent observations (AUER, 1987; AUER and SCHLEEHAUF, 1988), we obtained a fairly high specificity with the ELISA (about 95 %), in non-infected herds from tuberculosis-free and endemic areas. A similar figure (94.5 %) was found in animals with negative culture for mycobacteria and no visible lesions at necropsy (121/128). Besides, atypical mycobacterial infections and paratuberculosis did not seem to be the cause of false positive results. Lastly, significant spontaneous fluctuations in antibody levels were detected only in a small percentage of animals previously in contact with *M. bovis*. The discrepancies of our results with those of the above-mentioned authors could be due to the higher specificity of the bovine PPD as compared with the cell sonicate used by them.

The low sensitivity of this ELISA excludes its use as a single diagnostic tool in bovine tuberculosis eradication campaigns. However, although it did not identify all infected cattle, its high specificity and its capacity to identify a high proportion (73.6 %) of cattle with active tuberculosis make further studies worth considering. There are reasons to hope that studies in the field show the usefulness of this assay for the detection of bovine tuberculosis on a herd basis. This would make it a valuable tool for epidemiological surveillance.

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Zusammenfassung

Weitere Erkenntnisse über einen indirekten ELISA zur Diagnose der bovinen Tuberkulose

Zur Prüfung der Sensitivität und Spezifität eines ELISA zur Diagnose boviner Tuberkulose wurden Seren von Rindern mit bakteriologisch gesicherter Tuberkulose (n: 53), sowie von Tieren aus einem tuberkulosefreien Gebiet (n: 101) untersucht; die Resultate stimmten in 73,6 % bzw. 94,1 % der Fälle überein.

Des weiteren wurden die ELISA- und Tuberkulintest-Ergebnisse von 149 Rindern mit denen einer nachfolgenden pathologisch-anatomischen Untersuchung verglichen. Beide Testsysteme waren nicht in der Lage, zwei Tiere mit pathologisch/anatomisch und bakteriologisch gesicherter Tuberkulose zu erkennen; drei von 12 Tieren, aus denen *M. bovis* isoliert wurde und die keine morphologischen Veränderungen aufwiesen, sowie 2 von 7 Tieren, die mit atypischen Mykobakterien infiziert waren, zeigten zwar eine positive Tuberkulinprobe, aber es konnten keine Antikörper nachgewiesen werden; von 128 Rindern, bei denen weder pathologisch-anatomische Veränderungen beobachtet werden konnten noch Mycobakterien isoliert wurden, zeigten 6 eine positive Tuberkulinreaktion und 7 weitere Tiere wiesen Antikörper auf.

Bei 21 von 22 an Paratuberkulose erkrankten Rindern verliefen die ELISA-Untersuchungen negativ. Bei Rindern aus zwei Tuberkulin-negativen Herden (n: 697) konnten ebenfalls in 88,9 % und 96,4 % der Fälle keine Antikörper festgestellt werden. In einer Rinderherde mit nachgewiesener *M. bovis*-Infektion war die Verteilung seropositiver Tiere unter den Tuberkulin-positiven und Tuberkulin-negativen Tieren nahezu gleich.

Eine kutane Tuberkulinapplikation führte bei 4 experimentell infizierten Tieren zu einer Antigenantwort; ein vergleichbares Resultat konnte jedoch nur bei 2 von 10 gesunden Tieren nach

wiederholter PPD-Applikation beobachtet werden. Neun Kontrolltiere, denen Tuberkulin einmal bzw. nicht injiziert wurde, zeigten keinen Anstieg der Antikörperwerte.

Die geringe Empfindlichkeit dieses ELISAs läßt nur eine Anwendung als diagnostisches Mittel im Rahmen von Eradikationsprogrammen zur Bekämpfung der bovinen Tuberkulose zu. Möglicherweise könnte dieser ELISA aber auch bei epidemiologischen Überwachungsprogrammen Verwendung finden, sobald seine Brauchbarkeit bei der Erkennung infizierter Herden nachgewiesen ist.

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