Competitive and Indirect Enzyme-Linked Immunosorbent Assays for *Mycobacterium bovis* Infections Based on MPB70 and Lipoarabinomannan Antigens

Edward A. Sugden, Kathryn Stilwell, Elizabeth B. Rohonczy, and Paul Martineau

ABSTRACT

A competitive enzyme-linked immunosorbent assay (C-ELISA) using *M. bovis* BCG Tokyo culture filtrate as antigen and anti-MPB70 4C3/17 monoclonal antibody was developed for use in multiple animal species. An analysis of the C-ELISA data for cattle and bison serum panels revealed specificities of 68% to 85% and sensitivities of 85% to 89%. Receiver operater characteristics (ROC) of this data revealed areas of 81% to 92% for C-ELISA and demonstrated that C-ELISA as well as the indirect ELISA protocols, MPB70-ELISA and LAM-ELISA, discriminate *M. bovis* infected animals from non- infected animals for these particular panels. The kappa statistic values for agreement beyond chance between C-ELISA and MPB70-ELISA were determined after ELISA cutoffs were adjusted to minimize false positives. There were poor to excellent agreements between C-ELISA and MPB70-ELISA in all species tested (Bovidae, Cervidae, and Camelidae) that were consistently higher than the kappa statistic between C-ELISA and LAM-ELISA. The humoral response to one antigen and little or no response to the other in many animals argued for a parallel interpretation of C-ELISA and LAM-ELISA to increase sensitivity.

RÉSUMÉ

Une épreuve ELISA-compétitive (ELISA-C) utilisant comme antigène un filtrat de culture de la souche BCG Tokyo de *M. bovis* et l'anticorps monoclonal anti-MPB70 4C3/17 a été mise au point en vue de son utilisation chez plusieurs espèces animales. Une analyse des résultats de l'ELISA-C obtenus avec des sérums provenant de bovins et de bisons a démontré que les spécificités étaient de 68 % et 85 % et les sensibilités de 85 % et 89 %. Les résultats obtenus avec l'ELISA-C, de même que ceux obtenus avec les protocoles d'ELISA indirects ELISA-MPB70 et ELISA-LAM, permettent de distinguer les animaux infectés par *M. bovis* des animaux non-infectés. Les valeurs de la statistique kappa entre l'ELISA-C et l'ELISA-MPB70 ont été déterminées après que les valeurs-seuils eurent été ajustées pour minimiser les faux-positifs. La correlation entre les résultats de l'ELISA-C et l'ELISA-MPB70 variait de faible à excellente pour toutes les espèces testées (Bovidae, Cervidae, et Camelidae) et était constamment plus élevée que la statistique kappa entre l'ELISA-C et l'ELISA-LAM. La réponse humorale envers un antigène et très peu ou aucune réponse envers un autre chez plusieurs animaux militent en faveur d'une interprétation parallèle des résultats de l'ELISA-C et l'ELISA-LAM afin d'augmenter la sensibilité de l'interprétation.

(Traduit par docteur Serge Messier)

INTRODUCTION

There is a continuing need for serodiagnostic tests to detect animals infected with *Mycobacterium bovis* with improved sensitivity, specificity, reproducibility, and wider applicability to different animal species. Serology may detect animals in which the cell mediated immune (CMI) response is poor and thus complement skin tests or in vitro CMI tests for the detection of infected animals (1–7).

Traditional serologic procedures for diagnosing *M. bovis* infections (8,9) have been supplanted in recent years by more sensitive enzyme-linked immunosorbent assays (ELISA) (3–7,10–16). Earlier ELISA protocols used crude antigen preparations such as unheated crude culture filtrate (3) purified protein derivative (PPD) from heated-killed cultures (11,13–15), whole bacteria (16), sonicated bacteria (12), and phosphatide extracts (15) in conjunction with a variety of enzyme conjugated antibodies to anti-species immunoglobulins. In general, lack of specificity was reported using such antigens.

More recently, attempts to increase ELISA specificity and detect multiple animal species have focused on the use of purified antigens such as MPB70, a major secreted protein of *M. bovis* (17–19). MPB70 has been studied in ELISA protocols using both anti-bovine IgG conjugates (5,7,20) as well as a streptavidin conjugate in a protein-G assay (4). Other studies to improve specificity include: 1) The assessment of other purified antigens (21); 2) the removal of cross-reacting antibodies from sera (14); and 3) the specific detection of the more discriminatory IgG antibodies (22) using protein A (10) or protein G (4) based assays. While the use of purified antigens has increased specificity, the sensitivity has been compromised (5,21). However, higher sensitivities with ELISA have been achieved through the testing of post-skin test sera (4,9).

The present study focused on a competitive ELISA (C-ELISA) which involves the competition of a monoclonal antibody (McAB) with antibody in sera from infected animals,

Agriculture and Agri-Food Canada, Animal Diseases Research Institute, P.O. Box 11300, Station H, Nepean, Ontario K2H 8P9.

Received October 30, 1995.

followed by detection of the bound McAB. This approach is potentially highly specific, does not require purified antigen, and could be used for multiple animal species. While similar studies have been done for human tuberculosis and leprosy (23,24), there are no reports for bovine tuberculosis. A C-ELISA protocol may also provide an improved discrimination of infected from non-infected animals, as found in the case of a C-ELISA for Brucellosis (25).

It was decided to evaluate 4C3/17 (SB-10), a commercially available McAB (26), in the C-ELISA protocol. This reagent is specific for a MPB70 epitope not present in *Nocardium asteroides* (26), but which is close to an epitope reacting with polyclonal antibody in sera from cattle naturally infected with *M. bovis* (27). This protocol was compared to an indirect ELISA using MPB70 (MPB70-ELISA) antigen (4) and an indirect ELISA using a *M. paratuberculosis* lipoarabinomannan antigen (LAM-ELISA) (28,29) on panels of sera from a variety of animal species.

MATERIALS AND METHODS

ANTIGENS AND MONOCLONAL ANTIBODY

Culture filtrates from *M. bovis* BCG Tokyo (ATCC # 35737), cultured for 8–9 wk in modified Reids medium (30), were filter sterilized (0.2μm), preserved with 1 mM phenylmethylsulfonyl fluoride and 0.01% merthiolate, concentrated to 3–4 mg protein/mL with PM-10 ultrafilters (Amicon, Oakville, Ontario), and stored at −20°C. The MPB70 antigen was chromatographically purified from *M. bovis* AN-5 and evaluated by electrophoresis and amino acid sequence (19). This antigen was kindly donated by Drs. T. Fifis and P.R. Wood (Commonwealth Scientific and Industrial Research Organization, Parkville, Australia) and stored frozen at 4 mg/mL in 0.01 M sodium phosphate in saline, pH 7.0. The LAM antigen was chromatographically purified from *M. paratuberculosis* and was assessed by chemical and spectroscopic analyses for sugar and lipid components and protein contamination (29). It was dissolved to 0.2 mg/mL in water and stored frozen. The 4C3/17 anti-MPB70 McAB was also kindly donated by Drs. Fifis and Wood and is commercially available from Agen Biomedicals (Acacia Ridge, Australia).

TEST PROTOCOLS

For all ELISA protocols, the coating buffer was 0.06 M sodium carbonate (pH 9.6), the diluting and washing buffer was 0.01 M sodium phosphate in saline (pH 7.2) containing 0.05% Tween 20 (PBS-T), and the substrate/chromogen solution contained 4.0 mM H_2O_2 plus 1.0 mM ABTS (2,2'-azino-bis(3-ethylbenzthiazoline sulfonic acid) in 0.05 M sodium citrate (pH 4.5) (31). Certified polystyrene Nunc Plasticware Maxisorp (cat. no. 4-39454) microtitre plates (Life Technologies, Burlington, Ontario) plates were used in all protocols. A Flow Titertek shaker (Interscience, Burlington, Ontario) was employed. Washing was done with a M96V Flow Titertek Plus washer, and substrate development was determined using a MCC/340 Flow Titertek Multiskan reader.

For the C-ELISA, the concentrated *M. bovis* BCG Tokyo culture filtrate was diluted to 0.5 μg protein/mL in coating buffer and 100 μL was passively adsorbed on the plates overnight at 25°C. The plates were washed and 50 μL of serum diluted 1/12.5 was added, followed by 50 μL of 4C3/17 McAB diluted 1/25 000, to give final dilutions of 1/25 and 1/50 000, respectively. After shaking the plates for 2 min, incubating at 25°C for 3 h, and washing, 100 μL of a 1/8000 dilution of horseradish peroxidase labelled goat anti-mouse IgG (Bethyl Laboratories, Montgomery, Texas, USA) was added. Following a final 1 h incubation at 25°C, the plates were washed and 200 μL substrate/chromogen solution was added. The plates were developed according to a timing protocol (see below) and were read at 414 nm.

In the case of indirect MPB70-ELISA or LAM-ELISA, the antigen was similarly adsorbed after dilution in coating buffer to 1.0 μg protein/mL or 1.0 μg LAM carbohydrate/mL (29), respectively. After washing the next day, 100 μL of a 1/500 dilution of serum was added, and the plates were incubated overnight at 25°C. The plates were washed and then incubated for 2 h with 100 μL of biotinylated protein G (Pierce, Chromatographic Specialties, Brockville, Ontario), then washed and incubated for another 2 h with 100 μL of horseradish peroxidase labelled streptavidin (Kirkegaard and Perry Laboratories, Life Technologies, Burlington, Ontario). Both conjugates were diluted at 1/5000. The plates underwent a final wash, substrate/chromogen was added, and development assessed as for the C-ELISA.

QUALITY ASSURANCE OF TESTS

On each 96 well plate a cross-quadrant placement of diluted sera was used to minimize between well variation (32), with 16 wells being reserved for the placement of 1 reference "target" high titre serum (see below) and 3 other standard sera of high, medium, and low titres, respectively. Color development was timed until the target sera exhibited an average (4 wells) optical density (OD) value of 1.0, at which time the plate was read, thus reducing between plate variation (32).

The distribution of between well variability was determined at various OD values and specification limits (SL) for each ELISA protocol, and were chosen such that 97.5% of these values were accepted (SL = average between well variability + 2 standard deviations (SD) of this variability). The SL values were subsequently found to be inversely proportional to the various OD values according to the relationship SL = a+b/OD value, where a and b vary between ELISA protocols. For example, C-ELISA, MPB70-ELISA, and LAM-ELISA exhibited SL values (expressed as a percentage of OD value) of 30.9%, 23.4%, and 15.9% at an OD value of 0.1, and 19.9%, 17.2%, and 11.6%, respectively at an OD value of 1.0. These values are comparable to those of other studies (12).

Between plate standard deviations (SD) for the 3 standard sera were used to establish SL for acceptability of plates whereby SL = OD value ± 2 SD_{units}. An acceptable coefficient of variation (CV) for between plate variation should not exceed approximately 15%, in general, and for OD values less than 0.1, CV values of approximately 50% are acceptable (33).

The OD values were further corrected for the between plate variation of the standard sera for all accepted

TABLE I. Groups of animals from which serum samples were taken

Group	Species	No. of animals	Age range[a] (y)	Status and comments[b]
1	Cattle	20	4–7	Mixed breed; negative on culture and histopathology (also negative part of cattle panel).
2	Cattle	10	3–6	Negative for *M. paratuberculosis* infection; National Repository (35).
3	Cattle	244	A	A herd of Holstein cows was negative on repeated skin tests; *M. paratuberculosis* infection was present in the herd.
4	Cattle	43	A&Y	Mixed breed; suspicious on skin tests and/or histopathology; 10 infected with *M. smegmatis*, *M. avium*, or unknown Mycobacteria.
5	Cattle	15	2–9	*M. paratuberculosis* infected; National Repository (35).
6	Cattle	39	A&Y	Mixed breed; Experimental *M. bovis* (BM-228) infection of 14 calves; *M. bovis* isolated from 24 adults, the majority of which were also histopathology positive (includes the 20 animals of the positive part of cattle panel). 1 animal was sensitized with *M. bovis*.
7	Watusi (cattle)	19	2–8;Y	Suspicious on the basis of positive skin tests in all animals and gross lesions in 3 animals.
8	Bison	19	A&Y	Negative on culture and includes 11 lesion-free calves from *M. bovis* infected cows (also negative part of bison panel).
9	Bison	7	2–8	Suspicious on the basis of skin tests or gross lesions.
10	Bison	19	3–19	*M. bovis* isolated from all animals (also positive part of bison panel).
11	Other Bovidae	11	1–9	Mixed species; 4 elands; 5 nilgai; 2 gnus; *M. bovis* was isolated from 2 and the remainder were considered suspicious on the basis of skin tests and/or gross lesions.
12	Llamas	11	2–3	Negative on the basis of negative skin tests.
13	Llamas	4	3–14	Suspicious on the basis of gross lesions.
14	Llamas	5	2–3	Experimentally infected with *M. bovis* TB# 64/90, a field isolate from a llama. Serum samples were post skin-test.
15	Other Camelidae	10	3–20	Mixed species; 7 camels; 3 guanacos; suspicious on the basis of gross lesions.
16	Fallow deer	5	4–10;Y	Negative on the basis of negative culture and no gross lesions.
17	Fallow deer	68	2–3	Negative on the basis of the the skin test.
18	Elk	24	1–14;Y	Negative on the basis of no evidence of disease and 3 skin test negatives.
19	Elk	30	1–14;Y	Suspicious on the basis of gross lesions and 3 skin test positives.
20	Elk	3	A	*M. bovis* was isolated.
21	Other Cervidae	17	2–10	Mixed fallow deer and white-tailed deer; suspicious on the basis of a positive skin test and/or gross lesions.

[a] Where age was unknown animals were designated adults (A) or young (Y)
[b] Negative, positive, and suspicious refers to *M. bovis* infection status unless otherwise stated

plates. This was done by 1st comparing OD values for the 3 standard sera for individual plates (y values) to the means of these OD values for each serum for all plates (x values) in a linear regression analysis, and then correcting the data of each plate such that y = x. These corrected OD values were subsequently shown to follow a hyperbolic relationship of variability to OD values of the form y = a+b/x, similar to that for between well variation (see above). Optical density increments of this variability, unique for each ELISA protocol, were used to raise cutoff levels to minimize false positives before calculating the agreement between these protocols, rather than using arbitrary increments or fixing the specificity to higher values (see below). The CV for between plate variation of corrected data for C-ELISA, MPB70-ELISA, and LAM-ELISA were determined to be 19.6%, 14.4%, and 15.4% for an OD value of 0.1, and 4.5%, 3.4%, and 3.5% for an OD value of 1.0, respectively. Most readings exceeded 0.1, so this variability compared with previous studies (22).

ANALYSES

The discriminatory ability of the various ELISA protocols was estimated by the determination of the area under receiver operating characteristics curves (ROC curves) (34) and sensitivity and specificity for cattle and bison serum panels (see below). The kappa statistic (35) was used to measure agreement beyond chance between ELISA protocols based on detection of antibodies either to epitopes on one antigen (C-ELISA vs. MPB70-ELISA) or to epitopes on different antigens (C-ELISA vs. LAM-ELISA) after raising the cutoff OD value to minimize false positivity.

SERUM PANELS

A cattle serum panel was kindly donated by the Animal and Plant Inspection Service of the United States Department of Agriculture (APHIS/USDA) to be used to compare the capability of the ELISA protocols to discriminate *M. bovis* infected from non-infected animals. This included 20 samples each from animals in these categories judged on the basis of culture and histopathology (see also Groups 1 and 6, Table I). A 2nd panel of bison sera from a zoological park included 19 samples each judged to be from infected or non-infected animals primarily on the basis of culture (see also Groups 8 and 10, Table I).

ANIMAL GROUPS

For the purpose of determining agreement between ELISA protocols, animals from a variety of sources were grouped by species, or in the case of more than one species in a group, on the basis of animal families (Bovidae, Camelidae, or Cervidae) (Table I). Animals were considered to be infected on the basis of either *M. bovis* isolation or experimental infection, and may have shown typical histopathology or other positive tests (Table I). Animals exhibiting no signs of *M. bovis* infection, either due to their history or various results, were assured to be negative. However, where there was evidence of infection, in the absence of *M. bovis* isolation,

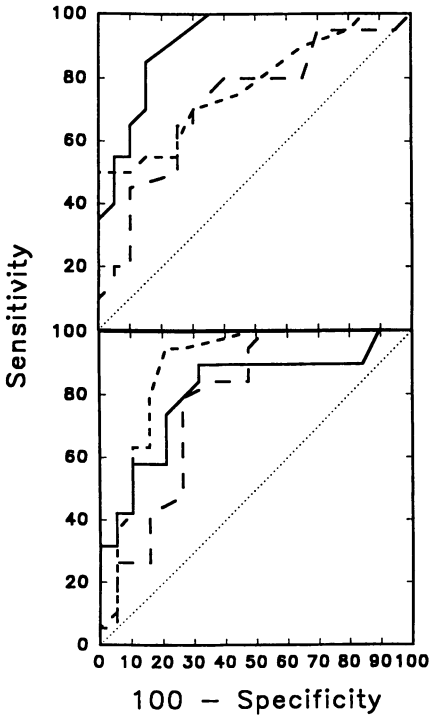

Figure I. Receiver operator curves (ROC) for a serum panel of cattle (top half) and bison (bottom half). ROC areas (for cattle and bison panels, respectively) = 92% and 81% for C-ELISA _____; 72% and 78% for MPB70-ELISA _ _ _; and 78% and 88% for LAM-ELISA _ _ _ _. All areas are significantly different from 50% (no discrimination)..... ($P < 0.001$), indicating the discrimination of *M. bovis* infected from non-infected animals.

animals were considered to be suspicious. This included those with any evidence of lesions, since previous work had demonstrated false positive results for a variety of other pathological conditions (36). Serum from cattle infected with *M. paratuberculosis* was available from the National Repository (37).

RESULTS

ELISA protocols were compared with respect to the discrimination of *M. bovis* infected animals from non-infected animals. Applying these protocols to the cattle and bison panels, specificity, sensitivity, and ROC curves were determined and are depicted in Table II and Figure 1. In Table II, the optimal specificity and sensitivity values for all protocols ranged from 68.4% to 85.0% and 55.0% to 94.7%, respectively, whereas the data for C-ELISA itself revealed specificities of 68.4% (bison) and 85% (cattle) and sensitivities of 85% (cattle) and 89% (bison).

TABLE II. Specificities and sensitivities for cattle and bison serum panels

Panel	ELISA	OD cutoff	Specificity[a]	Sensitivity[b]	Spec + Sens[c]
Cattle	C-ELISA	0.890	85.0(64.0,94.8)[d]	85.0(64.0,94.8)	170.0
	MPB70-ELISA	0.190	75.0(53.1,88.8)	65.0(43.3,81.9)	140.0
	LAM-ELISA	0.180	85.0(64.0,94.8)	55.0(34.2,74.2)	140.0
Bison	C-ELISA	0.830	68.4(46.0,84.6)	89.5(68.6,97.1)	157.9
	MPB70-ELISA	0.170	73.7(51.2,88.2)	78.9(56.6,91.5)	152.6
	LAM-ELISA	0.140	78.9(56.6,91.5)	94.7(75.3,99.1)	173.6

[a] Specificity is the percentage of *M. bovis* negative animals exhibiting a negative OD (above the OD cutoff for C-ELISA, or below the OD cutoff for MPB70-ELISA and LAM-ELISA)
[b] Sensitivity is the percentage of *M. bovis* positive animals exhibiting a positive OD (below the OD cutoff for C-ELISA, or above the OD cutoff for MPB70-ELISA and LAM-ELISA)
[c] Spec + Sens = Maximum value for Specificity + Sensitivity
[d] 95% confidence limits are given in parentheses

The relatively low specificity of 68.4% for the bison panel may be due to the presence of *M. bovis* infection or sensitization in lesion-free calves, from infected cows, in the negative part of the panel. Nevertheless, these calculations seem to reveal that all protocols are capable of discriminating, at the herd level, *M. bovis* infected from non-infected animals, at least for these particular panels. Indeed, a more thorough analysis using ROC curves proved that discrimination was significant in all cases with ROC area values of 81% (bison) and 92% (cattle) for C-ELISA (Fig. 1). However, in view of small sample numbers as reflected in the confidence intervals (Table II), it was not possible to differentiate between the protocols. The only significant difference between ROC curves was between C-ELISA (92%) and MPB70-ELISA (72%) for cattle ($P < 0.05$).

The next phase in assessing the various ELISA protocols was to determine the agreement beyond chance between them for the various animal groups (see Table I) using the kappa statistic. This was done after minimizing the occurence of false positives. Basic cutoff values for a fixed specificity of approximately 85%, that of the C-ELISA for cattle (Table II), were determined for the various animal species, followed by an adjustment of cutoff values based on ELISA variability (see Materials and Methods), such that the occurrence of false positives in groups considered to be negative was minimal.

Four basic cutoff sets were determined after pooling the ELISA data groups considered to be negative and fixing specificity as close to 85% as possible (Table III). The cutoff values of the Bovidae groups of cattle and bison were similar for all ELISA protocols, whereas the MPB70-ELISA and LAM-ELISA values were 3-5 fold higher in magnitude than those for the Camelidae and Cervidae groups, perhaps reflecting the presence of natural autoantibodies to *M. bovis* antigens, demonstrated in clinically healthy cattle (38). The proportion of animals for all groups showing positive ELISA results was calculated at the basic cutoff value (specificity of 85%) and after raising the cutoff by 2 and 4 SD units of ELISA variability based on the corrected between plate variability (see Materials and Methods), which is preferable to raising the cutoff to a higher fixed specificity, because differences in ELISA precision are thus accounted for. It was only after raising the cutoff by 4 SD units that false positive results were minimized (Table IV). From this Table it is shown that while negative and some suspicious groups show minimal positive results, other suspicious groups show more positives, and the highest proportion occurs in *M. bovis* infected groups. The high proportion of detected animals for group 14 is a reflection of the anemestic response known to occur in post-skin tested animals (4,9). The 2 indirect ELISA protocols, MPB70-ELISA and LAM-ELISA, were compared with C-ELISA on the basis of the proportion of detected infected animals and also by a calculation of kappa. This latter calculation was only done for those groups with positive reactors with the C-ELISA.

According to Table IV, the overall ability of the ELISA protocols to detect *M. bovis* infected animals

TABLE III. Average Optical Density (OD) cutoff sets for various animals at a specificity as close to 85% as possible

Cutoff Set	OD Cutoff		
	C-ELISA	MPB70-ELISA	LAM-ELISA
Bovidae (Cattle)[a]	0.847 ± 0.066(0.684)[e]	0.247 ± 0.015(0.318)	0.185 ± 0.007(0.254)
Bovidae (Bison)[b]	0.760(0.607)	0.250(0.321)	0.150(0.216)
Camelidae[c]	0.900(0.731)	0.050(0.103)	0.080(0.140)
Cervidae[d]	0.790 ± 0.040(0.634)	0.020 ± 0.014(0.071)	0.060 ± 0.010(0.118)

[a] Average OD cutoffs for Groups 1-3 (see Table 3)
[b] OD cutoffs for Group 8
[c] OD cutoffs for Group 11
[d] Average optimal OD cutoffs for Groups 16-18
[e] In parentheses are OD cutoffs modified by 4 SD units of ELISA variability (subtracted for C-ELISA and added for MPB70-ELISA and LAM-ELISA) and used for dichotomizing ELISA data for Table 4

appears to be in the order LAM-ELISA > MPB70-ELISA > C-ELISA with the exception of elk which did not appear to respond to LAM-ELISA. In the case of *M. paratuberculosis* infection there was essentially no detection by C-ELISA and MPB70-ELISA on comparing Groups 5 and 6. It was noted that LAM-ELISA and MPB70-ELISA, while detecting more infected animals, also detected more animals in negative and suspicious groups.

A comparison of MPB70-ELISA to C-ELISA according to kappa revealed agreements ranging from poor (0.35-0.38), to good (0.62), to excellent (1.00) (35). These were consistently higher in all groups than kappa for a comparison of LAM-ELISA to C-ELISA, which exhibits mainly poor values. The kappa value for Group 3 showed no agreement in keeping with the negative status.

DISCUSSION

The objectives of this work were to present an estimate of the ability of C-ELISA to discriminate *M. bovis* infected from non-infected animals in comparison to MPB70-ELISA and LAM-ELISA and to determine the agreement between C-ELISA and these protocols for indirect ELISA. Calculations of sensitivities, specificities, and ROC curve areas show that these protocols can discriminate groups of *M. bovis* infected from non-infected cattle and bison (Table II and Figure 1). Further validation using expanded serum panels will be needed to increase the accuracy and precision of these preliminary estimates.

Calculation of the kappa statistic (Table IV) permits a measurement of the agreement beyond chance between the ELISA protocols for several animal species. Comparing C-ELISA with MPB70-ELISA revealed higher kappa values than for a comparison with LAM-ELISA because both measure anti-MPB70 antibodies. Nevertheless, these were poor in some cases as evident in Table IV, and likely occur for a variety of reasons. Firstly, MPB70-ELISA could be detecting antibodies to more than one epitope on MPB70-ELISA, including perhaps one which cross-reacts with antigens of *Nocardium asteroides* (18) other mycobacteria, or other bacteria. This appeared to be a tendency in some negative groups (Groups 3, 8, 20). A previous investigation found that 9% of animals non-infected or infected with other mycobacterial species cross-reacted with indirect MPB70-ELISA (21). Secondly, the influence of McAB affinity on serum polyclonal antibody binding in C-ELISA, compared to the binding of only the polyclonal antibody in MPB70-ELISA, could alter the agreement. Finally, while it was presumed that there is no interaction of the McAB with epitopes on other antigens other than MPB70 antigen in the culture filtrate used to coat C-ELISA plates, this can only be ruled out by conducting C-ELISA using purified MPB70.

Notably, these factors could explain why an animal infected with *M. paratuberculosis* was positive on MPB70-ELISA but negative on C-ELISA (Table IV; Group 5), of interest in geographical areas where paratuberculosis is endemic. It is possible that this discrepancy is due to low affinity serum antibodies in such animals to epitopes on MPB70 common to *M. paratuberculosis* and *M. bovis* that are unable to compete with the McAB.

In contrast to the kappa values for the comparison of C-ELISA to MPB-70-ELISA (Table IV), there was very poor agreement between C-ELISA and LAM-ELISA, in spite of the fact that both ELISA protocols detect *M. bovis* infected animals (Table IV). These results suggest that in some animals there is a humoral response to one antigen in the absence of a response to the other. For example, Group 11 contains 2 adult female nilgai, one 2 year old and the other is a 4 year old from the same zoological park that had an outbreak of tuberculosis. Both showed prominent responses against one of the antigens in the absence of a response to the other. Another example is evident in Group 20 where 3 infected elk were detected by C-ELISA and MPB-70-ELISA but not by LAM-ELISA. Why, in some animals, is there differential response to 2 antigens rather than a parallel response? Certainly the answer to this question may throw some light on the immunopathogenesis of *M. bovis* infections. For Group 5, animals with *M. paratuberculosis* infection, the detection with LAM-ELISA in the absence of any detection with C-ELISA was a reflection of the relative specificities of the antigens. LAM is a polysaccharide antigen common to members of the *Mycobacterium* genus and also occurring in *Corynebacterium* spp. and *Nocardium* spp. (39), and thus capable of detecting both mycobacterial infections.

As also described elsewhere, the phenomenon of a differential response to purified antigens leads to the problematic occurrence of "blind spots" (5). Indeed, previous studies have demonstrated relatively low sensitivities for MPB70-ELISA as compared with PPD which contains a mixture of antigens (5,21). In this regard a parallel interpretation of C-ELISA and LAM-ELISA increased detection of *M. bovis* infected animals (Table 4), reinforcing previous studies advocating the use of dual antigens (40). In addition, the use of LAM antigen meets an important requirement of a good serodiagnostic assay, that of being capable of screening for other mycobacterial diseases (41).

In addition to a potentially greater specificity of C-ELISA as compared to MPB70-ELISA, a 2nd advantage of C-ELISA is its applicability to multiple animal species, since the enzyme conjugate detects the same McAB in all cases. While it is true that MPB70-ELISA and LAM-ELISA are also applicable to multiple animal species through the use of protein G conjugates (42), there could be a differential reaction with the IgG subclasses of some animal species. A 3rd advantage is that there seems to be less fluctuation in C-ELISA cutoff values between Bovidae, Camelidae and Cervidae than for the indirect ELISA protocols that exhibit a 3-5 fold less magnitude in basic cutoff value for these various animals. Finally, the use of McAB obviates a requirement for purified antigen, and finer adjustments of sensitivity and specificity can be made through the use of other McAB to the MPB70 antigen.

A possible disadvantage of C-ELISA is that experience showed evidence of a greater fluctuation in the percentage of false positives between some cattle groups than in the case of MPB70-ELISA and LAM-ELISA, although this was not evident when the cutoff OD value was lowered to minimize false positives (Table IV). A possible explanation may be the use of a low dilution of serum in C-ELISA (1/25) in contrast to a higher dilution (1/500) in the indirect protocols. While such a high amount of serum is necessary to balance the McAB competition, any non-specific tendency in the serum to alter the binding of serum antibodies, McAB, or goat anti-mouse conjugate binding to McAB could conceivably be magnified, leading to this observed variability. The use of non-purified antigen as an alternative to purified MPB70-ELISA may also be a factor. Future experimentation on C-ELISA should focus on an assessment of the effects of McAB selection, serum dilution, and antigen purity on this variability.

The variation that occurs between animal species, particularly for the indirect protocols of MPB70-ELISA and LAM-ELISA, but also somewhat for C-ELISA (Table II), may pose problems in diagnostic application, given that it may be difficult to obtain sufficient numbers of certain species to reliably determine accurate cutoff values. It may be more practical to rely on the determination of titre involving the application of these protocols to several dilutions of sera, even considering the greater cost and labour involved.

Finally, while these results reveal an ability for discrimination, it is generally recognized that the sensitivities and specificities of ELISA protocols for serodiagnosis of bovine tuberculosis are relatively poor in comparison to those for other diseases. Sensitivity, in particular, is substantially lowered when cutoff values are raised to minimize false positives as in Table IV, and although improved by such modifications as the use of more than one antigen (Table IV), leads to the question of how meaningful these tests are that miss a large proportion of *M. bovis* infected animals in many instances. While some dismiss serodiagnosis as a useful adjunct to diagnosis (7), there are others that are concerned that skin tests or other tests may miss severely infected animals which are detected by ELISA, due to an inverse relationship between humoral and cellular immunity (4-6). Also they appreciate the reinforcement, albeit slight in some cases where severe infection is minimal, that is provided by serodiagnosis.

TABLE IV. Percentage of ELISA positives[a] for the various animal groups and agreement of MPB70-ELISA and LAM-ELISA with C-ELISA beyond chance according to Kappa

Group	Animal species[b]	Status[c]	C-ELISA	MPB70-ELISA[d]	LAM-ELISA[d]	Par Inter[e]
1	Cattle (20)	NI	0	0	5.0	5.0
2	Cattle (10)	NI	0	0	0	0
3	Cattle (244)	NI	0.8	4.5 (-0.01)	ND	NA
4	Cattle (43)	S	0	4.6	9.3	9.3
5	Cattle (15)	MPI	0	6.7	33.3	33.3
6	Cattle (39)	MBI	20.5	23.1 (0.62)*	33.3 (0.17)	43.6
7	Cattle (Watusi) (19)	S	0	0	10.5	10.5
8	Bison (19)	NI	0	5.3	10.5	10.5
9	Bison (7)	S	0	0	0	0
10	Bison (19)	MBI	15.8	26.3 (0.38)*	42.1 (-0.06)	52.6
11	Other Bovidae (11)	MBIS	9.1	9.1 (1.00)*	9.1 (-0.10)	18.2
12	Llamas (11)	NI	0	0	0	0
13	Llamas (4)	S	0	0	25.0	25.0
14	Llamas (5)	MBI	60.0	60.0 (1.00)*	80.0 (0.54)	80.0
15	Other Camelidae (10)	S	10.0	0 (0)	10.0 (-0.11)	20.0
16	Fallow deer (5)	NI	0	0	0	0
17	Fallow deer (68)	NI	0	ND	2.9	2.9
18	Elk (24)	NI	0	4.2	0	0
19	Elk (30)	S	6.7	10.0 (0.35)*	0 (0)	6.7
20	Elk (3)	MBI	33.3	100.0 (0)	0 (0)	33.3
21	Other Cervidae (17)	S	0	23.5	5.9	5.9

[a] The ELISA data of groups 1-21 were dichomotized according to the appropriate modified (4 SD units) optimal OD cutoffs (see Table 3)
[b] Number of animals per group is indicated in parentheses
[c] Non-infected (NI), *M. bovis* infected (MBI), *M. paratuberculosis* infected (MPI), suspicious for *M. bovis* infection (S), a mixture of *M. bovis* infected and suspicious animals (MBIS)
[d] Kappa is indicated in parentheses. Minor negative values occur where agreement beyond chance is less than the agreement due to chance alone
[e] Par Inter = Parallel Interpretation whereby a positive ELISA in either C-ELISA or LAM-ELISA is interpreted as positive overall
ND — Not done
NA — Not applicable
* — ($P < 0.05$)

ACKNOWLEDGMENTS

The authors are grateful to Mr. D. McIntyre for the collection of blood samples, and to Ms. B. DuPree, Mr. G. Saumure, and Ms. B. Taylor for their participation in antigen preparation. We thank the following individuals for their suggestions and contributions of sera: Dr. M. Essey of USDA/APHIS; Dr. D.C. Sockett of the Wisconsin Department of Agriculture; Dr. R.H. Whitlock of the University of Pennsylvania; Dr. S. Stehman of the New York Diagnostic Laboratory, Cornell University; and Dr. F. Lord of the Animal Health Division, Agriculture and Agri-Food Canada.

REFERENCES

1. **MAHMOOD KH, ROOK GAW, STANFORD JL, STUART FA, PRITCHARD DG.** The immunological consequences of challenge with bovine tubercle bacilli in badgers (*Meles meles*). Epidemiol Infect 1987; 98: 155–163.
2. **THOEN CO, THROLSON KJ, MILLER LD, HIMES EM, MORGAN RL.** Pathogenesis of *Mycobacterium bovis* infection in American bison. Am J Vet Res 1988; 49: 1861–1865.
3. **PLACKETT P, RIPPER J, CORNER LA, SMALL K, de WITTE K, MELVILLE L, HIDES S, WOOD PR.** An ELISA for the detection of anergic tuberculous cattle. Aust Vet J 1989; 66: 15–19.
4. **HARBOE M, WIKER HG, DUNCAN JR, GARCIA MM, DUKES TW, BROOKS BW, TURCOTTE C, NAGAI S.** Protein G-based enzyme-linked immunosorbent assay for anti-MPB70 antibodies in bovine tuberculosis. J Clin Microbiol 1990; 28: 913–921.
5. **GRIFFIN JFT, NAGAI S, BUCHAN GS.** Tuberculosis in domesticated red deer: Comparison of purified protein derivative and the specific protein MPB70 for in vitro diagnosis. Res Vet Sci 1991; 50: 279–285.
6. **RITACCO V, LOPEZ B, de KANTOR IN, BARRERA L, ERRICO R, NADER A.** Reciprocal cellular and humoral immune responses in bovine tuberculosis. Res Vet Sci 1991; 50: 365–367.
7. **WOOD PR, CORNER LA, ROTHEL JS, RIPPER JL, FIFIS T, McCORMICK BS, FRANCIS B, MELVILLE L, SMALL K, DeWITTE K, TOLSON J, RYAN TJ, DeLISLE GW, COX JC, JONES SL.** A field evaluation of serological and cellular diagnostic tests for bovine tuberculosis. Vet Microbiol 1992; 31: 71–79.
8. **WALLACE R, CARRIERE J, DIENA BB, GREENBERG L.** The bentonite flocculation test in the serology of tuberculosis. II Detection of tuberculosis in cattle. Can J Microbiol 1968; 887–890.
9. **LEPPER AWD, PEARSON CW, OUTTERIDGE PM.** Assessment of the bentonite flocculation test for detecting tuberculosis in cattle. Aust Vet J 1973; 49: 445–450.
10. **THOEN CO, MILLS K, HOPKINS MP.** An enzyme-linked immunosorbent assay reagent for detecting antibodies in tuberculous exotic animals. Am J Vet Res 1980; 41: 833–835.
11. **SUTTON PJ, DeLISLE GW, WALL EP.** Serology of tuberculosis in red deer. Bull Royal Soc New Zealand 1985; 22: 155–158.
12. **AUER LA.** Assessment of an enzyme linked immunosorbent assay for the detection of cattle infected with *Mycobacterium bovis*. Aust Vet J 1987; 64: 172–176.
13. **RITACCO V, de KANTOR, BARRERA L, NADER A, BERNADELLI A, TORREA G, ERRICO F, FLEISS E.** Assessment of the sensitivity and specificity of enzyme-linked immunosorbent assay (ELISA) for the detection of mycobacterial antibodies in bovine tuberculosis. J Vet Med B 1987; 34: 119–125.
14. **HAMMAM H, REFAI M, BISPING W, KIRPAL G.** Studies on the efficiency of absorbed bovine PPD in tuberculin and serological tests. J Vet Med B 1989; 36: 175–179.
15. **HANNA J, NEILL SD, O'BRIEN JJ.** Use of PPD and phosphatide antigens in an ELISA to detect the serological response in experimental bovine tuberculosis. Res Vet Sci 1989; 47: 43–47.
16. **DOWLING LA, SCHLEEHAUF SM.** Specific antibody responses to *Mycobacterium bovis* in infected cattle analysed with six mycobacterial antigens in enzyme-linked immunosorbent assays. Res Vet Sci 1991; 50: 157–161.
17. **NAGAI S, MATSUMOTO J, NAGUSUGA T.** Specific skin-reactive protein from culture filtrate of *Mycobacterium bovis* BCG. Infect Immun 1981; 31: 1152–1160.
18. **HARBOE M, NAGAI S.** MPB70. A unique antigen of *Mycobacterium bovis* BCG. Am Rev Respir Dis 1984; 129: 444–452.
19. **FIFIS T, COSTOPOULOS C, RADFORD AJ, BACIC A, WOOD PR.** Purification and characterization of major antigens from a *Mycobacterium bovis* culture filtrate. Infect Immun 1991; 59: 800–807.
20. **RADFORD AJ, DUFFIELD BJ, PLACKETT P.** Cloning of a species-specific antigen of *Mycobacterium bovis*. Infect Immun 1988; 56: 921–925.
21. **FIFIS T, COSTOPOULOS C, CORNER LA, WOOD PR.** Serological reactivity to *Mycobacterium bovis* protein antigens in cattle. Vet Microbiol 1992; 30: 343–354.
22. **GRANGE JM, GIBSON J, NASSAU E.** Enzyme-linked immunosorbent assay (ELISA): A study of antibodies to *Mycobacterium tuberculosis* in the IgG, IgA, and IgM classes in tuberculosis, sarcoidosis, and Crohn's disease. Tubercle 1980; 61: 145–152.
23. **HEWITT J, COATES ARM, MITCHISON DA, IVANYI J.** The use of murine antibodies without purification of antigen in the serodiagnosis of tuberculosis. J Immunol Methods 1982; 55: 205–211.
24. **WILKINS E, BOTHAMLEY G, JACKETT P.** A rapid simple enzyme immunoassay for detection of antibody to individual epitopes in the serodiagnosis of tuberculosis. Eur J Clin Microbiol Infect Dis 1991; 10: 559–563.
25. **NIELSEN K, GALL D.** Advances in the diagnosis of bovine brucellosis: Use of enzyme immunoassays. Genet Engin Biotechnol 1994; 14: 25–39.
26. **WOOD PR, RIPPER J, RADFORD AJ, BUNDESON PG, RYLATT DB, COTTIS LE, JOHN M, PLACKETT P.** Production and characterization of monoclonal antibodies specific for *Mycobacterium bovis*. J Gen Microbiol 1988; 134: 2599–2604.
27. **RADFORD AJ, WOOD PR, BILLMAN-JACOBE H, GEYSEN HM, MASON TJ, TRIBBICK G.** Epitope mapping of the *Mycobacterium bovis* secretory protein MPB70 using overlapping peptide analysis. J Gen Microbiol 1990; 136: 265–272.
28. **HUNTER SW, GAYLORD H, BRENNAN PJ.** Structure and antigenicity of the phosphorylated lipopolysaccharide antigen from the leprosy and tubercle bacilli. J Biol Chem 1986; 261: 12345–12351.
29. **SUGDEN EA, SAMAGH BS, BUNDLE DR, DUNCAN JR.** Lipoarabinomannan and lipid-free arabinomannan antigens of *Mycobacterium paratuberculosis*. Infect Immun 1987; 55: 762–770.
30. **ANGUS RD.** Production of reference PPD tuberculins for veterinary use in the United States. J Biol Stand 1978; 6: 221–227.
31. **WRIGHT PF, KELLY WA, GALL DEJ.** Application of a timing protocol to the reduction of interplate variability in the indirect enzyme immunoassay for detection of anti-*Brucella* antibody. J Immunoassay 1985; 6: 189–205.
32. **STEMSHORN BW, BUCKLEY DJ, ST. AMOUR G, LIN CS, DUNCAN JR.** A computer-interfaced photometer and systematic spacing of duplicates to control within-plate enzyme-immunoassay variation. J Immunol Methods 1983; 61: 367–375.
33. **GALL D, NIELSEN K, BOSSE J.** Guidelines for the Development, Optimization, and Standardization of the Targeting ELISA Procedure. Version 1.0. Animal Diseases Research Institute, Nepean: Agriculture Canada, 1993.
34. **HANLEY JA, McNEIL BJ.** The meaning and use of the area under a receiver operating characteristic (ROC) curve. Radiology 1982; 143: 29–36.
35. **FLEISS JL.** Statistical Methods for Rates and Proportions, 2nd ed. Toronto: John Wiley & Sons, 1981.
36. **AUER LA, SCHLEEHAUF SM.** Antibodies to mycobacteria in cattle not infected with *Mycobacterium bovis*. Vet Microbiol 1988; 18: 51–61.
37. **SOCKETT DC, CARR DJ, RICHARDS WD, COLLINS MT.** A repository of specimens for comparison of diagnostic procedures for bovine tuberculosis. J Vet Diagn Invest 1992; 4: 188–191.
38. **MORRIS JA, THORNS CJ.** Evidence for the reaction of bovine autoantibodies with *Mycobacterium bovis*. Vet Rec 1985; 117: 169.
39. **YAMAMURA Y, MISAKI A, AZUMA I.** Chemical and immunological studies on polysaccharide antigens of mycobacteria, nocardia, and corynebacteria. Bull Int Union Tuberc Lung Dis 1972; 47: 181–191.
40. **RAHEMAN SF, WAGNER S, MAUCH H, VASUDEVA ND, INGOLE DL.** Evaluation of a dual-antigen ELISA test for the serodiagnosis of tuberculosis. Bull World Health Organ 1988; 66: 203–209.
41. **GRANGE JM.** The humoral immune response in tuberculosis: In nature, biological role and diagnostic usefulness. Adv Tuberc Res 1984; 79: 1–78.
42. **BJORCK L, KRONVALL G.** Purification and some properties of streptococcal protein G, a novel IgG-binding reagent. J Immunol 1984; 133: 969–973.