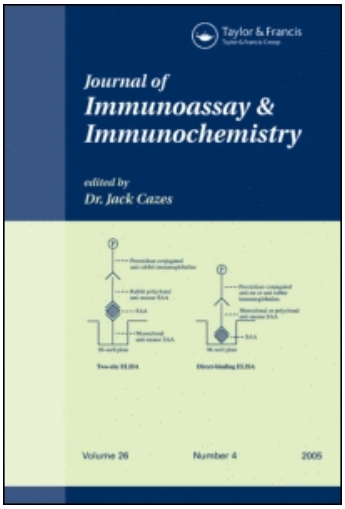

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Clavijo, Alfonso and Thorsen, Jan(1995) 'Serologic Diagnosis of Caprine Arthritis-Encephalitis by Elisa with Two Recombinant Proteins in a Parallel Testing Format', Journal of Immunoassay and Immunochemistry, 16: 4, 419 — 436

To link to this Article: DOI: 10.1080/15321819508013571
URL: http://dx.doi.org/10.1080/15321819508013571

JOURNAL OF IMMUNOASSAY, 16(4), 419-436 (1995)

SEROLOGIC DIAGNOSIS OF CAPRINE ARTHRITIS-ENCEPHALITIS BY ELISA WITH TWO RECOMBINANT PROTEINS IN A PARALLEL TESTING FORMAT

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ABSTRACT

A scheme for screening sera for antibodies to caprine arthritis-encephalitis virus (CAEV) was evaluated for its ability to identify positive and negative samples in a population with heterogeneous risk factors, using the criteria of sensitivity, specificity and positive predictive value. Five hundred caprine serum samples were tested using a transmembrane recombinant-based ELISA. Those that gave positive results were considered positive, while those with equivocal or negative results were retested with a core recombinant protein-based ELISA. Equivocal results after the second test were considered indeterminate and retesting is advised. Using this approach, a sensitivity, specificity and positive predictive value of 98.8 %, 97.2 % and 98.6 % were obtained. These values are superior to those obtained by these tests used individually. The high sensitivity, specificity and predictive value of this new scheme of CAEV screening make it an attractive addition to any control or eradication program.

(KEYWORDS: caprine arthritis-encephalitis virus, diagnosis, lentivirus, recombinant protein, serology)

INTRODUCTION

Caprine arthritis-encephalitis virus (CAEV) infection is an important disease of goats because of the high prevalence of both infection and disease (1). Production losses due to CAE in goats have not been studied sufficiently. In general, the economic costs of CAE centre around early culling, replacement costs and slower genetic improvement of the herd. The initial CAEV serological test, adopted for routine use and still the most widely used is the agar gel immunodiffusion test (AGID) (1) based on the immunodiffusion test for ovine progressive pneumonia (2) that detects mainly antibodies against the 28 kda core protein. Although there have been attempts to make a better antigen derived from CAEV (3), the commercially available AGID test uses ovine progressive pneumonia virus (OPPV) as antigen. This fact has affected the sensitivity of the assay considerably (4) and has limited its value in CAEV control programs (5). Although a positive diagnosis can be made with confidence by the AGID test, the method is rather insensitive and gives many false negative reactions (6).

At the present, there are several enzyme-linked immunosorbent assay (ELISA) protocols for screening CAEV infection and they have demonstrated the superior sensitivity and specificity of this test compared with the AGID test for diagnosis of CAEV infection (7).

Immunodiffusion and ELISA tests will not distinguish between CAEV and other closely related lentiviruses. If required, a more precise diagnosis can be made by means of the immunoblot technique, which will distinguish between

CAEV and maedi-visna virus (8). The immunoblot method has also proved to be more specific than ELISA. It allows the recognition of nonspecific reactions, minimizing false-positive results. Immunoblotting has been used as a validation test for the ELISA used in routine diagnosis of CAEV (9).

In general, repeated testing is advised, since the time required for seroconversion after infection is variable. Some goats take several months to seroconvert (10) and, in addition, the antibody response in some goats appears to be minimal.

Control programs for CAEV are based on preventing transmission from infected dams to their offspring by isolating the offspring at birth and depriving them of colostrum or milk from infected dams (11-13). In general, it is considered that feeding pasteurized milk and routine testing and segregation would be a substantially more effective means of control of the disease in dairy goat herds than would feeding pasteurized milk alone (14).

The use of recombinant protein-based ELISAs to identify CAEV-positive goats may be a valuable tool for control and eradication programs. Recombinant proteins can provide uniform, well defined antigens for antibody analysis, diminishing inter- and intra-assay variation. In addition, they have proved superior in sensitivity and specificity when compared with whole virus ELISA (15).

We report a strategy which uses two different recombinant proteins in parallel as an aid in detecting CAEV-antibody positive goats in populations with different prevalences of the disease.

MATERIALS AND METHODS

Serum samples

Serum samples from 500 goats were tested. Of these, 359 were from several commercial dairy goat herds in Ontario with a high prevalence of CAE, over 50 % positive by agar gel immunodiffusion test (AGID). The other 141 samples were obtained from Jamaican goat herds with a low prevalence of CAE, all negative by AGID test. Pools of serum samples from 10 goats that were negative or positive by AGID, whole virus ELISA and immunoblot were used as negative and positive controls respectively.

Recombinant core (p28) and transmembrane (p40) protein antigens

The production of CAEV recombinant proteins has been described in detail elsewhere (16). In brief, the genes for $p28^{gag}$ and $gp40^{env}$, corresponding to core and transmembrane proteins respectively, were amplified by the polymerase chain reaction (PCR). The PCR products were cloned into the vector pGEM3Z (Promega Co. Madison ,WI) previously digested with *sma*I. Inserts with the verified DNA sequence were subcloned in frame in the *Bam*HI and *Eco*RI restriction sites of plasmid pGEX-2T (Pharmacia Biotech Inc, Piscataway, NJ) and transformed into *E. coli* JM109.

E. coli strain JM109 with recombinant clone was cultured on LB medium and the recombinant proteins were purified by a sequence of three steps: a) enrichment of inclusion bodies; b) solubilization of the expressed protein, and

c) purification by electroelution. Using this procedure, about 700 μg/ml and 500 μg/ml of purified p40 and p28 were respectively obtained.

ELISA with purified recombinant fusion proteins

Recombinant protein-based ELISA procedures using core and transmembrane recombinant proteins have been described previously (16). Briefly, microplates (Nunc-Immunoplate, Roskilde, Denmark) were coated with 67 ng/well and 53 ng/well of purified p28 and p40 proteins, respectively. The serum was diluted 1:100 in PBS, 0.1 % Tween 20, 1 % BSA, 1 % gelatin and 100 μl were added to each well and incubated for 1 hour at 37 ^{o}C. After washing, 100 μl of anti-goat immunoglobulins conjugated with horseradish peroxidase were added to each well, and the plates were incubated at 37 ^{o}C for 1 h. Wells were rinsed again, and 100 μl of substrate solution [0.3 mg/ml 2.2-azino-di-3-ethyl-benzthiazoline sulphonate (ATBS), 0.1 μl/ml of 30% H_2O_2, 0.05 M citric acid, and 0.1 M disodium hydrogen phosphate (pH 5.0)] were added to each well. After 5-7 min, the reaction was stopped by the addition of 50 μl of 10 mM sodium azide and the optical density at 414 nm was read on a Titertek Multiskan microplate reader (Flow Laboratories, McLean, VI).

Samples were tested in duplicate. Those with greater than 30 % discrepancy between wells were repeated. Blanks and calibration standards were always run in the same assay as the unknown samples. The serological status of each test sample (P) was determined from the mean optical density of the duplicate sample divided by the mean optical density of eight replicates of the

reference negative serum pool (N), tested at the same dilution as test sera (1:100). The individual P/N ratio for each sample serum was calculated and the results expressed as positive to negative ratios (P/N). The immunoblot test was considered to be a confirmatory test for comparison of the results obtained with the recombinant antigen-based ELISA. In order to determine the optimal concentrations of reagents. Serum and antigens were titrated. The dilution that showed the highest P/N ratio was selected, figure 1 and figure 2.

Immunodiagnostic assay

Immunoblotting was performed as described for human immunodeficiency virus (HIV) by Thorpe and colleages (17) and adapted to CAEV with some modifications. Briefly, 10^6 GSM cells infected with CAEV were collected, washed with PBS and suspended in lysis buffer containing 0.1 M tris-HCl, (pH 7.0); 4% SDS; 4% 2-mercaptoethanol; 20% glycerol; and 0.001 % bromophenol blue. This mixture was heated in a boiling water bath for 5 min and electrophoresed in a 12% acrylamide minigel overlaid with a 5 % stacking gel and a wide slot in a minielectrophoresis apparatus (Bio-Rad Laboratories. Hercules Ca). The proteins were electroblotted for 1 hr in a Midget Multiblot electrophoretic transfer unit (LKB. Bromma, Sweden) onto a nitrocellulose sheet (Biotrace-NC, Gelman Sci. Ann Arbor, MI) in transfer buffer (0.192 M glycine, 0.025 M Tris, 0.1 % SDS and 30% methanol) at a constant current of 450 mA. Blots were incubated for 2 hr at room temperature with antisera diluted 1:100 and then incubated for 2 hr with peroxidase-conjugated anti-goat IgG (Kirkegaard

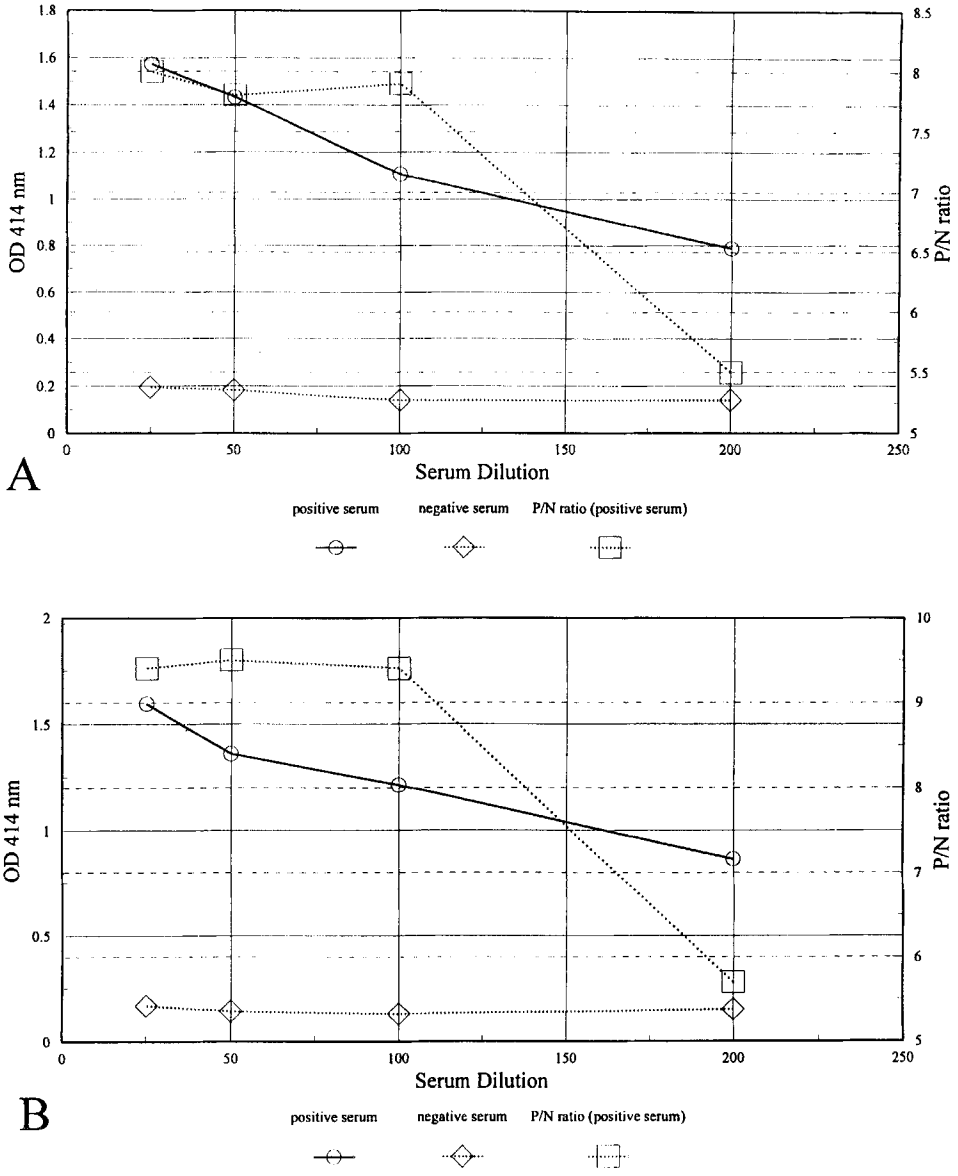

FIGURE 1. Titration curve for positive and negative control serum using P28 (A) and P40 (B) recombinant proteins as antigens in ELISA. The P/N ratio is also shown.

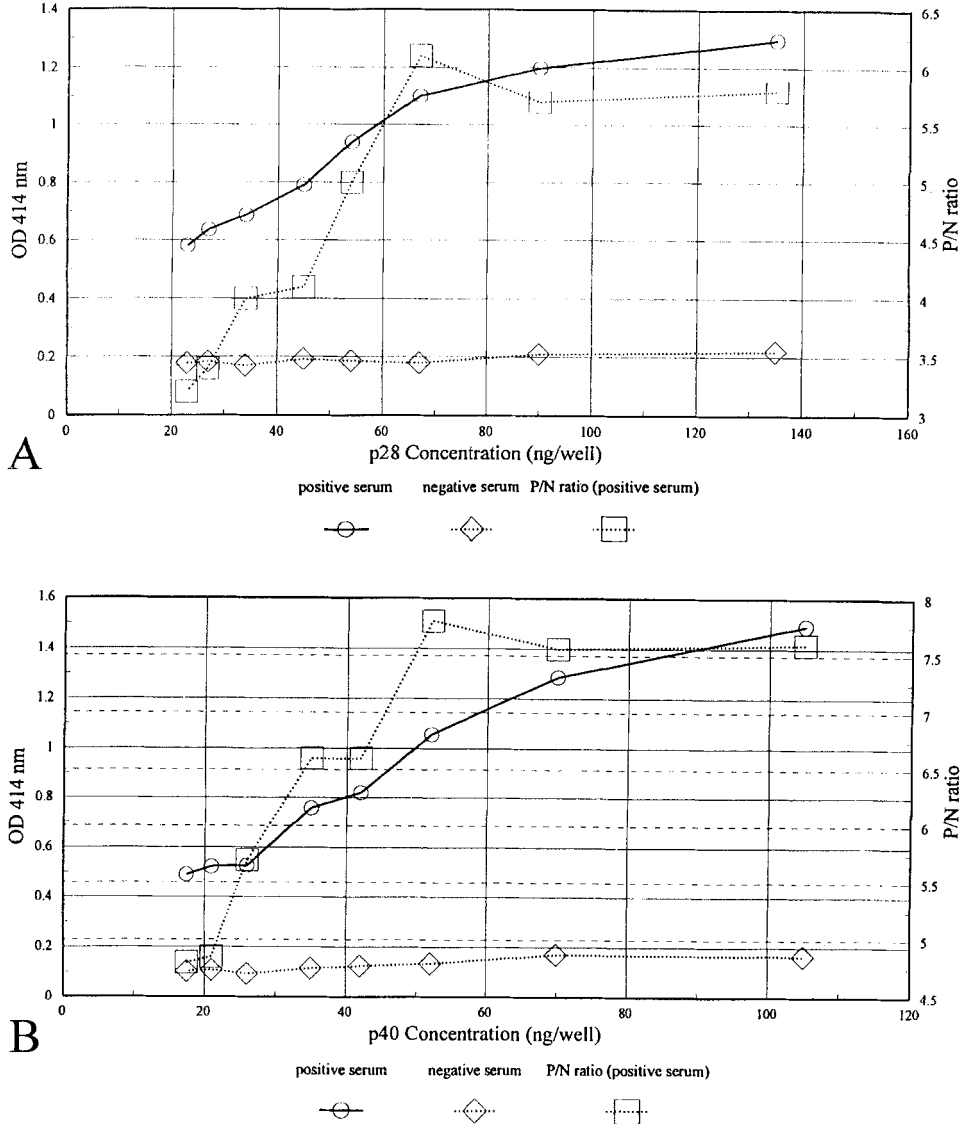

FIGURE 2. Optimal concentration of P28 (A) and P40 (B) recombinant proteins in ELISA using positive and negative control serum. The P/N ratio is also shown.

& Perry Laboratories Inc, Gaithersburg, MD). Detection was performed by standard protocols (18). A negative test result was defined as no antibodies being found against any of the CAEV gene products.

Antibody testing protocol

The testing protocol consists of two phases. Samples received during the first phase are tested in duplicate wells with the transmembrane (p40) recombinant-based ELISA, since previous studies showed that this antigen reacted with a higher number of seropositive goats (16). Sera that react with the transmembrane protein antigen are considered positive if the P/N ratio was ≥ 2, equivocal if the P/N ratio was 1.8 - 2 and negative if the P/N ratio was ≤ 1.8. In the second phase, the nonreactive and equivocal sera from the transmembrane ELISA were tested using the core (p28) ELISA. Again sera with P/N ratios $\geq$ 2 were considered positive, while those with P/N ratios 1.8-2 with either or both recombinant-based ELISAs were retested to ensure reproducibility. Samples initially giving equivocal results and then two negative results in separate tests were regarded as negative. Characteristic absorbances for the number of animal tested are shown in Table 1.

Statistical analysis

The relative sensitivity of the procedure was calculated as (true positive/ true positive+false negative) X 100, the relative specificity as (true negatives/ false positive+true negative)X 100, the positive predictive value as (true positive/

TABLE 1

	Number of Animals	Mean P/N ratio*±S.D.	P/N ratio ranges
Positive	272	7.4±3.2	2-18.9
Negative	228	0.9±0.2	0.3-1.9

* P/N = Ratio of mean optical density of sample serum to the mean optical density of reference negative serum pool.

true positive+false positive)X 100 and the negative predictive value as (true negative/true negative+false negative)X 100 (19).

A true positive or negative sample was defined as one which was positive or negative by immunoblot.

RESULTS

Sample distribution

In phase 1, all 500 serum samples were tested by the transmembrane ELISA. Then, in In phase 2, 248 serum samples were retested using the core ELISA. The distribution of the sera tested by this screening protocol is shown in Table 2. In flocks with a prevalence of infection of 60 % or more, the test had higher positive predictive value, as expected. The positive predictive value of a test was directly related to the prevalence of the disease in the population tested. Consequently, when the disease prevalence is low, a low positive predictive value is obtained, as observed in the group with a low risk of infection, which had a 12 % prevalence. In this case, more retesting will be necessary to verify ELISA-

TABLE 2

Distribution of sera from goats tested in phases 1 and 2.

Category	No (%) animals	Prevalence %	Immunoblot	Combined results from consecutive p40 and p28 r-based ELISAs					
				Positive	Negative	Total	Sensitivity %	Specificity %	Positive predictive value %
Flocks with high risk of infection	359 71.8 %	60 %	Positive	250	3	253	98.9 %	99.0 %	99.6 %
			Negative	1	105	106			
			Total	251	108	359			
Flocks with low risk of infection	141 28.2 %	12 %	Positive	17	0	17	100 %	96.7 %	81.0 %
			Negative	4	120	124			
			Total	21	120	141			

positive sera, especially those with P/N ratios close to the cut-off point, the majority of which will be false positives. Although the sensitivity and specificity of the test in this population was high, the positive predictive value was low.

Transmembrane and core recombinant-based ELISA

Of the 500 sera tested by transmembrane ELISA a total of 248 (49.6 %) required verification by core ELISA. Of these 248 samples, 21 (8.4 %) were positive and 227 (91.6 %) were negative by immunoblot. The distribution of these 21 true positive samples in the transmembrane ELISA was 13 (61.9 %) samples negative; 8 (30.1 %) equivocal.

In phase 2, the 248 samples that were equivocal or negative by the transmembrane ELISA were retested. Ten samples, 2 % of the initial 500 serum samples gave equivocal results. Rebleeding the goats at a future date would be required. None of these 10 samples were positive by immunoblot. A definite result was obtained with the remaining 238 serum samples; 218 negative (91.5 %) and 20 positive (8.4 %). Of these 238 samples, 21 (8.8 %) were true positive by immunoblot. Of 272 serum samples positive by one or both recombinant-based ELISAs, 267 (98.2 %) were true positive and of 218 serum samples negative by both tests 215 (98.6 %) were true negative.

Immunoblot was performed on all 500 serum samples and 270 samples were positive. Equivocal results were obtained with two samples with both ELISA tests. Both of these sera were negative by immunoblot. From 20 samples with

equivocal results in the transmembrane ELISA 10 were defined as positive or negative when they were retested with the core ELISA (Table 3).

Statistical evaluation

From the results of the transmembrane recombinant-based ELISA a specificity of 93.4 % a positive and negative predictive value of 98.8 % and 94.2 % respectively and a sensitivity of 92,2 % were calculated (Table 3).

When the core recombinant-based ELISA was used with the equivocal and negative serum samples from phase 1, a specificity and sensitivity of 99.1 % and 85,7 % respectively and a positive and negative predictive value of 90.0 % and 98.6 % were calculated.

The combined performance of transmembrane and core ELISAs is shown in Table 3. The overall specificity of 97.7 % was slightly lower than that with the core protein alone but better than with the transmembrane ELISA. The combined tests increase in the overall sensitivity to 98.8 %, compared to 92.2 % and 85.7 % in transmembrane and core ELISAs respectively.

DISCUSSION

The use of recombinant protein-based ELISA tests, adds to the sensitivity of the ELISA the specificity of the immunoblot, when individual viral proteins are used to determine the differential immune responses against these proteins.

From 500 serum samples that were tested with the transmembrane recombinant protein-based ELISA, half were defined as negative or positive

TABLE 3

Sensitivity, specificity and predictive values for p40-p28 ELISAs combined in a parallel testing.

Phase, Test and Result	True status of sample		Total	Sensitivity %	Specificity %	Positive predictive value %	Negative predictive value %
	No Positives	No Negatives					
PHASE 1							
p40 ELISA							
Positive	249	3	252	92.2 %	93.4 %	98,8 %	94.2 %
Equivocal	8	12	20				
Negative	13	215	228				
Total	270	230	500				
PHASE 2							
p28 ELISA							
Positive	18	2	20	85.7 %	99.1 %	90.0 %	98.6 %
Equivocal	0	10	10				
Negative	3	215	218				
Total	21	227	248				
p40-p28 ELISA in parallel							
Positive	267	5	272	98.8 %	97.7 %	98.2%	98.6 %
Equivocal	0	10	10				
Negative	3	215	218				
Total	270	230	500				

without further confirmation. The reason to use the transmembrane protein as the first antigen in this testing scheme is based on previous observations that most CAEV-infected goats react against this protein (16). This observation is supported by various authors (20-21). The ELISA using transmembrane protein as antigen has high sensitivity (92.2 %) when compared to the core protein-based ELISA. However, since antibodies against core protein appear earlier than those directed to transmembrane glycoproteins (22) some CAEV-infected goats will not be identified using only transmembrane glycoprotein as antigen. In this study a complementary ELISA based on core recombinant protein as antigen was used to test those samples that gave equivocal or negative results with the transmembrane antigen. With this approach the number of false negatives was substantially reduced. In contrast to the transmembrane recombinant protein-based ELISA, the core protein-based ELISA had lower sensitivity but considerably higher specificity (99.1 %). When these two test are combined sequencially, the high sensitivity of the transmembrane recombinant protein and the specificity of the core recombinant protein are combined. The immunoblot is still considered to be the "gold standard", and those samples that gave equivocal results in the combined test should be retested by the immunoblot test. Alternativelly, it would be advisable to rebleed the goats in one or two months later and retested, since apparent false negative results may be due to delayed seroconversion (10) or low antibody levels at the time of testing (23). This retesting would not represent a real problem in time or resources if it is considered that in the present study only

10 of 500 sera (2 %) were included in the category that should be retested in these way.

An additional advantage of this parallel testing scheme is that the cost of performing two ELISA tests for most of the serum samples was found to be very similar, if not identical, to that incurred by using one ELISA that combined both antigens in the same test. In addition the double testing of negative and equivocal samples with two antigens separately, increased the positive and negative predictive values considerably and reduced the possibility of error involved when a sample is tested only once.

In summary, this study clearly shows that using two recombinant protein-based ELISA test in parallel will identify true positive and negative samples. The procedure would benefit programs of control and eradication where sensitivity, specificity and predictive value are parameters of special importance. The occasional samples which give an equivocal result in the parallel testing can be verified by immunoblot or by future retesting of the goat.

ACKNOWLEDGEMENTS

The financial assistance of the Canadian International Development Agency, which provided a traineeship for Dr. Clavijo, and the Ontario Ministry of Agriculture and Food is gratefully acknowledged.

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