Evaluation of an Enzyme-linked Immunosorbent Assay for the Detection of Antibodies to Caprine Arthritis-Encephalitis Virus in Goat Serum

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ABSTRACT

An indirect enzyme-linked immunosorbent assay (ELISA), was evaluated for its ability to detect serum antibodies against caprine arthritis-encephalitis virus (CAEV). The ELISA was compared to three other serological immunoassays, agar gel immunodiffusion test (AGIDT), immunoblot assay (IBA), and a fixed-cell immunoperoxidase assay (FCIPA). A total of 511 samples, from 40 farms representing a variety of goat breeds and ages were tested. An estimate of the ELISA sensitivity and specificity was made, relative to combined test results of the three other CAEV serological assays. The degree of agreement of test results among these four assays was evaluated.

The number of positives detected by the ELISA, AGIDT, IBA and IPA tests was 193, 154, 204 and 163, respectively. Of the 511 sera tested, 172 were positive to any two or all three of these tests, and were defined as reference positive. A total of 237 samples were negative to all three reference tests, and were defined as reference negative. Relative to these references, the ELISA had a point estimate of 98.3% sensitivity and 97.9% specificity.

There was good agreement between the ELISA and the other three assays with a kappa statistic of agreement greater than 0.7 for all three comparisons. The ELISA is therefore considered a suitable assay, with high sensitivity and specificity, for detection of antibodies to CAEV in serum.

RÉSUMÉ

Une méthode ELISA (enzyme-linked immunosorbent assay) indirecte a été évaluée pour sa capacité à déceler des anticorps sériques dirigés contre le virus de l'arthrite-encéphalite caprine (VAEC). Cet ELISA a été comparé à trois techniques immunosérologiques soit l'immunodiffusion radiale double, le Western-blot et un test d'immunoperoxydase pour lequel la phase solide était constituée de cellules fixées. Un total de 511 échantillons provenant de 40 fermes ont été analysés. Parmi ces échantillons, une variété de races de chèvres, ainsi que différents groupes d'âge étaient représentés. Une évaluation de la sensibilité et de la spécificité de l'ELISA a été faite relativement aux résultats moyens obtenus avec les trois autres techniques immunosérologiques. La similarité entre les résultats obtenus avec les quatre tests a été analysée.

Le nombre d'échantillons positifs décelé par l'ELISA, par l'immunodiffusion radiale double, par le Western-blot et par le test d'immunoperoxydase a été respectivement de 193, 154, 204 et 163. Parmi les 511 sérums analysés, 172 se sont révélés positifs dans deux ou trois tests de référence et 237 ont été négatifs dans les trois tests. Ces échantillons ont constitué les témoins positifs et négatifs. En se servant de ces témoins, la sensibilité de l'ELISA a été évaluée à 98,3 % et la spécificité à 97,9 %.

En utilisant le test statistique kappa, pour comparer les résultats obtenus par la méthode ELISA à ceux obtenus avec chacun des trois tests de référence pris individuellement, une corrélation supérieure à 0,7 a été calculée pour chacune des trois comparaisons. Conséquemment, de par sa grande sensibilité et sa spécificité, cet ELISA peut être utilisé comme un test fiable pour déceler des anticorps sériques dirigés contre le VAEC. (*Traduit par Dr Pauline Brousseau*)

INTRODUCTION

Caprine arthritis-encephalitis virus (CAEV) is classified in the *Retroviridae* family, in the genus *Lentivirus* (1). It possesses certain properties in common with other lentiviruses, and particularly with maedi-visna virus (MVV) (2). The virus usually enters the body from the digestive tract by ingestion of infected colostrum, reaching target cells of the monocyte-macrophage line (2). The virus causes a persistent infection that is characterized by restricted viral replication, with only a small number of cells being infected, many of which carry the viral information as proviral DNA (3,4).

Caprine arthritis-encephalitis virus infection of goats does not always produce clinical disease, even though the infection persists throughout the life of the animal (5). Infection occurs most often during the first few months of life, leading to seroconversion against CAEV. A certain proportion of infected animals (5 to 75%) will develop clinical illness after a long incubation period (one to seven years). In kids (two to six months old), the primary clinical manifestation is posterior ataxia leading to a rapidly fatal quadriplegia due to leukoencephalomyelitis (6). In adult goats the primary clinical signs of infection are symmetrical arthritis and periarthritis ("big knee") often with sclerosis or induration of the udder ("wooden udder") (7,8). Despite seroconversion in most

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infected goats, neutralizing antibodies are absent, or present at only a low titer and they do not control virus replication (9). However, antibodies to an internal protein (p28) and or to an envelope protein (gp135) are produced, as detected by agar gel immunodiffusion (AGIDT) (10,11).

At present, diagnosis of CAEV infection in a herd of goats is confirmed by detection of antibodies against CAEV by AGIDT (10,11) or enzyme-linked immunosorbent assay (ELISA) (12,13,14). The ELISA is preferred because of its ease of application to a large number of samples and lower cost.

The first objective of this study was to estimate the sensitivity and specificity of a CAEV ELISA, relative to combined test results from three other CAEV serological assays, including an AGIDT, an immunoblot assay (IBA), and a fixed-cell immunoperoxidase assay (FCIPA). The second objective was to evaluate the agreement of test results among these four assays.

MATERIALS AND METHODS

CAPRINE ARTHRITIS-ENCEPHALITIS VIRUS PRODUCTION

Caprine arthritis-encephalitis virus was isolated from the lung of a goat showing clinical signs of arthritis. Infected macerated lung tissue was cocultured with primary cultures of fetal lamb cornea (FLCor) cells taken from a MVV-free sheep flock. Growth media consisted of Hanks' minimum essential medium, 0.1 mM nonessential amino-acids, 2 mM L-glutamine, 10% fetal bovine serum, 100 mg/mL streptomycin, and 100 IU/mL penicillin. The FLCor tissue culture had previously been shown to be free of bovine virus diarrhea virus and mycoplasma. After several passages (coculture of infected FLCor cells with uninfected FLCor cells), typical cytopathic effects of CAEV infection were seen. Caprine arthritis-encephalitis virus was positively identified by a FCIPA (as described below), using known CAEV positive reference serum. The antigens produced for the AGIDT, ELISA, and IBA all reacted positively with known CAEV polyclonal antisera or a monoclonal antibody.

TEST SYSTEM ANTIGEN PREPARATION

ELISA antigen: The antigen used in the ELISA was prepared similarly to that described for MVV ELISA antigen preparation (15). After clarification, the tissue culture supernatant was centrifuged (106,000 × *g* for 180 min). The pellet was then suspended in 1/10 the original volume with buffer B (50 mM Tris-HCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.1 M KCl, 1 mM DTT, pH 7.5) (16) and centrifuged again as described above. The pellet was resuspended in 1/100 of the original volume in TN buffer (50 mM Tris-HCl, 0.15 m NaCl, pH 7.5) and treated with 0.1% sodium dodecyl sulphate (SDS). This material was centrifuged as described above and the supernatant used to coat the ELISA plates.

Agar gel immunodiffusion antigen: The antigen used in the AGIDT test was prepared as described for the AGIDT antigen for detection of MVV antibodies (17). Infected FLCor cells were grown until the cytopathic effect was approximately 80% (usually 14 days), at which time the viral material was frozen, thawed and centrifuged at 1,000 × *g* for 15 min (Sorvall SS-34 rotor). The supernatant was placed into a dialysis membrane (12,000–14,000 molecular weight cutoff) and concentrated against polyethylene glycol (8,000 molecular weight) for 48 h at 4°C. After 100-fold concentration the resulting material was used directly in the AGIDT.

Immunoblot antigen: This antigen was prepared as the previously described MVV immunoblot antigen (18). Briefly, CAEV infected FLCor cells were frozen and thawed three times, clarified and concentrated by centrifugation (106,000 × *g* for 180 min) and suspended in TN buffer to 1/100 the original volume and then used directly in the immunoblot.

Fixed-cell immunoperoxidase assay antigen: A CAEV infected monolayer of FLCor cells was used to provide the antigen for the FCIPA test (see FCIPA test protocol below).

SEROLOGICAL TEST PROTOCOLS

Enzyme-linked immunosorbent assay: The CAEV antibody ELISA was done as described (19). Polystyrene 96-well flat bottom plates (Nunc, Gibco BRL, Burlington, Ontario) were coated with 100 μL/well of ELISA antigen diluted 1/400 in 0.05 M carbonate buffer, pH 9.6. The plates were incubated overnight at 4°C and stored frozen at −70°C until used. Upon use the plates were thawed and washed five times with wash buffer (0.145 M NaCl, 2.28 mM NaH_2PO_4, 7.75 mM Na_2HPO_4, 0.5% Tween-20, pH 7.2) in an automatic washer (Titertek, ICN Biomedicals Canada Ltd., Mississauga, Ontario). Serum samples, diluted 1/50 in diluent buffer (1% nonfat dry milk, 0.151 M NaCL, 2.28 mM NaH_2PO_4, 7.75 mM Na_2HPO_4, 0.5% Tween-20, pH 7.2), were added in duplicate to the plate (100 μL/well) and incubated for 1 h at 22°C. After washing five times, 100 μL/well of horseradish peroxidase conjugated rabbit antigoat IgG (H + L) (ICN Biomedicals) diluted 1/1000 in diluent buffer, was added and incubated for 45 min at 22°C. Finally, the plate was washed five times with wash buffer and once with ultra pure water, and 100 μL/well of 1 mM ABTS [2,2-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] (Sigma Chemical Co., St. Louis, Missouri) dissolved in 0.5 M citrate buffer pH 4 with 0.015% H_2O_2 was added. Optical density (OD) was measured at 414 nm in a photometer (Titertek, ICN Biomedicals) when a target positive control serum reached an OD of 1.0 (20). Each plate also contained duplicate samples of a weak known positive control serum, a known negative serum, and a well containing only buffer. The OD values obtained on each plate for these control sera (including the target control serum) were required to be within previously established acceptable OD ranges for these sera for the plate to be considered valid. Acceptable OD ranges (mean ± 3 standard deviations) were established from several hundred previous determinations of the OD values of these control sera (including the target control serum).

Agar gel immunodiffusion test: The AGIDT protocol was described previously (17). Briefly, glass slides were coated with 1% agar in borate buffer (0.9% boric acid, 8% NaCl, pH 8.0). Wells (2.0 mm) were cut in a pattern

of six peripheral around one central at 2.0 mm distance. Alternating peripheral wells were filled with positive reference serum (10 μL), and test serum (10 μL), and the central well was filled with CAEV antigen (10 μL). The reactions were interpreted by two independent readers. Sera were classified as positive if they gave rise to a complete line of identity with the reference sera or caused the ends of the reference lines to curve toward each other.

Immunoblot assay: Immunoblot antigen was mixed with an equal volume (500 μL) of sample buffer (4% SDS, 20% glycerol, 125 mM Tris, pH 6.8), heated to 100°C for 3 min and centrifuged at 10,000 × *g* for 5 min. This material was separated by electrophoresis (21), in a 10% preparative polyacrylamide gel (6.0 by 8.5 cm by 1.5 mm) at 20 mA of constant current until the dye reached the bottom of the gel.

Proteins were electroblotted (22), onto a 0.22 μm (pore size) nitrocellulose membrane (Bio-Rad Laboratories, Richmond, California) at 7 V/cm for 4 h in 25 mM Tris buffer (pH 8.6) containing 192 mM glycine, 20% (vol/vol) methanol and 0.01% SDS. The nitrocellulose sheet was then left overnight in Tris-buffered-saline plus tween (TBST, 25 mM Tris, 0.9% NaCl, 0.1% Tween-20, pH 8.0) at 4°C.

The nitrocellulose membrane was mounted in a miniblotter (Miniblotter, Immunetics, Cambridge, Massachusetts) apparatus placed on a rocker platform. The viral proteins were reacted with 26 different serum samples in separate lanes for 1 h at 22°C. Each blot had a known positive and known negative control serum. Serum samples were diluted 1/50 in 1% nonfat dry milk TBST. The nitrocellulose membrane was washed three times in TBST, and bound antibody was visualized by incubation for 1 h with affinity purified antigoat IgG (H + L) conjugated to horseradish peroxidase (ICN Biomedicals) diluted 1/200 in 1% nonfat dry milk TBST. The membrane was again washed three times in TBST, followed by tetramethylbenzidine with H_2O_2 (TMB Membrane, Peroxidase Substrate System, Kirkegaard and Perry Laboratories Inc., Gaithersburg, Maryland). Development was stopped by washing the membrane three times in distilled water.

Reaction at a location consistent with the known molecular weights of CAEV antigens and not observed in the negative control serum lane was considered positive. Those samples which were difficult to interpret were repeated and if still unreadable were dropped from the study.

Fixed-cell immunoperoxidase assay: For the FCIPA a CAEV infected monolayer of FLCor cells was obtained by coculture of 1×10^5 infected FLCor cells with 5×10^5 uninfected FLCor cells. The cells were seeded into 96-well tissue culture dishes (Falcon, Fisher Scientific Co., Nepean, Ontario) and incubated for nine days in Hanks' minimum essential medium, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 5% fetal bovine serum, 100 mg/mL streptomycin, and 100 IU/mL penicillin. After nine days the tissue culture supernatant was discarded, the cell monolayer was washed once with 200 μL/well phosphate buffered saline plus tween (PBST, 0.01 M phosphate, NaCl, 0.05% Tween-20, pH 7.6) for 5 min and fixed with 200 μL/well of 20% acetone/PBS/0.02% bovine serum albumin for 10 min. The plates were dried and either used immediately or frozen at −20°C for use later.

The plates were rehydrated with 150 μL/well of PBS for 5 min. Duplicate wells were incubated for 30 min with 50 μL/well of test sera, diluted 1/50 in buffer (0.5 M NaCl, 0.5% Tween-20). Known positive and negative control sera were included on each plate. Following removal of the sera and three 2-min washes with PBST, 50 μL/well of horseradish peroxidase labelled rabbit antigoat IgG (H + L) (ICN Biomedicals) diluted 1/500 in dilution buffer with 2% normal rabbit serum, was added to all wells for 30 min. After removal of the conjugate and three 2-min washes with PBST, 50 μL/well of substrate was added. The substrate consisted of 1 mL of chromogen [120 mg 3-amino-9-ethylcarbazole (Aldrich Chemical Co., Milwaukee, Wisconsin) in 15 mL of N,N-Dimethylformamide (Aldrich Chemical)] added to 19 mL of acetic acid/acetate buffer, pH 5.0, containing 66.6 μL of freshly added 3% H_2O_2 (Fisher Scientific). After 10 min the color development was stopped by removal of the substrate, three 2-min washes with PBST, and a final 20 second rinse with ultra pure water. After drying the plates were examined under a light microscope for peroxidase staining of infected cells. Staining above that of the negative control wells in a pattern consistent with cytoplasmic staining was considered positive.

TEST SERA

A total of 511 samples submitted from 40 farms for routine CAEV testing were used. These samples represented a variety of goat breeds and ages.

ASSESSMENT OF ELISA TEST PERFORMANCE

Caprine arthritis-encephalitis virus ELISA sensitivity and specificity were estimated, relative to the following definitions of reference positive and reference negative animals. A reference positive animal was defined as one that was positive to any two or all three of the AGIDT, IBA and FCIPA tests. The ELISA relative sensitivity was defined as the proportion of the reference positive animals that were classified as CAEV ELISA positive. A reference negative animal was defined as one that was negative to AGIDT, IBA and FCIPA tests. The ELISA relative specificity was defined as the proportion of reference negative animals that were classified as CAEV ELISA negative.

The performance of the ELISA was examined along the entire OD scale. The ELISA data were dichotomized at each 0.01 increment along the OD scale, between zero and one. The relative sensitivity and specificity, and the sum of sensitivity plus specificity, were estimated and graphed at each increment. The final critical OD value, that was selected to dichotomize the ELISA results for subsequent analysis, was that OD at which the sum of the sensitivity plus specificity was maximized (23).

Test results of the four immunological assays were analyzed for their agreement with one another, using the kappa statistic. This statistic is a measure of agreement, beyond that agreement which might be expected due to chance (24).

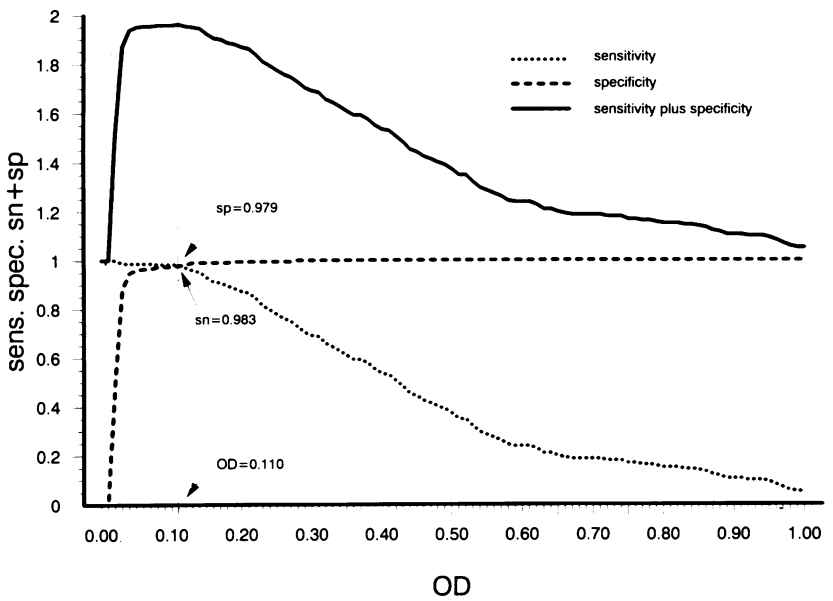

Fig. 1. Determination of the sensitivity (sn), specificity (sp), and sensitivity plus specificity (sn + sp) of the caprine arthritis-encephalitis antibody detection enzyme-linked immunosorbent assay, at various [0.01 optical density (OD) increment] cutoffs.

TABLE I. Summary of the results of the four immunological assays for antibodies to caprine arthritis-encephalitis virus in goat serum

	Positive	Negative	Total
ELISA[a]	193	318	511
AGIDT[b]	154	357	511
IBA[c]	204	281	485[d]
FCIPA[e]	163	348	511

[a]Enzyme-linked immunosorbent assay
[b]Agar gel immunodiffusion test
[c]Immunoblot assay
[d]Twenty-six samples were unreadable by IBA and were dropped from the study
[e]Fixed-cell immunoperoxidase assay

TABLE II. Kappa statistics of agreement between test pairs of immunological assays

	AGIDT	IBA	FCIPA[d]
ELISA[a]	0.71	0.72	0.73
AGIDT[b]	—	0.55	0.52
IBA[c]	—	—	0.53

[a]Enzyme-linked immunosorbent assay
[b]Agar gel immunodiffusion test
[c]Immunoblot assay
[d]Fixed-cell immunoperoxidase assay

RESULTS

Of the 511 sera tested by the AGIDT, IBA and FCIPA reference systems, 172 were positive in any two or all three tests. These samples were defined as reference positive and were used in the assessment of ELISA sensitivity. A total of 237 samples were negative in all three reference tests. They were defined as reference negative and were used in the assessment of ELISA specificity. The remaining 102 samples were positive in only one of the three tests. They were not included in further analysis.

Figure 1 illustrates the relative sensitivity, specificity and the sum of sensitivity plus specificity of the ELISA, as it was dichotomized at incremental values along the OD scale. The sum of sensitivity plus specificity was maximized at an OD of 0.11, corresponding to a point estimate of sensitivity of 98.3%, relative to the reference positive definition. The 95% confidence interval about this point estimate was from 94.6% to 99.6% (n = 172). Similarly, at that critical OD value, the point estimate of relative specificity was 97.9%, with a 95% confidence interval of 94.9% to 99.2% (n = 237). These nonsymmetrical confidence intervals about the proportions, are exact estimates according to Fleiss (24).

Table I shows the outcome of the four immunological assays applied to the 511 sera. The kappa statistic of agreement between the various test pairs is shown (Table II). In general, a kappa statistic greater than 0.7 reflects good agreement between tests.

DISCUSSION

Part of the evaluation of a new serological test should include the estimation of its diagnostic sensitivity and specificity. Ideally, this should involve a comparison of the results of the new test, to the true infection status of the animal, as defined by some biologically independent (nonserological) test system. Unfortunately, the true infection status of an animal is rarely known. This is especially true in CAE. Determination of true CAEV infection status is difficult because of the pathogenesis of this disease, in which restricted viral replication makes virus isolation from infected animals unreliable. We therefore evaluated a new serological test, relative to the results of other CAEV serological tests, which were used to define the reference status of animals.

In this study, we defined reference positive animals as those that were positive in any two or all three of the AGIDT, IBA or FCIPA tests. We defined reference negative animals as those that were negative to all three of the AGIDT, IBA and FCIPA reference tests. This approach allowed the estimation of sensitivity and specificity relative only to those reference definitions. These estimates would be biased from the true sensitivity and specificity, if the reference definitions did not represent the infection status, reasonably well.

Enzyme-linked immunosorbent assay test sensitivity and specificity vary with the critical OD cut off value, that is used to dichotomize the ELISA into a positive or negative result. This is illustrated in Fig. 1. In previous studies, the critical OD has often been set at two or three standard deviations above the mean OD among reference negative samples. An alternative method was used in this study, selecting the cut off at the point where the sum of sensitivity plus specificity was maximized. At this point, the test provided the most discriminatory information, without placing an a priori preference on either specificity or sensitivity.

Since the four immunological tests used in this study were not biologically independent of one another, we compared the agreement between different test pairs using the kappa statistic (Table II). The amount of agreement between the tests is likely to be at least partially attributable to the similarities and differences in the test mechanics

and antigens. Agreement implies only that the two tests are measuring the same or closely correlated factors. Therefore, good agreement does not necessarily imply correctness of test results relative to infection.

The four immunological assays used for detection of CAEV antibodies in this study differed in their mechanisms of detecting antigen-antibody reactions. The ELISA, IBA and FCIPA tests all involved systems of enzyme amplification, whereas the AGIDT has no such amplification. The AGIDT relies upon passive diffusion to bring the antigen and antibody together in the correct proportions to cause a line of precipitation and is the least sensitive (25).

The other three assays differ in antigen presentation and therefore may have different abilities in antibody detection. In the FCIPA, the CAEV antigens are presented in a native configuration (except for acetone labile epitopes which may be altered) as they are being presented in or on the cell. In the ELISA, the CAEV antigens are purified, processed chemically and finally bound to polystyrene, any or all of which factors may alter some of the epitopes on the various CAEV proteins. The immunoblot uses a less purified viral antigen, chemical solubilization of proteins, and electrophoretic separation of proteins.

These variations in assay mechanics, antigen preparation, and antigen presentation may cause the different tests to detect different subsets of CAEV antibodies. The AGIDT detects primarily anti-gp135 antibodies. The ELISA and FCIPA were both shown, with monospecific sera, to detect both anti-gp135 and anti-p28 antibodies, and the IBA was shown to detect reactivity to the p28, p70 and gp135 proteins (data not shown). Therefore, the tests might be expected to show reasonable, but not complete agreement as is illustrated (Table II).

The application of these established immunological assays in combination, detects a broader base of immunological responses than just one test. Therefore, it seems reasonable to use combinations of these established immunological assays to define the reference populations and to evaluate the relative sensitivity and specificity of a new test, in the absence of a practical, biologically independent reference assay.

In conclusion, the CAEV ELISA described in this study demonstrated good sensitivity and specificity relative to a panel of other serological tests. Furthermore, the good agreement between the CAEV ELISA and the AGIDT, IBA and FCIPA test, suggests that the ELISA could replace these tests, and would provide similar results.

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