Development of an Enzyme-Linked Immunosorbent Assay for Caprine Arthritis-Encephalitis Virus

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Because relatively few caprine arthritis-encephalitis virus (CAEV)-infected animals exhibit clinical signs of illness, efforts to control and eradicate this virus will depend heavily on a sensitive diagnostic test that can be easily carried out. The currently utilized tests are of limited usefulness because of relatively low sensitivity or because of incomplete cross-reactivity of goat sera with heterologous test antigens. An enzyme-linked immunosorbent assay (ELISA) with purified CAEV antigen and biotin-avidin amplification steps was therefore developed and compared with a radioimmunoassay (RIA) against CAEV p28. Of over 500 sera tested, there was 99% concordance between the two tests. On the other hand, 23 of 24 sera obtained from animals with clinical signs of disease that were negative by agar gel immunodiffusion test (with ovine progressive pneumonia virus antigen) were positive by ELISA and RIA. These results suggest that an ELISA with CAEV antigen is superior to the agar gel immunodiffusion test and is easier and faster than an RIA, and therefore may be the method of choice for diagnosing CAEV infection.

Caprine arthritis-encephalitis virus (CAEV) is a member of the subfamily Lentivirinae of the Retroviridae family, together with maedi-visna virus and ovine progressive pneumonia virus (OPPV). CAEV causes a slowly evolving complex syndrome in domestic goats that is observed worldwide and is characterized by progressive arthritis, especially in older animals, leukoencephalomyelitis, and mastitis (6, 15, 22). CAEV infection is followed by lifelong persistence in the face of significant immunity to viral proteins. The majority of virus carriers fail to develop clinically apparent disease (2).

Identification of infected symptomatic and healthy carrier goats has been achieved by using an agar gel immunodiffusion test (AGID) in which antigen was prepared from OPPV-infected sheep cell cultures (8). A similar AGID test has been described in which the antigen was ether-disrupted CAEV (18). Reciprocal immunoprecipitation studies demonstrated that maedi-visna virus, OPPV, and CAEV contain cross-reactive p28 major structural proteins and, to a lesser extent, cross-reactive glycoproteins (9, 12). However, the AGID in which maedi-visna antigens were used appeared to be more sensitive in detecting lentiviral antibodies in goat sera compared with the AGID test employing ether-disrupted CAEV (10). This may be due to the removal of viral glycoprotein by ether in the latter case, thus precluding being able to detect antibodies to the gp135.

On the other hand, enzyme immunoassays have been developed for measuring antibodies against several viruses of veterinary interest (5) and have proved to be more sensitive than classical tests. Furthermore, such assays are simple and rapid, avoid the radioactive isotopes needed for radioimmunoassays (RIAs), and allow processing of large numbers of sera at one time. An indirect enzyme-linked immunosorbent assay (ELISA) for serum antibody determination against lentiviruses in sheep has been reported in which maedi-visna virus was used as the test antigen (18). This test has recently been improved by the use of a double-antibody sandwich blocking procedure, using a monoclonal antibody directed to the major core protein (p28) of maedi-visna virus (19). These tests have also been used to detect antibodies to CAEV in goat sera (1, 3, 7, 14), but to date there has been no report of a CAEV-specific ELISA with CAEV as the test antigen. This report describes the development of an indirect ELISA which employs sucrose gradient-purified CAEV as the test antigen and compares this assay with an RIA to CAEV p28.

MATERIALS AND METHODS

Antigens. The strain of CAEV used for antigen in the ELISA experiments was obtained from a goat from Florida with a natural case of arthritis (9) and was propagated in Himalayan tahr ovary or lung cells (American Type Culture Collection designations are HJ1.0v, CRL 6274 and HJ11Lu, CRL 6273, respectively). Approximately 30-liter lots of supernatant from virus-infected cells were concentrated 100-fold by pressure filtration. The virus was pelleted, suspended in TNE (10 mM Tris hydrochloride [pH 7.8], 100 mM NaCl, 1 mM EDTA), and isoprecipitantly banded in 17 to 55% (wt/wt) sucrose (in TNE) gradients. Virus bands were removed, diluted 1:3 in TNE, pelleted, and resuspended at a final 10,000-fold concentration, and the protein level was determined before aliquoting and freezing at −80°C. A control antigen was prepared by ammonium sulfate precipitation of culture supernatants obtained from uninfected tahr cells incubated 3 to 4 days in medium containing 1% serum.

Test procedures. The indirect ELISA was based on a previously published procedure (21), using a biotin-avidin system to increase the sensitivity of the test (20). Optimal volumes and reagent dilutions were determined by checkerboard titrations, using negative and known weak and strong positive sera as determined previously by RIA. Flat-bottom 96-well microELISA plates (Immunoplate I; Nunc, Copenhagen, Denmark) were shown in preliminary tests to be equal or superior to other ELISA plates and were used throughout. Antigen was diluted in 50 mM carbonate (pH 9.6) to a final level of 2 μg/ml, and 200 μl was added to each
well (400 ng per well) and incubated overnight at 4°C. Additional wells received either no antigen or control antigen (400 ng per well). Before the addition of each reagent, the plates were washed three to five times (20× wash solution [Kirkegaard and Perry, Inc., Gaithersburg, Md.] diluted with deionized water and adjusted to 0.05% Tween 20). All reagents (Kirkegaard and Perry) were diluted in Blotto (2% nonfat dried milk solids, 1% Triton X-100, 50 mM Tris hydrochloride [pH 7.5], 0.05% Tween 20, 10 mM EDTA; Advanced Biotechnologies, Inc., Bethesda, Md.).

The order, volumes, dilution, and incubation conditions used for each reagent were as follows: (i) test serum, 100 μl, 1:100, 60 min at 37°C; (ii) biotin-anti-goat immunoglobulin G, 100 μl, 1:8,000, 60 min at 25°C; (iii) peroxidase-streptavidin, 100 μl, 1:1,000, 30 min at 25°C; (iv) ABTS (2,2′-azino-di-[3-ethyl-benzthiazoline sulfate]-H₂O₂) solution, 150 μl, 30 min at 25°C; (v) 1% sodium dodecyl sulfate (no prior wash), 150 μl. Plates were read at 405 nm, using a microplate ELISA reader (model 580; Dynatech Laboratories, Inc., Alexandria, Va.). Negative, weak, and strong control sera were included in each assay. The criteria for determining whether a serum was positive or negative are discussed below. When serum titers were determined, serial two-fold dilutions were carried out in Blotto and 100-μl aliquots were transferred to wells of a test plate. Subsequent steps were done as described above.

To define negative and positive sera, an RIA with iodinated CAEV p28 was used. CAEV p28 was purified by established procedures (16) and iodinated by the chloramine-T method (13) to a specific activity of 60 to 80 μCi/μg. Serial twofold dilutions of goat sera were incubated with approximately 10,000 cpm of 125I-labeled p28 for 3 h at 37°C and for 18 h at 4°C in a 200-μl reaction volume containing 0.1 M Tris hydrochloride (pH 7.8), 13 mM EDTA, 1% bovine serum albumin, 0.05% sodium azide, 0.4% Triton X-100, and 10 mM NaCl. This was followed by the addition of 25 μl of porcine anti-goat immunoglobulin G and 0.4 ml of 10 mM Tris hydrochloride, pH 7.8. After 3 h at 4°C, the reaction mixtures were centrifuged at 2,000 × g for 15 min at 4°C and the radioactivity in the pellets was measured. Positive sera were defined as those which bound at least 20% of the probe at a dilution of 1:20, and titers were calculated by determining the reciprocal of the dilution resulting in binding half of the maximum amount of the probe bound under conditions of excess antibody.

AGID was done with OPPV (strain WLC-1) as the test antigen according to published procedures (8, 10).

Sera. Goat sera were obtained from several sources. The largest group was obtained from several herds monitored by the Veterinary School of the University of California, Davis (N. East). These animals, representing a variety of breeds including alpine, la mancha, nubian, saanen, and togenburg, were primarily young animals that were involved in an eradication program. The other sera were obtained primarily from older animals located in Colorado, Florida, and Israel.

**RESULTS**

**Optimization of the ELISA.** Preliminary experiments were undertaken to determine the concentration of viral antigen capable of adequately detecting weak positive sera and the antisera dilution necessary to minimize nonspecific background while allowing detection of weak reactivity to the virus antigen. This was achieved by using sera known to be strongly and weakly reactive by RIA (titers of 2,500 and 20, respectively), as well as a known negative serum. The best results were obtained when 400 ng of viral antigen was added to each well for the overnight absorption and an initial dilution of 1:100 for the test sera was used (data not shown).

**Determination of minimal positive OD value.** Defining the optical density (OD) representing the minimal value for a positive serum first required an analysis of a large number of negative sera, as defined by RIA. A total of 303 goat sera that were negative by RIA were tested in the ELISA against wells containing no antigen, 400 ng of CAEV, and 400 ng of control antigen. The mean OD at 405 nm (ODₘₙₜ) values for the 303 sera were 0.088, 0.163, and 0.100, respectively (Table 1). The values obtained with the California sera were consistently lower than those seen with the other sera, particularly with the wells containing control antigen, in which the California sera averaged about one-fourth the values of the other sera. Table 1 summarizes the results of subtracting control values (either wells without antigen or wells with control antigen) from the values obtained with viral antigen for the two groups of sera, as well as the combined sera.

It is apparent that adjusting the OD obtained in wells with virus antigen by subtracting values obtained in the wells with control antigen is not useful, principally because the mean corrected values of the California sera remain much different from those for the other sera. This was principally due to some of the sera in the heterogeneous group exhibiting very high reactivity toward the control antigen, leading to both the very low mean corrected OD and the high standard deviation. Nevertheless, the use of wells with control antigen represents an important control, since they identify sera with high nonspecific background.

Adjusting values obtained with wells containing virus by subtracting the OD obtained from wells without antigen was more useful. This resulted in the two sets of sera having mean ODs much closer to each other compared with uncorrected values, with modest standard deviations (Table 1). Subsequent analysis of data with both positive and negative sera involved the correction of the OD in this manner.

**Analysis of distribution of negative OD values.** Figure 1A presents histograms showing the distribution of uncorrected
TABLE 2. Comparison of RIA and ELISA in detecting antibodies to CAEV

<table>
<thead>
<tr>
<th>Origin of sera</th>
<th>No.</th>
<th>No. tested by RIA</th>
<th>No. tested by ELISA</th>
<th>% Concordance</th>
</tr>
</thead>
<tbody>
<tr>
<td>California</td>
<td>313</td>
<td>52 261</td>
<td>52 261</td>
<td>100</td>
</tr>
<tr>
<td>Other</td>
<td>207</td>
<td>109 98</td>
<td>105 102</td>
<td>97.1</td>
</tr>
<tr>
<td>Totals</td>
<td>520</td>
<td>161 359</td>
<td>156 364</td>
<td>98.8</td>
</tr>
</tbody>
</table>

deviations \(0.075 \pm 5 \times 0.042\), with an OD of 0.285 representing the upper limit of negative sera. The only caveat is that in the case of sera with very high reactivity toward control antigen and borderline positive reactivity to viral antigen, the result must be considered ambivalent.

Analysis of positive and negative goat sera by ELISA and RIA. A total of 520 sera were analyzed by both RIA and ELISA. All sera were diluted 1:100 and tested in the ELISA at that single dilution, while sera tested by RIA were diluted either 1:20 or 1:50 followed by six to eight serial twofold dilutions. For the ELISA data, sera exhibiting a corrected OD greater than 0.285 were considered positive, while sera binding greater than 20% of the iodinated CAEV p28 were considered positive in the RIA. Table 2 summarizes the results on the 520 sera and indicates that about 30% of all the sera tested were positive for antibodies to CAEV. This is lower than is seen for many goat populations (2), but this is largely due to the majority of the sera having been obtained from herds in California which are involved in a long-term control project in which most of the animals were known or assumed to be negative. There were six instances of discordance between the two assays; 5 of 176 non-California sera were positive by RIA and negative by ELISA, and 1 of the same group was positive by ELISA but negative by RIA.

Titration of sera by ELISA and RIA. Figure 2 illustrates titration curves obtained by ELISA with both weak and strong positive sera. For comparison, the average OD values of 32 negative sera diluted in the same way (starting at 1:100) are shown in the lower curve. If an OD of 0.285 is used as an endpoint, these two sera have titers of 1:400 and 1:20,000.

FIG. 1. Distribution of OD values of goat sera negative for antibodies to CAEV by RIA. The number of sera with an OD of 0.05 within each range indicated in the abscissa is indicated by a solid bar (California sera), open bar (sera other than those from California), or cross-hatched bar (total sera). (A) Distribution of raw values obtained in wells with virus antigen. (B) Distribution of values obtained by subtracting the OD obtained in wells without antigen from that of wells with virus antigen. (C) Distribution obtained by combining the two sets of data shown in panel B. In this case, the upper limit of the value on the abscissa represents the mean OD plus 5 standard deviations.

FIG. 2. Use of the ELISA to determine the titers of goat sera. Serial twofold dilutions of a strongly positive (○), weakly positive (△), and 32 negative sera were added to the wells of plates containing 400 ng of CAEV antigen per ml. The ODs for the negative sera were averaged (▲) and indicate that what little background exists at 1:100 rapidly disappears. The titer of a serum is defined as the reciprocal of the dilution which leads to an OD of 0.285 or greater.
respective. Titers on all the positive sera were determined by ELISA and RIA. For the ELISA, titers varied between 1:100 and 1:12,800, while for the RIA, titers varied between 1:20 and 1:3,200. While the ELISA titers are somewhat higher than the RIA titers overall, there is excellent correlation between the two sets of data (Fig. 3). Nevertheless, since each assay is using a different antigen preparation, many sera show significantly different titers in the two assays.

Comparison of ELISA, RIA, and AGID test. Although no systematic comparison of the ELISA to the AGID test was done, a limited comparison was useful in showing the greater sensitivity of the ELISA. A group of 24 sera were obtained from animals which demonstrated clinical signs of CAE disease (typically swollen joints). None of these sera were positive in an AGID with OPPV antigen, but 23 of the sera were positive in both the ELISA and RIA. Although this group of sera was atypical in its lack of reactivity in the AGID test, these results indicate that the ELISA is superior to AGID in detecting low antibody levels.

DISCUSSION

In the present study, an indirect ELISA with biotin-avidin amplification was developed which was capable of sensitively detecting antibodies to CAEV in goat sera. It was previously demonstrated that utilization of biotinylated antibodies and avidin conjugates increased the sensitivity of an ELISA in detecting antibodies to murine hepatitis surface antigen by a factor of almost 80 in comparison with an assay with anti-species peroxidase conjugate as the second reagent (20). Using the more sensitive detection system allowed a higher initial dilution of the goat sera without compromising our ability to detect weak positive sera. At the same time, however, this higher initial dilution had a profound effect on lowering the background of the assay and minimizing false-positive and false-negative values.

Analysis of the ELISA data obtained from sera known to be negative by RIA analysis revealed significant variation, particularly with regard to sets of sera from different parts of the country. Whether this represents variations in storage conditions, time between collection and assay, or general health of the animals is not clear, but it was necessary to make a correction for background before determining whether a serum was positive or negative. As shown in Table 1 and Fig. 1, this was done by subtracting the OD obtained from wells receiving no antigen from the OD obtained from wells with viral antigen. This adjustment had the effect of normalizing all sera into essentially a single group and allowed the establishment of criteria which were universally useful in identifying positive sera. Such corrections have been shown to be useful in other cases as well (4, 11, 17). Wells containing control antigen obtained from ammonium sulfate precipitation of tissue culture fluids from mock-infected tahr cultures exhibited a much wider variation in OD than the other wells. Although relatively few sera exhibited extremely high reactivity toward the control antigen, this was statistically disastrous, since attempting to correct values with these data led simultaneously to a very low mean OD and a very high standard deviation (Table 1). Nevertheless, wells with control antigen are useful in identifying sera with high nonspecific background, so that, if their reactivity toward viral antigen is weakly positive, the results can be considered equivocal.

Although the ELISA can be used for rapid screening of many sera by testing them at the single dilution of 1:100 (one person can easily screen several hundred sera in a day), it can also be used to determine the titers of the positive sera (Fig. 2). An important measure of the sensitivity of the ELISA is to determine the correlation to RIA titrations. Table 2 indicates that of 520 sera tested in both assays, 98.8% tested the same. There were six exceptions, with five being positive by RIA and negative by ELISA and one being negative by RIA and positive by ELISA.

The conventional field test for CAEV is AGID, typically with antigen prepared from OPPV-infected sheep cells (8). This test typically develops a single precipitin line which appears to be to the virus glycoprotein (12). While the glycoprotein represents the antigen which induces the greatest amount of antibody, it also represents the most antigenically variable protein of the virus (9, 12). Thus, while use of glycoprotein as the antigen for the AGID test ensures great sensitivity as long as genetic variation is not excessive between the virus used as the test antigen and the virus infecting the test animals, it is likely that different sources of antigen would be required in different parts of the world. Indeed, when a number of sera from goats which demonstrated clinical signs of disease but which were AGID negative were analyzed by RIA and ELISA, all but 1 of the 24 sera were positive by both tests. To some extent it is due to the considerably greater sensitivity of both of these tests, but it also is due to the use of the more genetically stable gag antigens as the major component of the ELISA antigen preparation (and the sole component being assayed by RIA). Thus, despite the lower titers typically seen in goats to the gag antigens, the greater sensitivity of the ELISA allows the detection of a higher percentage of seropositive goats. Furthermore, the test is simple and rapid, does not use radioactive probes, allows hundreds of samples to be screened per day, and is as sensitive as an RIA.

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