

Detection of Serum Antibodies to Ovine Progressive Pneumonia Virus in Sheep by Using a Caprine Arthritis-Encephalitis Virus Competitive-Inhibition Enzyme-Linked Immunosorbent Assay

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A competitive-inhibition enzyme-linked immunosorbent assay (cELISA) for detection of antibodies to the surface envelope (SU) of caprine arthritis-encephalitis virus (CAEV) was recently reported (L. M. Herrmann, W. P. Cheevers, T. C. McGuire, D. Scott Adams, M. M. Hutton, W. G. Gavin, and D. P. Knowles, *Clin. Diagn. Lab. Immunol.* 10:267-271, 2003). The cELISA utilizes CAEV-63 SU captured on microtiter plates using the monoclonal antibody (MAb) F7-299 and measures competitive displacement of binding of the anti-CAEV MAb GPB 74A by goat serum. The present study evaluated the CAEV cELISA for detection of antibodies to ovine progressive pneumonia virus (OPPV) in sheep. Three hundred thirty-two sera were randomly selected from 21,373 sheep sera collected throughout the United States to determine the sensitivity and specificity of cELISA and agar gel immunodiffusion (AGID) based on immunoprecipitation (IP) of [³⁵S]methionine-labeled OPPV antigens as a standard of comparison. A positive cELISA test was defined as >20.9 percent inhibition (% I) of MAb 74A binding based on two standard deviations above the mean % I of 191 IP-negative sheep sera. At this cutoff, there were 2 of 141 false-negative sera (98.6% sensitivity) and 6 of 191 false-positive sera (96.9% specificity). Sensitivity and specificity values for IP-monitored AGID were comparable to those for cELISA for 314 of 332 sera with unambiguous AGID results. Concordant results by cELISA and IP resolved 16 of the 18 sera that were indeterminate by AGID. Additional studies evaluated cELISA by using 539 sera from a single OPPV-positive flock. Based on IP of 36 of these sera, there was one false-negative by cELISA among 21 IP-positive sera (95.5% sensitivity) and 0 of 15 false-positives (100% specificity). We conclude that the CAEV cELISA can be applied to detection of OPPV antibodies in sheep with high sensitivity and specificity.

Ovine progressive pneumonia virus (OPPV) is the North American equivalent of maedi/visna virus, a monocyte/macrophage-tropic lentivirus which causes multiorgan inflammatory disease in sheep (14). Based on agar gel immunodiffusion (AGID) serology, the prevalence of OPPV is as high as 90% in sheep flocks of the western United States (4). A majority of OPPV-infected sheep are lifelong carriers without clinical signs; however, both asymptomatic carriers and sheep with clinical disease are potential sources of OPPV transmission through colostrum and milk (19). Therefore, serologic tests with high sensitivity and specificity are needed for successful eradication of OPPV.

We recently reported a competitive-inhibition enzyme-linked immunosorbent assay (cELISA) which detects antibodies to the surface envelope (SU) of caprine arthritis-encephalitis virus (CAEV) in goat sera (8, 15). The CAEV cELISA utilizes native CAEV-63 SU captured on microtiter plates with the monoclonal antibody (MAb) F7-299 and measures the ability of undiluted goat serum to displace binding by a second anti-SU MAb (GPB 74A) (15). Validated against immunoprecipitation of [³⁵S]methionine-labeled CAEV-63 antigens, the

CAEV cELISA exhibits high sensitivity (100%) and specificity (96.4%) for diagnosis of CAEV infection in goat herds (8).

The major structural proteins of CAEV and OPPV share antigenic determinants in immunoprecipitation assays (7). The degree of antigenic cross-reactivity between CAEV and OPPV is unknown; however, the sensitivity of AGID using OPPV antigen for detection of antibodies to CAEV in goat sera is low (11). In the present study, we evaluated the CAEV cELISA for detection of anti-OPPV antibodies in sheep using (IP) of [³⁵S]methionine-labeled OPPV-WLC1 as a standard of comparison. In addition, we compared the performance of the CAEV cELISA with that of a commercial AGID test that also utilizes OPPV-WLC1 as antigen (3).

MATERIALS AND METHODS

Animals. Sheep sera (21,373) were collected from 22 different states in the United States for the National Animal Health Monitoring System sheep 2001 study. Three hundred thirty-two of the 21,373 sheep sera were randomly selected and analyzed using CAEV cELISA, OPPV-WLC1 AGID, and IP of [³⁵S]methionine-labeled OPPV-WLC1. Additional studies to evaluate the CAEV cELISA utilized sera from 539 sheep in an OPPV-positive flock at the U.S. Sheep Experiment Station in Dubois, Idaho. Most sheep from this flock originate from Dubois, Idaho, except for the occasional ram originating from the Dubois vicinity. Thirty-six of these sera were tested independently by IP of [³⁵S]methionine-labeled OPPV-WLC1.

cELISA. Undiluted sheep sera were evaluated for CAEV SU cross-reactive antibodies using a CAEV antibody test kit (VMRD, Inc., Pullman, Wash.). Each

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test kit included positive and negative goat sera verified by IP of CAEV antigens. Results were expressed as percent inhibition (% I) of Mab GPB 74A binding calculated by $[1 - OD_{620}(\text{test sample})/OD_{620}(\text{negative kit control})] \times 100$, where OD_{620} is the optical density at 620 nm (15). To verify the accuracy of results based on negative goat sera in the test kits, positive and negative sheep sera verified by IP of OPPV-WLC1 antigens were included in all cELISA tests. Based on 273 plate readings, the OD_{620} values for IP-negative goat serum (mean = 0.823) and IP-negative sheep serum (mean = 0.837) were not significantly different (paired *t* test, $P = 0.19$). In addition, the positive control sheep serum registered positive by cELISA in all tests (mean $OD_{620} = 0.036$) corresponding to 95.6% I based on the negative goat serum control and 95.7% I based on the negative sheep serum control.

AGID. The Washington State Animal Disease Diagnostic Laboratory performed the AGID assays on sheep sera using a CAEV/OPPV antibody test kit (Veterinary Diagnostic Technology, Inc., Wheat Ridge, Colo.), which utilizes OPPV-WLC1 as the antigen (2, 3). AGID test results were defined as positive or negative based on concordant results by two independent evaluators. If two independent evaluators recorded discordant results, the AGID status was defined as indeterminate.

IP. Antibodies to OPPV structural antigens in serum were detected by IP of lysates of OPPV-WLC1 labeled with [35 S]methionine followed by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate. Protocols for production of labeled OPPV-WLC1 in goat synovial membrane cells, preparation of viral lysates, and IP with protein G have been described (7, 10). After IP washes, 40 μ l of 2 \times Nupage sodium dodecyl sulfate sample buffer containing 10% (vol/vol) sample reducing agent (Invitrogen, Carlsbad, Calif.) was added to each sample. Samples were mixed, heated at 75°C for 10 min, clarified by brief centrifugation (10,000 \times g), and loaded onto a 4 to 12% gradient Tris-Bis Nupage Gel (Invitrogen) using 1 \times morpholinepropanesulfonic acid as a running buffer with 1 \times morpholinepropanesulfonic acid-0.25% (vol/vol) antioxidant (Invitrogen) in the inner chamber. Gels were fixed in 10% acetone-40% methanol for 30 min, rinsed three times with double-distilled water, and treated with Enlightening solution (Perkin-Elmer Life Sciences, Boston, Mass.) for 30 min with rocking. Gels were dried for 1.5 h and exposed to X-ray film for 14 days at -80°C. Positive IP results were defined by detection of OPPV gp135 SU and/or gp90 oligomeric transmembrane Env proteins (7). Antibodies to three additional low-molecular-mass Gag proteins were also detected in most SU-positive sera. However, none of 368 sera evaluated (332 from the National Animal Health Monitoring System and 36 from Dubois) immunoprecipitated a Gag protein in the absence of SU or transmembrane.

Statistical analysis of cELISA sensitivity and specificity. The 95% confidence intervals (CI) for the sensitivity and specificity of cELISA compared to IP were determined in the following way: $95\% \text{ CI} = [p(1 - p)/n] \times 1.96$, where p = proportion of cELISA and IP concordant samples and n = total number of samples (20). The kappa statistic was utilized to assess agreement between cELISA and IP (13, 20).

RESULTS

cELISA testing of sheep sera. cELISA results in relation to IP status of 332 sheep sera are shown in Fig. 1A. There were 191 IP-negative sera, with cELISA values ranging from -26.9 to 46.8% I of Mab 74A binding, and 141 IP-positive sera, with cELISA values from 5.1 to 100% I with a sharp distinction between IP-negative and IP-positive sera. Histogram analysis also indicated a distinct bimodal distribution of % I values corresponding to IP-negative and IP-positive sera (Fig. 1B). The mean \pm standard deviation (SD) % I for the 191 IP-negative sera was 2.04 ± 9.41 , and the mean \pm SD % I for the 141 IP-positive sera was 72.93 ± 18.83 . A cELISA cutoff value of 20.9% I was defined by 2 SD above the mean % I for the 191 IP-negative sera. Based on this cutoff, the sensitivity of cELISA was $98.6 \pm 1.9\%$ CI (139 of 141 IP-positive sera with cELISA values of >20.9% I), and the specificity was $96.9 \pm 2.5\%$ CI (185 of 191 IP-negative sera with cELISA values of \leq 20.9% I). Therefore, there were 2 of 141 false-negative cELISA tests and 6 of 191 false-positive cELISA tests. The kappa statistic was

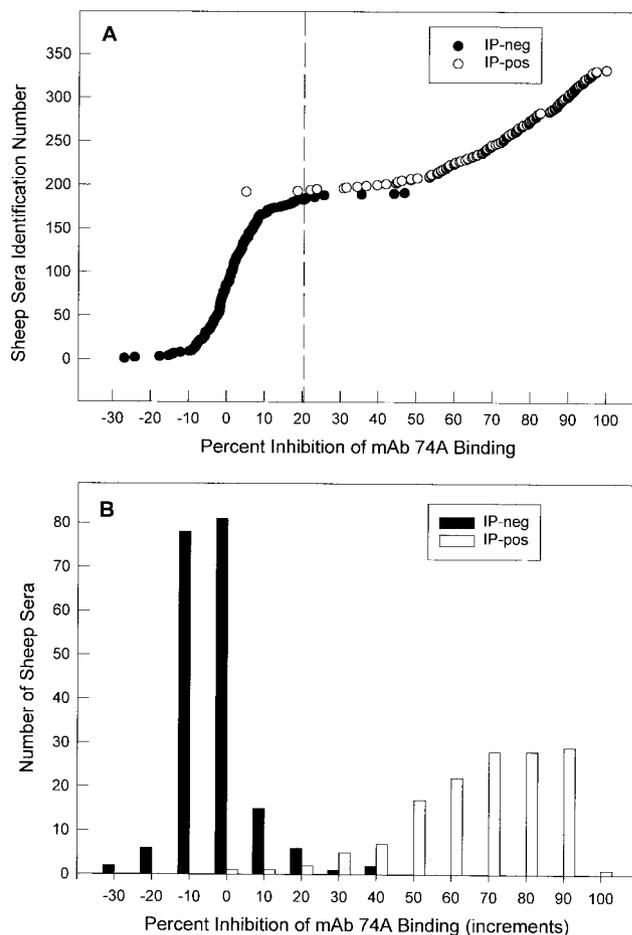


FIG. 1. Evaluation of 332 sheep sera by cELISA and IP. (A) Distribution of cELISA values (% I of Mab 74A binding) in relation to IP status of sheep sera. Identification numbers for sheep sera (1 to 332) were assigned arbitrarily. The dashed line indicates the cELISA cutoff value (20.9% I) defined by 2 SD above the mean % I of the 191 IP-negative sera. (B) Frequency distribution of the number of sheep, with cELISA values in 10% increments corresponding to IP status.

0.95, indicating excellent agreement between cELISA and IP (13).

AGID testing of sheep sera. The 332 sheep sera in Fig. 1 were evaluated for anti-OPPV antibodies using AGID. Unambiguous AGID results were obtained for 314 sera. The sensitivity and specificity of AGID for these 314 sera compared to IP were $99.3 \pm 1.4\%$ CI (136 of 137 IP-positive sera) and $99.4 \pm 1.1\%$ CI (176 of 177 IP-negative sera). cELISA results in relation to IP status for 18 indeterminate AGID results are shown in Fig. 2. Based on the cutoff of 20.9% I established in Fig. 1, cELISA and IP results were concordant for 16 sera. Thus, concordant cELISA and IP results resolved 16 of 18 AGID-indeterminate sera.

Independent evaluation of the cELISA cutoff value. To extend the cELISA results for 332 randomly chosen sheep sera, we evaluated an additional 539 sera from a single OPPV-positive flock (U.S. Sheep Experiment Station). Based on the 20.9% I cutoff established in Fig. 1, the distribution of % I values for these 539 sera indicated a sharp distinction between cELISA-negative and -positive sera (Fig. 3A). To assess the

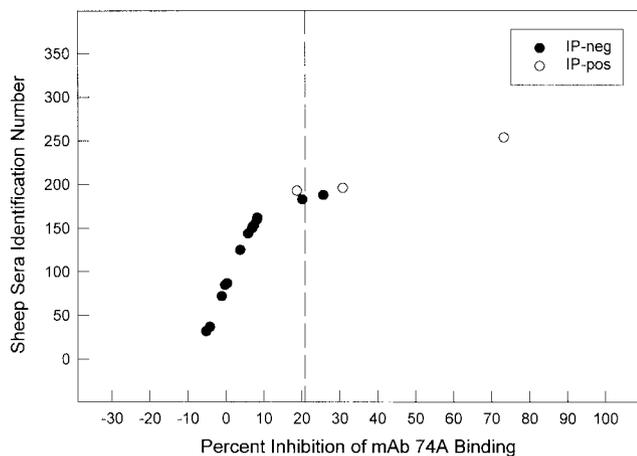


FIG. 2. Distribution of cELISA values in relation to IP status of 18 AGID-indeterminate sheep sera. The cELISA cutoff value (20.9% I) is indicated by the dashed line.

accuracy of the cELISA cutoff value (20.9% I), 36 of 539 sera were tested by IP. Eleven sera were chosen on the basis of cELISA values near the cutoff. Twenty-five sera with cELISA values of <10% I and >40% I were selected randomly. The IP results for these 36 sera are shown in Fig. 3B. There was one false-negative serum by cELISA among 21 IP-positive sera (95.5% sensitivity) and 0 of 15 false-positive sera (100% specificity). Thus, the efficacy of cELISA for randomly chosen sera at the 20.9% I cutoff was applicable to a single OPPV-infected flock.

DISCUSSION

We previously described a cELISA for detection of antibodies to CAEV in goat sera based on inhibition of MAb 74A binding to the CAEV-63 SU (8, 15). In the present study, we evaluated the CAEV cELISA for detection of OPPV antibodies using 332 sheep sera from across the United States. Using a cELISA cutoff value of 20.9% I for sheep sera, the sensitivity and specificity of the cELISA compared to IP were 98.6% and 96.9%, respectively. Therefore, our results show that most sheep sera that immunoprecipitate OPPV-WLC1 also have antibodies that cross-react with CAEV-63 SU as measured by inhibition of MAb 74A binding. In this context, we confirmed that sera from OPPV-infected sheep immunoprecipitate SU of two strains of CAEV (63 and Co) as well as OPPV-WLC1 (data not shown).

The high sensitivity of cELISA for detection of cross-reactive antibodies in sheep is in contrast to findings of a previous study which evaluated detection of OPPV-WLC1 cross-reactive antibodies in goat sera by AGID (11). In that study, OPPV-WLC1 AGID detected CAEV-63 IP-positive antibodies in goat sera with a sensitivity of only 56%. The reason for enhanced sensitivity of cELISA compared to that of AGID for detection of cross-reactive antibodies is unknown. The sensitivity of AGID relies on cross-reactive antibodies precipitating OPPV WLC1 viral proteins in agar, whereas the sensitivity of cELISA relies on cross-reactive antibodies inhibiting the binding of MAb 74A to a single conformational epitope on SU. We

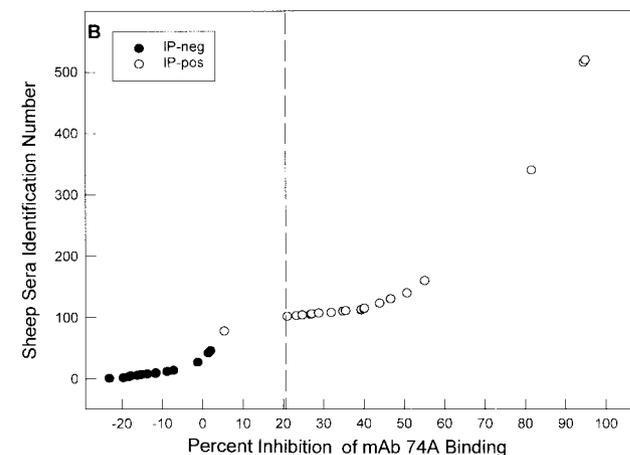
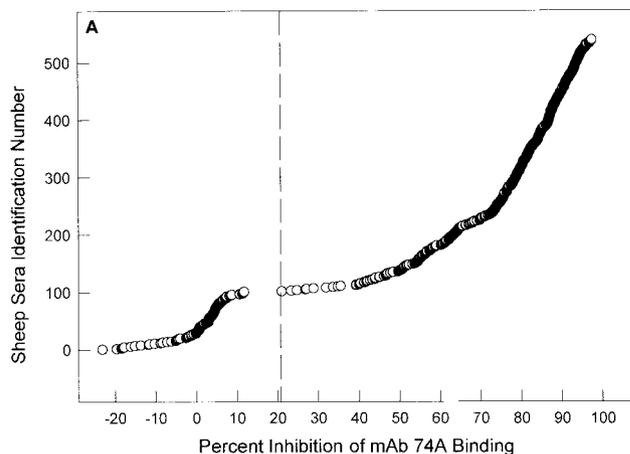


FIG. 3. Evaluation of sheep sera from the Dubois OPPV-positive flock. (A) Distribution of cELISA values of 539 sheep sera. Sheep identification numbers were assigned arbitrarily. The cELISA cutoff value (20.9% I) is indicated by the dashed line. (B) Distribution of cELISA values in relation to IP status of 36 sheep sera selected from 539 sera in panel A. The cELISA cutoff value (20.9% I) is indicated by the dashed line.

speculate that anchoring of CAEV-63 SU by MAb F7-299 in the cELISA results in a conformational orientation of SU that increases exposure of cross-reactive epitopes.

In the CAEV cELISA validation using sheep sera, there were 6 of 368 false-positive sera (IP negative, cELISA positive). We have shown previously that cELISA has a similar prevalence of false-positives with goat sera and that cELISA is more sensitive than IP for detection of anti-CAEV SU antibodies in goat sera (8). Therefore, the high sensitivity of cELISA compared to IP may contribute to the prevalence of apparently false-positive sera.

Of 368 sheep sera evaluated, there were three false-negative cELISA tests (cELISA negative, IP positive). Thus, a few OPPV-infected sheep produce antibody to OPPV SU epitopes which are not conserved between CAEV-63 and OPPV-WLC1. We have recently sequenced the *env* gene of 11 North American isolates of OPPV and determined that the SU amino acid sequence heterogeneity between CAEV-63 and these iso-

lates is ~35%, equivalent to 193 of 550 SU amino acids. Therefore, SU amino acid sequence diversity allows for OPPV-specific antibodies that register as false negative in the CAEV cELISA. Nevertheless, our results show that cELISA detects OPPV SU antibodies with a sensitivity of >98%.

The present results show that unambiguous AGID results and cELISA performed similarly when monitored by IP. However, 18 of 332 sera (5%) were indeterminate by AGID, and cELISA monitored by IP resolved the status of 16 of these sera. Because AGID is subject to discordant results between readers, we conclude that cELISA is a more reliable diagnostic test for detecting OPPV antibodies based upon the IP standard.

Several indirect ELISA tests utilizing both disrupted whole virus and recombinant viral proteins have been developed for the detection of OPPV antibodies (1, 5, 12, 16–18, 21, 22). However, only two indirect ELISA formats have shown >95% sensitivity and specificity when compared to AGID (12, 18). In addition, two other competitive ELISA tests have been reported for the detection of OPPV antibodies; one was validated by AGID with 88% specificity (9) and the other was validated against indirect ELISA with 82% specificity (6). To our knowledge, none of the previously described indirect or competitive ELISA tests have been validated by Western blot or IP. Therefore, the performance of these assays compared to the cELISA described here cannot be adequately assessed.

In summary, the present study applies a CAEV cELISA to field sheep sera for the detection of serum antibodies to OPPV in sheep. One advantage of the cELISA is its use of undiluted serum, which allows detection of sera with low titers of antibody and minimizes the prevalence of false negatives. In addition, cELISA is superior to AGID because it measures SU-reactive antibodies without subjective assessment.

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