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Comparison of Monoclonal Antibody-Based Sandwich Enzyme-Linked Immunosorbent Assay and Virus Isolation for Detection of Peste des Petits Ruminants Virus in Goat Tissues and Secretions

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A monoclonal antibody-based sandwich enzyme-linked immunosorbent assay (S-ELISA) was developed for specific detection of peste des petits ruminants virus. Compared with virus isolation in Vero cell cultures using 89 paired tissue and secretion samples from six experimentally infected goats, S-ELISA was significantly more sensitive (71.9% versus 65.2%; $P < 0.05$). The S-ELISA is a suitable alternative to virus isolation.

Peste des petits ruminants (PPR) is a severe, rinderpest-like disease of goats and sheep characterized by pyrexia, catarrhal inflammation of the ocular and nasal mucous membranes, erosive stomatitis, enteritis, and pneumonia (22). PPR virus (PPRV) is a paramyxovirus of the *Morbillivirus* genus (7). Other morbilliviruses are measles virus (MV) of humans, canine distemper virus (CDV) of dogs, rinderpest virus (RPV) of cattle and buffalo (10), and the recently identified phocine distemper virus (PDV1) of seals (3). RPV is the only morbillivirus whose host range overlaps with that of PPRV. In goats and sheep, the two viruses produce clinical disease and pathology that are indistinguishable (8, 13). There is also serologic cross-reactivity between PPRV and RPV (7). Consequently, development of a diagnostic test for PPR must address the question of differential diagnosis between PPRV and RPV. The standard techniques for differential diagnosis of PPR and rinderpest—cell culture isolation, inoculation into goats and cattle, differential neutralization (7), and differential electrophoretic profile of the N protein (5)—require virus isolation, which is time-consuming, costly, and often insensitive.

To circumvent these shortcomings, new techniques based on direct detection of virus or viral components in diagnostic samples are being developed. Recently, ^{32}P-radiolabeled or biotinylated nucleic acid (cDNA) probes corresponding to a variable region of the N genes of PPRV and RPV have been used to selectively hybridize RNA from the homologous virus (4, 18). The use of such probes makes a differential diagnosis on field samples possible within 3 days, but highly specialized technical training is required for proper testing to be accomplished. The objective of the present study was to use a PPRV-specific neutralizing monoclonal antibody (MAb) in a simple and rapid double-antibody sandwich enzyme-linked immunosorbent assay (S-ELISA) for specific detection of PPRV antigen in goat tissues and secretions. The S-ELISA was compared with a single-passage virus isolation procedure in Vero cell cultures routinely performed for PPRV isolation.

Tissues and secretions obtained from six adult mixed-breed PPRV-infected goats were used. The goats were housed together and fed pelleted hay ad libitum in a level 3 biological containment facility. They were inoculated subcutaneously with 1.25 ml of a lymph node-spleen suspension containing approximately 1,000 50% tissue culture infective doses ($TCID_{50}$) of virulent PPRV Nigeria 75/1 (23). Five of the six goats (goats 1 and 3 through 6) had fever (40 to 41.5°C) that was first observed at 5 days postinfection (d.p.i.). Physical examination revealed, at various times after 5 d.p.i., coughing, sneezing, nasal and lachrymal discharges, oral cavity erosions, and diarrhea in all infected goats except goat 2. Lachrymal, nasal, and oral swabs were collected from all goats at 6 d.p.i., when the five symptomatic animals had a serous nasal discharge. Heparinized blood was also collected at 6 d.p.i. Swabs were stored frozen at −70°C in 0.8 ml of minimum essential medium with Earle's salts (EMEM) supplemented with 2% fetal bovine serum and antibiotics (40 μg of gentamicin sulfate and 2.5 μg of amphotericin B per ml). Peripheral blood lymphocytes (PBL) were isolated from part of the heparinized blood using Ficoll-Paque (Pharmacia LKB Biotechnology, Alameda, Calif.) as described previously (1). The remainder of the blood was stored at 4°C until used. The goats were euthanatized at 8 d.p.i. (goats 1, 2, 5, and 6) or 11 d.p.i. (goats 3 and 4), and the following tissues were collected and stored at −70°C: brain, tonsil, lung, mesenteric lymph nodes, liver, spleen, small intestine, colon, cecum, and kidney. Significant postmortem lesions in the five symptomatic goats were oral erosions, necrotic areas in the colon and cecum, submucosal hemorrhages of the ileocecal region, and hemorrhagic mesenteric lymph nodes. The observed clinical and pathological findings were suggestive of PPRV infection.

Heparinized blood was used for virus isolation and S-ELISA within 24 h of collection. Following a preliminary observation of negative immunofluorescence staining with freshly prepared PBL in contrast to mitogen-stimulated lymphocytes (unpublished observations), isolated PBL were used only after 2 days' stimulation with pokeweed mitogen (PWM) (Sigma Chemical Co., St. Louis, Mo.). The PBL were resuspended at 5×10^6 cells per ml in RPMI 1640 with 20% fetal bovine serum and 50 μg of gentamicin sulfate per ml, and PWM was then added to a final concentration of 5 μg/ml. Flasks (25 cm^2) were seeded with 6 ml of PBL suspension and incubated vertically in a

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humidified incubator at 37°C and 5% CO_2 for 2 days. These stimulated PBL were sonicated for 1 min prior to use. Tissue extracts were obtained from frozen specimens after they were allowed to thaw at room temperature (18 to 20°C) and homogenized in EMEM plus antibiotics in Pyrex tissue grinders to obtain 10% (wt/vol) suspensions. After centrifugation at 3,000 × g for 10 min, the supernatants were collected and tested. Similarly, frozen swabs were allowed to thaw and then expunged into sterile tubes before testing.

Isolation of PPRV was done in African green monkey (Vero) cell cultures (CCL 81; American Type Culture Collection, Rockville, Md.). Tissue homogenates and whole blood were inoculated in 1-ml volumes onto 1-day-old monolayers of Vero cells in 25-cm^2 flasks. For expunged swabs, 1-day-old Vero cell monolayers in 24-well plates were inoculated with 0.25 ml per well. After virus adsorption for 2 h at 37°C, the inoculum was removed and replaced with EMEM plus 5% fetal bovine serum plus antibiotics (8 ml and 1 ml for 25-cm^2 flasks and 24-well plates, respectively). Virus isolation from lymphocytes was done by coculturing 1-ml suspensions (5×10^6 cells) of PWM-stimulated PBL with freshly trypsinized Vero cells in 25-cm^2 flasks. All cultures were examined daily for development of cytopathic effect (rounding of cells, clumping into grape-like clusters, and formation of syncytia). The assay was stopped after 10 days of incubation. Virus isolation was recorded as successful if cells from an inoculated flask showed typical cytopathic effect and/or fluoresced specifically when stained with a PPRV-specific MAb followed by fluoresceinated goat anti-mouse immunoglobulin G (Sigma Chemical Co.) (19). No attempt was made to improve the sensitivity of virus isolation by blindly subculturing negative cultures, since the objective was to compare the S-ELISA against a one-step virus isolation procedure.

The double-antibody S-ELISA relies on the ability of antibody immobilized on the surface of microtiter wells to bind with antigen in a test sample. The bound, or "captured," antigen is then detected with a second antibody that is specific for the target antigen. Three previously described MAbs, designated B_2G_3, B_2G_6, and B_9G_3, were chosen for the development of an S-ELISA on the basis of their PPRV specificity, their immunoglobulin G isotype, and their recognition of three nonoverlapping epitopes on the hemagglutinin protein of PPRV (19). MAb B_2G_3 was chosen as the detecting antibody on the basis of its high reactivity in indirect ELISA and its PPRV specificity (21). The MAbs and a polyclonal mouse anti-PPRV ascites antibody (20) were purified from mouse ascitic fluids with Bakerbond ABx beads (J. T. Baker Inc., Phillipsburg, N.J.) and Sephadex G-25 desalting-buffer exchange columns (Pharmacia LKB Biotechnology) following the manufacturers' instructions. Aliquots of purified MAb B_2G_3 and the polyclonal antibody were biotinylated by using Sulfo-NHS-Biotin (Pierce, Rockford, Ill.). The S-ELISA was developed for specific detection of PPRV antigen by following a modification of established procedures (9). Immulon-2 flat-bottom microtiter plates (Dynatech Laboratories, Chantilly, Va.) were coated by incubation overnight at 4°C or for 1 h at 37°C with 1 μg of crude ascites or purified MAb diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6) per well (volume, 100 μl per well). Unbound antibody was removed by washing four times with phosphate-buffered saline containing 0.05% Tween 20 (PBST). The plates were then allowed to react for 1 h at 37°C with 100 μl of test sample per well. Following four washes in PBST, the plates were incubated for 1 h at 37°C with 100 μl of a 1:500 dilution of biotinylated MAb B_2G_3 in PBST plus 2% bovine calf serum (PBST-BCS) per well. After four more washes, plates were reacted with a 1:2,000 dilution of peroxidase-conjugated streptavidin (Zymed Laboratories Inc., San Francisco, Calif.) in PBST-BCS. After four final washes, the enzymatic reaction was developed by adding 100 μl of a substrate-chromogen mixture consisting of 0.01% hydrogen peroxide and 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma Chemical Co.) at 0.1 mg/ml in 0.05 M citrate-phosphate buffer (pH 5.0) per well. Color development was stopped after 25 min by adding 50 μl of 2 M H_2SO_4 per well, and the optical density (OD) was read at 450 nm with a Titertek Multiskan reader (ICN-Flow, Irvine, Calif.).

The S-ELISA was standardized by using both gradient-purified PPRV prepared as described previously (21) from the prototype Nigeria 75/1 isolate (23) and an infected Vero cell culture supernatant of the same virus. Negative controls included gradient-purified virus preparations of the four other morbilliviruses (RPV RBOK vaccine strain, MV Edmonston strain, CDV Onderstepoort strain, and PDV1 2-6A isolate) and infected Vero cell culture supernatants of the same viruses. The PPRV and RPV came from the Foreign Animal Disease Diagnostic Laboratory, Plum Island, repository; CDV and MV were provided by Max J. G. Appel of Cornell University; and PDV1 was a gift from Albert D. M. E. Osterhaus of the National Institute of Public Health and Environmental Protection (Bilthoven, Netherlands). The culture, titration, and gradient purification of all those viruses have been described elsewhere (21). Eight serial fivefold dilutions starting at 10^5 $TCID_{50}$ per well and 4,000 ($10^{3.6}$) ng per well for cell culture supernatants and purified viruses, respectively, were used in standardizing the assay (Fig. 1). Of the four antibodies (polyclonal anti-PPRV ascites and MAbs B_2G_3, B_2G_6, and B_9G_3) used for initial solid-phase coating, the MAb B_2G_3 captured the most antigen as evidenced by high OD values. Also, the biotinylated MAb B_2G_3 was a better detector of captured antigen than biotinylated polyclonal anti-PPRV ascites antibody. The sensitivity of S-ELISA was estimated by graphically interpolating the detection limit (Fig. 1), defined as the smallest amount of positive control virus or viral antigen that would give an OD at least twice the mean OD of the negative controls at a corresponding dilution. The detection limits were 65 $TCID_{50}$ per well (Fig. 1a) and 12 ng per well (Fig. 1b) for PPRV-infected cell culture supernatant and purified PPRV, respectively. The viral protein content of the purified antigen was estimated at 40% (data not shown); the limit of detection for viral antigen was therefore about 5 ng per well. The four other morbilliviruses (RPV, MV, CDV, and PDV1) failed to react in the assay (Fig. 1), confirming its specificity for PPRV antigen. To save time after plates are coated with solid-phase antibody, the test sample, the detecting antibody, and the streptavidin-peroxidase conjugate may all be added in one step and incubated for 2 h. This single-step format was nonstatistically compared with the standard multiple-step procedure described above (Fig. 2). Application of the single-step S-ELISA procedure led to a reduction in sensitivity of only 0.35 $\log_{10}$ (i.e., 2.24 $TCID_{50}$ per well) (Fig. 2), which is of small practical importance when weighed against a decrease of about 90 min in running time.

Pairs of the goat samples described above (i.e., heparinized blood; lachrymal, nasal, and oral secretions; and sonicated PWM-stimulated PBL suspensions collected at 6 d.p.i. and homogenates from various tissues collected at necropsy) were used for PPRV isolation in Vero cells and for detection of specific antigen by S-ELISA. A cocktail of homogenates from archived RPV-infected goat tissues (spleen and mesenteric lymph nodes) from which RPV could be readily isolated in Vero cells was used as a negative control. Results of S-ELISA were expressed as a binding ratio, computed as the OD of the

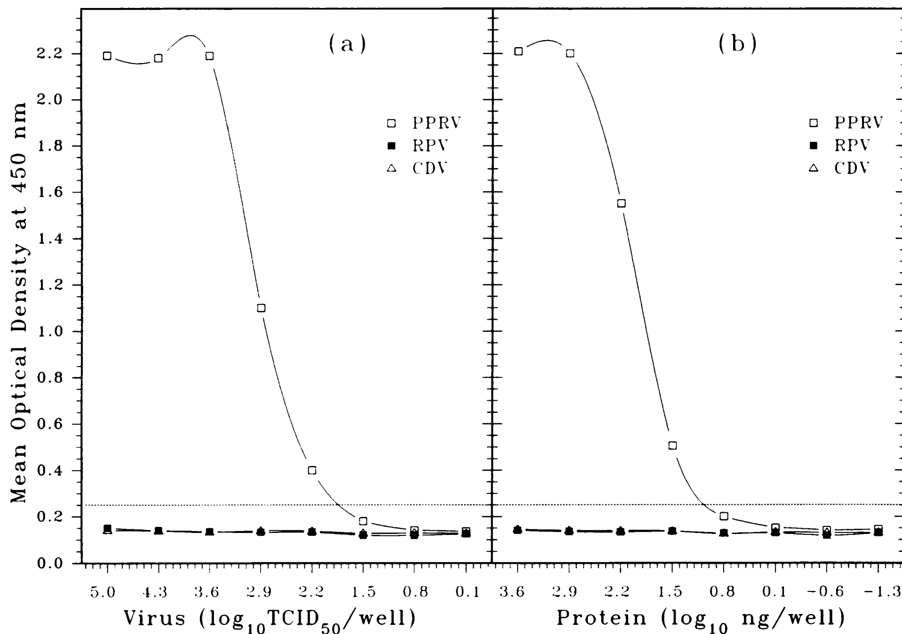

FIG. 1. Estimation of S-ELISA sensitivity and specificity. Titration of PPRV-, RPV-, and CDV-infected culture supernates containing 10^5 $TCID_{50}$/100 μl (a) or gradient-purified viruses starting at 4 μg/100 μl (b). The dotted line indicates the negative cutoff value. Data for MV and PDV1 are not shown because they parallel those for RPV and CDV.

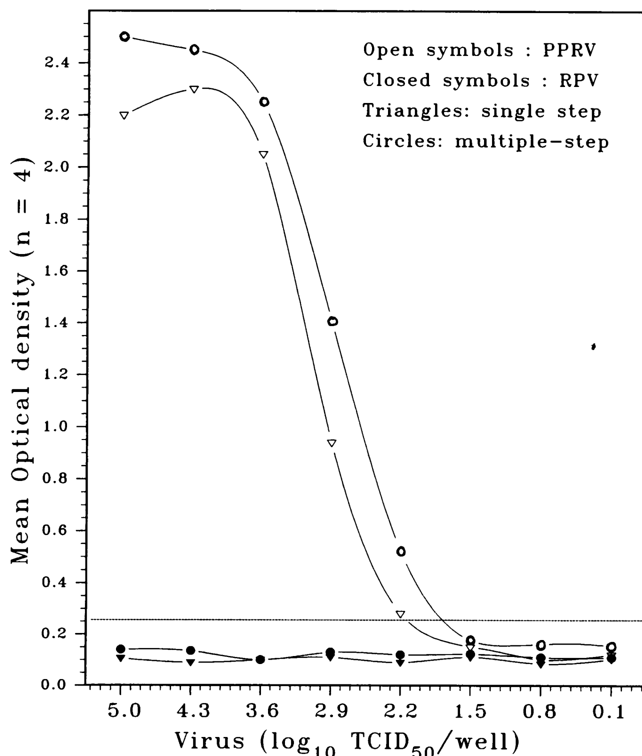

FIG. 2. Comparison of single-step and multiple-step S-ELISA. Titration of quadruplet fivefold serial dilutions of virus containing 10^5 $TCID_{50}$/100 μl. The dotted line indicates the negative cutoff value.

test sample divided by the OD of the negative control. Cell culture isolation was scored as positive or negative. The results are presented in Table 1. S-ELISA was positive for 64 of 89 (71.9%) samples, while virus isolation was positive for 58 of 89 (65.2%) samples. All six goats had detectable virus and antigen in at least five of their tissues. Although goat 2 did not show any clinical signs, it was clearly infected, as evidenced by virus isolation and antigen detection (the amount of input virus alone, 100 $TCID_{50}$, was judged to be too low to be detectable). Working on the premise that all six goats were infected and considering 15 samples per goat (14 for goat 2), comparison of the two methods using McNemar's test for matched samples (6) indicates that S-ELISA was significantly ($P < 0.05$) more sensitive than virus isolation.

The S-ELISA procedure described here uses the same MAb in the solid phase and in detecting the captured antigen. Most other studies of S-ELISAs for viruses (11, 14, 17) have used at least two different MAbs recognizing different epitopes for solid phase and detection. Our attempt to use various combinations of three MAbs directed against three nonoverlapping epitopes yielded much lower sensitivity than when a single MAb (B_2G_3) was used throughout. The difference may be explained by the properties of the solid-phase antibody and the captured antigen. On the one hand, MAb B_2G_3 has a very high avidity for the antigen (endpoint titer, 1:10^7); on the other hand, the neutralizing epitope recognized by that MAb on the surface hemagglutinin protein of PPRV may be a dominant surface epitope (19). Assuming that PPRV is roughly spherical and that captured antigen consists mainly of intact virions or protein aggregates, it is easy to understand how the same MAb works for capture and for detection.

The S-ELISA procedure can be completed in less than 7 h. The running time is even shorter if the single-step alternative is used. In comparison, virus isolation and identification (when successful on first passage) takes at least 6 days to complete. The short turnaround time of S-ELISA and a sensitivity at

TABLE 1. Comparison of virus isolation and antigen detection by S-ELISA with goat tissues and secretions

Sample[a]	Result for[b]:											
	Goat 1		Goat 2		Goat 3		Goat 4		Goat 5		Goat 6	
	VI	EL	VI	EL	VI	EL	VI	EL	VI	EL	VI	EL
Brain	−	−	ND	ND	−	−	−	+	−	+	−	+
Blood	−	−	−	−	−	−	−	−	−	−	−	−
PBL	+	++	−	−	+	+++	+	+++	+	+++	+	+++
Tonsil	+	+++	−	−	+	+	+	++	+	+++	+	++
Lung	−	−	−	−	+	+	−	−	+	+	−	−
MLN	+	++	+	++	+	++	+	+	+	+++	+	+++
Liver	−	+	+	++	+	++	+	++	+	++	+	++
Spleen	+	+	+	+	+	+	+	+	+	++	+	+
SI	+	+++	+	+	+	++	+	+	+	+++	+	+++
Colon	+	+++	−	−	+	+++	+	+++	+	++	+	+++
Cecum	+	+++	−	+	+	+++	+	+++	+	+++	+	+++
Kidney	+	++	+	++	+	++	+	+++	+	++	+	++
NS	−	−	−	−	+	+	−	−	+	++	+	++
LS	−	−	−	−	+	+	−	−	−	+	+	+
OS	−	−	−	−	−		−	−	+	+	+	+

[a] MLN, mesenteric lymph node; SI, small intestine; NS, nasal swab; LS, lachrymal swab; OS, oral swab.
[b] Virus isolation (VI) was scored as positive (+) or negative (−). S-ELISA (EL) results were expressed as binding ratios, and the ratios were scored as follows: −, <2.5; +, 2.5 to 5.0; ++, 5 to 10; and +++, >10. ND, not done.

least equal to that of cell culture isolation make it a suitable alternative to virus isolation. Although the sensitivity of virus isolation may be improved by using primary lamb or goat cells, those cells pose the problem of variability among batches; consequently, most diagnostic laboratories prefer continuous cell lines such as the Vero cells used in this study. Other assays that have been described for detection of PPRV antigen, such as dot immunoassay (15), complement fixation (8), agar gel immunodiffusion (16), and immunohistochemical staining (2), which are based on polyclonal antisera, lack specificity because of cross-reactivity with RPV. MAb-based techniques circumvent that shortcoming. Antigen capture assays using MAbs have recently been described for several viral diseases (11, 12, 14, 17). The simplicity and rapidity of S-ELISAs (or capture ELISAs) make them attractive alternatives to long, expensive, and often insensitive virus isolation procedures. Moreover, MAb-based assays offer the additional advantage of allowing standardization between laboratories using the same reagent.

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