An Enzyme-linked Immunosorbent Assay for Detection of Antibodies to Maedi-Visna Virus in Sheep I. A Simple Technique for Production of Antigen Using Sodium Dodecyl Sulfate Treatment

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ABSTRACT

We report the efficacy of an anionic detergent, sodium dodecyl sulfate (SDS) for preparing maedi-visna antigens for an indirect enzyme-linked immunosorbent assay (i-ELISA). Ovine maedi-visna virus (MVV) pelleted by differential centrifugation followed by liquid chromatography was treated with SDS or one of three lipid solvents: ethyl ether, chloroform or fluorocarbon. The SDS-treated antigen resulted in higher optical density values with positive serum and better discrimination between positive and negative serum samples from specific-pathogen-free (SPF) sheep experimentally inoculated with the virus. Optimal results were obtained when MVV was treated with concentrations of 0.25% and 0.125% of SDS. A viral antigen prepared by centrifugation and treatment of a viral pellet with SDS was also suitable for the i-ELISA. This latter technique may facilitate the production of MVV antigens for use in the i-ELISA.

RÉSUMÉ

Nous rapportons l'efficacité d'un détergent anionique, le dodécyle sulfate de sodium (SDS) pour la préparation d'antigènes du virus maedi-visna (MV) utilisés dans la technique ELISA. Le virus MV mis en culot par ultracentrifugation et purifié par chromatographie en phase liquide a été traité par le SDS ou l'un des trois solvants lipidiques suivant: l'éther, le chloroforme ou le fluorocarbone. La préparation traitée par le SDS donne une valeur de densité optique plus élevée pour les sérums positifs et une discrimination accrue entre les sérums négatifs et positifs provenant de moutons exempts d'agents infectieux, expérimentallement inoculés par le virus MV. Des résultats optimaux ont été obtenus avec des concentrations de 0.25% et de 0.125% de SDS. La préparation virale préparée par centrifugation et traîtement du culot de virus avec le SDS était également convenable pour la technique ELISA. Cette dernière technique peut faciliter la production des antigènes du virus MV pour leur utilisation dans la technique ELISA.

INTRODUCTION

Since its first application in 1971 by Engvall and Perlmann (1), the indirect enzyme-linked immunosorbent assay (i-ELISA) technique has been widely used for the serodiagnosis of various infectious diseases. For animal lentiviral infections, the i-ELISA has been applied to detect antibodies against caprine-arthritis encephalitis (CAE) (2), equine infectious anemia (EIA) (3), ovine maedi-visna viruses (MVV) or ovine progressive pneumonia (OPP) (4, 5).

As a first step in performing the technique, a suitable preparation of the viral antigen is required to prevent undesirable background which might affect the sensitivity and specificity of the test (6). Houwers and Gielkens (7) were the first to report the use of the conventional i-ELISA for MVV. Their antigen preparation required high speed centrifugation on a cushion of sucrose followed by ether extraction. Ethyl ether is an organic solvent used to disrupt lipid viruses but is fairly ineffective in removing nonviral components (8). In order to reduce the amount of cellular proteins interfering with the test, Vitu *et al* (5) stressed the importance of a more purified preparation of the viral antigen. They applied consecutive zonal centrifugations in density gradients such as sucrose to obtain good discrimination between positive and negative serum samples. McGrath *et al* (9) indicated that retroviral activity is sensitive to the osmotic shock of suspension in sucrose gradients and that the conventional pellet/banding technique had an adverse effect on the virus population.

In order to reduce the problems associated with the production of MVV antigen for use in an i-ELISA, a novel approach was attempted. An anionic detergent (sodium dodecyl sulfate or SDS) which is commonly used to dissociate viral subunits (10) was compared with organic solvents (ethyl ether, chloroform and fluorocarbon). A simple technique for production of MVV antigen was also evaluated with the i-ELISA.

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MATERIALS AND METHODS

VIRUS

The maedi-visna virus (MVV) used in this experiment was isolated from a field case and used after numerous passages in ovine choroid plexus cells. The virus showed an identity line in the agar gel immunodiffusion test (AGID) with the OPP strain supplied by Dr. R.C. Cutlip, Ames, Iowa, USA and was morphologically indistinguishable from other lentiviruses by electron microscopy.

The virus was cultivated in 850 cm^2 roller bottles of newborn lamb lung cells prior to use as inoculum. The growth medium was composed of Hanks' minimal essential medium (MEM), 2 mM L-glutamine, 0.1 mM nonessential amino-acids, 10% fetal bovine serum (FBS), 100 mg/mL of streptomycin and 100 IU/mL of penicillin. The maintenance medium was devoid of FBS. After 80% of cytopathic effect (CPE) was observed (three or four days postinoculation), the cells were frozen once at -70° C and thawed. The virus pool had a titer of approximately 10^6 median tissue culture infectious doses ($TCID_{50}$)/mL.

ANTIGEN PREPARATIONS

1. *Extraction with anionic detergent or lipid solvents*

The medium harvested from the roller bottles was clarified at 1,000 x *g* (Sorvall type SS34 Rotor) for 10 min, then the virus was pelleted by ultracentrifugation at 106,000 x *g* (max) for 180 min at 4° C using a fixed angle rotor (Beckman type 30). The pellet was resuspended in 1/100 of the original volume in buffer B described by Verwoerd *et al* (11). The supernatant obtained was processed by chromatography (Pharmacia, Dorval, Quebec). The column used (1.5 x 9 cm) was packed with Sepharose CL4B in buffer B and eluted with 250 mL of the same buffer at a flow rate of 2 mL/min. The virus peak was monitored by optical density measurement at 280 nm. The fractions containing the virus were pooled and reintroduced into a similar column packed with Sephadex G-200 (Pharmacia, Dorval, Quebec) and eluted under the same conditions as above. The fractions of the peak activity were pooled and the material was distributed into four aliquots. An equal volume of ethyl ether (Allied Fisher Scientific Ltd, Dartmouth, Nova Scotia), fluorocarbon (1,1,2-Trichlorotrifluorethane (TTE) (Matheson Coleman & Bell, Norwood, Ohio)), 10% chloroform (Allied Fisher, Dartmouth, Nova Scotia) or 2% SDS (Schwartz, Mann., Orangeburgh, New York) doubly washed with ethanol were respectively added into aliquots 1-4. After a short incubation period of 10 min at room temperature (RT) with frequent shaking, the mixtures were clarified at 1,000 x *g* for 15 min (Sorvall type SS34 rotor). Similar preparations of controls were obtained from cell cultures free of MVV. The aqueous phase of each preparation was used for comparative studies in a conventional i-ELISA.

2. *Simple antigen preparation using SDS*

The medium harvested from the roller bottles was clarified as indicated previously and then concentrated by two consecutive differential centrifugations performed at 4° C in a fixed angle rotor (Beckman 30) at 106,000 x *g* for 90 min. The pellet of the first centrifugation was resuspended in 1/10 of the original volume with buffer B (11). The pellet of the second centrifugation was resuspended in 1/100 of the original volume in TN buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl) then treated with 0.1% SDS prior to being recentrifuged as previously described. The aqueous phase obtained was used directly in the i-ELISA after dilution in 0.05 M carbonate buffer, pH 9.6.

i-ELISA

The i-ELISA used to compare the antigen preparations was essentially similar to the technique described by Voller *et al* (6). Standard 96-well polystyrene flat bottom plates (Linbro, Titertek, Flow Laboratories, McLean, Virginia; Cat. #76-301-05) were coated with 100 μL of the viral antigen or the antigen control diluted in 0.05 M carbonate buffer pH 9.6, as required. After an overnight incubation at 4° C in a humidified box, the wells were washed three times with the ELISA buffer (0.14 M NaCl, 1.5 mM KH_2PO_4, 0.8 mM $Na_2HPO_4.H_2O$, 3 mM KCl) containing 0.05% Tween 20. A volume of 100 μL/well of the serum was added at a dilution of 1/50 in ELISA buffer and incubated at room temperature (RT) for 2 h. After washing three times, 100 μL/well of horseradish peroxidase (HRP)-conjugated rabbit antisheep immunoglobulin, heavy and light chains (Cappel, Organon Teknika Corp., West Chester, Pennsylvania) diluted 1/2000 in the ELISA buffer were added and incubated for 1 h at RT. To reveal the reaction, 100 μL/well of 1 mM ABTS (2,2 -Azinobis [3-ethylbenzthiazoline sulfonic acid], Sigma Chemical Co., St. Louis, Missouri), prepared in 0.5 M citrate buffer pH 4 and containing 0.015% H_2O_2 were used. Optical density was measured by a photometer (Titertek, Multiskan, Flow Laboratories, McLean, Virginia) at a wavelength of 414 nm, 30 min after the addition of the substrate. The i-ELISA was slightly modified later by the inclusion of 7.5% FBS and 0.2 M NaCl in the buffer used for diluting the serum samples and the conjugate which was used at 1/1000 dilution.

SERUM CONTROLS

Serum control samples were obtained from two specific-pathogen-free (SPF) sheep experimentally infected with approximately 10^6 $TCID_{50}$/mL of MVV inoculated by the intratracheal route. Preinfection (Pre-I) serum samples were retained for negative controls. They were both negative by AGID. The positive sera were collected at five months (sheep A) and seven months (sheep B) postinoculation (Post-I). The guidelines of the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care were followed. The AGID reaction was strongly positive for sheep A. Sheep B showed a weaker reaction in AGID.

RESULTS

ANTIGEN EXTRACTION

For each lot of antigen, the i-ELISA was performed using Pre- and Post-I serum samples from sheep A and B. Figure 1 shows the optical density (O.D.) values of the positive serum (P)

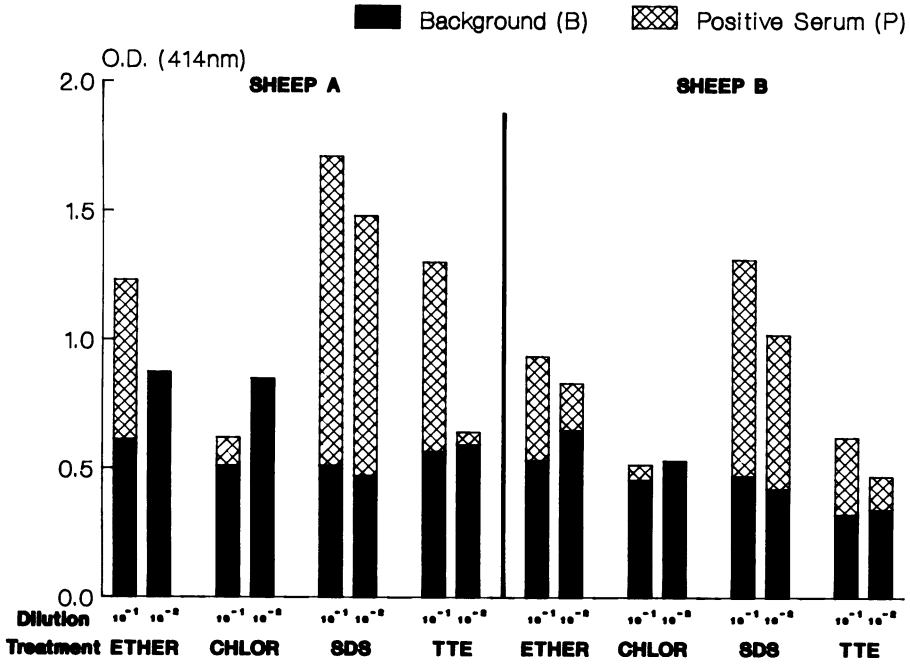

Fig. 1. Optical density (O.D.) of MVV positive serum (P) and the background (B) of sheep A and B obtained by i-ELISA following treatment of the MVV antigen with ether, chloroform (CHLOR), sodium dodecyl sulfate (SDS) and fluorocarbon (TTE).

and the O.D. values of the background (B) for each animal at two dilutions of the antigens. B corresponds to the mean of the O.D. obtained for the negative serum incubated with the viral antigen and the pre- and post-I serum samples incubated with the preparations free of MVV. Figure 2 represents the mean ratios P/B of both animals at antigen dilutions 10^{-1} to 10^{-4}. The SDS treatment gave higher O.D. values with positive serum and better discrimination between the positive and negative controls. The optimum P/B ratio of 3.1 was obtained at a dilution of 10^{-1}. At a dilution of 10^{-2}, the P/B ratio was 2.8 and decreased rapidly at higher dilutions of the antigen. The chloroform treatment yielded a high background and no difference between the positive and the negative serum was obtained. The ether and fluorocarbon treatments gave comparable results. The maximum P/B ratio obtained for both preparations was 1.9 at a 10^{-1} dilution.

In order to determine the concentration of SDS to be used for optimum results, the viral preparation was treated with 0.025% up to 1% of SDS. An untreated preparation was included as well. Figure 3 shows the P/N ratios of the positive (P) and the negative (N) serum samples of animal A at four dilutions of the antigens. The untreated antigen gave the lowest P/N ratios at all the dilutions studied. The P/N ratios increased gradually with the increasing concentration of SDS up to 0.25%. At this concentration, an optimum difference between the positive and the negative serum samples (P/N of 4.55) was observed for an antigen diluted at 1/160. Antigen treated with 0.125% gave the next highest P/N with a value of 4.34 at a dilution of 1/160. Concentrations higher than 0.25% of SDS in the viral preparation reduced the P/N ratios accordingly.

SIMPLE ANTIGEN PREPARATION USING SDS

The effectiveness of the SDS treatment on a simplified preparation of MVV antigen obtained by differential centrifugation was evaluated. Pre- and Post-I serum samples of sheep A were used. The P/N and the P/B ratios of 3.69 and 3.49 respectively at a 1/100 dilution of the viral antigen and the control preparation free of virus were determined, using the conventional i-ELISA. To decrease the background in the i-ELISA, the diluent buffer was modified to include FBS and increased NaCl molarity. Table I shows the optical densities obtained using the modified i-ELISA at increasing dilutions of the antigens. The P/N and the P/B ratios of the Pre-I and Post-I serum samples are also shown.

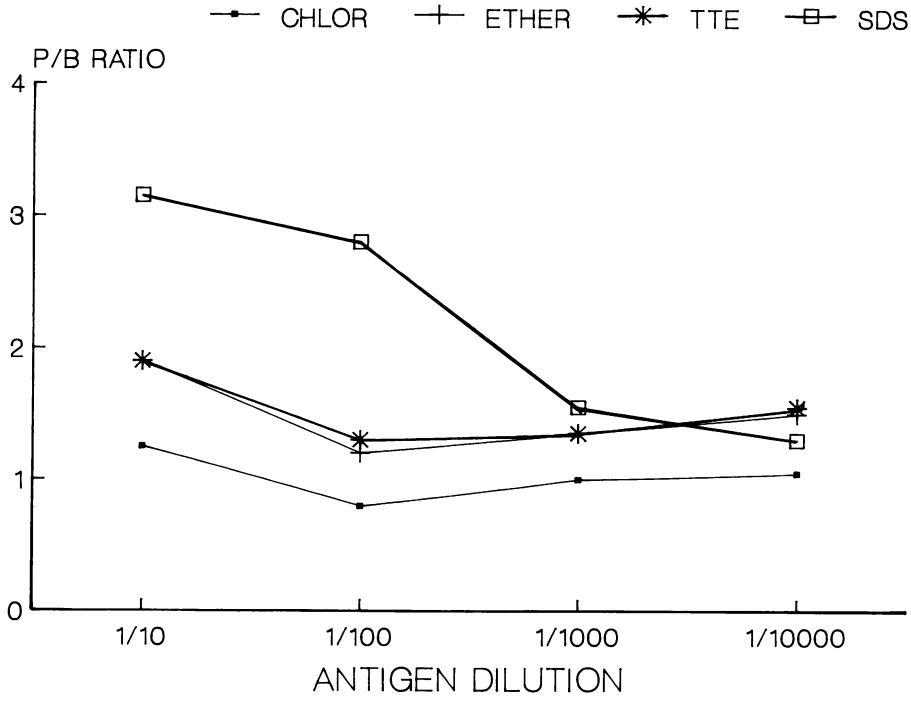

Fig. 2. Mean positive/background (P/B) [ratio of positive serum O.D. with MVV Ag (P) to the mean O.D. of negative serum with MVV Ag and the positive and negative serum with MVV-free Ag (B)] ratios of sheep A and B at four dilutions of the antigen, following chemical treatment with chloroform (CHLOR), ether, fluorocarbon (TTE) and sodium dodecyl sulfate (SDS).

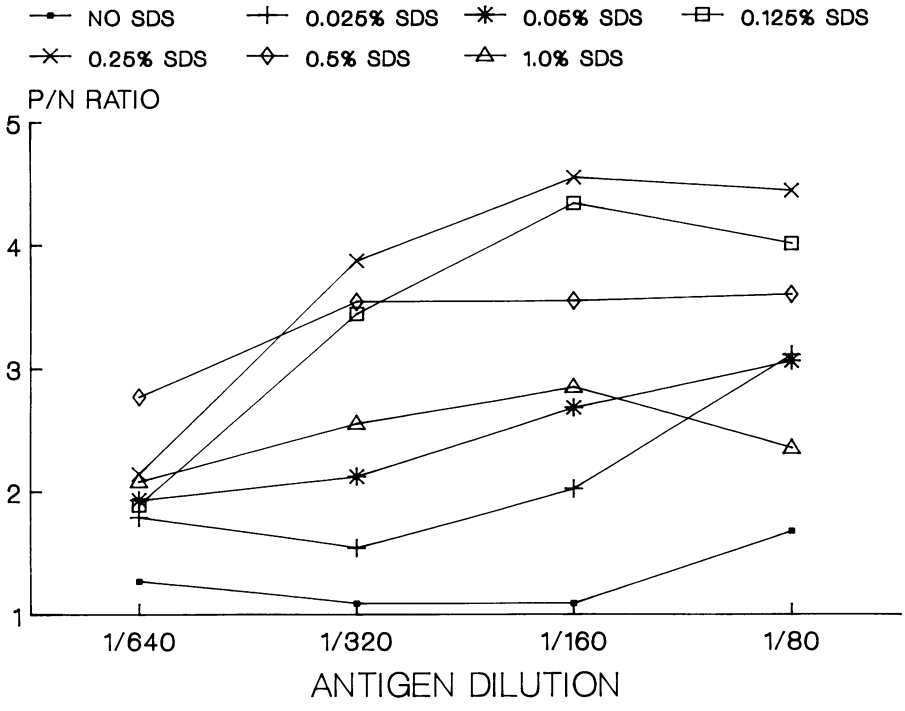

Fig. 3. Optical density (O.D.) ratios of positive (P) and negative (N) serum samples from sheep A obtained by i-ELISA at four dilutions of the maedi-visna virus antigen and varying concentrations of sodium dodecyl sulfate (SDS).

DISCUSSION

Our data suggest that SDS is more suitable than lipid solvents to treat MVV for use as an i-ELISA antigen. This anionic detergent has been used to dissociate a number of viruses into their protein and nucleic acid components. The resulting proteins however are usually denatured by the action of the detergent (10). Marel and Wezel (12) reported that the biological activity and antigenicity of the virus sub-units were often decreased considerably by the action of ionic detergents. Loss of activity after SDS treatment and recovery after its removal are not predictable, and some antigenic properties may remain after the action of SDS (13). According to our results, it seems that the antigenic activity of the antigen preparation was retained after SDS treatment as shown by the antigen-antibody (Ag-Ab) reaction revealed by the i-ELISA. The concentration of SDS in the viral preparation was an important factor to consider. Concentrations of 0.25% and 0.125% gave optimum discrimination between our positive and negative serum whereas at higher or lower concentrations, the sensitivity of the i-ELISA was reduced considerably. Friedmann *et al* (14) used different concentrations of SDS at various temperatures to disrupt MVV particles and to release internal nucleic acid components. When 1% SDS was applied, disrupted particles outnumbered intact virus particles and complex coiled and twisted structures were observed by electron microscopy. At 0.1% SDS, fewer particles were disrupted but clearly defined structures such as simple rings and short curvilinear rods were obtained. Treatment with 0.05% SDS released mostly rings of RNA. At this point, it has not been determined which of the viral components obtained after SDS treatment were implicated in the Ag-Ab reaction detected by the i-ELISA. Viral infections result in the formation of antibodies directed against the intact virion and a variety of viral sub-units and even to other macromolecules such as enzymes that are synthesized during viral replication. (15). Many of these antibodies are not protective but serve as useful indicators of viral infection. Our SDS treated antigen did not yield an immunoprecipitate line in the AGID test which usually detects a glycoprotein and/or a major internal protein p25 of MVV (16). It is possible that the concentration of SDS antigen was insufficient to give a reaction in the AGID. Nevertheless we have, on rare occasions, observed strong AGID reactors from field animals which were negative by the i-ELISA using the SDS-treated antigen. If these sheep were truly infected with MVV and the strong reactions in AGID were specific, these data suggest that SDS may expose other antigens and the i-ELISA may detect other antibodies than those which are detected by the AGID. It is quite possible that more than one antigen is implicated in the serological reaction.

One problem we encountered during the SDS treatment was the formation of a precipitate in both the viral and the control preparations. Sodium dodecyl sulfate does not usually precipitate proteins since they are dissolved in or surrounded by micelles of detergent (10). Insoluble protein-SDS complexes and precipitates from large inorganic monovalent (Cs^+, K^+) and multivalent cation (Ca^{+2}) salts in solution with dodecyl sulfate are reported (13). The precipi-

TABLE I. Optical density (414 nm), P/N and P/B ratios of pre- and post-infection (I) serum samples of sheep A at various dilutions of sodium dodecyl sulfate (SDS)-treated maedi-visna virus (MVV) antigen (Ag) and Ag free of MVV in the modified indirect enzyme-linked immunosorbent assay

	Antigen dilution				
	1/40	1/80	1/160	1/320	1/640
SDS-treated Post I	0.875	0.858	0.720	0.465	0.249
MVV Ag Pre-I	0.059	0.046	0.040	0.026	0.022
SDS-treated Post-I	0.065	0.059	0.049	0.036	0.027
MVV-free Ag Pre-I	0.069	0.049	0.045	0.036	0.015
P/N[a]	14.8	18.6	18.0	17.9	11.3
P/B[b]	13.7	16.8	16.0	14.1	11.8

[a]Ratio of positive serum O.D. to negative serum O.D.
[b]Ratio of positive serum O.D. with MVV Ag to the mean O.D. of negative serum with MVV Ag and positive and negative serum with MVV-free Ag

tates were removed from our preparations by centrifugation without modification of the antigenic activity. The SDS treated antigen was quite stable. Several freeze/thaw cycles did not modify the activity of the antigen in the i-ELISA. The SDS treated antigen precoated polystyrene plates used in the test were also kept frozen at -70° C for many months without loss of activity.

In the following experiment, we found that the results obtained for the antigen prepared after SDS treatment of the viral pellet obtained by differential centrifugation only were comparable to the antigen treated after purification by chromatography. A fairly large pellet which did not exhibit i-ELISA activity was recovered and discarded at the end of this technique. Besides having a direct effect on the viral particle, it seems that the SDS can be used to remove a certain amount of cellular material present in the preparation which may compete with the viral antigen during the passive attachment to the polystyrene plate and consequently be responsible for higher background. It is reported that SDS has been used to lyse cells infected by viruses (10). The removal of such material may also explain the increased efficiency of the anionic detergent SDS over the lipid solvents. Ethyl ether is often used to extract retroviral antigen for the i-ELISA (2,4,17-19). This is a lipid solvent of poor efficiency and is fairly ineffective in denaturating proteins and cannot be used to remove nonviral components (8). Fluorocarbon and chloroform are lipid solvents which are capable of removing extraneous proteins and lipids from viral preparations but the risk of irreversible denaturation or alteration of the viral protein conformation remains possible (8). From our results, fluorocarbon gave similar results to ether but chloroform gave too high a background. To increase the differentiation between positive and negative serum samples by reducing the nonspecific binding on the ELISA plates, FBS and increased molarity of NaCl were added to the diluent buffer as reported by Schroeder *et al* (2). Various assays indicated that, in our system, 7.5% FBS and 0.2 M NaCl gave optimum results (data not shown). At this concentration of FBS and NaCl, the P/N and P/B ratios were almost four times higher than when the "blocking" agents were omitted. Using this modified i-ELISA, a virus pellet obtained by ultracentrifugation was treated in parallel with 0.1% of SDS and ethyl ether as described in Materials and Methods. Whereas the P/N ratio of the SDS-treated antigen reached 14.7 at a 1/100 dilution of the antigen, the ether-treated antigen gave almost no reaction. The SDS-treated antigen gave much better results in our experiments and avoided the use of highly toxic and/or flammable chemicals. To our knowledge, this is the first time that SDS has been used in the production of a retroviral antigen for an i-ELISA. Even though the data we reported here are based on only two sheep experimentally inoculated with MVV, thousands of serum samples from a number of sheep producers have been tested at our laboratory by the technique we reported. Data from large scale monitoring are presented in a companion publication (20).

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