An Enzyme-linked Immunosorbent Assay for Detection of Antibodies to Maedi-Visna Virus in Sheep
II. Comparison to Conventional Agar Gel Immunodiffusion Test

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ABSTRACT

A study was conducted to compare the indirect enzyme-linked immunosorbent-assay (i-ELISA) test using antigen prepared by a simple technique using sodium dodecyl sulfate (SDS) treatment to the conventional agar gel immunodiffusion test (AGID). Ten specific-pathogen-free (SPF) sheep were inoculated with maedi-visna virus (MVV) and serum antibody titers compared over a period of 14 weeks. All the sheep seroconverted by the i-ELISA compared to 90% by the AGID. The i-ELISA detected antibody at a mean of 2.6 weeks prior to the AGID. In both tests, fluctuations were observed in the serum antibody response of two sheep. The i-ELISA had a specificity of at least 98.8% and an increased relative sensitivity of 15.5% compared to the AGID, based on the analysis of sera from experimental sheep with MVV free status and sera from sheep from various sources. Of the sera from a seronegative flock which had been monitored with the AGID after a "test and remove" eradication program, 10.2% were positive by the i-ELISA. It was concluded that the AGID test may not be adequate to monitor samples for an eradication scheme.

RÉSUMÉ

Une étude a été menée afin de comparer la technique ELISA utilisant un antigène traité par le dodécyle sulfate de sodium à la technique de l'immuno-diffusion en gel d'agar (IDGA). Dix moutons exempts d'agents pathogènes spécifiques ont été inoculés avec le virus maedi-visna (MV) et les titres d'anticorps ont été déterminés sur une période de 14 semaines, utilisant les deux techniques sérologiques. Tous les moutons ont démontré une séroconversion par la technique ELISA comparativement à 90% d'entre-eux par la technique de l'IDGA. La technique ELISA a détecté la présence d'anticorps environ 2.6 semaines avant qu'ils ne soient par la technique de l'IDGA. Des fluctuations dans le taux des anticorps sériques ont été notées indépendamment de la technique utilisée. La technique ELISA a une specificité d'au moins 98.9% et une sensitivité relative supérieure de 15.5% comparativement à l'IDGA. Les données de spécificité et de sensitivité sont basés sur l'analyse de sérums provenant de troupeaux déclarés seronégatifs et de troupeaux provenant de différentes sources. Parmi les sérums de moutons provenant d'un troupeau déclaré négatif par un programme d'éradication du MV, 10.2% des animaux se sont avérés positifs lorsque testés par la technique ELISA. Ceci nous amène à conclure que la technique de l'IDGA ne convient pas aux programmes d'éradication de la maladie.

INTRODUCTION

Maedi-visna is a slow evolving disease of sheep characterized by interstitial pneumonia, meningoencephalitis, indurative mastitis and nonsuppurative arthritis (1). Although the infection has been reported to be of high prevalence in many countries (2), only some of the infected animals develop the chronic progressive lesions and eventually die (3). The causal agent is the prototype of the *Lentivirinae* sub-family of the *Retroviridae*. These are RNA viruses that contain reverse transcriptase and replicate by way of DNA intermediates. The lentiviruses have the ability to evade the defence mechanisms of their natural host, thus causing persistent infection (4). The infected animals remain carriers of the virus for life, contributing to the propagation of the infection within flocks (5). Viral transmission occurs principally between the dam and her progeny, via the colostrum and milk (6). The degree of transmission between the ewes and their lambs is high. De Boer and Houwers (2) reported that a contact of only 10 h between the infected dam and her offspring resulted in 28% cross-transmission. In order to control the spread of the virus, the viral carriers have to be detected and eradicated as no vaccine or treatment is yet available (6). As most of the infected sheep develop a humoral response, the demonstration of anti-maedi-visna virus (MVV) antibodies by serological techniques is indicative of viral infection (7). The serological tests available for the diagnosis of maedi-visna are essentially the agar gel immunodiffusion test (AGID) and the enzyme-linked immunosorbent-assay (ELISA). The latter is much more sensitive than the former (8,9) and should be the technique of choice, especially for the large scale monitor-

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ing of flocks participating in eradication programs. However, the production of the antigen for the ELISA is a critical step and might negate the use of the technique. Vitu *et al* (9) found that the purity of MVV antigen has a considerable impact on the performance of the indirect ELISA (i-ELISA). Increasing the purity of the MVV antigen preparation helps to reduce the background which reduces diagnostic sensitivity and specificity, but the complex manipulations and expensive apparatus required for purification are not always feasible in some laboratories. We demonstrated recently (10) that a crude viral preparation of MVV can be used for an i-ELISA after treatment with sodium dodecyl sulfate (SDS). The SDS-treated antigen gave high differentiation between MVV positive and negative serum samples from specific pathogen-free (SPF) sheep experimentally inoculated with MVV virus. In this paper we compare the conventional AGID technique with the i-ELISA using the SDS-treated antigen on several hundred ovine serum samples originating from various sources.

MATERIALS AND METHODS

SERUM SAMPLES

Sera from four groups of sheep were collected for the comparison studies.

Group 1: Sheep experimentally inoculated with MVV

Fifteen six month old SPF Finncross sheep kept in isolation facilities were used for this experiment. Each of the ten animals in group A received 5 mL of the MVV inoculum by the intratrachael route (IT), using a 23½ gauge needle. Group B was composed of three sheep inoculated IT with culture medium free of virus. In group C were two sheep which were not inoculated. Groups B and C were housed together. Blood was collected by jugular venepuncture at two weeks intervals (from all three groups). Sample collection started one month prior to the inoculation and continued up to 14 weeks postinoculation (PI). All of the serum samples were kept frozen at -20°C until tested. The procedures followed the guidelines of the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care.

Group 2: Sheep from MVV seronegative flocks

Serum samples (335) were obtained from two experimental sheep flocks which had been declared free of MVV. Flock A' had been monitored twice a year for five years by MVV AGID with consistently negative results. A total of 60 serum samples were collected from this flock. An additional 275 serum samples were collected from flock B'. The sheep of this flock were various generations of lambs from cesarean-derived, colostrum deprived F_1 parental ewes, raised in isolated facilities. The flock of origin was heavily infected with MVV as determined by AGID.

Group 3: Sheep from various commercial flocks

A total of 896 serum samples were collected from 16 different premises. They were located geographically distant from each other.

Group 4: Sheep from a "test and remove" program of eradication

A sheep flock heavily infected with MVV was monitored at regular intervals of four to six months by MVV AGID. The seropositive animals and their offspring were separated or culled in order to eradicate the disease as proposed by Houwers *et al* (3). After three years of monitoring, the serum samples from this flock were all negative by MVV AGID for three consecutive tests. The i-ELISA was performed in parallel with the AGID at the time of the last test.

ANTIGEN PREPARATION

Maedi-visna virus

The maedi-visna virus used in this experiment was originally provided by the Animal Diseases Research Institute, Nepean, Ontario. The virus was passaged many times in ovine choroid plexus (OCP) cells. It showed an identity line in AGID with the ovine progressive pneumonia (OPP) strain supplied by Dr. R.C. Cutlip (Ames, Iowa, USA), gave a specific fluorescence with MVV antisera, produced characteristic cytopathic effect (CPE) of MVV in sheep cells and was morphologically indistinguishable from other lentiviruses by electron microscopy.

MVV inoculum

To prepare the viral inoculum used to infect the sheep of group 1, 10 mL of MVV was inoculated onto confluent monolayers (850 cm^2) of newborn lamb lung cells. The growth medium was composed of Hanks' minimum essential medium, 0.1 mM nonessential amino-acids, 2 mM L-glutamine, 10% fetal bovine serum (FBS), 100 mg/ML of streptomycin and 100 IU/mL of penicillin. Fetal bovine serum was omitted from the maintenance medium after inoculation of the cells with the virus. When the cytopathic effect was approximately 80% (usually four days postinfection), the viral material was frozen, thawed and clarified at 1,000 x *g* for 15 min (Sorvall SS-34 rotor). The viral infectivity titer obtained was $10^{6..66}$ median tissue culture infectious doses ($TCID_{50}$)/mL in OCP cells.

AGID antigen

The clarified material was poured into membrane tubing (Spectrapor, Fisher Scientific, Dartmouth, Nova Scotia) with a cutoff of 12,000-14,000 molecular weight (MW) and was concentrated against polyethylene glycol 8,000 MW for 48 h at 4°C. The antigen obtained was used directly in the AGID test at a preevaluated dilution.

ELISA antigen

The i-ELISA antigen was obtained after treatment of a viral pellet with 0.1% SDS, as previously described (10). The antigen was used directly in the i-ELISA after the optimal dilution was determined by checkerboard titration.

SEROLOGICAL TESTS

AGID technique

The technique used for the AGID test was similar to the microimmunodiffusion test described by Winward *et al* (11). The test substrate was composed of 1% Noble agar (Difco) dissolved in 0.05 M Tris-HCl, pH 7.5 and 8% NaCl. The agar was layered onto microscope slides previously cleaned and stored in alcohol. A template cutter was used to cut the

TABLE I. Comparison of enzyme-linked immunosorbent assays and agar gel immunodiffusion tests on serum from ten experimentally infected sheep (Group 1A)

Weeks PI	Sheep number																			
	1262		1291		0138		0509		0540		1292		1285		1280		1294		1295	
	E[a]	A[b]	E	A	E	A	E	A	E	A	E	A	E	A	E	A	E	A	E	A
0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	–	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	–	–	–	–
3	+	–	+	–	+	+	+	–	+	–	+	+	+	–	+	+	+	–	–	–
4	+	–	+	–	+	+	+	–	+	s	+	s	+	–	+	+	+	–	–	–
5	+	+	+	+	+	+	+	s	+	+	+	+	+	–	+	+	+	–	+	–
6	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	–
8	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	s
10	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	–
12	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	–
14	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	–

[a]ELISA test
[b]AGID test
+ = positive, – = negative, s = suspect
PI = postinoculation

solidified agar. The pattern consisted of three rosettes per slide. Each rosette was created by six peripheral wells (3 mm diameter) distanced 2 mm apart and an identical central well. The latter received 10 μL of the AGID antigen whereas alternate peripheral wells received the same volume of a positive MVV sheep antiserum control. The sera to be tested were added individually to the remaining peripheral wells. The slides were incubated in a humidified box and were incubated at room temperature (RT) for 48 h before reading.

ELISA technique

A conventional i-ELISA was used to test all serum samples. The wells of flat bottom polystyrene microplates (Linbro, Titertek, Flow Laboratories, McLean, Virginia) received 100 μL/well of the ELISA antigen diluted in 0.05 M carbonate buffer, pH 9.6. After overnight incubation at 4° C in a humidified box, the plates were washed three times with a machine washer (Titertek, Microplate Washer 120, Flow Laboratories, McLean, Virginia) with the ELISA buffer (0.14 M NaCl, 1.5 mM KH_2PO_4, 0.8 mM $Na_2HPO_4 \cdot 12\ H_2O$, 3 mM KCl, 0.05% Tween 20). Serum samples were diluted 1/50 in serum-conjugate diluting fluid (SCDF), the ELISA buffer to which 0.2 M NaCl and 7.5% FBS were added. After 2 h incubation at RT, the plates were again washed three times. Horseradish peroxidase-conjugated rabbit antisheep immunoglobulin, heavy and light chains (Cappel, Organon Teknika, West Chester, Pennsylvania) diluted 1/000 in SCDF was added to each well (100 μL/well) and the plates were incubated for 1 h at RT. After washing procedures, substrate ABTS (2,2-azinobis [3-ethylbenzthiazoline sulfonic acid], Sigma Chemical Co., St. Louis, Missouri), diluted in 0.5 M citrate buffer, pH 4.0 and containing 0.015% H_2O_2 was added to reveal the reaction. The optical density (O.D.) was evaluated using a photometer (Titertek Multiscan, Flow Laboratories, McLean, Virginia) at a wavelength of 414 nm after 30 min incubation at RT.

CUTOFF VALUE

For the sheep of all groups, the cutoff value of the i-ELISA was the mean O.D. plus three standard deviations (SD) of the negative sera. For group 1, the cutoff was calculated from the O.D.'s obtained from subgroups B and C during the whole monitoring period. For this group, sera were tested against MVV antigen and medium free of MVV (control antigen) treated with 0.1% SDS. The mean of the corrected O.D. (O.D. corresponding to viral antigen minus O.D. corresponding to medium free of virus) was used to compare sub-

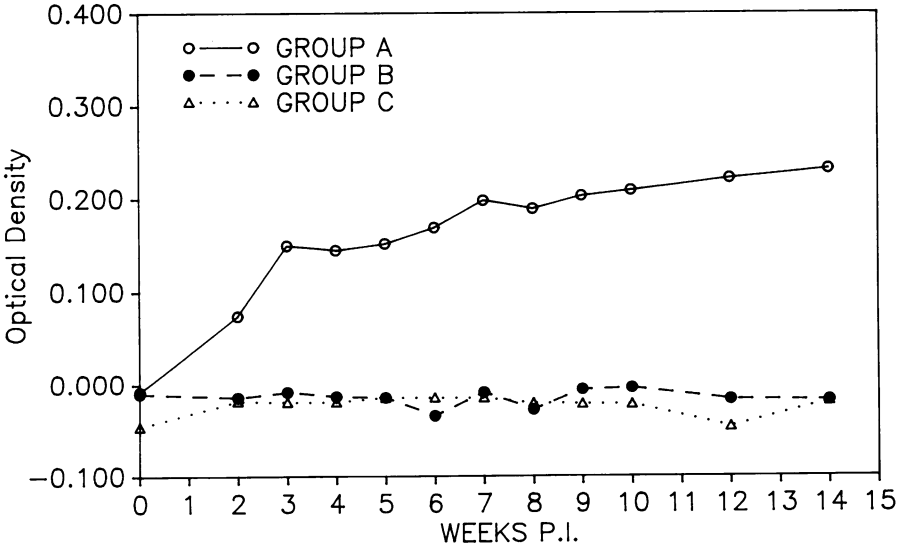

Fig. 1. Mean of corrected optical density $(O.D.)_{414}$ (O.D. of sample on positive antigen minus O.D. of sample on negative antigen) for group A (ten sheep inoculated with maedi-visna virus (MVV), B (three sheep inoculated with MVV-free medium) and C (two uninoculated sheep).

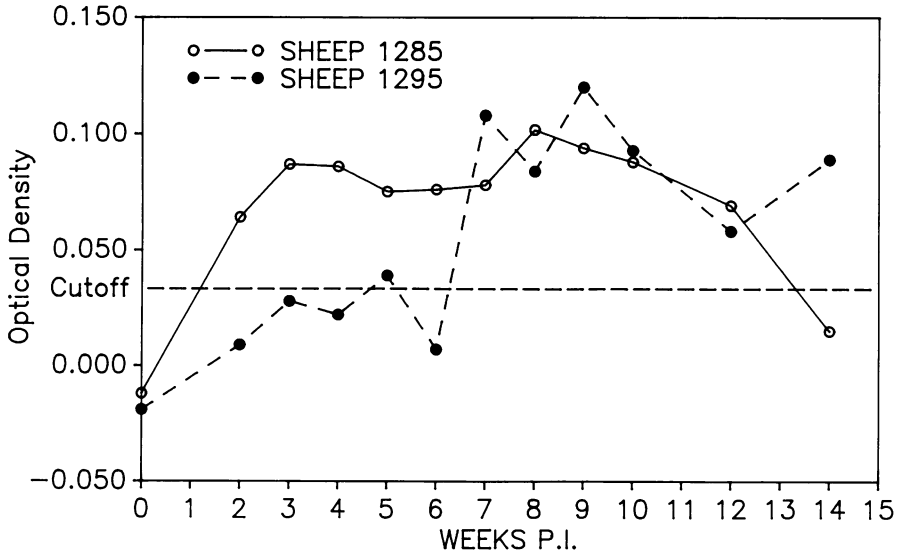

Fig. 2. Corrected optical density $(O.D.)_{414}$ of sheep 1285 (a) and sheep 1295 (b) over the 14 weeks of monitoring period.

groups A, B and C. For groups 2-4, a cutoff value of 0.311 was determined from 330 serum samples collected from the MVV seronegative flock (Group 2). In all groups tested by the i-ELISA, duplicates were performed.

RESULTS

GROUP 1

The sheep of groups A and B received respectively MVV inoculum and the medium free of MVV at week 0. Sheep of group C remained uninoculated. Figure 1 compares the mean corrected O.D. of the antibody response of each sub-group tested by the i-ELISA. A cutoff value of 0.033 was obtained. Sera with an O.D. value higher than the cutoff were considered positive whereas sera with lower O.D. values were negative. According to the Student's *t* test (two-tailed), groups B and C were not significantly different ($p > 0.05$). In group A, significant antibody titers were observed after two weeks PI. The antibody response increased gradually with time up to the end of the experiment. Table I summarizes the ELISA and the AGID results obtained for each of the sheep in group A. All serum samples from groups B and C were negative by both tests. At two weeks PI, the ELISA showed a positive-response for seven of the ten sheep whereas none of the animals had detectable antibodies in the AGID. At three weeks PI, only three of the ten sheep had detectable antibodies by AGID whereas the ELISA was able to detect antibodies in nine. All of the sheep had seroconverted in the ELISA by five weeks PI, but at 14 weeks PI, antibody was not detected in two of the infected sheep by the AGID test. Ewe 1285 remained negative in the AGID for the duration of the monitoring period, but antibody was detected by the ELISA up to 12 weeks PI. This animal also displayed a drop in antibody at week 14 with the ELISA (Fig. 2). These fluctuations in antibody levels were also observed in the AGID as well as the ELISA results for sheep 1295 (Fig. 2). For the animals which seroconverted, the ELISA detected MVV antibodies at a mean of 2.4 weeks PI compared to a mean of 5.0 weeks PI for the AGID test.

GROUP 2

All 335 serum samples from the sheep of flocks A′ and B′ were negative by AGID. The ELISA detected one positive reactor from flock A′. This sheep had been routinely checked at approximately six month intervals by AGID with constant negative results for five years. It was originally obtained from a MVV seropositive flock. From the ELISA testing of flock B′, three positive reactors were detected. As indicated previously, these sheep were descendants of cesarean-derived colostrum-deprived lambs from ewes heavily infected with MVV.

GROUP 3

A total of 896 serum samples from 16 premises were evaluated by AGID and ELISA. Table II summarizes the results obtained. The ELISA detected 269 positive reactors of which 210 were also positive or suspicious by the AGID test. A low percentage (5.3%) were found positive or suspicious by AGID but not by ELISA. Of the 284 serum samples which were positive or suspicious by either test, 94.7% were positive by ELISA whereas only 79.2% were positive or suspicious by AGID, for a difference of 15.5%.

GROUP 4

An AGID seronegative flock was obtained by culling the positive reactors and their offspring. The last three consecutive AGID tests performed at intervals of six months were negative. Of the 117 sheep tested using the ELISA at the third trial, 12 (10.1%) were found to be positive.

DISCUSSION

In this experiment, we compared the i-ELISA, using a simple preparation of SDS-treated antigen, to the conventional AGID test. According to our results, the ELISA was analytically more sensitive than the AGID. In the group of sheep experimentally inoculated with MVV, the ELISA was on average able to detect MVV antibodies 2.6 weeks prior to the AGID test. All infected sheep seroconverted on the ELISA test by week 5 PI whereas 20% of the animals remained negative in the AGID test by week 14 PI. When we compared both tech-

TABLE II. Comparison of enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID) test results on 896 ovine serum samples from 16 different premises

	ELISA		
AGID	Positive	Negative	Total
Positive	199	8	207
Negative	59	612	671
Suspect	11	7	18
Total	269	627	896

niques using field cases, we found that the ELISA was able to detect 15.5% more positive reactors than the AGID test. Previous studies have also demonstrated the increased relative sensitivity of the i-ELISA for the diagnosis of MVV. Houwers *et al* (8) used an ether-extracted partly purified antigen in their i-ELISA and found that it was 11.5% more sensitive than the AGID test. Vitu *et al* (9) used a much more purified MVV antigen and found similar results. In their experiment, the i-ELISA was able to detect a positive reactor at seven weeks PI whereas the AGID test was clearly detecting it only at 26 weeks PI. Our serological tests were able to detect positive reactors much earlier. The differences observed could be attributed to one or more factors; differences in strain of MVV used to produce ELISA antigen (8), variation in susceptibility of sheep breeds to MVV infection (12), age at time of inoculation, virus dose and route of inoculation. The early detection of antibodies by the i-ELISA might also be attributable to the exposure of other antigen(s) by the use of SDS which were not exposed in the methods reported previously (8,9). This anionic detergent-treated-antigen may detect antibodies which appear earlier during the evolution of the disease.

The higher relative sensitivity of the ELISA should be taken into consideration particularly when eradication programs in infected flocks are initiated. The use of the AGID test, which demonstrates a lower relative sensitivity, might impair the success expected. Higher numbers of infected sheep may remain as undetected carriers (silent carriers) if an assay of lower diagnostic sensitivity is used for monitoring. Some animals are also less likely to be detected because of fluctuating antibody levels such as were observed in group 1A. Houwers and Nauta (13), using immunoblotting analysis, reported a decline of antibody activity over time in MVV-diseased sheep. The MVV-infected seronegative animals, especially the ewes, might continue to propagate the infection. Because the detection of the antibody response in their progeny is often quite long (3), the percentage of infectivity within the flock might continue to increase. In our experiment, 10.2% of the sheep on a "test and remove" program for eradication were still positive by ELISA after three consecutive negative AGID tests.

In the testing of serum samples from two experimental sheep flocks declared free of MVV, we found four positive reactors (1.2%) using the ELISA technique. The seropositive ewe of flock A′ was frequently tested by AGID with negative results. Because the flock was used for experimental purposes, her progeny were culled and therefore could not be evaluated. The ewe was possibly a silent carrier since she was originally from a seropositive flock. The other three sheep which were positive by the ELISA were from flock B′ originating from cesarean-derived, colostrum-deprived lambs. If we consider that the *in-utero* transmission of MVV is possible, as reported previously (14), these sheep might also have been silent carriers. In all four cases, however, no attempt was made to isolate MVV to confirm this possibility. If these animals were not infected by MVV and the serological reactions observed were false, the i-ELISA using an SDS-treated antigen would have a specificity of 98.8%. For the 896 serum samples from field cases analysed, 5.3% of serum samples which were positive or suspicious by AGID were negative by the ELISA. The relative sensitivity of the i-ELISA was determined by dividing the total number of i-ELISA reactors by the total number of reactors (positive and suspicious) in both tests. The relative sensitivity of the AGID test was determined by dividing the total number of AGID reactors (positive and suspicious) by the total number of reactors in both tests (positive and suspicious). The relative sensitivity of the ELISA was 94.7% compared to 79.2% for AGID.

The antigen used for the i-ELISA we performed was treated with 0.1% SDS after the virus was pelleted twice by ultracentrifugation. This technique was very simple compared to that used by Vitu *et al* (9) to produce highly purified material. They used complex purification procedures to obtain a suitable antigen with reduced background for their i-ELISA. In our test, the background was not a problem even though we used a much cruder antigen. As we had surmized previously (10), it seems that the SDS removes cellular material present in the viral preparation which may be responsible for nonspecific attachment to the polystyrene plate. Also, the inclusion of FBS and increased molarity of NaCl in the dilution buffer for the serum and conjugate reduces nonspecific binding on the ELISA plates (15). The plates were precoated with the antigen and frozen at -70° C prior to being used, but even though SDS can crystallize in the frozen state (16), no problems were encountered. A large number of plates were frozen and thawed without any alterations. Also, the activity of the antigen remained after long-term storage at -70° C.

The use of the technique reported here is advantageous in that 1 L of infected medium, the product of ten infected roller bottles, was sufficient to coat approximately 100 plates (our optimal dilution of the antigen was 1/100). This would allow the testing in duplicate of approximately 4,800 serum samples, including positive and negative serum controls for each plate.

In conclusion, the i-ELISA, using an SDS treated MVV antigen, has proved to be far superior to the AGID test for diagnosis of maedi-visna and should be used especially when eradication programs are undertaken.

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