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COMPARATIVE EVALUATION OF THE AGAR GEL IMMUNODIFFUSION TEST AND RECOMBINANT ELISA TESTS FOR THE DIAGNOSIS OF OVINE PROGRESSIVE PNEUMONIA

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Abstract

The accurate identification of ovine progressive pneumonia virus (OPPV)-infected animals is a critical issue for the sheep industry. Serological tests, including the agar gel immunodiffusion (AGID) and ELISA tests, are the most frequently used methods to identify OPPV-infected animals. Although the sensitivity of the AGID test has been questioned, in many cases the sensitivity and specificity of serological tests have been determined by using serum samples collected from the general population in which the precise state of infection of the animals cannot be confirmed by other means. In order to compare the sensitivity and specificity of the AGID test with that of recombinant ELISA, serum samples from OPPV or placebo experimentally inoculated lambs were collected before inoculation and weekly for 26 weeks thereafter. Serum samples were tested for the presence of OPPV antibodies by the AGID test, a recombinant p24-OPPV protein ELISA (rp24-ELISA) or a recombinant transmembrane-OPPV protein ELISA (rTM-ELISA). Paired serum samples were also submitted to a private diagnostic laboratory that uses a caprine arthritis encephalitis virus (CAEV) envelope recombinant protein ELISA (rCAEV-ELISA) for the identification of OPPV-infected sheep. To confirm the OPPV-infected or -free status of the experimental animals, virus isolation was attempted every other week from blood mononuclear cells.

OPPV was reisolated from all virus inoculated lambs but never from the placebo controls, thus confirming the infectious- or free-OPPV status of the experimental animals. The specificity of the AGID test was always 100%, and the sensitivity ranged from 11% on post-inoculation week 2 to 100% from post-inoculation week 5 until the end of the experiment (average 91.5). The specificity of the recombinant ELISA test varied depending on the recombinant OPPV protein used. While the sensitivity and specificity of the rp24-ELISA varied from 22.2 to 100 (average 87.7) and 50 to 100 (average 94.6), respectively, the sensitivity and specificity of rTM-ELISA test ranged from 5.5 to 100 (average 86) and from 62.5 to 100 (average 94.9). Surprisingly, the CAEV-ELISA missed all OPPV-infected cases.

Our results indicate that the OPPV AGID has high sensitivity and speci-

ficity for the diagnosis of OPP in this experimental setting. Recombinant ELISA tests based on OPPV recombinant proteins had good sensitivity and specificity. The lower sensitivity of the rTM-ELISA compared to the AGID test was partially due to the lower percent of infected cases detected by the rTM-ELISA in the early stages of infection; however, the sensitivity of this test after week 8 post-inoculation was always 100%. The ELISA test based on CAEV recombinant antigens performed very poorly. Therefore, each test must be carefully standardize before they can be recommended for diagnostic purposes.

To determine the effect of OPPV inoculum size on the time of seroconversion by the AGID test, pairs of lambs were inoculated with 10-fold dilutions of OPPV from 10^5 to 10^1 $TCID_{50}$. The 2 lambs inoculated with 10^1 $TCID_{50}$ of OPPV seroconverted by 8 weeks after inoculation. Lambs inoculated with 10^2 to 10^5 $TCID_{50}$ seroconverted between 4 and 6 weeks post-inoculation. Therefore, the amount of virus inoculum seem to have only a minor effect on the time of seroconversion. However, the sensitivity and specificity of serological tests using different strains of OPPV needs to be further investigated.

Introduction

Ovine lentivirus (OvLV) comprises a subgenus of the lentivirus genus of exogenous, non-oncogenic retroviruses. Maedi-visna, the prototype species of OvLV, was first isolated by Sigurdsson et al., in Iceland in 1960. The North American strains of OvLV are usually referred as ovine progressive pneumonia virus (OPPV).[1] Ovine lentiviruses are widespread throughout most of the sheep producing areas of the world. In the United States, the reported seroprevalence is 26% but a wide variation exists between states and between farms.[2]

Lentiviral infection of sheep may lead to a disease complex characterized by cachexia and chronic active inflammation of the lungs, lymph nodes, joints, mammary gland and the central nervous system.[3-5] In the United States, pulmonary disease is the most important cause of morbidity and mortality in lentivirus infected sheep.[5] The financial loss attributed to OPP may be due to animal deaths, depressed lamb growth due to agalactia of ewes with mastitis, losses from secondary infections and loss of trade due to restrictions in the international export market. Ovine lentiviruses have been eradicated from Iceland and several countries of the European community have eradication campaigns underway. Sheep infected with OPPV remain infected for life; therefore, early detection of infected animals is critical for the control of this infection.[1]

Agar gel immunodiffusion (AGID) test is the most commonly used serological technique to identify infected animals because of its low cost, simplicity and high specificity. However, generally the sensitivity of the AGID test is considered low. More recently, indirect ELISA tests using either whole virus

or recombinant OPPV proteins have come to the scene claiming a high degree of sensitivity.[6-8] In many cases the sensitivity and specificity of serological tests are based on the used of serum samples collected from the general population in which the precise state of infection cannot be confirmed by other means. In this paper, we compare the sensitivity and specificity of an AGID test with those of two recombinant ELISA tests using serum samples collected chronologically after experimental OPPV or placebo inoculation. In addition, paired serum samples were submitted to a private veterinary diagnostic laboratory that runs an ELISA test for the identification of OPPV infected animals using a caprine arthritis encephalitis virus recombinant protein (rCAEV-ELISA).

Materials and Methods

Animals and animal inoculation. Twenty-six Rambouillet or Rambouillet x Suffolk newborn lambs from seronegative ewes were allowed to ingest colostrum and then separated from their mothers and raised on an artificial diet. Newborn lambs were randomly allocated into two groups. The first group consisting of 18 lambs was inoculated intratracheally with $1x10^6$ $TCID_{50}$ of OPPV strain 85/34 (8 of these 18 lambs received daily treatment with recombinant ovine interferon- [roIFN] for 30 days as part of another experiment). The second group consisted of 8 lambs inoculated with a non-infected cell culture supernatant. Serum samples collected before inoculation and weekly for 26 weeks after inoculation were assigned a code and tested blindly for the presence of OPPV antibodies by the AGID test, by two different recombinant ELISA tests, or were submitted to a private diagnostic laboratory. Blood samples collected every other week starting before experimental inoculation until the end of the experiment were tested for the presence of infectious virus by standard virus isolation in tissue culture.

Ten additional one month-old Rambouillet lambs were inoculated in the same way as above with OPPV dilutions (2 lambs/dilution) ranging from $1x10^5$ to $1x10^1$ $TCID_{50}$, and serum samples collected weekly were tested by the AGID test.

Agar Gel Immunodiffusion Test. Serum samples were tested for the presence of OPPV antibodies by the AGID test using a commercially available kit and following recommendations by the manufacturer (Veterinary Diagnostic Technology, InC. 4890 Wheat Ridge, CO 80033).

ELISA. An ELISA test was used to determine the antibody responses to the transmembrane (TM) and p24 OPPV-structural proteins, as previously described.[8] Briefly, microtiter plates were coated with 120 g/well recombinant TM or p24 proteins in 0.1 sodium bicarbonate buffer (pH 9.6) and kept refrigerated until further use. The plates were then washed 3 times in ELISA washing solution (0.15 M NaCL, 0.05% Tween 20), and excess binding sites were saturated with 100 l of 1% bovine serum albumin (BSA) in phosphate-buffered saline (pH 7.2, 0.15 M) for 1 hr at 37 C. After 3 washes, 100 l diluted

sheep serum (1:50) in 1% BSA buffer was added to each well, and plates were incubated at 37 C for 1 hr. Following a subsequent washing of the wells, 100 l of anti-sheep immunoglobulins conjugated with horse radish peroxidase was added to each well, and plates were incubated at 37 C for 1 hr. Wells were washed again, and 100 l of substrate solution (citric acid, 2,2'-azinobis, 3-ethyl bensthiazoline sulfonic acid, H_2O_2) was added. The color reaction was allowed to proceed at room temperature for 30 min, and the absorbance of each well at 405 nm was recorded in an automatic ELISA plate reader.

Virus Isolation. Blood samples were collected in EDTA-containing vacuum tubes and blood mononuclear cells (BMNC) were separated by centrifugation on a Ficoll-Hypaque gradient as previously described.[9] Subsequently, a total of 4×10^6 separated BMNC were cocultivated with semiconfluent monolayers of goat synovial membrane (GSM) cells in 25 cc tissue culture flasks for 12 days. At the end of this period, cell cultures were rinsed in Hank's balanced salt solution (HBSS), fixed in methanol, stained with Giemsa and evaluated for the presence of syncytia. A positive score was given when at least one cell containing at least five nuclei was found.[9]

Data Analysis. Due to their quantitative character, the results obtained from the rTM-ELISA and the rp24-ELISA were corrected for between-plate variability by dividing the OD reading of the test samples by the OD reading of the positive control in their respective plate. Initially, cut-off points that had been determined previously[8] were used as the positive-negative threshold for the rp24- and rTM-ELISAs. However, after realizing that under this criteria too many infected animals were scored as negative during the initial weeks after infection, new cut-off values that results in a better trade-off between sensitivity and specificity were established. (Figures 1 and 2).

Results

Ovine lentivirus was re-isolated at least in two occasions from all OPPV-inoculated lambs. Virus isolation in individual lambs ranged from 2 to 12 times during the course of the experiment and the frequency of isolation in the OPPV-inoculated experimental group was higher between post-inoculation weeks 2 and 8. Ovine lentivirus was never isolated from any of the placebo-inoculated controls.

The results of the sensitivities and specificities of the AGID, the rp24-ELISA and the rTM-ELISA are presented in table 1. Positive reactions to the core protein in the AGID test were first seen in two OPPV-inoculated lambs by 2 weeks post inoculation. On week 3 after inoculation, 8 OPPV-inoculated lambs showed a weak positive reaction against the envelope (env) protein, while in 8 OPPV-inoculated lambs the predominant reaction was against the core protein. By 4 weeks post-inoculation, only one OPPV-inoculated animal was still negative, 10 lambs showed clear anti-p24 bands and the remaining 7 reacted with different levels of intensity to the env protein. All

lambs infected with 1 x 10^6 $TCID_{50}$ were positive by the AGID test by 5 weeks post-inoculation and remained positive for the rest of the experiment. None of the placebo-inoculated controls showed any positive reactions by the AGID test during the experiment. Based on these results the specificity of the AGID test was always 100%, on the other hand the sensitivity ranged from 11% on post-inoculation week 2 to 100% from post-inoculation 5 week until the end of the experiment (average 91.5%).

The initial cutoff points between positive and negative rp24- and rTM-ELISA OD readings were taken from the experience of a previous report; however, because under this criteria many infected animals were scored as negative during the initial phases of infection, new cutoff values, to obtain the optimal trade-off between sensitivity and specificity, were established for each ELISA format by plotting the percent accuracy in the detection of infected and non-infected animals in each of the OD values (Figure 1 and 2). Based on this system a cutoff value of 0.4 for the rp24-ELISA and of 0.08 for the rTM-ELISA were selected, respectively.

The specificity of the rp24-ELISA ranged from 50 to 100 (average 94.6%) and the sensitivity from 22.2 to 100 (87.7%). The average specificity of the rTM-ELISA was 94.9 (range 62.5 to 100) and the average sensitivity was 86% (range 5.5 to 100). In this experiment, preinoculation serum samples had a particularly high OD reading in both ELISA formats, regardless of the experimental group, resulting in specificities of 50.0 for the rp24-ELISA and of 62.5 for the rTM-ELISA during the experimental week 0.

The time of seroconversion in lambs inoculated with 10^5 or 10^4 $TCID_{50}$ of OPPV occurred by week 4 post-inoculation. One of each lamb inoculated with 10^3 and 10^2 $TCID_{50}$ seroconverted at weeks 5 and 4, respectively, and the other 2 lambs in these groups seroconverted by week 6 post-inoculation. The two lambs inoculated with 10^1 $TCID_{50}$ seroconverted by 8 weeks post-inoculation.

Discussion

For the most part the specificity of the AGID test for the detection of lentivirus infected sheep has been found to be 100%; however, its sensitivity has been reported to be lower than that of ELISA tests.[7,8] Using experimentally lentivirus-inoculated sheep, Simard and Briscoe,[10] found that by 2 weeks post-infection, 70% of the experimental animals could be detected by an indirect whole virus ELISA while none could be detected by AGID. The average sensitivities of these tests from week 3 to 14 were 96% for the ELISA test and 70% for the AGID test. Similarly, increased sensitivities of an indirect whole virus ELISA and a recombinant ELISA tests over the AGID test have been found by Houwers et al.,[7] and by Kwang et al,[8] respectively. However, false positives reactions, that affect the sensitivity of some recombinant ELISA tests, have been reported and ascribed to reactions to *E. coli* antigens that contaminate the recombinant viral proteins during purification.[6]

In our study, we compared the sensitivity and specificity of a commercially available AGID test with 2 recombinant ELISA tests using sheep serum samples collected sequentially after OPPV or placebo inoculation. OPPV was reisolated from all virus-inoculated sheep but never from the placebo inoculated animals, thus confirming the infectious- or free-OPPV status of the animals in each of the 2 experimental groups. The average sensitivity (91.5) and specificity (100) of the AGID test was slightly superior to the rELISAs. The average sensitivities and specificities of the rp24ELISA (87.4 and 94.6, respectively) and the rTM-ELISA (86 and 94.9, respectively) were not significantly different between each other. In our experiment, we used the OPPV strain 85/34 for animal inoculation. This strain of OPPV is a biological clone and therefore is constituted by a genetically diverse pool of OPPVs or quasispecies.[11] Both antigens used for the rELISAs were originally cloned from an OPPV infectious molecular clone.[12,13] Although the OPPV p24 and TM proteins carry conserved epitopes, it is possible that some immunogenic differences in the critical epitopes between strain 85/34 and the recombinant OPPV proteins existed, thus resulting in lower cross reactivity between these 2 OPPV strains and decreased sensitivity of the rELISA tests. This observation is supported by the fact that the relative sensitivity and specificity of the rTM-ELISA using serum samples of OPPV naturally infected sheep (where a wide range of strains may exist) were 97.6 and 100% respectively.[8] Furthermore, this theory also could explain the reasons why the rCAEV-ELISA failed to detect all infected animals, since differences in epitope immunological cross reactivity between small ruminant lentiviruses exists. In a recent publication, the sensitivity of the CAEV AGID was higher (91.0) than that of the OPPV AGID test (56.0) for the detection of caprine antibody to CAEV.[14] Similarly, some CAEV recombinant antigens might fail to detect ovine antibody to OPPV. Variability in the sheep immune response to different regions of OPPV env protein has been found.[15] The private veterinary diagnostic laboratory that runs the rCAEV-ELISA indicated that the antigen used for their test was a recombinant CAEV envelope protein but did not specified the characteristics. It is known that many of the epitopes in the env protein of lentiviruses are poorly conserved. Therefore, if the immunological epitopes present in the recombinant env protein of the CAEV-ELISA are different from those in the OPPV strain 85/34, the test would fail to detect infected animals. Further evidence that different proteins from small ruminant lentiviruses have different immunological cross reactivity comes from the fact that the OPPV rTM-ELISA test was more effective than the OPPV rp24-ELISA and the OPPV AGID test in identifying CAEV antibodies in the goat population.[16]

Our results indicate that the OPPV AGID has a high sensitivity and specificity for the diagnosis of OPP. Other advantages of the AGID test include its low cost and simplicity. Recombinant ELISAs varied greatly in their effectiveness to detect infected animals. Recombinant ELISA tests based on OPPV recombinant proteins had good sensitivity and specificity. The slight lower

sensitivity of the rTM-ELISA compared to the AGID test was partially due to the lower percent of infected cases detected by the rTM-ELISA in the early stages of infection; however, the sensitivity of this test after week 8 post-inoculation was always 100%. This delayed detection by the rTM-ELISA may be explained by the fact that ELISA tests detect only the IgG antiviral response, while the AGID test detects both IgM and IgG antibodies. It is well known that the initial antibody response is by IgM, subsequently switching to IgG.

Because ELISA tests may be easier to perform when a large number of animals are screened, this test may be the one of choice for eradication campaigns; however, when individual infected animals need to be identified, the AGID test could be chosen. In addition, the sensitivity and specificity of ELISA tests can be manipulated by moving up or down the cutoff value depending on particular needs in each diagnostic situation. Because ELISA tests can quantify the amount of antibody, these tests are ideal in research situations in which the kinetics of the host immune response to particular lentivirus proteins needs to be studied. It is not clear why the OD readings in all serum samples collected at week 0 (before inoculation) were higher than the readings at week 1, but this suggests that serum samples from newborn lambs have a higher affinity for non-specific binding and therefore result in false positive reactions. The ELISA test based on CAEV recombinant antigens performed very poorly, indicating that each test must be carefully standardize before they can be recommended for diagnostic purposes.

In one study, in which 10% of the animals in a group of 20 sheep were found seropositive by ELISA, 70% were found positive by in situ hybridization, PCR and cocultivation, suggesting that latent OPPV infections may occur.[17] In our study, all experimental lambs infected with 1×10^6 $TCID_{50}$ became seropositive by the AGID test by 5 weeks post-inoculation, indicating that delayed seroconversion or latency did not occur. Furthermore, the amount of virus inoculum seemed to have only a minor effect on the time of seroconversion. Lambs inoculated with 10^6, 10^5 or 10^4 $TCID_{50}$ of OPPV seroconverted between 2 and 5 weeks post inoculation. One of each lamb inoculated with 10^3 and 10^2 seroconverted at weeks 5 and 4, respectively, and the other 2 lambs in these groups seroconverted by week 6 post-inoculation. The two lambs inoculated with 10^1 seroconverted by 8 weeks post-inoculation. Nevertheless, the role of genotypically and phenotypically diverse OPPV strains in latency and time of seroconversion needs to be further investigated.

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Table 1 Sensitivity and specificity of the agar gel immunodiffusion, rp24- and rTM-ELISA tests.

	Agar gel immunodifusion		rp24-ELISA		rTM-ELISA	
Week	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
0		100.00		50.00		62.50
1	0.00	100.00	22.22	100.00	16.67	100.00
2	11.11	100.00	27.78	87.50	5.56	100.00
3	34.21	100.00	77.78	100.00	33.33	100.00
4	94.44	100.00	38.89	87.50	38.89	100.00
5	100.00	100.00	94.44	100.00	77.78	100.00
6	100.00	100.00	94.44	87.50	33.33	100.00
7	100.00	100.00	94.44	100.00	100.00	100.00
8	100.00	100.00	94.44	100.00	94.44	100.00
9	100.00	100.00	100.00	100.00	100.00	100.00
10	100.00	100.00	100.00	100.00	100.00	87.50
11	100.00	100.00	94.44	100.00	100.00	100.00
12	100.00	100.00	88.89	100.00	100.00	100.00
13	100.00	100.00	38.89	100.00	100.00	100.00
14	100.00	100.00	94.44	87.50	100.00	100.00
15	100.00	100.00	88.89	100.00	100.00	100.00
16	100.00	100.00	88.89	100.00	100.00	87.50
17	100.00	100.00	83.33	87.50	100.00	100.00
18	100.00	100.00	88.89	100.00	100.00	100.00
19	100.00	100.00	83.33	87.50	100.00	87.50
20	100.00	100.00	100.00	100.00	100.00	87.50
21	100.00	100.00	100.00	100.00	100.00	100.00
22	100.00	100.00	100.00	100.00	100.00	85.71
23	100.00	100.00	100.00	100.00	100.00	100.00
24	100.00	100.00	100.00	85.71	100.00	71.43
25	100.00	100.00	100.00	100.00	100.00	100.00
26	100.00	100.00	100.00	85.71	100.00	100.00
Average	91.5904	100	87.7768	94.642692	86	94.986154