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Reliable Confirmation of Antibodies to Bovine Respiratory Syncytial Virus (BRSV) by Enzyme-Linked Immunosorbent Assay Using BRSV Nucleocapsid Protein Expressed in Insect Cells†

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The nucleocapsid (N) protein of bovine respiratory syncytial virus (BRSV) in the baculovirus expression system was evaluated as a source of antigen in an enzyme-linked immunosorbent assay (ELISA) for the detection of respiratory syncytial virus (RSV) antibodies. The recombinant N protein was purified from infected-cell extracts by sucrose gradient centrifugation and used in the ELISA for the detection of antibodies to various RSV strains. The ELISA was compared with the virus neutralization (VN) test for determining BRSV antibodies in 10 consecutive serum samples from four calves vaccinated with a live modified BRSV vaccine and from two nonvaccinated control calves. The ELISA compared favorably with the VN test for detecting serological responses. All serum samples which were positive in the VN test were also positive in the ELISA. None of the serum samples collected from the two nonvaccinated calves reacted in the ELISA. To determine the usefulness of the ELISA for epidemiological studies, 58 cattle serum samples were tested in the ELISA and the VN test. Approximately 94% (42 of 45) of field serum samples which were positive in the ELISA were also positive in the VN test. No case was found in which the ELISA result was negative and the VN test result was positive. Thirteen of the serum samples were negative in both methods. Our results indicate that the ELISA with the baculovirus-expressed N protein as an antigen is an efficient, sensitive, and specific method for detecting serum antibodies to RSV.

Bovine respiratory syncytial virus (BRSV), a member of the genus *Pneumovirus* of the family Paramyxoviridae, is closely related to human respiratory syncytial virus (HRSV). It is a major cause of lower respiratory tract disease in cattle (21). Seroepizootiologic studies have demonstrated that exposure of cattle to BRSV is widespread in many countries (3, 9). BRSV infections can be diagnosed by virus isolation or serology. However, BRSV is labile, and virus isolation fails on many occasions (14, 20). Therefore, serology is more commonly used to detect BRSV infections by virus neutralization (25), immunofluorescence (15), complement fixation (23), and enzyme-linked immunosorbent assay (ELISA) (6, 26). Among the available methods, ELISA is highly sensitive and rapid. However, BRSV-infected cells are used as the ELISA antigen, and owing to the slow replication characteristics of BRSV, the infectivity titer of the virus may vary. Therefore, there is a need for developing an alternative means for antigen production.

High-level expression of recombinant proteins in a baculovirus system provides a good opportunity to produce a standard, high-titer source of antigen for both research and diagnostic applications (22). The nucleocapsid (N) protein appears to be an ideal candidate for expression, as it is the most abundant viral protein in BRSV-infected cells (11, 13) and sera from infected calves contain high levels of antibody to the N protein (27). Furthermore, the viral N protein has been used to diagnose mumps (12), measles (7), Hantaan virus (19), influenza virus (17), Lassa fever virus (4), rabies virus (16), and vesicular stomatitis virus (1). We previously expressed the BRSV N protein by using a baculovirus expression system (18). We report here the immunogenicity and utility of the recombinant N protein in a diagnostic ELISA. For this purpose, 10 consecutive serum samples from six calves were tested. The ELISA results were compared with those of virus neutralization (VN) tests to investigate the consistency of the immunity level as measured by these two methods. We then used the ELISA to investigate the seroepidemiology of BRSV infections in cattle.

MATERIALS AND METHODS

Cells and viruses. A continuous cell line (Sf9) derived from the fall armyworm, *Spodoptera frugiperda*, was cultivated in Grace's medium (22) containing 10% bovine fetal serum. Wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant baculoviruses were propagated as described by Summers and Smith (22). The construction and characterization of the recombinant baculovirus encoding BRSV strain A51908 N protein were accomplished by established methods (22) as described elsewhere (18). The BRSV N protein produced in insect cells was indistinguishable from the authentic BRSV N protein by sodium dodecyl sulfate (SDS) gel electrophoresis (Fig. 1). BRSV strains A51908 (ATCC VR-794) and FS1 and a caprine respiratory syncytial virus strain (GRSV) were grown in cultures of bovine turbinate (Btu) cells as described previously (13). HRSV strain Long (ATCC VR-26) was grown in HEp-2 cells.

Purification of BRSV N protein expressed in Sf9 cells for the ELISA. To produce antigen for the ELISA, a purification procedure previously described for NS1 protein of blue-

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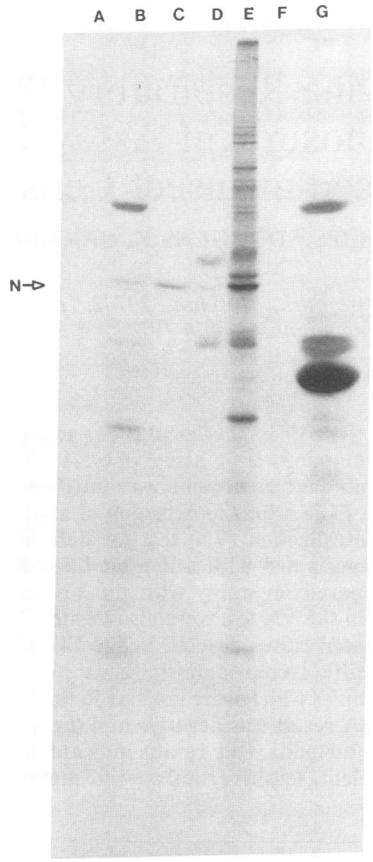

FIG. 1. Expression of N protein by recombinant baculovirus and confirmation of the authenticity of recombinant N protein. Lanes: A, uninfected *S. frugiperda* cells immunoprecipitated with BRSV-specific antiserum; B, recombinant baculovirus-infected cell lysate; C, recombinant baculovirus-infected cell lysate immunoprecipitated with BRSV antiserum; D, BRSV-infected Btu cell lysate immunoprecipitated with BRSV antiserum; E, BRSV-infected Btu cell lysate; F, wild-type AcNPV-infected cell lysate immunoprecipitated with BRSV antiserum; G, wild-type AcNPV-infected cell lysate. The position of the N protein is indicated.

tongue virus (24) was used to purify the N protein expressed in Sf9 cells. In brief, monolayer Sf9 cells in 150-cm^2 flasks were infected at a multiplicity of infection of 3 to 5 with recombinant baculovirus, harvested 4 or 5 days after infection by centrifugation at 500 × *g* for 15 min at 4°C, and washed in phosphate-buffered saline. Cell pellets were resuspended in 1 ml of 10 mM Tris-HCl (pH 7.4)–150 mM NaCl containing 0.5% Nonidet P-40 per 150-cm^2 flask and left on ice for 30 min. The cells were then recentrifuged at 500 × *g* for 5 min to remove cell debris. The supernatants were recovered and layered on 10 to 50% (wt/vol) continuous sucrose gradients in 10 mM Tris-HCl (pH 7.4). The gradients were centrifuged at 197,000 × *g* for 3 h at 4°C in an SW41 rotor. After centrifugation, 1-ml fractions were collected from the top of each gradient. A portion of each fraction was subjected to gel electrophoresis. The fractions containing BRSV N protein were pooled and pelleted by centrifugation for 2 h at 197,000 × *g* in an SW41 rotor. The pellet was resuspended in 10 mM Tris-HCl (pH 7.4) and stored at 4°C.

Preparation and characterization of an antiserum to the N protein expressed in Sf9 cells. Monospecific antisera against the baculovirus-expressed N protein and wild-type AcNPV cell proteins were prepared by use of mice. In brief, each mouse received one intraperitoneal injection of either 50 μg of purified N protein or 100 μg of wild-type AcNPV cell proteins in Freund's incomplete adjuvant on day 0 and then three injections of 50 μg of antigen, on days 7, 14, and 21. Blood was collected 7 days after the last injection, and serum samples were tested for the presence of antibodies by immunoprecipitation (8).

ELISA with the purified N protein. A solid-phase ELISA was used to test the ability of the purified N protein to react with various polyclonal RSV antisera and a monoclonal antibody to BRSV N protein. ELISA plates (Immulon-2; Dynatech) were coated by overnight treatment at 4°C with approximately 100 ng of the N protein in 0.1 M sodium bicarbonate (pH 9.5) per well. After adsorption of the antigen, the plates were saturated with blocking buffer (5% skim milk powder in TBS-Tw [144 mM NaCl in 25 mM Tris-HCl {pH 7.6}–0.1% Tween 20]) for 30 min at room temperature. Various dilutions of different polyclonal RSV antisera or an N protein monoclonal antibody in blocking buffer (100 μl) was incubated in antigen-coated wells for 2 h at 37°C. After a wash with TBS-Tw, 100 μl of affinity-purified goat anti-rabbit immunoglobulin M-immunoglobulin G-, conjugated with horseradish peroxidase (1 μg/ml), was added for 1 h at 37°C. ABTS [2,2′-azino-di-(3-ethylbenzothiazoline sulfonate)] substrate (100 μl) was added after a wash with TBS-Tw. The A_{410} was recorded with an automated spectrophotometer (Titertek Multiscan; Flow Laboratories, Vienna, Va.).

For the determination of BRSV antibody titers, serum samples were tested at serial twofold dilutions, starting at 1:10. A single test dilution of 1:10 and a substrate reaction time of 60 min were selected for subsequent assays of unknown serum samples. ELISA results were expressed as the net absorbance. The cutoff value for a positive test was taken as the mean absorbance plus 3 standard deviations for a panel of five control serum samples with no detectable VN antibodies.

Vaccination of calves and serological testing. Six purebred Angus calves from the Wye herd, Wye Research and Education Center, Queenstown, Md., were used in this study. Calves were weaned at an average age of 205 days. All calves were found BRSV free by both virus isolation and VN tests. Four calves (vaccinated group; calves 94, 95, 97, and 98) were inoculated intramuscularly with an attenuated BRSV vaccine strain as directed by the manufacturer (SmithKline Beecham Animal Health, Lincoln, Nebr.). A booster vaccination was given 4 weeks later. The two remaining calves served as nonvaccinated controls. On the day of weaning, the nasal mucosa of each calf was swabbed for virus isolation. Nasal swabs were swirled in transport medium, and the eluates were inoculated onto Btu cells for virus culturing. The specimens were considered negative if no cytopathic effects developed within two subpassages.

Serum was collected from each calf for the determination of ELISA and VN antibody titers against BRSV on 10 occasions: days 0 (day of weaning), 7, 14, 28, 35, 42, 56, 84, 112, and 140. All serum samples were heated to 56°C for 30 min and stored at −20°C prior to use. For the VN test, serum samples were serially diluted twofold in microtitration plates. BRSV strain A51908 (100 50% tissue culture infective doses) was added to each serum dilution. After incubation for 1 h at 37°C, cells were added in amounts sufficient to form a monolayer. The plates were incubated for 5 days. Cytopathic effects were examined microscopically. The reciprocal of the highest serum dilution that completely inhibited

cytopathic effects was recorded as the VN titer. A titer of >4 was judged sufficient to consider the calf BRSV seropositive.

Serum samples. A total of 58 cattle serum samples used for the ELISA and the VN test were obtained from a herd in Maryland that had a history of respiratory tract disease. All serum samples were heat inactivated at 56°C for 30 min. For the determination of BRSV antibody titers by the ELISA, serum samples were tested at serial twofold dilutions, starting at 1:10. For the VN test, serum samples were serially diluted twofold, starting at 1:4.

RESULTS

Purification of the N protein produced in insect cells. Analysis of fractions from the sucrose gradient by SDS-polyacrylamide gel electrophoresis showed that the BRSV N protein was mainly localized in fractions from the middle of the gradient. The peak fractions containing N protein were pooled and pelleted. A purity of greater than 90%, as estimated by visual inspection of Coomassie blue-stained SDS-polyacrylamide gels, was achieved for recombinant BRSV N protein (data not shown).

Characterization of antisera produced to the expressed N protein. To assess the ability of the N protein produced by the recombinant baculovirus to induce antibodies which could react with the N protein present in BRSV-infected cells, a mouse antiserum was produced by use of the N protein purified from infected Sf9 cells by sucrose gradient centrifugation. The ability of the antiserum to recognize N protein was tested by immunoprecipitation. Preimmune serum and antiserum to the wild-type AcNPV-infected Sf9 cell proteins did not react with any viral or cellular proteins in the BRSV-infected Btu cell lysates, whereas antiserum to the purified N protein specifically precipitated the BRSV N protein. The anti-N protein serum reacted in an ELISA with several BRSV strains and with the Long strain of HRSV (data not shown). Therefore, this antiserum could be used for RSV detection. However, the anti-N protein serum did not neutralize BRSV infectivity when tested in a plaque reduction assay (data not shown).

ELISA with recombinant virus-derived N protein for identification of BRSV antibodies. Since postinfection animal sera contain abundant antibodies to the N protein (27), we investigated the usefulness of the N protein produced by the recombinant baculovirus for antibody detection by an ELISA. The reactivity of purified recombinant N protein in the ELISA with polyclonal rabbit antisera made against BRSV strain A51908, BRSV strain FS1, a caprine RSV strain, and HRSV strain Long, and with a monoclonal antibody to BRSV N protein was examined. Normal horse serum was used as a negative control. All four RSV antisera and the monoclonal antibody reacted with the purified N protein in proportion to the endpoint titer of the antiserum (Fig. 2). The baculovirus-expressed N protein did not react with antisera to parainfluenza virus type 3 and infectious bovine rhinotracheitis virus, indicating specificity for RSV antibodies. Reactivity was not detected when each RSV antiserum was tested with wild-type AcNPV-infected cell extracts (data not shown).

To determine whether nonpurified N protein produced by the recombinant baculovirus could be used in the ELISA, recombinant virus-infected *S. frugiperda* cell extracts were adsorbed to microtiter plates and reacted with homologous and heterologous BRSV antisera as described in Materials and Methods. The results indicated that purified N protein reacted with higher dilutions of RSV antisera than nonpurified N protein.

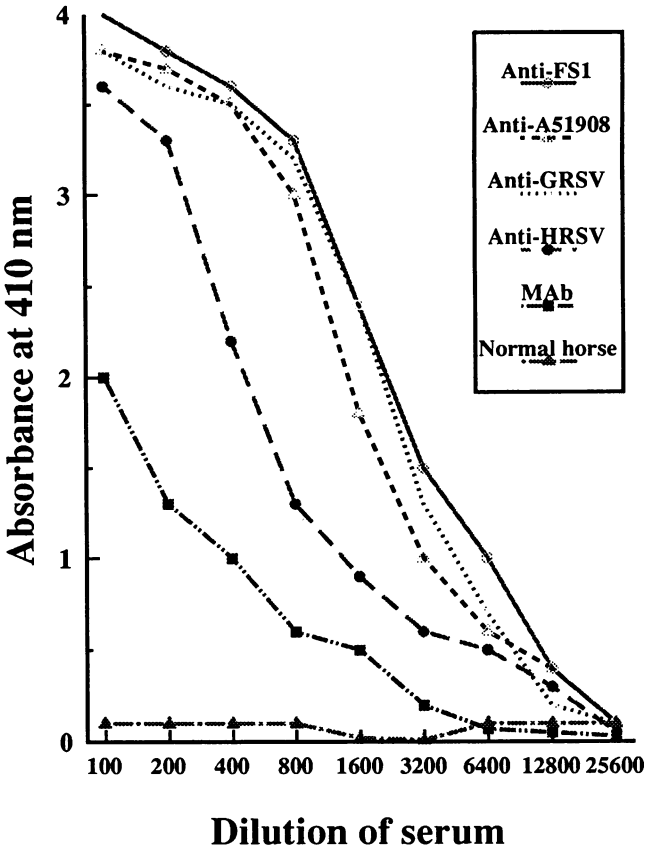

FIG. 2. Reaction of N protein purified from recombinant baculovirus-infected *S. frugiperda* cells as an antigen in an ELISA for BRSV antibodies. Purified N protein was adsorbed to the solid phase and examined with 1:100 to 1:25,600 dilutions of polyclonal rabbit antisera made against BRSV strain A51908, BRSV strain FS1, a caprine RSV strain (GRSV), and HRSV strain Long and a monoclonal antibody to BRSV N protein (MAb). Normal horse serum was used as a negative control.

Comparison of the ELISA and the VN test for the detection of BRSV antibodies in vaccinated calves. All four vaccinated calves and two nonvaccinated control calves remained clinically normal throughout the course of the study. Virus isolation attempts failed to identify any calf harboring BRSV on day 0. The mean ELISA titer value for five VN-negative, prevaccination serum samples was 0.172 (standard deviation, 0.038). Serum samples were considered positive for BRSV when the A_{410} was >0.286 (mean ± 3 standard deviations).

Serum samples from all six calves were tested in the ELISA and the VN test. The results of the ELISA and the VN test for the vaccinated calves are presented in Fig. 3. There was agreement between the ELISA and the VN test. All VN-positive serum samples from vaccinated calves were positive in the ELISA. As shown in Fig. 3, serum samples collected on days 0 and 7 were negative in both the ELISA and the VN test. An antibody response was detected for the first time by the ELISA on day 14 for three of the four vaccinated calves (calves 94, 95, and 98). The VN test first detected antibodies in two calves (94 and 98) on day 14 but failed to detect antibodies in two other calves (95 and 97)

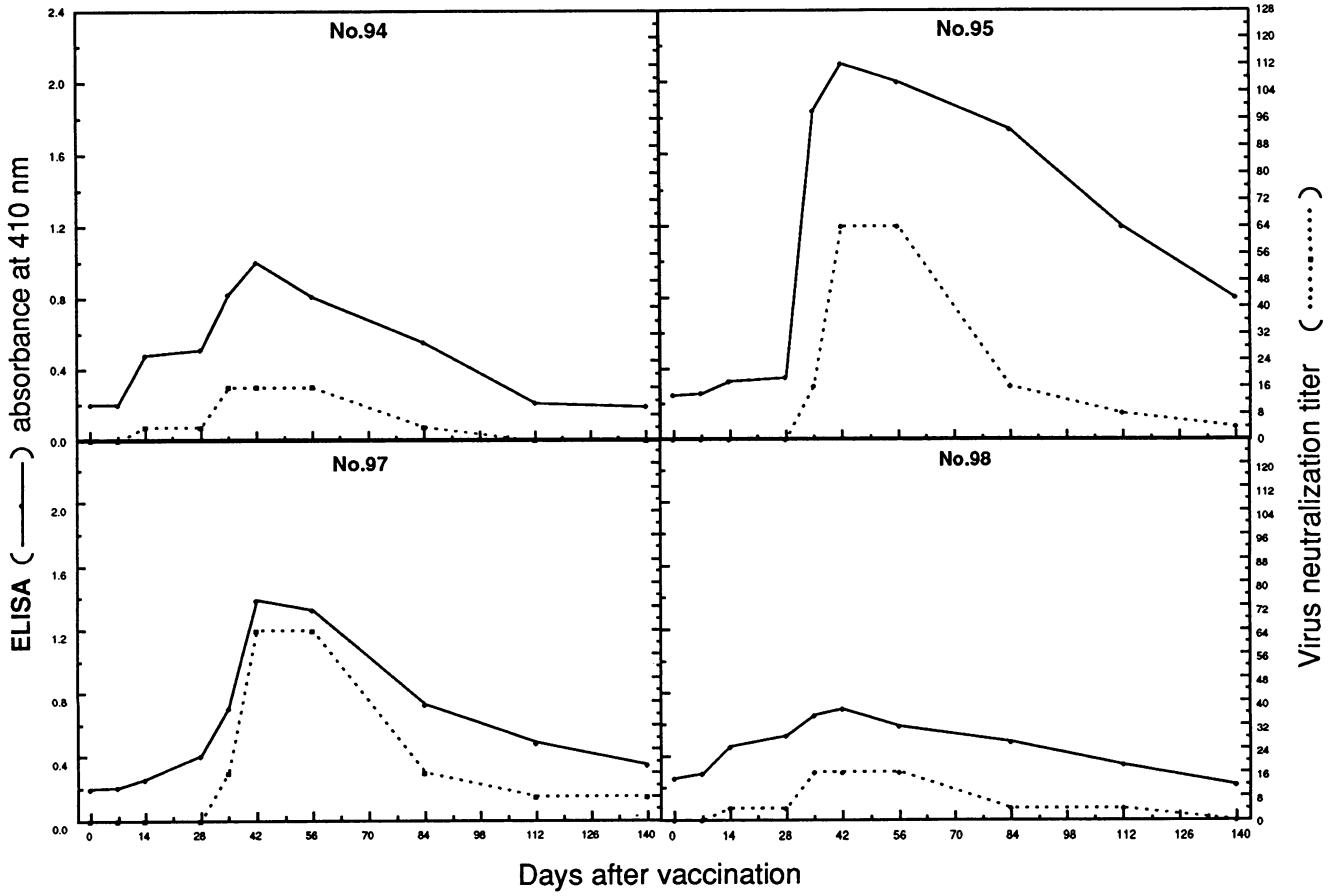

FIG. 3. ELISA and VN test results obtained with serum samples from four vaccinated calves. Calves were inoculated intramuscularly on day 0 with an attenuated BRSV vaccine, and a booster vaccination was given after 4 weeks. The ELISA and the VN test were performed as described in the text.

until day 35. In all vaccinated calves, there was a significant rise in antibody titer after booster vaccination on day 28. The antibody titers of the postvaccination sera were higher between 35 and 84 days in both tests. The antibody titers dropped sharply after day 56 in all vaccinated calves and were undetectable on day 112 in two calves (94 and 98). All serum samples from two nonvaccinated calves were negative for BRSV antibodies in both the ELISA and the VN test (data not shown).

Comparison of the ELISA and the VN test for the detection of BRSV antibodies in field serum samples. A total of 58 serum samples from a herd with a history of respiratory tract disease were tested for BRSV antibodies by the ELISA and the VN test. The ELISA appeared to be highly sensitive and detected antibodies in all 42 VN-positive serum samples. There were three cases in which ELISA results were positive but VN test results were negative. The VN titers in positive serum samples varied between 4 and 64. In general, an increase in the VN titer of serum samples was accompanied by an increase in the ELISA titer. However, nine serum samples with a low VN titer had a high ELISA titer; two serum samples had the opposite pattern. Thirteen serum samples were negative in the ELISA and the VN test.

ELISAs with the recombinant N protein and purified BRSV strain A51908 as antigens were compared by use of all 58 field serum samples. BRSV antibodies were detected by the ELISA with recombinant N protein in 45 of 47 (96%) serum samples positive in the ELISA with purified BRSV. The two discrepant serum samples were weakly positive in the ELISA with purified BRSV. All 11 serum samples without detectable antibodies to BRSV in the ELISA with purified BRSV were also negative in the ELISA with recombinant N protein.

DISCUSSION

The BRSV N protein was expressed at high levels in Sf9 cells with a recombinant baculovirus vector. The level of expression of BRSV N protein was estimated to be approximately 60 mg per 2×10^9 infected Sf9 cells. The baculovirus-expressed N protein was similar in size to the authentic viral protein and was precipitated with polyclonal antibodies raised against BRSV strain A51908 (Fig. 1). The N protein could be purified from recombinant baculovirus-infected cells by sucrose gradient centrifugation. The procedure used for purification of the N protein was similar to that used for purification of the NS1 protein of bluetongue virus (24). The purified N protein was over 90% pure. Antibodies made against the baculovirus-expressed N protein reacted with N protein in BRSV-infected cells. The N protein has been shown to be highly conserved (97%) between BRSV strains A51908 and 391-2 (2). In general, the most conserved gene or gene product is used as a diagnostic reagent because it can detect a wide variety of related strains. Therefore, we chose

the N protein as an antigen to detect RSV antibodies in an ELISA. We found that antibodies to recombinant N protein could be used to detect BRSV and HRSV strains. However, antibodies to recombinant N protein failed to neutralize BRSV in a plaque reduction assay. Similar results were obtained with a recombinant vaccinia virus expressing HRSV N protein (5, 10).

The ELISA with BRSV-infected cell lysates as antigens has been shown to be a specific and reproducible method for detecting serum antibodies to BRSV (6, 26). However, the ELISA has not been routinely used to detect BRSV antibodies because of difficulties in reproducing high-titer assay antigens. Furthermore, some serum samples react with control cell antigens in the ELISA (6). In our study, the recombinant N protein was evaluated as a potential source of antigen for a diagnostic ELISA because antibodies to the N protein appear early and predominate during the BRSV infection (27). The recombinant BRSV N protein could supply an abundant and pure source of antigen necessary for the ELISA.

In the vaccination study, measurable BRSV antibodies did not exist at the beginning of the experiment, and control calves never developed antibodies to BRSV. Therefore, there was no indication that the calves were exposed to BRSV during the course of the study except through vaccination. There was a close correlation between ELISA and VN test results. Although the ELISA detected antibodies only to the N protein and the VN test detected antibodies mainly to the F protein (5), our results are not surprising, because the antibody response to BRSV is predominantly directed to both F and N proteins (27). The ELISA titer correlated well with the VN titers of serum samples from vaccinated calves. None of the 20 serum samples collected from two nonvaccinated calves reacted in the ELISA, indicating that false-positive reactions did not occur in this test. There was also a close correlation between ELISA and VN test results in field serum samples. Our results demonstrate that the ELISA is as sensitive as the VN test in detecting RSV antibodies.

We conclude that N protein produced in insect cells can be used in the ELISA and that this test is sensitive, rapid, and reliable. The results of this test compared well with those of the VN test for detecting serum antibodies to BRSV. The VN test provides the best estimate of protection from BRSV infection, but it is unsuitable for screening large numbers of serum samples for epidemiological studies or for early and rapid detection of antibodies to BRSV. Although the ELISA does not necessarily indicate a protection level, it is rapid and equal in sensitivity to the VN test in detecting RSV antibodies. With the availability of large quantities of recombinant N protein for use as an assay antigen, a standardized ELISA for RSV is now possible.

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