

Rapid, Sensitive, Competitive Serologic Enzyme-Linked Immunosorbent Assay for Detecting Serum Antibodies to Bovine Herpesvirus Type 1

CYNTHIA A. RIEGEL, VIRGINIA K. AYERS, AND JAMES K. COLLINS*

Diagnostic Laboratory, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado 80523

Received 23 March 1987/Accepted 9 September 1987

An enzyme-linked immunosorbent assay (ELISA) for the detection of cattle antibodies to bovine herpesvirus type 1 was developed on the basis of competition between serum antibody and a virus-neutralizing mouse monoclonal antibody. The assay showed improved sensitivity over the virus neutralization (VN) test and over an enhanced VN test in which incubation of antibody-virus mixtures was carried out for 24 h. With the ELISA, antibodies in sera from experimentally infected cattle were detected earlier after infection and showed more rapid increases in levels. A comparison of the ELISA with the VN tests by using a set of 85 field sera with low levels of antibodies demonstrated that the ELISA was the most sensitive test, detecting 10 positive serum samples that were negative by the VN tests. The ELISA was inexpensive, rapid, and highly reproducible and showed a significant improvement in sensitivity over VN tests.

Bovine herpesvirus type 1 (BHV-1) is a widespread and economically important pathogen causing a variety of disease manifestations, including respiratory and genital tract infections, abortion, conjunctivitis, and encephalitis (12). Infected herds usually have high morbidity and low mortality, although the loss of fetuses in pregnant cows can approach 60%. Mortality in adult animals is rarely due to BHV-1 and is more often the result of a secondary bacterial respiratory tract infection.

Diagnosis of BHV-1 infection can be difficult for a variety of reasons. Subclinical infections are common, and like other herpesviruses, BHV-1 can result in latent infection and carrier status (2, 12). Diagnosis is frequently not attempted until secondary disease has become evident, by which time demonstrable virus is no longer present. Several laboratory methods are available for diagnosing BHV-1, including virus isolation, direct fluorescent-antibody examination of infected tissues (11, 13) and antigen detection by enzyme-linked immunosorbent assay (ELISA; 10). Serologic diagnosis is also available and widely used, most often in the form of a virus neutralization (VN) test. Although VN tests have been the assays of choice for many years, they are insensitive, costly, and time-consuming (1, 6, 7, 13). A serologic test of increased sensitivity would be advantageous not only for diagnostic utilization but also for screening large numbers of animals to detect low levels of antibodies and to establish BHV-1-free herds.

Described here are the development and utilization of a highly specific and highly sensitive competitive ELISA for the detection of BHV-1 antibody. The assay is based on the competition between a horseradish-peroxidase-conjugated mouse monoclonal antibody and bovine serum antibody. Previous studies (7) have demonstrated that this competitive ELISA correlates with VN titers. In the present study, ELISA is compared with and found to be more sensitive than a routine VN test and an enhanced VN test (1).

ELISA was carried out with serial serum samples obtained from animals experimentally infected with BHV-1 (6).

Additional sera were obtained from samples submitted to the Colorado State University Diagnostic Virology Laboratory for routine BHV-1 serologic assay. The VN test was carried out on all serum samples by standard procedures (4, 6) and, in addition, with a modification of the assay as described by Bitsch (1), in which a 24-h incubation of virus with serum was carried out at 37°C prior to addition of cells.

For the competitive-ELISA procedure, Immulon II microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 400 ng of density-gradient-purified BHV-1 per well in 0.1 ml of 0.01 M sodium borate buffer, pH 9.0 (6, 10). Plates were held at 37°C until dry and blocked with 0.2 ml of 2% bovine serum albumin (American Research Products Co., South Euclid, Ohio) in TNE buffer (0.05 M Tris, 0.15 M NaCl, 0.001 M EDTA; pH 7.4) for 1 h at 37°C. Wells were then emptied and either used immediately or frozen in a sealed, humidified chamber at -20°C. For the assay, 0.05 ml of undiluted serum was added to one well and incubated at 37°C for 0.5 h. Without being washed, 0.05 ml of horseradish-peroxidase-conjugated BHV-1-specific D9 monoclonal antibody (9) diluted at 1:500 to 1:1,000 in 0.1% bovine serum albumin in TNE buffer was added. Plates were then incubated for an additional 0.5 h at 37°C. After incubation, wells were washed four to five times with 0.05% Tween 20 in TNE buffer, and 0.1 ml of freshly prepared 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) was added. Color was allowed to develop for 15 min and then stopped with 0.05 ml of 0.5% sodium dodecyl sulfate. A_{405} was read spectrophotometrically with a Titertek Multiskan ELISA reader (Flow Laboratories, McLean, Va.). The conjugate dilution was selected to give an A_{405} of approximately 0.5 to 0.6 with fetal calf serum for competition; a decrease in absorbance of greater than 50% with competing serum was defined as positive.

Binding of monoclonal antibody in the ELISA was inhibited by BHV-1 convalescent-phase serum (Fig. 1). Preimmune control serum did not compete with the monoclonal antibody in the assay. The competition was linear with respect to dilution of the convalescent-phase serum and thus

* Corresponding author.

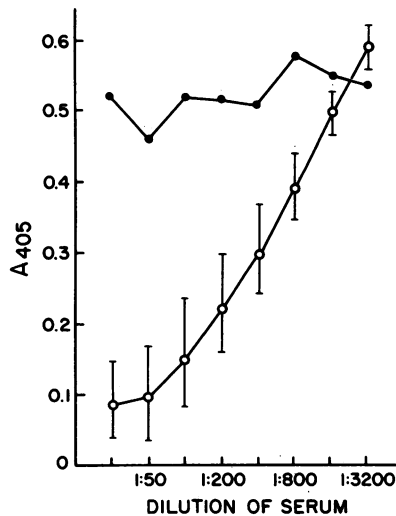


FIG. 1. Competition between horseradish-peroxidase-conjugated monoclonal antibodies and BHV-1 preimmune serum (●) or convalescent-phase serum (○). Bars represent standard deviations obtained with five repetitions of the assay.

correlated with VN titer as previously shown (7). The assay was highly reproducible, since the standard deviations (bars; Fig. 1) were obtained from assays performed on five different days by three individuals.

Serum samples from experimentally infected cattle gave earlier antibody responses to BHV-1 by ELISA compared with those by VN tests. After showing no VN titers and no ELISA competition in their sera for several days postinfection, all the infected animals (11 of 11) showed significant increases (65% of maximum) in ELISA competition on day 8, and this correlated well with the decline in virus shedding from animals (Fig. 2). On the same day, all titers (11 of 11) by routine VN test were negative, and titers obtained with the enhanced VN test averaged 1:2, with 7 of 11 showing

TABLE 1. Serologic test results with sera from 11 infected cattle

| No. of days after infection | No. of positive ($n = 11$) results in: | | |
|-----------------------------|------------------------------------------|-------------------------------|--------------------------------|
| | Routine VN ^a test | Enhanced VN ^a test | Competitive ELISA ^b |
| 6 | 0 | 0 | 0 |
| 8 | 0 | 7 | 11 |
| 11 | 11 | 11 | 11 |

^a Positive result, titer $\geq 1:2$.

^b Positive result, $\geq 50\%$ competition.

positive titers (Table 1). With the routine VN test, animals did not seroconvert until day 11, at which time the average titer was 1:8. At later times after infection, increasing antibody titers were detected by all assays. The ELISA reached maximal competition by day 10, whereas the VN titers were not maximal until days 14 to 18.

To further examine the specificity of the ELISA, 30 cattle which had been maintained free of BHV-1 were tested, vaccinated with a modified live BHV-1 vaccine (Tech America, Inc., Elwood, Kans.), and retested. Results (Table 2) with these 30 cattle of known BHV-1 status indicate that the ELISA was 100% specific.

To compare the three assays further, 85 clinical serum samples submitted for routine BHV-1 VN testing and with low or negative VN titers (negative to 1:16) were analyzed by both the competitive ELISA and the enhanced VN test. With the enhanced VN test, 6 of 35 sera which were negative by routine VN test were found to be positive, and all sera which were positive by routine VN test showed enhancements of titers by 2- to 8-fold (average, 3.9-fold) with the enhanced VN test (data not shown). In a comparison of the enhanced VN test with the competitive ELISA, 41 serum samples were positive in both assays and 34 serum samples were negative in both assays (Fig. 3). No serum sample was positive by the enhanced VN test and negative by ELISA. However, 10 serum samples that were negative by both enhanced and routine VN tests were positive by competitive

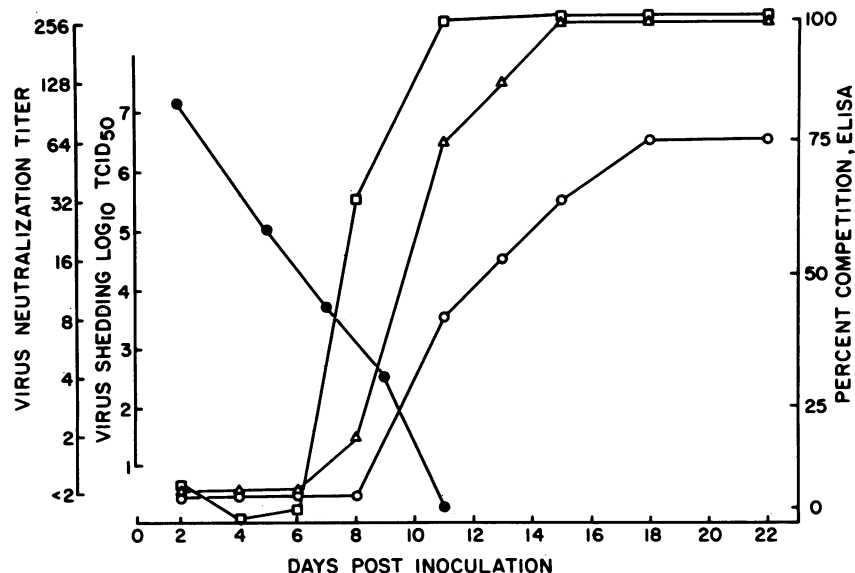


FIG. 2. Virus shedding and antibody responses of experimentally infected animals. Antibody responses were measured by competitive ELISA (□), 24-h enhanced VN test (△), and standard VN test (○). ●, Virus shedding. Results are the mean values from 11 animals. TCID₅₀, 50% Tissue culture infective dose.

TABLE 2. Serologic test results with sera from 30 vaccinated cattle

| Result by enhanced VN test | No. of results by competitive ELISA (positive/negative) | |
|----------------------------|---------------------------------------------------------|------------------------------|
| | Prevaccination | Postvaccination ^a |
| Positive | 0/1 | 25/0 |
| Negative | 0/29 | 0/5 |

^a 14 days postvaccination.

ELISA. These 10 samples had A_{405} values of 0.10 to 0.23, which would place their competition values in the range of 65 to 85% of maximum competition, similar to those competition values seen with sera from experimentally infected animals 8 days after infection. Additional VN-negative sera from field animals may also have had antibodies even though they did not compete at the 50% level (Fig. 3), but since the exposure history of the animals from which the sera came was not known, no definitive conclusion could be reached.

These results demonstrate the feasibility of using a competitive ELISA for the detection of antibody to BHV-1. When sera with known specificities (i.e., from experimentally infected or vaccinated cattle) were used, the assay was specific and more sensitive than VN tests in that antibody was detected earlier and more dramatically after experimental infection. With such easily detectable increases in antibody responses in serum samples taken just a few days apart, the ELISA would be very useful for diagnostic testing.

The applicability of a new diagnostic test is usually based on determinations of specificity and sensitivity that are compared with those of an established assay which provides acceptable results. In this case, however, the referent assays could not be used for such determinations, and instead, the determination of the true negative or positive statuses of serum samples from experimentally infected or vaccinated animals was based on the known infection history of the animal from which the samples were taken.

The development of sensitive serologic assays for BHV-1 has been sought to establish with the highest accuracy whether an animal has been previously exposed to BHV-1 and therefore may be a latent carrier (1, 2, 14). Several tests have been developed to improve sensitivity. The addition of complement to the neutralization reaction has increased sensitivity (15). The development of the enhanced VN test in which neutralization was carried out for 24 h showed that sensitivity could be increased at least fourfold (1). These results were confirmed in this study. In addition, the competitive ELISA was even more sensitive than the enhanced VN test, in that several field sera and sera from experimentally infected cattle which were negative by the VN test were positive by ELISA.

The success of this competitive ELISA may have been due to the selection of the monoclonal antibody used for competition. The selection was based on the finding that the glycoprotein which this monoclonal antibody recognized was one of the first recognized by cattle after infection and was also recognized consistently by cattle sera (8). Previous results suggested that this monoclonal-antibody-defined epitope was recognized by cattle antibodies (7) and that the epitope was involved as a major determinant of neutralization (9).

The competitive ELISA had distinct advantages over VN tests with respect to ease of performance. It was also easier to perform than conventional indirect ELISAs for detecting

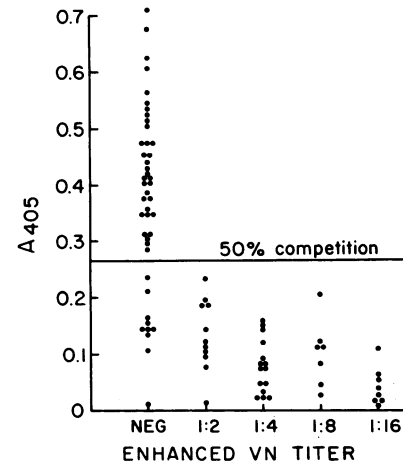


FIG. 3. Competitive ELISA versus the 24-h enhanced VN test for 85 selected clinical sera having low or negative neutralization titers. Each point is the average of two determinations. NEG, Negative titer.

bovine antibody bound to coated wells (3, 5, 6). The competitive ELISA required only one well of a microtiter plate and was carried out with undiluted serum. There was no evidence that the use of undiluted serum caused problems in terms of background or nonspecific reactions. The incubation of serum and conjugate was done sequentially, without the need for a washing step. Results were obtained in slightly more than 1 h compared with 3 days for VN testing. In addition, the assay plates were stable at -20°C , enabling storage of several months. The assay would be highly suitable for screening large numbers of sera with great sensitivity.

LITERATURE CITED

- Bitsch, V. 1978. The P_{34}^{37} modification of the infectious bovine rhinotracheitis virus-serum neutralization test. *Acta Vet. Scand.* **19**:497-505.
- Bitsch, V. 1984. On the latency of infectious bovine rhinotracheitis virus infection and its significance, especially with regard to the possibility of controlling infection, p. 163-170. *In* G. Wittman, R. M. Gaskell, H. J. Rziha (ed.), *Latent herpes virus infections in veterinary medicine*. Martinus Nijhoff Publishing, The Hague, The Netherlands.
- Bolton, D. C., H. J. Chu, A. A. Ardans, B. Kelly, and Y. C. Lee. 1981. Evaluation of the critical parameters of a sensitive ELISA test using purified infectious bovine rhinotracheitis virus antigens. *Vet. Microbiol.* **6**:265-279.
- Carbrey, E. A. 1972. Recommended standard laboratory techniques for diagnosing infectious bovine rhinotracheitis, bovine virus diarrhea, and shipping fever. *Proc. U.S. Anim. Health Assoc.* **75**:629-648.
- Cho, H. J., and J. G. Bohac. 1985. Sensitivity and specificity of an enzyme-linked immunosorbent assay for the detection of infectious bovine rhinotracheitis viral antibody in cattle. *Can. J. Comp. Med.* **49**:189-194.
- Collins, J. K., G. A. Bulla, C. A. Riegel, and A. C. Butcher. 1985. A single dilution enzyme-linked immunosorbent assay for the quantitative detection of antibodies to bovine herpesvirus type 1. *Vet. Microbiol.* **10**:133-147.
- Collins, J. K., A. C. Butcher, and A. F. Fountain. 1985. A one-step competitive serologic ELISA for detecting neutralizing antibodies to bovine herpesvirus type 1, p. 361-368. *In* 28th Annual proceedings, American Association of Veterinary Laboratory Diagnosticians. American Association of Veterinary Laboratory Diagnosticians, Inc., Tucson, Ariz.

8. **Collins, J. K., A. C. Butcher, and C. A. Riegel.** 1985. Immune response to bovine herpesvirus type 1 infections: virus-specific antibodies in sera from infected animals. *J. Clin. Microbiol.* **21**:546-552.
9. **Collins, J. K., A. C. Butcher, C. A. Riegel, V. McGrane, C. D. Blair, Y. A. Teramoto, and S. Winston.** 1984. Neutralizing determinants defined by monoclonal antibodies on polypeptides specified by bovine herpesvirus 1. *J. Virol.* **52**:403-409.
10. **Collins, J. K., A. C. Butcher, Y. A. Teramoto, and S. Winston.** 1985. Rapid detection of bovine herpesvirus type 1 antigens in nasal swab specimens with an antigen capture enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* **21**:375-380.
11. **Howard, D. R.** 1979. Fluorescent antibody techniques for diagnosing animal disease conditions, p. 283-302. *In* 22nd Annual proceedings, American Association of Veterinary Laboratory Diagnosticians. American Association of Veterinary Laboratory Diagnosticians, Inc., Tucson, Ariz.
12. **Kahrs, R. F.** 1977. Infectious bovine rhinotracheitis: a review and update. *J. Am. Vet. Med. Assoc.* **171**:1055-1064.
13. **Kahrs, R. F.** 1982. Viral diseases of cattle, p. 135-156. Iowa State University Press, Ames.
14. **Muller, H. K., I. Ascherl, H. U. Kuperschied, L. Bruckner, M. Ackermann, and U. Kihm.** 1986. Detection of antibodies to BHV-1 in bovine semen by a modified ELISA, p. 806-809. *In* IVth International Symposium on Veterinary Laboratory Diagnosis, June 2-6, 1986, Amsterdam, The Netherlands.
15. **Potgieter, L. N. D.** 1975. The influence of complement on the neutralization of infectious bovine rhinotracheitis virus by globulins derived from early and late bovine antisera. *Can. J. Comp. Med.* **39**:427-433.