JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1987, p. 1705–1710
0095-1137/87/091705-06$02.00/0

Vol. 25, No. 9

Comparison of Competitive and Indirect Enzyme-Linked Immunosorbent Assays for Detection of Bluetongue Virus Antibodies in Serum and Whole Blood

AHMAD AFSHAR,[1]* FREDERICK C. THOMAS,[1] PETER F. WRIGHT,[1] JANET L. SHAPIRO,[1] PARKALA T. SHETTIGARA,[1] AND JOHN ANDERSON[2]

Animal Diseases Research Institute, Agriculture Canada, Nepean, Ontario K2H 8P9, Canada,[1] and Animal Virus Research Institute, Pirbright, Woking, Surrey GU24 0NF, United Kingdom[2]

Received 5 March 1987/Accepted 22 May 1987

An indirect (I) enzyme-linked immunosorbent assay (ELISA) and a competitive (C) ELISA, using a group-specific monoclonal antibody against bluetongue virus (BTV), are described for the detection of antibodies to BTV in cattle and sheep sera. The performance of these assays in detecting anti-BTV antibody in sequential serum samples and eluates from whole blood (WB) dried on filter paper from three calves and four sheep experimentally infected with type 10 BTV was evaluated. The C-ELISA was superior to the I-ELISA in the detection of anti-BTV antibody in the sera and WB samples from both cattle and sheep early after infection with BTV. BTV antibodies were demonstrable by C-ELISA in all the bovine and ovine sera and WB eluates by 9 days postinfection; whereas the I-ELISA results for sheep sera and WB eluates were similar, anti-BTV antibody was not detected in bovine serum and WB eluates until 26 and 14 days postinfection, respectively. While both ELISAs proved reliable, under the present test conditions involving detection of early postinfection reactions of experimentally infected animals, the C-ELISA was always as sensitive or more sensitive than the standard agar gel immunodiffusion test, the modified complement fixation test, and the plaque neutralization tests in the detection of anti-BTV antibodies. Unlike observations with the immunodiffusion test, no reaction was seen between BTV antigen and bovine epizootic hemorrhagic disease virus antiserum in either ELISA. The results suggest that either ELISA may be suitable for routine diagnostic testing and may have the potential to replace other tests for detection of anti-BTV group-specific antibodies and that the C-ELISA may have the most potential.

Bluetongue virus (BTV) is an arthropod-borne orbivirus that causes disease in domestic and wild ruminants (17, 19). At present, 24 internationally recognized serotypes of the virus have been reported (6, 13). Infection is most often detected by the presence of BTV antibodies in serum. Several assays are described in the literature that detect serotype-specific or group-specific antibodies (7, 9). The agar gel immunodiffusion (AGID) test is the most widely used assay for detection of group-specific anti-BTV antibodies (14, 15). The AGID test is not highly sensitive, specific, or quantitative, and interpretation of the results is often subjective. As an alternative, enzyme-linked immunosorbent assays (ELISAs) have been developed for large-scale testing. An indirect (I) ELISA was first described by Manning and Chen (11), who used partially purified BTV antigen to quantitate antibodies in sera from sheep experimentally infected with BTV. Later, the I-ELISA with purified BTV antigen was applied to detect group-specific antibody in sera from cattle naturally exposed to BTV and sheep and cattle experimentally infected with BTV (8, 16). In 1984, Anderson (2) described a blocking ELISA in which the immobilized BTV antigen is first reacted with a test serum and then with a group-specific murine monoclonal antibody (MAb). Anti-BTV antibody, if present in the test serum, blocks the antigen, preventing reaction with the MAb in the subsequent step of the test. The blocking ELISA was found to be highly sensitive and specific in the detection of antibodies to 22 serotypes of BTV (2).

* Corresponding author.

Since the initial evaluation of these two ELISAs was carried out independently by separate laboratories (2, 8, 11, 16), we adapted the techniques and compared their performance in measuring the humoral immune response to BTV infection in cattle and sheep. In addition, a comparative study of the ELISA results with those of the AGID, modified complement fixation (MCF), and plaque neutralization (PN) tests was carried out. This report also describes the successful use of eluates from whole blood, collected and dried on filter paper, for the detection of antibodies to BTV by both ELISAs.

MATERIALS AND METHODS

BTV antigen preparation. A stock ELISA antigen was prepared from baby hamster kidney (BHK-21) cells infected under serum-free medium with plaque-purified BTV serotype 11 by a method described previously (2). This BTV antigen (13.71 mg of total protein per ml) and a stock of control antigen (13.09 mg of total protein per ml), which was prepared in the same way from uninfected BHK-21 cells, were used throughout this study.

Serum samples. Sera and whole blood from serial bleedings of three cross-bred, 6- to 20-month-old calves and four adult sheep experimentally infected with BTV type 10 were studied. Each animal was inoculated by both intravenous and subcutaneous routes with washed and sonicated infected bovine erythrocytes (21), containing approximately 10^2 PFU. The animals were housed in separate pens in a maximum containment (level E) facility. They were observed and examined for clinical changes, and their rectal temperatures were recorded daily. Serum samples were

obtained prior to inoculation and on days 2, 5, 7, 9, 12, 14, and 20 and thereafter at weekly intervals after infection. Whole blood taken from the jugular vein was allowed to diffuse into and saturate 9-cm-diameter filter paper disks (Whatman no. 4; Whatman, Clifton, N.J.) and placed in sterile plastic petri dishes. The filters were allowed to dry at room temperature in a biocontainment hood, and disks of 6-mm diameter were punched and stored in screw-cap vials at −20°C until used. Each small disk retained approximately 5 μl of serum, and the required dilution of eluate was made by soaking the appropriate number of disks in phosphate-buffered saline (PBS), pH 7.2, containing 0.05% Tween 20 (PBST). The blood eluate was used for the ELISA after storage overnight at 4°C and gentle shaking at room temperature for 10 min.

Sera from serial bleeds of four cattle, two experimentally infected with epizootic hemorrhagic disease of deer virus (EHDV) and two vaccinated with inactivated EHDV (2), were tested for their reactivity against BTV in the PN, MCF, AGID, I-ELISA, and C-ELISA tests.

ELISA procedures. I-ELISA was based on previously described methods (11, 16) and adapted according to our routine ELISA procedures (1, 12). In brief, flat-bottomed 96-well polystyrene plates (Linbro; Flow Laboratories, Mississauga, Ontario, Canada) were optimally coated by passive adsorption with 100 μl of BTV stock antigen and the control cell antigen, diluted 1:200 in 0.05 M carbonate buffer, pH 9.6, per well for 18 h at 4°C. The wells were emptied and washed four times with PBST. Sera or blood eluates were tested at a single dilution of 1:100 in PBST containing 3% bovine serum albumin (BSA), and 100-μl samples were applied in duplicate in a diagonal sample placement pattern (12, 18). Following an incubation period of 3 h at 25°C, the wells were again washed and filled with 100 μl of either 1:2,000 dilution of horseradish peroxidase-labeled rabbit anti-bovine immunoglobulin G (IgG) (H+L) (lot 2225; Miles Laboratory Ltd., Rexdale, Ontario, Canada) or 1:3.000 anti-ovine IgG (H+L) conjugate (lot 23410; Cappel, Cooper Biomedical, West Chester, Pa.) in PBST. After incubation for 60 min at 37°C, the conjugate solution was discarded and the plates were washed with PBS without Tween. The wells were filled with 100 μl of substrate solution containing 1.0 mM ABTS [2,2-azino-di-(3-ethylbenzthiazoline-sulfonic acid)]–4 mM H_2O_2 in 50 mM sodium citrate, pH 4.0, and the plates were then shaken continuously at 25°C for precisely 30 min. Optical density (OD) values were then recorded at 414 nm with a Titertek Multiskan MC plate reader (Flow Laboratories), interfaced with a computer. By means of a custom computer program developed at this institute, the OD value for each serum sample was expressed as the mean OD value resulting from the reaction with BTV antigen minus the mean OD value resulting from the reaction with the control antigen. For comparative and not necessarily diagnostic applications, samples were considered positive if their OD values, as calculated above, were higher than the mean OD values for the corresponding preinoculation serum of the animal when tested against the BTV antigen only.

The C-ELISA was a modification of the blocking ELISA described previously (2) except that the MAb was added immediately after the test serum. The MAb (3-17-43) to BTV (hybridoma cell culture supernatant) was received from Animal Virus Research Institute, England, and stored at −20°C until use. Plates, diluent, and enzyme substrate were as used in the I-ELISA. Following BTV antigen adsorption to the wells and a wash, as described above, 50 μl of serum or blood eluate sample diluted 1:5 in PBST with 3% BSA was added to duplicate wells in a diagonal pattern (12, 18). Immediately, each well received 50 μl of MAb at a predetermined dilution of 1:50 in PBST with 3% BSA. After incubation at 25°C for 3 h and washing, the wells were filled with 100 μl of a 1:500 dilution of horseradish peroxidase-labeled rabbit anti-mouse IgG (H+L) (lot 30306; Zymed Laboratory, Inc., South San Francisco, Calif.) in PBST with 2% normal bovine serum. Following incubation for 60 min at 37°C, the conjugate solution was discarded, and then plates were treated as described above for I-ELISA.

Results were expressed as percent inhibition and were derived from the mean OD values for each sample by the following formula: % inhibition = 100 − [(OD test sample)/(OD negative serum)] × 100.

For comparative purposes and not necessarily diagnostic applications, samples were considered positive if they inhibited 40% or more of the MAb activity (12).

Other serological tests. The AGID (15), MCF (4), and PN (20) tests were the standard methods used at this institute for detection of antibodies to BTV.

RESULTS

Moderate pyrexia and depression were the only clinical signs observed among the experimentally infected calves and sheep. All animals responded serologically to BTV infection, and the humoral antibody profiles as found in their sera and whole blood by both ELISAs are shown in Fig. 1 and 2. The comparative seroconversion time for each animal by the C-ELISA, I-ELISA, AGID, MCF, and PN tests are shown in Table 1.

BTV antibody was demonstrable by C-ELISA in serum and blood eluate samples as early as 7 days postinfection (DPI) for one sheep (no. 52) and blood eluate of one calf (no. 444) and one sheep (no. 53). Samples collected 9 DPI and thereafter from all animals were positive by C-ELISA (Table 1). The I-ELISA detected BTV antibody by 9 DPI in the sera and blood eluates of all sheep except no. 52, which became positive at 16 DPI. However, the I-ELISA became positive at 14 and 26 DPI for the blood eluates and sera of cattle, respectively. Seroconversion was demonstrable by the AGID test beween 9 and 12 DPI in all animals, with the exception of one calf (no. 445), which seroconverted by 26 DPI (Table 1). Relative to the ELISA and AGID tests, seroconversion as detected by the MCF test was delayed. Apart from two sheep (nos. 52 and 55) that seroconverted on 16 and 19 DPI, sera from the rest of the animals were not positive by MCF until 26 DPI. By the PN test, all animals had seroconverted by 14 DPI except one calf (no. 4), which was positive by 26 DPI (Table 1). For both cattle and sheep, maximal antibody activity was evident earlier in the C-ELISA than in the I-ELISA and remained relatively stable throughout the 6-month period of the experimental infection (data after 75 DPI are not shown). Relative to C-ELISA, the rate of BTV antibody increase during the first month as measured by I-ELISA appeared to be slower in both cattle and sheep and peaked later during the course of infection (Fig. 1 and 2).

The antibody profiles measured by C-ELISA in whole blood eluate samples from sheep and cattle were comparable to those measured in the serum samples (Fig. 1 and 2). Slightly higher antibody activity was observed for blood in the I-ELISA than for serum, especially for samples collected later during infection (Fig. 1 and 2).

The results of the AGID and PN tests for serum samples collected from sheep and cattle are summarized and shown

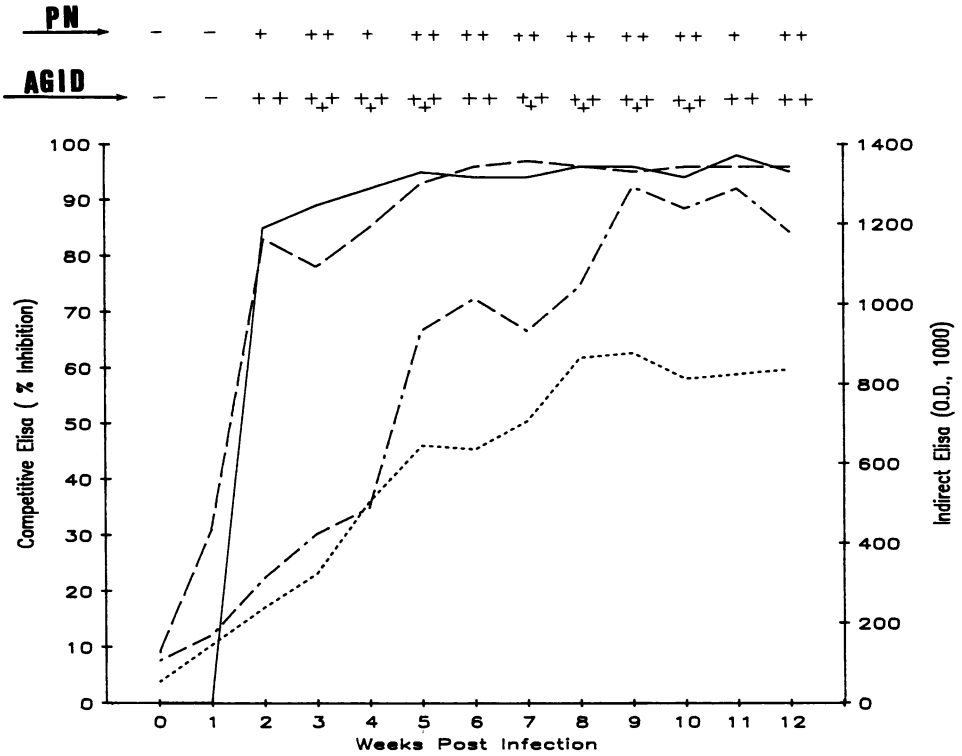

FIG. 1. Comparative development of BTV antibody measured by C-ELISA (——, serum samples; — —, whole blood eluates) and I-ELISA (– – –, serum samples; — · —, whole blood eluates), AGID (−, negative; +, curved precipitating line; ++, weak identity line; +++, strong identity line), and PN (−, negative; +, titer of 1:100 to 1:500, ++, titer of ≥1:500) tests in samples from three cattle infected with type 10 BTV.

in Fig. 1 and 2 along with ELISA antibody profiles. Antibody to BTV was demonstrated by the AGID test on 9 and 12 DPI in sheep and cattle sera, respectively (Table 1), when weak lines of identity (2+) with the reference BTV antiserum were observed. Strong identity lines (3+) developed when sheep and cattle sera were collected at 14 and 19 DPI, respectively (Fig. 1 and 2). Anti-BTV antibody was not demonstrable by the MCF test until 16 and 26 DPI in sheep and cattle sera, respectively (Table 1). At this time sera had relatively low MCF antibody titers (1:10 to 1:80), which increased (1:160 to 1:320) only in samples collected from sheep at 40 DPI or more (Fig. 3 and 4). Sera collected from one sheep (no. 54) from 9 to 19 DPI were unfit for the MCF test, although no anticomplementary activity was detected. Anti-BTV antibodies were demonstrable by PN tests from 12 and 14 DPI in sera collected from sheep and cattle, respectively (Table 1). The PN titers increased (≥1:500) and remained stable from 14 DPI in both sheep and cattle sera (Fig. 1 and 2). Generally the humoral response in sheep was found to be less variable than in cattle when assayed by the PN or AGID tests (Fig. 1 and 2).

None of the pre- or postinoculation sera collected from EHDV-infected cattle demonstrated false-positive reactions in the ELISA, MCF, or PN tests. However, false-positive reactions were observed in the AGID test in some postinoculation serum samples from three of four cattle.

DISCUSSION

The group-specific serological tests that are commonly used for diagnosis of BTV are the AGID and MCF tests (7). The AGID test, while simple and rapid, may be difficult to interpret, may lack sensitivity, is not quantitative, and may cross-react with other orbiviruses (e.g., EHDV) (7). The MCF test, while quantitative, is cumbersome to perform and cannot be used with anticomplementary or hemolysed sera (7, 20). Two types of ELISA, namely an indirect (11, 16) and a blocking (2) test, have recently been described and are potential candidates to replace the widely used AGID test. We have modified and successfully adapted these two ELISAs for measurement of BTV antibody in the sera of experimentally infected cattle and sheep.

In the C-ELISA, by simultaneous incubation of the test serum and the group-specific MAb, we have reduced the time requirement of the test to that of the I-ELISA. In addition to earlier detection of anti-BTV antibody by the C-ELISA in cattle and sheep sera (Table 1), a sharp rise in anti-BTV antibody activity was evident in the sera of these animals between the first and second week after infection (Fig. 1 and 2). Relative to the gradual increase of anti-BTV antibody as measured by the I-ELISA, this sudden increase in antibody activity as demonstrable by the C-ELISA may reflect the characteristics of early serum antibodies to BTV (e.g., affinity, avidity, immunoglobulin class, etc.). In a recent report on the application of a C-ELISA for detection of human cytomegalovirus antibody, lower affinity of antibody produced in the early stage of disease was incriminated in discrepancies between results obtained with I-ELISA (22).

The I-ELISA, an adaptation of a routine ELISA technique at this institute (1, 12), appears to be more sensitive in detecting anti-BTV antibody in sheep sera than in cattle sera collected during the early phase after experimental infection. In three of four sheep, BTV antibody was demonstrable as

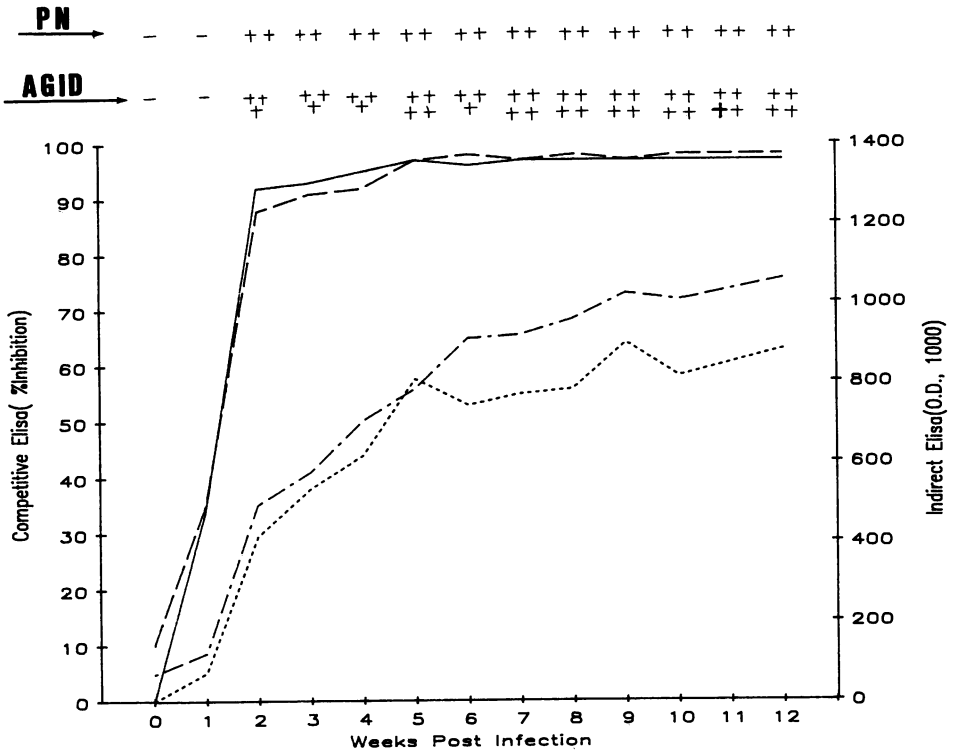

FIG. 2. Comparative development of BTV antibody measured by C-ELISA, I-ELISA, AGID, and PN tests in samples from four sheep infected with type 10 BTV. See the legend to Fig. 1 for symbols.

early as 9 DPI (Table 1), but in cattle seroconversion was evident only at 26 DPI. In addition to the possibility that bovine antibody produced in the early stage of infection is of low affinity or of a different isotype, other dissimilarities between BTV infection in cattle and sheep may explain the delayed demonstrability of BTV antibody in bovine sera.

As was reported with the blocking ELISA (2), we found that cattle sera having anti-EHDV antibody (e.g., complement fixation titers of 1:5 to 1:40) did not cross-react with BTV antigen in either the C-ELISA or I-ELISA. Unlike the AGID test, it appears that the I-ELISA for BTV is as antigen specific as the C-ELISA and may be applied to field sera without interference by anti-EHDV antibodies.

A comparison of several standard serological assays used at this institute for the detection of BTV is shown in Table 1 and Fig. 1 to 4. The C-ELISA is immunologically the most sensitive test with which we have been able to detect anti-BTV antibody. The I-ELISA compares favorably with the AGID test; however, changes in the characteristics of the precipitation lines on testing sequential serum samples would indicate that the AGID test is less reliable. Transient positive AGID results and false-negatives with this group-specific test have also been reported elsewhere (9, 19). Similar to a previous report (16), our preliminary results with bovine and sheep field sera indicate that the I-ELISA detects anti-BTV antibody in a number of sera that are AGID negative (data are not shown). However, complete agreement was observed between AGID-positive reactors when tested by ELISA. Apart from minor differences in the detection of seroconversion during the early phase after experimental infection, our study shows, in general and similar to other reports (8, 11), that the immunological sensitivity of both ELISAs is comparable to that of the PN or MCF test. However, like other reports (7, 20), we found that the PN test was very time-consuming and required cell cultures and considerable technical expertise to conduct. A number of serum samples from sheep were also unsuitable for the MCF test.

In this study, anti-BTV antibody was demonstrable in whole blood eluates earlier than in sera collected from cattle and sheep (Table 1). The anti-BTV antibody profiles from the testing of whole blood eluates in both ELISAs were similar to those of corresponding serum samples from cattle as well as sheep (Fig. 1 and 2). The slightly higher enzymatic activity that was observed in the I-ELISA with blood eluates compared with serum samples could be due to the additional

TABLE 1. Comparison of seroconversion time in cattle and sheep after BTV infection as demonstrated by ELISA and other standard tests

Animal no.	Seroconversion detected (DPI)				
	ELISA[a]		AGID	MCF	PN
	C	I			
Calves					
4	9 (9)	26 (14)	12	26	26
444	9 (7)	26 (14)	12	26	12
445	9 (9)	26 (14)	26	26	14
Sheep					
52	7 (7)	16 (9)	9	19	12
53	9 (7)	9 (9)	9	26	14
54	9 (9)	9 (9)	12	26	9
55	9 (9)	9 (9)	9	16	12

[a] Values shown are for serum and, in parentheses, for whole blood samples.

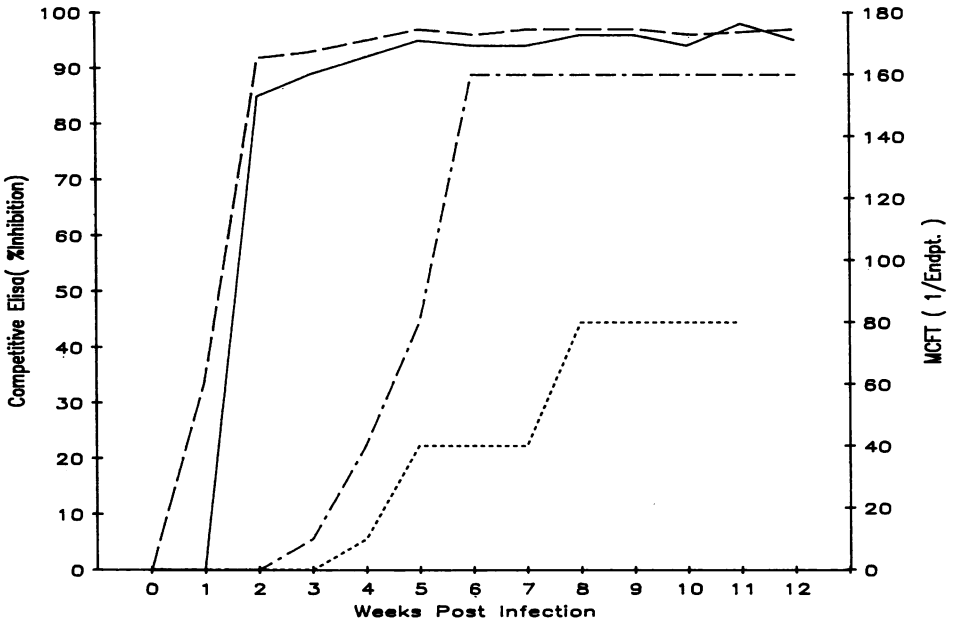

FIG. 3. Comparative development of BTV antibody measured by C-ELISA in three cattle sera (— — —) and four sheep sera (——) and by the MCF test in cattle sera (– – –) and sheep sera (— · —).

BTV antibodies in the blood cells. The usefulness of whole blood collected and dried on filter paper as a substitute for serum in the ELISAs has been previously reported for other antiviral antibody assays (3, 10). The successful use of whole blood eluates in detecting BTV antibody by ELISA could eliminate the need for collection of large volumes of blood, serum separation, bacterial contamination through excess handling and transportation, and the need for excessive freezer space for storage of sera. Several reports (5, 10) have demonstrated satisfactory storage of blood dried onto filter paper disks for up to 1 year at 4°C. In the present study no loss of activity could be detected in the ELISA after 6 months of storage at −20°C.

The performance of both the C-ELISA and I-ELISA in detecting anti-BTV antibody under the present test conditions would suggest that either test may be suitable as a routine diagnostic method and may have the potential to replace conventional tests. Furthermore, the use of an MAb in C-ELISA not only facilitates the standardization of BTV diagnostic tests between laboratories, it makes it possible to

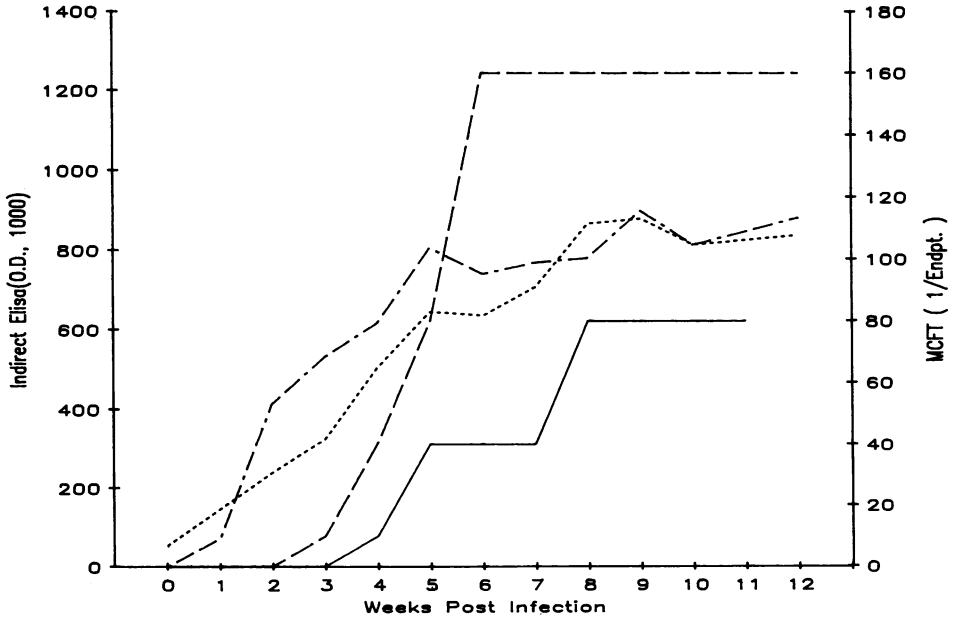

FIG. 4. Comparative development of BTV antibody measured by the I-ELISA for three cattle sera (– – –) and four sheep sera (— · —) and the MCF test for cattle sera (——) and sheep sera (— — —).

test sera from animals, e.g., wild ruminants, for which no antispecies conjugates are available. An extensive field survey is now under way to determine and compare the diagnostic sensitivity and specificity of both the C- and I-ELISAs.

ACKNOWLEDGMENTS

We thank Gilles Dulac and Peter Ide for their advice and encouragement in the course of this work. We acknowledge the technical assistance of Alain Tremblay, Richard Charron, Barbara Leung, Rita Groulx, and Jean-Marc Lapointe for their technical help. We also thank David Gall and Walter Kelly for their computer expertise.

LITERATURE CITED

1. **Afshar, A., P. F. Wright, and G. C. Dulac.** 1986. Development and evaluation of an indirect enzymeimmunoassay for detection of porcine antibodies to pseudorabies virus. Can. J. Vet. Res. **50**:422–426.
2. **Anderson, J.** 1984. Use of monoclonal antibody in a blocking ELISA to detect group specific antibodies to bluetongue virus. J. Immunol. Methods **74**:139–149.
3. **Banks, M.** 1985. Detection of antibodies to Aujeszky's disease virus in whole blood by ELISA disc. J. Virol. Methods **12**:41–48.
4. **Boulanger, P., G. M. Ruckerbauer, G. L. Bannister, D. P. Gray, and A. Girard.** 1967. Studies on bluetongue. III. Comparison of two complement fixation methods. Can. J. Comp. Med. **31**: 166–170.
5. **Brugh, M., and C. W. Beard.** 1980. Collection and processing of blood samples dried on paper for microassay of Newcastle disease virus and avian influenza virus antibodies. Am. J. Vet. Res. **41**:1495–1498.
6. **Campbell, C. H., and M. J. Grubman.** 1985. Current knowledge on the biochemistry and immunology of bluetongue, p. 58–79. *In* R. Pandey (ed.), Progress in veterinary microbiology and immunology, vol. 1. Karger, Basel.
7. **Della-Porta, A. J., I. M. Parsons, and D. A. McPhee.** 1985. Problems in the interpretation of diagnostic tests due to cross-reactions between orbiviruses and broad serological responses in animals, p. 445–453. *In* T. L. Barber, M. M. Jochim, and B. I. Osburn (ed.), Bluetongue and related orbiviruses. Alan R. Liss, Inc., New York.
8. **Hübschle, O. J. B., R. J. Lorenz, and H. D. Matheka.** 1981. Enzyme-linked immunosorbent assay for detection of bluetongue virus antibodies. Am. J. Vet. Res. **42**:61–65.
9. **Jochim, M. M.** 1985. An overview of diagnostics for bluetongue, p. 423–433. *In* T. L. Barber, M. M. Jochim, and B. I. Osburn (ed.), Bluetongue and related orbiviruses. Alan R. Liss, Inc., New York.
10. **Lana, D. P., W. W. Marquardt, and D. B. Snyder.** 1983. Comparison of whole blood dried on filter paper and serum for measurement of the temporal antibody response to avian infectious bronchitis virus by enzyme-linked immunosorbent assay. Avian Dis. **27**:813–821.
11. **Manning, J. S., and M. F. Chen.** 1980. Bluetongue virus: detection of antiviral immunoglobulin by means of enzyme-linked immunosorbent assay. Curr. Microbiol. **4**:381–385.
12. **Nielsen, K., and P. F. Wright.** 1984. Enzyme immunoassay and its application to the detection of bovine antibody to *Brucella abortus*, p. 1–121. Agriculture Canada, Animal Diseases Research Institute, Nepean, Ontario, Canada.
13. **Oberst, R. D., K. R. E. Squire, J. L. Stott, R. Y. Chung, and B. I. Osburn.** 1985. The coexistence of multiple bluetongue virus electropherotypes in individual cattle during natural infection. J. Gen. Virol. **66**:1901–1909.
14. **Pearson, J. E., E. A. Carbrey, and G. A. Gustafson.** 1985. Bluetongue and related orbivirus diagnosis in the United States, p. 469–475. *In* T. L. Barber, M. M. Jochim, and B. I. Osburn (ed.), Bluetongue and related orbivirus. Alan R. Liss, Inc., New York.
15. **Pearson, J. E., and M. M. Jochim.** 1979. Protocol for the immunodiffusion test for bluetongue. Am. Assoc. Vet. Lab. Diag. Proc. **22**:463–471.
16. **Poli, G., J. L. Stott, Y. S. Liu, and J. S. Manning.** 1982. Bluetongue virus: comparative evaluation of enzyme-linked immunosorbent assay, immunodiffusion, and serum neutralization for detection of viral antibodies. J. Clin. Microbiol. **15**: 159–162.
17. **Sellers, R. F.** 1981. Bluetongue and related diseases, p. 567–584. *In* E. P. J. Gibbs (ed.), Virus diseases of food animals, vol. 2. Academic Press, Inc., New York.
18. **Stemshorn, B. W., D. J. Buckely, G. St.-Amour, C. S. Lin, and J. R. Duncan.** 1983. A computer interfaced photometer and systematic spacing of duplicates to control within-plate enzyme immunoassay variation. J. Immunol. Methods **61**:367–375.
19. **Stott, J. L., and B. I. Osburn.** 1985. Bluetongue virus infection of cattle: current status and research trends. Proc. Annu. Meet. U.S. Anim. Health Assoc. **89**:121–129.
20. **Thomas, F. C., A. Girard, P. Boulanger, and G. M. Ruckerbauer.** 1976. A comparison of some serological tests for bluetongue virus infections. Can. J. Comp. Med. **40**:292–297.
21. **Thomas, F. C., N. G. Willis, G. M. Ruckerbauer, A. Girard, and P. Boulanger.** 1975. A comparison of methods for monitoring viremia in a bluetongue virus infected bovine. Am. Assoc. Vet. Lab. Diag. Proc. **18**:175–185.
22. **Wreghitt, T. G., J. Hicks, J. J. Gray, and C. O'Connor.** 1986. Development of a competitive enzyme-linked immunosorbent assay for detecting cytomegalovirus antibody. J. Med. Virol. **18**:119–129.