Bluetongue Virus: Comparative Evaluation of Enzyme-Linked Immunosorbent Assay, Immunodiffusion, and Serum Neutralization for Detection of Viral Antibodies

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Comparative studies on the detection of bovine serum immunoglobulin G antibodies to bluetongue virus with an enzyme-linked immunosorbent assay, an immunodiffusion method, and a serum neutralization assay demonstrated complete concordance between the enzyme-linked immunosorbent assay and the serum neutralization assay results. However, the immunodiffusion method failed to detect bluetongue virus antibody in a substantial number of sera found to possess bluetongue virus immunoglobulin G with the enzyme-linked immunosorbent assay.

Bluetongue virus (BTV) is an arthropod-borne orbivirus (2, 10, 11). The virus is widely distributed and is responsible for a number of disease syndromes of ruminants (1, 3, 7). Infection with BTV is most often monitored by detection of BTV antibodies in serum. Recently, we adapted the enzyme-linked immunosorbent assay (ELISA) system to the detection of anti-BTV immunoglobulin G (IgG) in sheep serum (9). Our findings have been confirmed in a study which also reported the detection of antiviral IgG in two bovine sera (5). In the present paper, we describe studies on the ELISA for the detection of anti-BTV IgG in bovine serum and compare ELISA results with those obtained with immunodiffusion and serum neutralization assays.

The preparation of the BTV antigen for the ELISA was modified from that previously reported (9). Preliminary studies indicated that a higher degree of antigen purity was required for ELISA detection of BTV antibody in the cattle serum than was necessary for sheep serum. Briefly, the preparation of the BTV antigen was as follows. Vero cells infected with BTV serotype 10 or 11 were harvested for 24 to 36 h postinfection, pelleted, and sonicated in lysis buffer (1% Triton X-100, 0.2 M KCl, 0.02 M MgCl$_2$, 0.0072 M CaCl$_2$, 0.002 M Tris-hydrochloride [pH 8.6]). Cell extracts were cleared of large debris by centrifugation at 11,000 × g for 10 min. The pellet was resuspended, sonicated, and cleared as before. The supernatant fluids were combined and layered onto a 40% sucrose cushion and centrifuged at 85,000 × g for 100

min. The pellet was resuspended in 0.002 M Tris-hydrochloride buffer (pH 8.6), applied to a 10 to 40% sucrose density gradient, and centrifuged for 70 min at 113,000 × g. The light scattering band from gradients was collected, diluted in Tris-hydrochloride buffer, pelleted at 195,000 × g for 90 min, resuspended in Tris-hydrochloride buffer, and assayed for protein concentration (8). Antigen was stored at 4°C.

The purity of the antigen preparation was assessed by negative-stain electron microscopy. BTV particles appeared to be free of contaminants (Fig. 1). The morphology of the particles, a diffuse outer capsid layer surrounding a less-distinct core, is consistent with previous reports (4, 10).

Bovine sera were obtained from cattle grazing in the foothills of the Sierra Nevada Mountains and from cattle which had been experimentally infected with BTV serotype 10 or 11. In addition, sera obtained from 30 cattle from Switzerland (a BTV-free country) were used as negative controls. These cattle were maintained at the Veterinary Institute, University of Zurich, Switzerland.

The ELISA procedure used was as follows. A Gilford EIA-50 (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) was used. BTV antigen was diluted in 0.1 M NaHCO$_3$, pH 9.6, and 150 ng of antigen was added to each well of the EIA-50 cuvette plate. After incubation at 37°C for 3 h or at 25°C for 16 h, wells were washed four times with saline-Tween 20 buffer (0.15 M NaCl, 0.05% Tween 20). Serum samples were diluted with serum buffer (0.15 M NaCl, 2 mM EDTA, 1% bovine serum albumin, 0.02% NaN$_3$, 0.05% Tween 20, and 50 mM Tris-hydrochloride [pH 7.4]), and 100 µl of diluted serum

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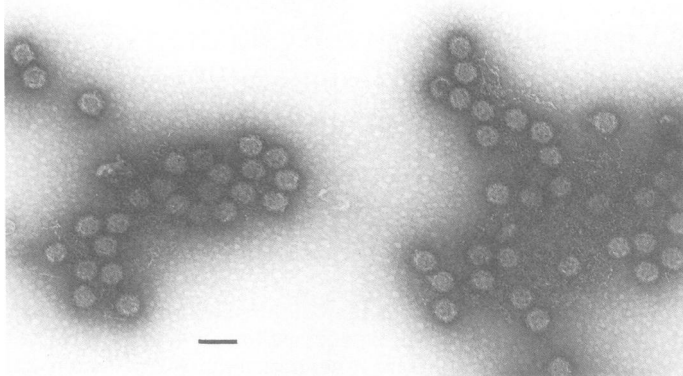
FIG. 1. Photoelectron micrograph of BTV antigen preparation. Virus was isolated as described in the text, absorbed to a Formvar-coated electron microscope grid, and immersed in a drop of 2.5% phosphotungstic acid before being air dried and examined in a Zeiss electron microscope. Bar indicates 100 nm.

was added to the wells. Duplicate wells were assayed. After incubation for 2 h at 37°C, the wells were emptied and washed four times as described above. To detect bovine IgG attached to the antigen-charged wells, 100 μl of antibovine IgG conjugated with horseradish peroxidase (Cappel Laboratories, Downingtown, Pa.) which had been diluted 1:100 with serum buffer was added. Serial dilution of conjugated serum demonstrated that optimal results were obtained with a serum dilution of 1:100. After an incubation period of 60 min at 37°C, the unbound conjugate was removed and the wells were washed four times with saline-Tween 20 buffer. Peroxidase bound to the wells was detected by adding 300 μl of freshly prepared substrate [0.2 mM 2,2-azino-di-(3-ethyl)benthiazoline sulfonic acid, 2 mM H_2O_2, 50 mM citric acid (pH 4)] and allowing the reaction to proceed for 10 min before measuring the absorbance at 405 nm.

The immunodiffusion assay used was the micro-agar gel precipitin (AGP) test developed for serodiagnosis of BTV infection (6). Serum neutralization assays were carried out by using dilutions of heat-inactivated sera obtained from cattle experimentally infected with BTV serotype 11. Serum samples were diluted in Eagle minimum essential medium and mixed in wells of microtiter plates with an equal volume of serotype 11 virus suspension containing 100 50% tissue culture infective doses to give a total volume of 100 μl. Four replicate wells were used for each serum dilution. After incubation for 90 min at 37°C, 100 μl of tissue culture medium containing 120,000 L cells per ml was added to each well, and the cultures were incubated at 37°C. After 5 days, the monolayers were stained with crystal violet, and the serum neutralization titers were calculated.

Establishment of a negative cutoff ELISA absorbance value was essential to a comparison between ELISA, AGP, and serum neutralization results. To obtain this value, sera from 30 BTV-free cattle yielding a negative response in serum neutralization tests were used to assess the ELISA values for BTV-seronegative cattle. The absorbance values for these sera gave a mean value and a standard deviation of 0.195 ± 0.035 absorbance units. The maximum ELISA absorbance interpreted as negative was three standard deviations above the mean, or 0.3 absorbance units.

One goal of the present study was to determine the correlation between the anti-BTV IgG content and ELISA absorbance. A series of assays was performed by using serial twofold dilutions of 12 bovine sera. The result (Fig. 2) demonstrated the expected relationship between absorbance values and serum dilutions. Absorbance values for 10 of 12 test serum samples at a 1:100 dilution were in excess of the cutoff value of 0.3 absorbance units and therefore were determined to be BTV seropositive. Tests with serum dilutions below 1:100 appreciably increased the background or base-line absorbance values of BTV-antibody-negative sera. Based on these results, a working dilution of bovine sera of 1:100 was selected and, as will be shown later, it was found that at this dilution both weakly positive sera and strongly positive sera gave rise to readily detectable ELISA absorbance values. The above studies demonstrated that BTV-ELISA can provide quantitative information from an assay at a fixed dilution.

The relative discriminatory abilities and sensitivities of the ELISA and AGP were compared for 126 serum samples. Results of the ELISA and AGP were in agreement for 111 of the 126
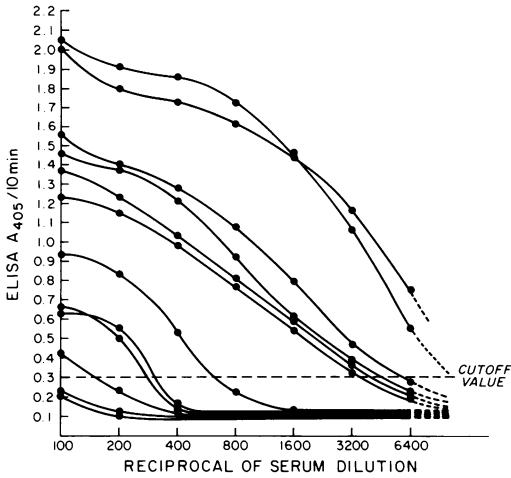
FIG. 2. ELISA absorbance profiles for serial dilutions of 12 serum samples. The cutoff value represents three standard deviations above the mean absorbance value determined for 30 BTV seronegative samples. A_{405}, Absorbancy at 405 nm.

FIG. 3. Comparison of the ELISA and AGP test. ELISA absorbance values for 126 serum samples which had been determined to be AGP seronegative (−), positive (+), or strongly positive (++). The strength of the positive AGP reactions relates to the intensity of the precipitin band. The cutoff value is the same as that shown in Fig. 2. A_{405}, Absorbancy at 405 nm.

sera. However, 15 AGP-seronegative samples were found to yield absorbance values higher than the 0.3 absorbance ELISA negative cutoff value (Table 1). In contrast, no ELISA-seronegative samples were found to be AGP seropositive. The correlation between ELISA absorbance values and AGP test results for the 126 serum samples is shown in Fig. 3. An increase in ELISA absorbance values with AGP band intensity was clearly apparent. The variation in absorbance values within a given AGP group is as expected, since ELISA absorbance values are a quantitative measure of anti-BTV IgG concentration. Since the results of these studies demonstrated that the ELISA did not perfectly correlate with the AGP test and indicated that AGP could yield false negatives and that the ELISA was the more sensitive of the two methods, it was of interest to examine the correlation between the ELISA and serum neutralization.

TABLE 1. Comparison of the AGP test and the ELISA

AGP test[a]	ELISA[b]	
	Positive	Negative
Positive	62	0
Negative	15	49

[a] Sera giving lines of identity with the positive reference serum were scored as positive regardless of the intensity or position of the band.

[b] Mean absorbance units ± standard deviations, 0.67 ± 0.035 for positive results; mean absorbance units ± standard deviations, 0.090 ± 0.019 for negative results.
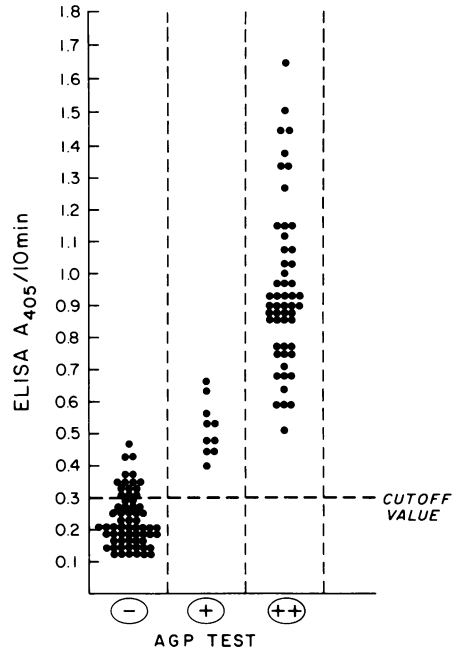
The correlation between ELISA and serum neutralization was determined by performing the serum neutralization assays on 30 selected sera which at a dilution of 1:100 had yielded negative or positive ELISA absorbance values. When the results of the two tests were compared, there was complete correlation (Fig. 4). Serum samples in which no neutralizing antibody could be detected had ELISA absorbance values that were less than the BTV ELISA negative cutoff value of 0.3 absorbance units. As neutralization titers increased, ELISA absorbance values increased as well. Two of the samples tested which were AGP negative but ELISA positive had a serum neutralization titer of 100.

For the present study, we established optimal conditions for an ELISA system for the measurement of bovine IgG to BTV. Our results on 126 cattle sera support and extend the previous report on the detection of BTV antibody in bovine sera (5). Clearly, the ELISA is demonstrated to be a sensitive and reliable method for the detection of BTV IgG in cattle. In addition, we found concordance between the results obtained with the ELISA and serum neutralization over a wide range of dilutions.
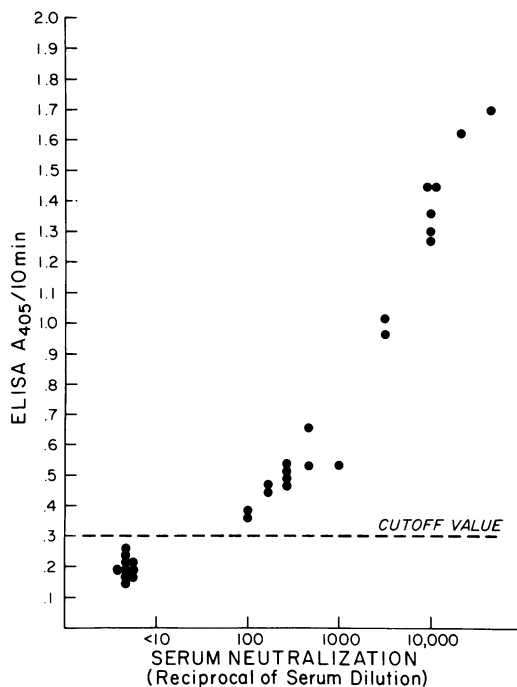
FIG. 4. Comparison of ELISA and serum neutralization. ELISA absorbance values for 30 serum samples as a function of titer of serum-neutralizing antibody are shown. The cutoff value is the same as that shown in Fig. 2. A_{405}, Absorbancy at 405 nm.

when assayed by ELISA (data not shown). These results, together with those previously reported (5, 7), indicate that the ELISA method, using any serotype as antigen, will most likely detect serum antibody to any of the 20 internationally recognized BTV serotypes.

The development of the ELISA for BTV makes it feasible to readily quantitate the immune status of infected animals. We are presently using the ELISA for quantitation of the kinetics of the humoral antibody response to BTV infection.

Although there was substantial agreement between ELISA and AGP test results, the concordance was not perfect in that 23% of the serum samples determined to be negative for BTV antibody by AGP were found to be positive by ELISA. On the basis of our comparative assays, the micro-AGP test, like the ELISA, appears to detect BTV antibody in cattle serum where the antibody is present in relatively high titers; however, unlike the ELISA, the AGP method does not appear to be sufficiently sensitive to detect viral antibody present at low concentrations.

We reported in a previous paper that the BTV-ELISA did not allow a distinction to be made between BTV serotypes 10, 11, 13, and 17, but rather that BTV antibody reacted with BTV antigen common to all four serotypes (9). A similar finding was reported when serotypes 1, 4, 10, and 20 were compared (5). A preliminary study in which BTV serotypes 10 and 11 were used as antigen demonstrated that BTV antibody in cattle sera showed heterotypic reactivity

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