Simple Procedure for Preparation of Bluetongue Virus and Epizootic Hemorrhagic Disease Virus Antigens for Agar Gel Immunodiffusion

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A simplified procedure was developed for preparing soluble antigen from two related orbiviruses, bluetongue and epizootic hemorrhagic disease viruses, for agar gel immunodiffusion. The antigens gave excellent results in both micro-agar gel diffusion (agar gel precipitin) and macro-agar gel diffusion (bluetongue immunodiffusion). Minor modification in the spatial arrangement of reference antisera, commonly utilized in the agar gel immunodiffusion tests, was employed to reduce the possible development of false-positive reactions. The procedures for antigen preparation were inexpensive and did not require elaborate filtration or high-speed centrifugation. Stability of antigen preparations at 5°C was excellent (in excess of 3 years for bluetongue virus and 2 years for epizootic hemorrhagic disease virus).

Serological diagnosis for previous exposure to bluetongue virus (BTV) has been the subject of numerous scientific communications in recent years. Various tests have been adapted, with various degrees of sensitivity and specificity, to detect BTV antibodies (1-4, 6, 11-15, 18-22). Klontz et al. first described an agar gel immunodiffusion (AGID) test for BTV utilizing antigen preparations from several different sources (mouse brain, chicken embryo, and cell culture) (14). A micro-AGID (agar gel precipitin) test was later described that utilized cell culture antigen (10, 11). A macro-AGID (bluetongue immunodiffusion) test for BTV (16) has recently been adopted by the U.S. Department of Agriculture as a routine serological test for the detection of BTV antibodies (17).

Owing to the widespread acceptance of AGID for detecting antibodies to BTV, an inexpensive and simple procedure for the preparation of BTV soluble antigen was developed. This procedure was adapted for preparation of soluble antigen for epizootic hemorrhagic disease virus (EHDV), another orbivirus related to BTV, which commonly infects wild ruminants, goats, and cattle.

MATERIALS AND METHODS

Viruses. BTV (serotypes BTV-10, -11, -13, and -17) and EHDV (serotypes EHDV-1 and -2) were kindly supplied by T. L. Barber, U.S. Department of Agriculture Agricultural Research Service, Denver, Colo. Virus stocks were prepared in baby hamster kidney (BHK-21) cells and were stored in the presence of buffered lactose peptone at -80°C (5).

Antisera. Reference antisera for the BTV AGID test were prepared by subcutaneous and intradermal inoculation of sheep with (i) 10⁶ PFU of cell culture source virus prepared in BHK-21 cells and (ii) 2.5 × 10⁵ embryo lethal doses of sheep blood source virus. Four serotypes of BTV (10, 11, 13, and 17) were utilized as inoculum. A second inoculation of virus was given at 42 days. Sera were collected weekly through 28 days after the second inoculation.

Reference antisera for the EHDV AGID test was obtained from M. Jochim (Agricultural Research Service, Denver, Colo.). Additional reference sera were prepared by inoculation of sheep, mature cattle, and fetal calves (90 days gestation) with 10⁶ PFU of either EHDV-1 or EHDV-2. Sheep and cattle were given a second inoculation at 42 days; sera were collected weekly through 28 days after the second inoculation. Sera from fetuses were collected approximately 180 days post-inoculation.

Soluble antigens. Preparation of BTV and EHDV soluble antigen was identical. Four serotypes of BTV (10, 11, 13, and 17) and two serotypes of EHDV (1 and 2) were utilized to make soluble antigen. Viruses were propagated in African green monkey kidney (VERO Maru) (Agricultural Research Service, Denver, Colo.) cells in 150-cm² tissue culture flasks. Cell monolayers of 90% confluency were washed twice with Eagle minimal essential medium followed by adsorption (90 min at 37°C) of 6.0 ml of virus suspension which had a titer of 10⁶-⁵ PFU/ml. After adsorption, 60 ml of maintenance medium (Eagle minimal essential medium with 2% fetal bovine serum, 290 µg of streptomycin per ml, 200 U of penicillin per ml, and 2.5 µg of amphotericin B per ml) was added to each flask.

Cell culture medium was harvested when the cytopathic effect reached 90% (approximately 48 h for BTV and 72 h for EHDV). Cell debris was removed by centrifugation at 8,000 × g for 2 h at 5°C. During the...
initial development of the viral soluble antigen, the clarified supernatant was subjected to further centrifugation at 100,000 × g for 4 h to remove complete viral particles and cellular debris. After clarification of the supernatant, the viral proteins were concentrated 50 times by negative pressure dialysis. S and S collodion bags (nitrocellulose) with a molecular weight exclusion of 25,000 (Schleicher & Schuell Co., Keene, N.H.) were utilized to concentrate the viral antigens; a volume of 300 ml was concentrated per bag. A side-arm flask connected to the concentration apparatus with sterilized amber latex IV tubing (0.5 by 0.125 in. [1.27 by 0.32 cm]) was utilized as a reservoir for the unconcentrated antigen. After concentration (48 to 72 h at 25°C), the antigen preparations were stored at 5°C in the presence of 0.01% sodium azide (final concentration).

**AGID.** Two AGID procedures were utilized to test the BTV and EHDV soluble antigen preparations. The micro-AGID test (10, 11) and macro-BTID test (17) were used with minor modification in the placement of reference antisera in the gel template (Fig. 1). Positive reference BTV antiserum was placed in the top and bottom wells only rather than in alternate wells as with the standard placement. The reason for utilizing reference serum in a well adjacent to the test sera was to enhance the detection of small amounts of antibody in test sera. The template for the macro-BTID test was supplied by J. Pearson (Veterinary Services Laboratory, Ames, Iowa). Both tests utilized 0.9% agarose in physiological saline. Whereas the micro-AGID test was stained with amido black (0.2% in 5% acetic acid), the macro-AGID test was observed with an indirect light source. Positive reference sera, test sera, and antigen were placed in both tests, as illustrated in Fig. 1.

**FIG. 1.** BTV micro-AGID test. The four test sera, which were negative in this example, were placed in wells B, C, E, and F. The positive reference serum was placed in wells A and D. The antigen was placed in the center well (Ag). Note the precipitin line which forms equidistant between the reference serum and the antigen wells.

**RESULTS**

Preparation of BTV and EHDV soluble antigen, regardless of serotype, by the method described gave excellent results in both the micro- and macro-AGID tests. The ultracentrifugation step, initially used to clarify the soluble antigen preparation further, was not necessary. Results obtained with antigen which was only clarified by low-speed centrifugation followed by concentration were similar (sharp precipitin line) to those obtained with the antigen subjected to high-speed centrifugation. Soluble antigen preparations were concentrated to various degrees to determine the lowest amount required to yield good antigen. A 50-fold concentration of the antigen was determined to be the most satisfactory. This concentration of antigen permitted subsequent dilution (1:1 to 1:4) of the stock antigen to obtain a degree of standardization for AGID by using a standard reference BTV antiserum. Storage of the antigen preparations at 5°C appeared very satisfactory; shelf life in excess of 3 years for the BTV antigen and in excess of 2 years for the EHDV antigen.

All four BTV serotypes worked equally well in the BTV AGID tests, as did the two serotypes of EHDV in the EHDV AGID tests. The quality of the BTV antigen (i.e., ability to form a sharp precipitin line) was compared with that of antigen preparations kindly supplied by the U.S. Department of Agriculture Veterinary Services Laboratory (J. Pearson) and the Arthropod-borne Animal Diseases Research Laboratory (M. Joichim). Results of at least equal quality were obtained with this antigen.

Production of reference BTV antiserum in sheep, for the BTV-AGID tests, was by inoculation of virus from cell culture or viremic sheep blood. BTV antiserum were considered good for reference purposes in the AGID test if they formed sharp precipitin lines. Antisera which formed faint or diffuse precipitin lines were considered unacceptable. A great deal of variability was observed in the quality of antisera (ability to form a sharp precipitin line) from different animals. Also, a difference was observed in the ability of different animal breeds to respond to BTV infection. Warhill sheep consistently responded with production of good BTV antigen antisera, but the cross-bred Suffolk and Hampshire sheep did not. Both BTV inocula were effective in eliciting production of BTV antisera. In some instances, reference antisera were obtained on days 21 through 42 after a single inoculation, whereas other animals did not yield good antisera until after the second virus inoculation. A significant number of animals, especially the cross-bred Suffolk and Hampshire sheep, yielded antisera which were
not acceptable as reference sera, regardless of the source of BTV antigen.

Attempts to obtain reference EHDV antisera by inoculation of adult cattle and sheep were unsuccessful. Production of reference EHDV antisera in fetal calves (90 days gestation) was complicated by abortion. EHDV reference antiserum was obtained in one of two fetal calves recovered; however, the serum was hemolyzed because the fetus was aborted. The appearance of false-positive reactions in the micro-BTV AGID test was observed on several occasions, whence the need for changing the placement of reference antisera in the wells. When placing reference antisera in alternate wells, it was observed that strong reference sera would lead to the development of false-positive lines of identity between the bordering reference antisera (Fig. 2). By placing reference serum in only the top and bottom wells (Fig. 1), this complication was eliminated. However, weak false-positive reactions could still appear if a test serum was unusually strong. False-positive reactions were not observed in the macro-BTID test.

A minimal one-way cross-reaction was observed between the BTV and EHDV soluble antigens. EHDV antiserum obtained from one fetal calf inoculated with EHDV-2 gave a weak precipitin line with the BTV antigen, but the reverse was not observed. This cross-reaction appeared to be an exception, since many serum samples obtained in the field were EHDV positive but BTV negative.

FIG. 2. BTV micro-AGID. Positive reference BTV antiserum was in wells B, C, and F; saline was in well A; negative sheep serum was in wells C and E; BTV soluble antigen was in the center well (Ag). Note the development of false-positive reactions in front of wells A, C, and E. This false-positive reaction is the result of a line of identity being formed between the reference serum wells.

DISCUSSION

The BTV and EHDV soluble antigen preparations gave excellent results in the AGID tests. The procedure for antigen preparation requires minimal laboratory equipment. Antigens were stable at refrigerator temperatures (5°C) for more than 3 years for BTV and more than 2 years for EHDV. The AGID test is an effective, inexpensive test for determining previous exposure to BTV and EHDV, but certain limitations must be recognized. (i) A positive AGID test indicates that the animal has been exposed to the viral agent, but it should not be used to indicate current or persistent viral infections. (ii) The AGID test is better suited to determine viral exposure on a herd basis than on an individual basis since recent reports have demonstrated that viremic animals can often be serologically negative by both complement fixation and AGID tests (16). (iii) False-positive results can occur in the micro-AGID test (this was not observed with the macro-BTID test, and the use of alternate wells of reference sera probably presents no problem) if exceptionally strong reference sera are used in association with weak antigen preparations or if a test serum sample is placed between an exceptionally strong test reactor and a reference serum. (iv) A certain degree of standardization of the reagents (reference serum and antigen) must be done if the test is to be reliable. When reference sera of good quality were chosen selectively, the BTV and EHDV AGID tests consistently gave reproducible results in our laboratory. All new antigen preparations were diluted so that the precipitin line formed equidistant between the standard reference serum and antigen wells.

The quality of reference antisera for the BTV AGID tests varied with individual animals and with breeds of sheep. Suffolk and Hampshire sheep routinely produced poor reference sera after infection with BTV and therefore should not be used. In contrast, Warhill sheep produced good antisera, although a certain degree of variability among animals was observed. Acceptable EHDV precipitating and neutralizing antibodies were not obtained after inoculation of sheep. This was attributed to a lack of virus replication. It is not understood why reference antisera from the two adult cows were poor since good neutralizing antibodies were detected. Acceptable reference antiserum was obtained from one of three fetal calves which received virus at 90 days of gestation. Fetal calves appear to produce good EHDV antibodies which can serve as reference antisera, but the fetuses should be infected in the third trimester to avoid abortion or stillbirth.

All four serotypes of BTV and two serotypes
of EHDV gave good soluble antigen for the AGID tests. Early reports indicated that the test detects antibodies to a group antigen (10, 11, 22). Gumm and Newman (3) recently reported that P7, an internal structural protein, was responsible for the precipitin band in an AGID test when sheep antisera were used against BTV heterologous in serotype to the virus antigen. When homologous sheep antisera against BTV were used, up to three bands were often observed; one band contained P7, one contained P5, and one contained all seven structural proteins (3). Immunoprecipitation of BTV proteins P3, P7, and P6A with heterologous guinea pig BTV antisera and immunoprecipitation of BTV protein P5 with heterologous rabbit BTV antisera have also been reported (8). The viral antigen utilized in this study undoubtedly contains many viral proteins because of the minimal purification. This could be an advantage in recognizing the variety of antibodies elicited in response to infection with the different serotypes of BTV and EHDV.

The limited cross-reaction observed between BTV and EHDV in the AGID test has previously been reported (10). This cross-reaction is not surprising in view of recent studies demonstrating immune precipitation of BTV and EHDV proteins with homologous and heterologous antibodies. Polyacrylamide gel electrophoresis of these immunoprecipitates revealed BTV and EHDV proteins with common antigenic determinants (7, 9).

LITERATURE CITED


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