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IDENTIFICATION OF BLUETONGUE ANTIBODIES BY THE TECHNIQUE OF IMMUNODIFUSION IN AGAR GEL

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SUMMARY

A soluble antigen of the bluetongue virus (BTV) was prepared for use in immunodifusion in agar gel tests (IDAG). The antigen is group specific and is capable of detecting antibodies induced by any of the 24 BTV serotypes. It was produced from type 4 BTV serotype and controlled in IDAG against reference antigens and sera (NVSL, Ames, USA; LARA, Campinas, Brazil; Veterinary Diagnostic Technology, Inc., USA) and by the enzyme-linked immunosorbent assay (ELISA) with 3-17-A3 monoclonal antibody (IADR, Pirbright, England). All tests yielded a reaction of total identity with the control reagents.

INTRODUCTION

Bluetongue (BT) is a viral disease in ruminants. It is transmitted by arthropods and is characterized by congestion, edema and hemorrhage. The etiological agent belongs to the *Reoviridae* family, Orbivirus genre, which includes 24 serotypes.

The disease is widely distributed throughout the world, and affects mainly sheep, in which the following clinical symptomatology is observed: inflammation of anterior digestive and respiratory mucosas which causes a bluish color. Serious cases may evolve into ulcerations. Inflammation of the feet and fetal deformation may occur. Mortality is low. Clinical symptoms are generally not observed in other species of ruminants, but they may act as virus reservoirs for long periods of time (2).

DIAGNOSIS

The bluetongue virus (BTV) is isolated by intravenous inoculation of 8-12 day-old chicken embryos with blood samples collected from animals in the viremia stage.

The serological diagnosis (identification of antibodies in the serum) is done by complement fixation (CF), immunodifusion in agar gel (IDAG), seroneutralization in cells (SN) and, recently, also by the enzyme-linked immunosorbent assay (ELISA) technique. The CF, IDAG and ELISA tests reveal the presence of BT group-specific antibodies.

For the import/export movement of animals, serum is previously tested for the presence of anti-BTV antibodies. The IDAG is one of the tests used for this purpose.

The IDAG detects antibodies in the serum beginning from 10 days post-infection. The detected antibodies are group specific, i.e., they have been induced by any of the 24 BTV serotypes. Cross reactions can be observed with sera from animals that have had epizootic hemorrhagic disease of deer.

MATERIALS AND METHODS

Antigen (Ag): Roller bottles containing BHK_{21} clon 13 cells grown at 37°C were inoculated with BTV serotype 4 (1) and returned to the incubator at 37°C. Cytopathic effect was observed 72 hours later and the bottles were treated with 3% chloroform, then frozen, thawed and

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clarified by centrifugation at 10,000g for 30 minutes. They were immediately inactivated with 5mM of binary ethylenimine at 26°C for 24 hours.

The inactivated virus suspension was concentrated with 100,000 NMWL Amicon filter, followed by 2 hours of ultracentrifugation at 200,000g. Later, 0.02% NaN_3 was added. The suspension was titered in IDAG and stored as 2 ml volumes at 4°C until used.

Before titration, specificity controls are performed and the Ag produced is studied against Ags and reference sera. The Ag is controlled by IDAG with reference reagents from NVSL, Ames, USA, LARA, Campinas, Brazil, and the Veterinary Diagnostic Technology Inc., USA; and by ELISA against the specific monoclonal antibody 3-17-A3, according to protocol of IADR, Pirbright, England. The Ag is titered when specificity of the group is confirmed.

Positive Control Serum (PCS): The PCS is a mixture of sera taken from cattle in the state of Rio de Janeiro, Brazil, which was positive to the IDAG test for BT. The sera were concentrated four times by precipitation of the immuno globulins with ammonium sulfate. Then 0.02% of NaN_3 was added, and the sera were stored as 2 ml volumes at -20°C.

2% Agar: 20 grams of purified agar (Difco) are dissolved in 1100 ml of demineralized distilled water and sterilized for 15 minutes at 15 pounds pressure. The solution is then poured into a tray, left to solidify, and cut into cubes with sides measuring approximately 2 cm. The cubes are immersed in distilled water and stored at 4°C until utilized.

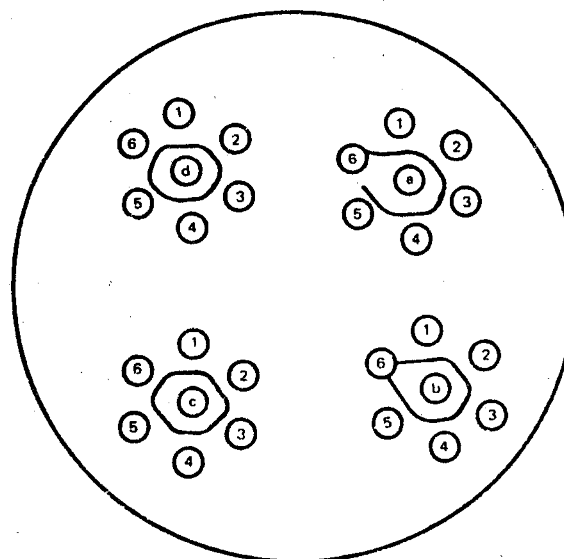
Borate Buffer (BB): A buffer is prepared with 0.05 M of sodium hydroxide (NaOH), 0.15 M boric acid (H_3BO_3), 1% of NaN_3, pH 8.6.

Preparation of 1% Agar Plates: The 2% agar is mixed with equal parts of BB and blended by heat in a water bath at boiling temperature. 16 ml of the BB-agar mixture are then poured into disposable plastic or glass Petri plates with a diameter of 90 mm. The plates are kept partially covered at room temperature (25-30°C) for a minimum of two hours to ensure acceptable agar gelling. Plates not used immediately may be stored in a cooler at 4°C for one week.

The wells in the agar gel are formed with a mold so as to make seven wells, one in the center and six forming a ring around it. All wells have an external diameter of 4 mm, and are equidistant 2mm for each other and from the center well. The agar can be removed from the well holes by suction using a vacuum pump, right before placing the reagents on the plate.

Titration of the Ag and the PCS: Each batch of Ag and PCS is titered to determine the optimal dilution to be used. The Ag and PCS are diluted twofold (1:1 to 1:8) in BB and each dilution of Ag is placed in the central well of each mold. The PCS dilutions are placed in four of the surrounding ring wells. A reference PCS is put into the two remaining wells (Fig. 1). Care must be taken to put in enough reagent to fill the wells

FIGURE 1. Titration of antigen (Ag) and positive control serum (PCS) for bluetongue



a, b, c, and d = Dilutions of Ag 1:1, 1:2, 1:4 and 1:8, respectively.
1 and 4 = Positive reference serum.
2, 3, 5 and 6 = Dilutions of PCS 1:1, 1:2, 1:4 and 1:8, respectively.

completely. Graduated micropipettes or Pasteur pipettes can be used to ensure good distribution of the reagents.

The plates are incubated for 48 hours at room temperature on a level surface. The reading is then made and the optimal dilutions are those which, when analyzed jointly, yielded sharpest line.

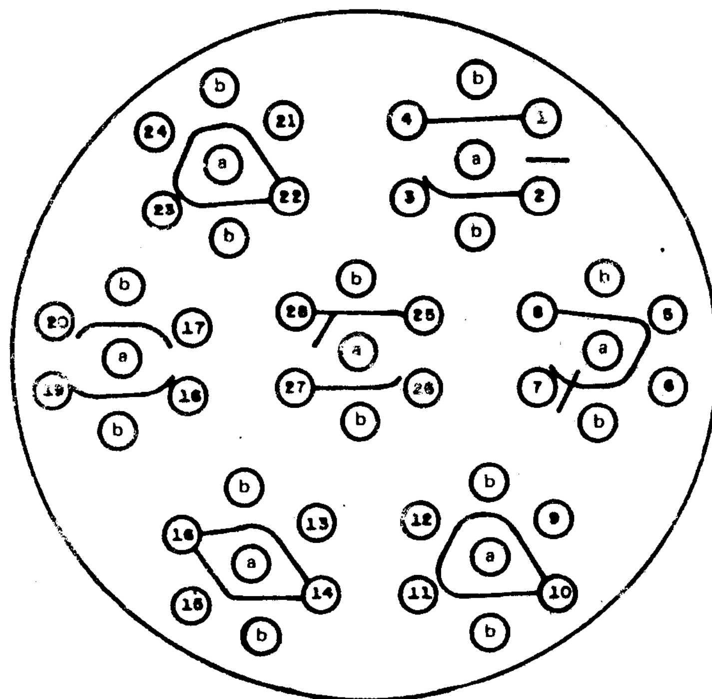
Specificity and Sensitivity of Ag and PCS: After determination of the optimal dilution of each batch of Ag and PCS, both are compared to standard Ag and PCS, to determine their specificity and sensitivity. This comparison utilizes a series of positive and negative sera from several species having different intensity of reaction.

Sera Analysis: The sera to be examined are recorded, appropriately numbered and placed in well identified plates. The sera are distributed in clockwise sequence in the molds and on the plate. Four undiluted sera placed in four ring wells of each mold. The PCS is placed in the two remaining wells. The Ag is deposited in the central well, as shown in Fig. 2. The plates are then immediately incubated at room temperature on a level table.

Reading the Plates: The plates are placed over an indirect light source with a black background and read from 24 to 48 hours after the reagents were placed on the plates. During this time the precipitation lines are clear. Readings taken after 48 hours are difficult to interpret because the lines become diffused.

When working with very hemolyzed sera or sera having high concentrations of lipides, or other substances, nonspecific difusions sometimes appear and may interfere with the reading. This problem can be solved by washing the agar gel with a 10x physiological solution before conducting the reading.

FIGURE 2. Distribution of sera to analyzed, Ag and PCS on a Petri plate and their reaction



a and b = Ag and PCS, respectively.
1 to 28 = Test sera.

TABLE 1. Interpretation of the reactions provided by the sera analyzed in Fig. 2

No. serum	Result	No. serum	Result
1	–	15	++
2	–	16	–
3	+	17	+
4	–	18	+
5	+	19	?
6	++	20	+
7	+	21	++
8	–	22	–
9	++	23	+
10	–	24	++
11	+	25	?
12	++	26	+
13	++	27	–
14	–	28	–

– negative, + positive,
++ intense positive, ? dubious.

RESULTS

All the sera to be examined are contiguous to PCS. This enables the observer to see whether the originated precipitation lines maintain identity with the PCS precipitation line. It should be remembered that some sera yield lines or reactions with identity or nonspecific for BT Ag, that cross or touch the specific precipitation line originated by the BT Ag and PCS (see Fig. 2 and Table 1):

(a) **Negative Serum (—):** These are those in the line of reference, that is, the line originated by the Ag and the PCS enter the well of the serum under study.

(b) **Positive Serum (+):** The reference line shows a slight curvature near the serum under study.

(c) **Intense Positive Serum (++):** The situation and appearance of the precipitation line originated by the serum under study are similar in intensity to the PCS line.

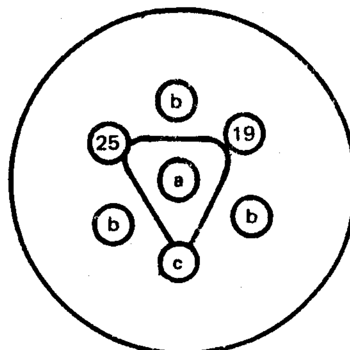
(d) **Dubious Serum (?):** The sera that for any reason can not be classed as —, + or ++, are reexamined using the following procedure:

Repetitition of the Dubious Sera. These are retested by IDAG. The PCS is placed in three alternate wells of the ring of wells, the dubious sera in two remaining wells, and a negative serum in the third well. In this way the reaction in the two contiguous reference lines is observed and compared with the negative serum (see Fig. 3).

The interpretation is conducted as indicated in the preceding section.

When sera again do not yield results enabling them to be classed in category + or —, it is advisable to repeat the test with a new serum.

FIGURE 3. Analysis of sera that provided dubious results in the test shown in Fig. 2 (sera No. 19 and 25)



a and b = Ag and PCS, respectively.
c = Negative control serum.
19 and 25 = Sera under study (19+ and 25?).
Request new sample serum No. 25.

REFERENCES

1. GROOCOCK, C.M., CAMPBELL, C.H. Isolation of an exotic serotype of bluetongue virus from imported cattle in quarantine. *Can. S. Comp. Med.*, 46:160-164, 1982.
2. OBDEYN, I.M. Bluetongue. A review of the disease. Rio de Janeiro, Pan American Foot-and-Mouth Disease Center, 1987. (*Scien. Tech. Monog.*, 16).