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A HAEMAGGLUTINATION AND HAEMAGGLUTINATION INHIBITION TEST FOR BLUETONGUE VIRUS

N. T. VAN DER WALT, Veterinary Research Institute, Onderstepoort, Republic of South Africa 0110

ABSTRACT

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Haemagglutination of bluetongue virus (BTV) was demonstrated for the first time by making use of a purified preparation of the virus. The reaction was found to be independent of variations in the pH, temperature, buffer system and origin of the erythrocytes used in the test. A haemagglutination inhibition test, subsequently developed, was demonstrated to be serotype specific. The storage of the virus for indefinite periods was facilitated by lyophilization of BTV in the presence of a low concentration of sucrose.

Résumé

UN TEST D'HAEMAGGLUTINATION ET D'INHIBITION D'HAEMAGGLUTINATION POUR LE VIRUS DE LA PESTE CATARRHALE OVINE

L'haemagglutination du virus de la peste catarrhale ovine (BTV) a été démontré pour la première fois en utilisant une préparation purifiée du virus.

La réaction fut trouvée être indépendante des variations en pH, température, du système de tamponnage et de l'origine des erythrocytes utilisés dans le test. Un test d'inhibition haemagglutination développé subséquemment se démontra spécifique de sérotype.

La conservation du virus pour ses périodes indéfinies fut facilitée par la lyophilisation du BTV en présence d'une basse concentration de sucrose.

INTRODUCTION

An assay for bluetongue virus (BTV) antibodies is important in epidemiological studies for the identification of BTV carriers, and also in virological and immunological studies of the virus.

The serological tests that are currently most commonly employed for the determination of BTV antibodies are the plaque reduction neutralization (PN) test, the complement fixation (CF) test and the agar gel precipitin test. The choice between these tests depends on the particular application for which it is to be used (Thomas, Girard, Boulanger & Ruckenbauer, 1976). The passive haemagglutination test that has been introduced for the BTV system (Blue, Dawe & Gratzek, 1974) has found limited application, probably because it s more laborious than the PN test. The haemagglutination inhibition (HI) test has not been used since previous attempts to obtain haemagglutination with BTV have been unsuccessful (Howell & Verwoerd, 1971). The HI test is used in many other systems (Tyrrell, 1973), however, and is often the method of choice for the detection of viral antibodies (Joo, Donaldson-Wood & Johnson, 1976), since as an *in vitro* test it has the advantage of being fast and easy to perform.

In view of these advantages a further attempt was made to obtain haemagglutination with BTV. This paper reports a successful procedure to obtain haemagglutination with purified BTV. An HI test for determining BTV antibodies is also described and was shown to be serotype specific.

MATERIALS AND METHODS

Buffers

The following buffers were used: TSAG buffer pH 9,0 consisting of 0,02 M Tris (hydroxymethyl) methylamine, 0,14 M NaCl, 0,001 M $CaCl_2.2H_2O$, 0,2% bovine albumin fraction V and 0,0025% gelatin. HSAG buffer pH 6,2 consisting of 0,025 M N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (Hepes), 0,14 M NaCl, 0,001 M $CaCl_2.2H_2O$, 0,2% bovine serum albumin and 0,0025% gelatin (Iwasa & Hori, 1976). BSA (borate-saline-albumin) consisting of 0,05 M borate, 0,12 M NaCl and 4% of bovine serum albumin. PBS (phosphate buffered saline) consisting of 0,15 M NaCl and 0,2 M phosphate (Clarke & Casals, 1958). Tris buffer pH 9,0 consisting of 0,002 M of Tris (hydroxymethyl) methylamine.

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Erythrocytes

Red blood cells from various species (indicated under Results) were collected in an equal volume of Alsever's solution (2,05% dextrose, 0,42% sodium chloride, 0,8% sodium citrate, 0,055% citric acid), washed 3 times in the same solution, made up to a 50% suspension and stored at 4 °C for up to 2 weeks. Before use the cells were washed twice in saline and then diluted with saline to obtain a 40% suspension. This solution was diluted to 0,25% in the desired buffer for use in the test.

Virus

Attenuated type 10 BTV (BTV 10A) was used as antigen in the test.

Propagation of the virus was carried out by infecting BHK cells with approximately 1,5 BTV plaque-forming units (PFU's) per cell and incubating them for 48 h at 37 °C before harvesting the virus by collecting the tissue culture fluid.

The virus was purified according to a modification of the method of Verwoerd (1969). Infected tissue culture harvests were centrifuged for 1 h at 1 400 g. The supernatant was discarded and the pellet suspended in 0,002 M Tris buffer (1,5 mℓ per 1×10^8 cells). The suspension was homogenized in an Ultra-Turrax homogenizer for 30 seconds. After the addition of a 1/100 volume of 5% Sephadex G200 and a 1/3 volume of Freon 113 (Trichloro-trifluoro-ethane) it was homogenized for 30 seconds and centrifuged for 15 min at 1 400 *g*. The Freon-Sephadex phase was washed twice with 30 mℓ of Tris buffer by homogenization and centrifugation as described above. Three more Freon extractions were then carried out on the combined water phases. The virus was concentrated by centrifugation through a 5 mℓ cushion of 40% sucrose in 0,002 M Tris buffer for 90 min at 78 000 *g*. The pellets were resuspended in 9 mℓ of 0,002 M Tris buffer. This preparation was referred to as the partially purified antigen.

For complete purification, 3 mℓ of the partially purified antigen suspension was layered on a 15–30% sucrose gradient and centrifuged for 50 min at 78 000 *g* in a SW 27 rotor. The virus band from each

tube was collected and the pellet containing virus aggregates suspended and re-centrifuged on a sucrose gradient as described above. The virus band that was collected from this gradient was pooled with the others and the virus then concentrated by centrifugation for 90 min at 78 000 *g*. The pellets were suspended in about 3 mℓ of Tris buffer and used as purified antigen.

Haemagglutination (HA) test

The microtitre equipment described by Takátsky (1955) as modified by Sever (1962) was used. In routine tests sheep red blood cells were used as indicator and TSAG buffer was used as diluent. Doubling dilutions of 0,025 mℓ of serum were made. A further 0,025 mℓ of diluent was then added to each well, followed by 0,05 mℓ of a 0,25% suspension of sheep red blood cells. Suitable controls were included in all the tests. The test was read after incubation for 3 h at room temperature. The highest antigen dilution producing complete haemagglutination was taken as 1 haemagglutinating unit.

Determining the optimum pH for the haemagglutination test

This was done by using BSA buffer (pH 9,0) as diluent and then adjusting the pH with sheep red blood cells suspended in PBS buffer at different pH's as described by Clarke & Casals (1958).

Removal of non-specific inhibitors from serum

Absorption with kaolin and treatment with dextran-sulphate-$CaCl_2$ (DS-C) and heparin-$MnCl_2$ has been described (Clarke & Casals, 1958; Liebhaber, 1970; Iwasa & Hori, 1976).

The haemagglutination inhibition (HI) test

The equipment and reagents were the same as described for the haemagglutination test.

Serial twofold dilutions of serum were made in TSAG buffer. To each 0,025 mℓ of dilution an equal volume of diluent containing 4 HA units of antigen (see Storage of antigen) was added. A serum control and a titration of the antigen suspension were included in each test. After incubation at room temperature for 10 min, 0,05 mℓ of a 0,25% sheep red blood cell suspension was added to each well. The tests were read after another incubation period of 3 h at room temperature. The HI titre was defined as the highest dilution that completely inhibited agglutination.

Storage of antigen

A stock suspension of purified antigen was diluted to a concentration of 240 μg/mℓ in a solution of 2% sucrose and 0,1% albumin in Tris buffer. This suspension was lyophilized in 0,5 mℓ aliquots. One aliquot was restored in 10 mℓ TSAG buffer to obtain 4HA units (12 μg of protein per mℓ) which were immediately used in HI tests.

BTV antisera

Antiserum was prepared by giving rabbits a single subcutaneous injection of 500 μg of purified BTV suspended in Tris-saline buffer. This had been emulsified in an equal volume of complete Freund's adjuvant. Serum samples were obtained by bleeding the animals at weekly intervals.

Plaque-neutralization test

The PN test was carried out as described by Thomas & Trainer (1979).

Virus concentration

The concentration of virus suspensions was calculated from optical density measurements, taking 1 unit of optical density at 260 nm to represent 200 μg of virus (Verwoerd & Huismans, 1972).

RESULTS

Haemagglutination by bluetongue virus

An attempt was made to obtain haemagglutination with unpurified, partially purified and completely purified antigen. A low level of agglutination (1:4) was obtained with unpurified virus whereas a high level of haemagglutination (1:512–1:1024) was measured when partially purified or equivalent amounts of purified virus were used (Table 1). As the contaminating material in the partially purified extract did not seem to interfere with the haemagglutination reaction, partially purified extracts could be used as antigen in haemagglutination tests.

TABLE 1 Haemagglutination with BTV

Antigen	Concentration		Haemagglutinating titre
	μg virus/mℓ	PFU/mℓ	
BTV infected tissue culture homogenate	—	$3,6\times10^9$	1:4
Tissue culture homogenate control	—	—	1:2
Partially purified BTV	240	1×10^9	1:512
Purified	240	$2,4\times10^9$	1:1024

Factors influencing the haemagglutination reaction

The effect of various factors on the haemagglutination reaction was studied by comparing the haemagglutinating titre of an antigen suspension under various conditions. The results are summarized as follows:

(a) *Antigen concentration*

The minimum concentration of antigen required to obtain haemagglutination was about 4 μg of protein/mℓ. This concentration was diluted 4 times in each test well, giving a final concentration of 1 μg protein/mℓ and representing 1×10^6 PFU's of virus/mℓ.

(b) *pH*

In the range between 6,0 and 9,0 no pH effect on the haemagglutination titre could be detected. Since BTV is known to be most stable at pH's above 8,0 (Verwoerd, 1969) HA and HI tests were routinely performed at pH 9,0.

(c) *Temperature*

As changes in temperatures between 4 °C and 37 °C did not affect the haemagglutination titre, the tests were carried out at room temperature.

(d) *Erythrocytes*

BTV agglutinable red blood cells were obtained from sheep, geese, rabbits and humans. Those of geese settled much more quickly than those of sheep. Tests performed with the red blood cells of geese could be read within half an hour, while those of sheep required a minimum of 3 hours.

(e) *Buffer system*

The BS-PBS, TSAG and HSAG buffer systems gave similar results in HA tests. TSAG was chosen for routine tests because it is well suited for work at pH 9,0 and is economical.

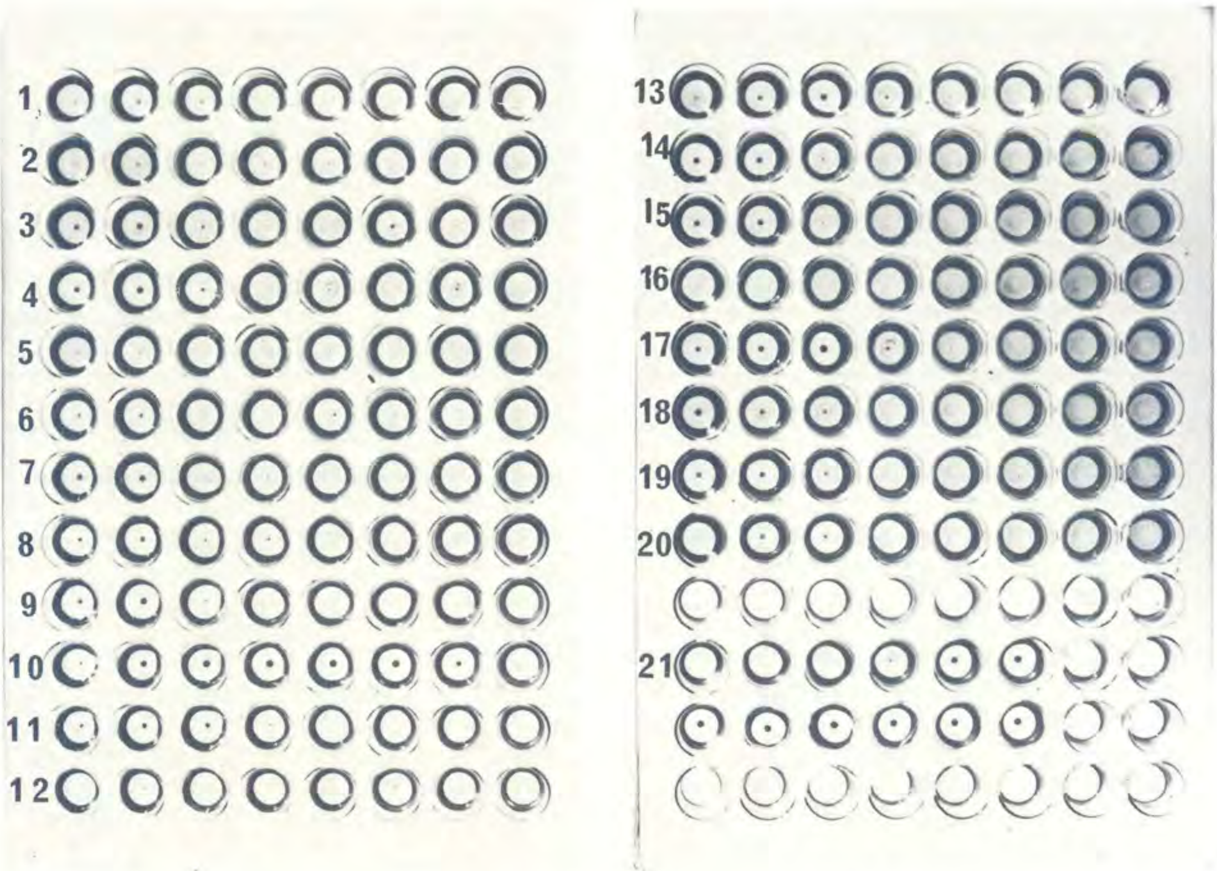

FIG. 1 The serotype specificity of the HI test. In rows 1–20 are dilutions of antisera to BTV types 1–20 incubated with 4–8 HA units of BTV type 10 A as indicated by the titration in row 21. Haemagglutination is shown by sheets of cells on the bottom of the wells, whereas unagglutinated cells form a button. The most pronounced inhibition of haemagglutination is seen in row 10 where BTV type 10 A was incubated with its homologous serum.

Natural agglutinins in control guinea-pig and rabbit sera

The demonstration of natural agglutinins in control guinea-pig and rabbit sera depended on the indicator cells that were used. When those of sheep were used, no natural agglutinins could be detected in guinea-pig sera and very low titres were obtained in rabbit sera (Table 2). Since absorption of 0,2 mℓ of rabbit serum with 0,1 mℓ of a 40% suspension of sheep cells did not cause a significant reduction in the natural agglutinin titre, rabbit serum was not absorbed before use in the HI tests.

TABLE 2 Titres of natural agglutinins present in control rabbit and guinea-pig sera

Origin of serum	Indicator cell	
	Goose red blood cells	Sheep red blood cells
Rabbit 1	1:16	1:2
2	1:64	1:4
Guinea-pig 1	1:2	0
2	1:2	0

When the red blood cells of geese were used as indicator cell, low concentrations of natural agglutinins could be detected in guinea-pig sera 1:2 at the maximum, but much higher levels could be demonstrated in rabbit sera (1:16–1:64).

Non-specific inhibitors of haemagglutination in control rabbit and guinea-pig serum

The detection of non-specific inhibitors of haemagglutination in rabbit and guinea-pig sera also depended on the type of indicator cell used. The mean of at least 4 different samples that were tested in each case is shown in Table 3. When goose red blood cells were used, the titres of non-specific inhibitors in rabbit and guinea-pig sera were in the order of 1:128, but when those of sheep were used, no non-specific inhibitors were detected in rabbit serum and only low titres (1:8) in guinea-pig serum. Since goose red blood cells have the advantage of settling more quickly than those of sheep, an investigation was made into their use in HA and HI tests. The main obstacle was to find a procedure for removing non-specific inhibitors from both rabbit and guinea-pig sera. As can be seen from Table 3, the only success was obtained by absorption of serum with kaolin.

Serotype specificity of the HI test

The HI test was shown to be serotype specific. This was determined by HI testing of 20 serotype specific anti-sera* against BTV type 10A. Only the homologous serum (Fig. 1) inhibited the virus specific haemagglutination.

* Obtained from the Section of Virology, Onderstepoort

TABLE 3 The titre of non-specific inhibitors in untreated and treated control rabbit and guinea-pig sera

Origin of serum	Titre of non-specific inhibitors in serum				Indicator cell
	Untreated	After treatment			
		Kaolin	DS-C	Mn-heparin	
Rabbit	1:128	1:32	1:128	1:128	Goose red blood cells
Guinea-pig	1:128	1:16	1:128	1:128	
Rabbit	0				Sheep red blood cells
Guinea-pig	1:8				

TABLE 4 Comparison of the antibody titres measured by the plaque reduction neutralization (PN) and haemagglutination inhibition (HI) tests

Day	Titre	
	PN	HI
0	< 1:16	0
3	< 1:16	0
8	< 1:16	0
11	< 1:16	1:8
17	1:32	1:16
24	1:128	1:64
32	1:256	1:64
39	1:256	1:64
51	1:256	1:64
60	1:512	1:64

HA titre 1:4–1:8

TABEL 5 The efficacy of different procedures for the preservation of BTV haemagglutinating activity

Treatment of antigen	Buffer	Additions	Titre before treatment	Titre after reconstitution
Freezedrying	BSA	—	1:128	< 1:2
Freezedrying	0,002 M Tris	—	1:128	< 1:2
	0,002 M Tris	5% bovalbumin	1:128	< 1:2
	0,002 M Tris	2% sucrose	1:128	1:128
Fast freezing	0,002 M Tris	—	1:128	< 1:2
Slow freezing	0,002 M Tris	—	1:128	< 1:2
Slow freezing	BSA	—	1:128	< 1:2

Comparison of the antibody titres measured by the PN and HI tests

In order to compare the 2 tests, the respective antibody titres of serum samples which had been prepared in rabbits were measured. The HI test compared favourably with the PN test for the detection of BTV antibodies (Table 4). The titres measured with the HI test were about four times lower than those measured in the PN test.

Storage of the haemagglutinating antigen

Since it is important to have antigen available for daily use and the stability of BTV in terms of its haemagglutinating property is limited to 14 days when stored in 0,002 M buffer at 4 °C, an investigation was made into various procedures for the preservation of the haemagglutinating property of the antigen. After suspensions of 240 μg virus/mℓ in different buffers were tried out, the haemagglutinating activity proved best preserved when lyophilized in buffers with low ionic concentration, such as 0,002 M Tris. Complete preservation of its activity was secured when 2% sucrose was added to the Tris buffer. On the other hand the addition of as much as 5% albumin failed to preserve the haemagglutinating activity of the antigen and slow freezing destroyed it altogether. The antigen was thus lyophilized in 0,5 mℓ aliquots of a suspension containing 240 μg of protein/mℓ in 2 mM Tris buffer and 2% sucrose and at the same time 0,1% albumin was added to provide "body" without affecting the stability.

DISCUSSION

Haemagglutination with BTV could be demonstrated by using purified or partially purified virus. The fact that virtually no haemagglutination was observed with unpurified virus, explain why haemagglutination with BTV was previously reported to be negative (Howell & Verwoerd, 1971). This negative result could perhaps be ascribed to the tendency of

unpurified virus to remain cell-bound (Verwoerd, 1969), thereby blocking the receptors on the virus that are necessary for the binding of the erythrocytes.

Since the haemagglutination reaction with BTV was not significantly influenced by changes of pH, temperature or the buffer system used, the choice of conditions for the test was based on practical considerations and the stability of the virus (Verwoerd, 1969). These factors are important variables in some virus systems (Liebhaber, 1970; Halonen, Ryan & Stewart, 1967). The fact that they do not play an important role in the BTV system was not surprising, however, as their influence varies considerably in different systems (Rosen, 1969).

BTV agglutinated red blood cells from various species to the same extent, but in serological tests much more non-specific interference was detected when goose red blood cells were used instead of those of sheep. The use of the latter is therefore of considerable advantage, since the laborious and often inefficient procedures that are necessary to remove non-specific interfering substances are eliminated. Nevertheless, the cells of geese were investigated because of the advantage that they settle much more quickly than those of sheep and the time required for the test is thus reduced.

When red blood cells of geese were used, the main problem was the removal of the non-specific interfering substances from the serum. This could only be effected by absorption with kaolin. Since this procedure is based on non-specific absorption, further studies will be necessary to identify these substances.

The HI test and the PN test compared well in the detection of BTV antibodies. The fact that the titres measured by the HI test were generally 2-4 times lower than those measured by the PN test, however, makes it less sensitive for the detection of low levels of antibody.

The usefulness of the test was considerably enhanced when it was found that the addition of 2% sucrose to the Tris buffer completely preserved the haemagglutinating activity of the virus during lyophilization and thus made possible storage of the antigen for a longer period than the 14 days in 0,002 M Tris buffer or 4 °C.

The addition of external proteins which have been shown to stabilize the virus under various conditions (Verwoerd, 1969) did not protect the haemagglutinating activity during lyophilization.

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