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# Bluetongue Virus Hemagglutination and Its Inhibition by Specific Sera

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With 1 Figure

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## Summary

Bluetongue-virus (BTV) was found to agglutinate a variety of erythrocytes including sheep-, chicken-, guinea pig- and mouse-erythrocytes. Hemagglutination was inhibited specifically with type specific serum. A temperature dependence was only found for chicken erythrocytes, which showed a hemagglutination optimum at 37° C. The hemagglutination was lost upon treatment of the virus with 0.4 per cent trypsin as well as after treatment with 0.01 M $KJO_4$. Heating of the virus preparation to 56° C resulted in the loss of the HA-activity. Gelchromatographic studies indicated that the hemagglutinating capacity is associated with the complete virion. Whereas virulent strains of BTV hemagglutinate a number of different erythrocytes the avirulent type tested produced only a slight hemagglutination with sheep red blood cells. However, specific antiserum produced with the avirulent strain yielded strong hemagglutination inhibition (HI) with the corresponding virulent strain. Treatment of sera prior to their use in the HI proved necessary to remove nonspecific inhibitors. The efficiency of $KJO_4$ in removing nonspecific inhibitors indicates that carbohydrates represent the major group of nonspecific inhibitors. The data represented recommend the hemagglutination inhibition test as a new method to identify the various BTV serotypes.

## Introduction

Bluetongue-virus (BTV) has been shown to possess a close relationship to reovirus (14) which supports its classification in the family of reoviridae. Though hemagglutination has been reported for reoviridae (4, 6) as well as for a member of the orbivirus-group (9) no hemagglutination with BTV has been found. On the other hand the strong affinity of BTV to sheep erythrocytes as demonstrated by Pini *et al.* (10) lends support to the possibility of an *in vitro* hemagglutination. A similar type specificity as observed for the reovirus hemagglutination would facilitate undoubtedly the serotype differentiation presently achieved with the rather laborious plaque inhibition method (5).

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## Materials and Methods

### *Virus*

Bluetongue-virus virulent types 3, 5, 8 and 10 were kindly supplied by Dr. B. J. ERASMUS (VRI Onderstepoort, South Africa). When received these strains had undergone 1 or 2 egg passages and additional 2—4 baby hamster kidney cell (BHK) passages. The attenuated type 10, designated BT 8 vaccine, was purchased from the Denver Serum Comp. (Denver, Col. U.S.A.).

A stock preparation of the different strains was stored at $-70°$ C after two additional BHK passages.

Unless otherwise stated the experiments were performed with the virulent type 3 virus.

### *Cell Cultures*

BHK-21 cells were passaged in roller bottles (6.5 cm diameter, 35 cm length) in modified Eagles medium enriched with 10 per cent calf serum. Confluent BHK monolayers had an average cell number of $3 \times 10^8$ cells per tube. For production of virus Eagles medium was replaced by Lavit medium without serum.

### *Virus Production*

Stock virus was used to infect BHK-cells at a multiplicity of infection of 0.5 plaque forming units. Simultaneously 50 ml of Lavit medium per tube were added. After incubation for 24—48 hours the cell layer showed complete destruction and was vigorously shaken off the glass wall. On the average $5 \times 10^7$ PFU/ml were obtained in the supernatant.

The virus containing cell suspension was clarified by a low speed centrifugation step. The resulting cell pellet was taken up in 0.002 M Tris buffer, pH 8.8, and treated according to the method described by VERWOERD *et al.* (14) with the difference that no ether-tween treatment was performed. Instead, the 5 times fluorcarbon extracted virus was pelleted through a $\frac{1}{3}$ volume of a 40 per cent (w/v) sucrose cushion containing 3 mg/ml Tween 80 in a SW 27 rotor for 150 minutes at 24,000 rpm at 4° C (Beckman L 5 ultracentrifuge). The resulting pellet was dissolved in 1.5 ml Tris buffer 0.002 M and layered onto a 10—40 per cent w/v sucrose gradient in Tris buffer. Upon centrifugation for 50 minutes in a SW 27 rotor at 24,000 rpm at 4° C the clearly visible virus band was recovered by puncturing the tube wall. Finally the obtained band material was diluted 1:5 in Tris buffer and pelleted in the same rotor as above for 40 minutes at 25,000 rpm at 4° C. The resulting pellet was dissolved in 0.5 ml Tris buffer and stored at $-70°$ C. In addition, the virus containing tissue culture fluid was mixed with solid $(NH_4)_2SO_4$ to obtain a 40 per cent saturation. The pH was adjusted immediately to pH 7.0 by dropwise addition of 5 N NaOH. Precipitation at 4° C for 60 minutes was followed by centrifugation for 30 minutes at $2{,}200 \times g$ at 4° C. The resulting precipitate was resolved in 1/100th the original volume in Tris 0.002 M, pH 8.8, and dialysed overnight against the same buffer. The dialysed preparation was treated three times with fluorcarbon and finally combined with the cell-extracted virus for further purification.

### *Infectivity Titration*

For the plaque-suspension technique (1) BHK cells grown in roller bottles were suspended in Earle's medium with 2 per cent calf serum after trypsination at a concentration of $2 \times 10^7$ cells per ml.

0.1 ml virus in phosphate buffered saline (PBS), pH 7.4, in serial tenfold dilutions was added to 0.9 ml cell suspension.

After incubation for 15 minutes at room temperature with occasional agitation 1 ml agarose medium was added and the mixture poured onto freshly prepared baselayers of agarose medium in plastic dishes. Agarose medium consisted of Earle's basic salt solution enriched with 0.5 per cent lactalbumin hydrolysate, 0.1 per cent yeast extract, 0.1 per cent bovine serum albumin and 0.55 per cent agarose. Plates were incubated for 3 days at 37° C in an atmosphere with 5 per cent $CO_2$. Plaques were

counted after staining with neutral red 1:10,000. In addition infectivity titration were performed in BHK-tissue culture tubes and the infectivity titer was calculated according to the method of REED and MUENCH (11).

*Preparation of Sera for Hemagglutination Inhibition (HI)*

To remove nonspecific HA-inhibitors sera were inactivated at 56° C for 30 minutes, whereupon one part of serum was mixed with two parts of a 0.01 M solution of $KJO_4$ in 0.005 M PBS, pH 7.4. After an incubation for 15 minutes at room temperature two parts of 1 per cent glycerol in the same buffer were added to stop the action of $KJO_4$.

Alternatively, the sera were treated with neuraminidase (Behring, Marburg) by mixing 4 parts of neuraminidase containing 0.17 U per ml and 1 part serum. Incubation at 37° C overnight of this mixture was followed by heat inactivation for 30 minutes at 56° C.

For the treatment of sera with trypsin 1 part of freshly prepared 0.4 per cent trypsin (Difco) in PBS was mixed with an equal volume of serum, and incubated for 30 minutes at 56° C. For use in the HI-test all sera were appropriately diluted with 0.05 M PBS, pH 7.4, to have a starting dilution of 1:5.

*Hemagglutination (HA) and HI Tests*

The microtiter method was used exclusively. Flexible polyvinylchloride plates (Dynatech, Plochingen, Federal Republic of Germany) with U-shaped wells proved most satisfactory for the test. To each serial twofold BTV antigen dilution in borate saline, pH 9.0, an equal amount of guinea pig erythrocytes 1 per cent in virus adjusting buffer, pH 7.0, (3) was added. The mixture was shortly agitated on a laboratory shaker and incubated at 37° C for 60 minutes. The reciprocal of the highest virus dilution showing complete hemagglutination was considered as the HA-titer.

In hemagglutination inhibition (HI)-tests, 4—8 HA-units of antigen in 25 µl were added to 25 µl of each serial twofold dilution of serum in borate saline, pH 9.0.

After incubation for 60 minutes at 37° C, 25 µl of the red blood cells (RBC) suspension was added. The plates were again incubated at 37° C for 60 minutes and the highest serum dilution showing complete inhibition of hemagglutination was considered as the HI-titer.

*Gel Filtration of BTV Hemagglutinin*

Freon-extracted BTV hemagglutinin was run over a Sepharose 4B column. Fractions were collected with an Isco fraction collector. All fractions were passed through a flow cell monitored by an ultraviolet absorbence monitor C Isco Model UA-5 connected to an ultraviolet analyser. Selected fractions were tested for virus infectivity and HA-activity.

*Treatment of BTV Hemagglutinin with $KJO_4$ or Enzymes*

One volume of purified antigen was mixed with 2 volumes of 0.01 M $KJO_4$ in 0,005 M PBS, pH 7.4, and incubated for 15 minutes at 37° C, this was followed by the addition of 1 per cent glycerin whereupon the mixture was included in the test. Similarly, 1 volume of 0.4 per cent trypsin was mixed with 1 volume of antigen which mixture after incubation for 30 minutes at 37° C was included in the test. Neuraminidase treatment was performed as advised by SENTSUI (12) by mixing 0.17 units RDE/ml with an equal volume of purified antigen. Incubation for 30 minutes at 37° C followed, whereupon the mixture was tested in HA as well as in infectivity titrations.

For control purposes, antigen samples without treatment were titrated in parallel for HA-activity and infectivity.

## Results

*Hemagglutination with RBC from Various Species*

RBC from guinea pig, mouse, goose, chicken, cows and sheep were subjected to HA-test. Upon virus purification with Freon, only guinea pig and sheep RBC were agglutinated. Yet further purification led to an additional agglutination of

mouse, goose and chicken red blood cells (Table 1). The results presented in Table 1 were obtained with BTV serotype 3V, yet the same pattern was found for serotypes 8V and 10V. In particular guinea pig RBC were agglutinated consistently to the highest titer. In contrast to the different virulent BTV types examined, which exhibited identical HA-values with RBC of a given species, the avirulent BTV type 10 yielded hemagglutination with sheep red blood cells only. In the latter case HA-values remained considerably lower when compared to values obtained with the corresponding virulent strain.

Table 1. *The influence of the purification upon BTV hemagglutination with various RBC*

| Degree of purification | | PFU/ml |
|---|---|---|
| 1) | supernatant fluid | $6 \times 10^7$ |
| 2) | supernatant after hypotonic cell lysis | $9.6 \times 10^8$ |
| 3) | 5 times Freon-extraction of step 2 | $2 \times 10^8$ |
| 4) | centrifugation of step 3 through a 40% sucrose cushion with 3 mg/ml Tween 80 | $2.2 \times 10^9$ |
| 5) | density gradient centrifugation of step 4 through a 10—40% sucrose gradient | $6.5 \times 10^8$ |

| | RBC (1%) | | | | |
|---|---|---|---|---|---|
| Step | Guinea pig | Mouse | Goose | Chicken | Sheep |
| 1 | — | — | — | — | — |
| 2 | — | — | — | — | — |
| 3 | 1:64 | — | — | — | 1:16 |
| 4 | 1:2048 | 1:2 | 1:64 | 1:64 | 1:256 |
| 5 | 1:320 | 1:80 | 1:160 | 1:80 | 1:20 |

Table 2. *The influence of the erythrocyte concentration upon BTV hemagglutination titer*[a]

| Ery.-conc. | SRBC | ChRBC | GPRBC |
|---|---|---|---|
| 0.5% | 1:320 | 1:1280 | 1:2560 |
| 0.75% | 1:80 | 1:640 | 1:1280 |
| 1.0% | 1:40 | 1:160 | 1:320 |

SRBC: sheep red blood cells, ChRBC: chicken red blood cells, GPRBC: guinea pig red blood cells. BTV type 3V was used in the test.

[a] Values represent titers from 3 different tests

Table 3. *The influence of the incubation temperature upon BTV hemagglutination titer*[a]

| Incubation temp. | SRBC | ChRBC | GPRBC |
|---|---|---|---|
| 4° C | 1:160 | 1:40 | 1:1280 |
| 22° C (RT) | 1:320 | 1:1280 | 1:2560 |
| 37° C | 1:320 | 1:1280 | 1:1280 |

[a] Particulars as for Table 2

*Effect of RBC Concentration and Incubation Temperature on the Hemagglutination Titer*

As expected, a gradual increase of the HA-titer with decreasing RBC concentration was observed. Yet despite optimal titers were obtained with 0.5 per cent TBC concentration, most HA-tests were performed with 1 per cent RBC concentrations, as more definite end points were observed (Table 2). No difference in HA titers was observed for sheep and guinea pig RBC when the incubation was performed at 4° C, room temperature, and 37° C. However, a marked decrease of the HA-titer was seen with chicken RBC at 4° C (Table 3). In addition, the HA-titer did not change after holding the test plate with guinea pig or sheep RBC at room temperature for 24 hours whereas a significant HA-titer loss was found for chicken RBC kept under the same conditions, indicating elution of the hemagglutinin.

*Influence of pH, Buffer Composition and Various Reagents on HA*

Phosphate buffer was adjusted to pH-values ranging from pH 5.75 to pH 9.0. Hemagglutination was lost completely at pH-values lower than pH 6.0 whereas over the pH range from pH 6.0 to 9.0 the same HA-titer was observed. Similarly, no change of the HA-titer occurred when the borate-saline-phosphate buffer system was replaced by the following diluents: dextrose-veronal-gelatine buffer, pH 7.4, M/90 phosphate-NaCl-buffer, pH 7.4, PBS, or saline.

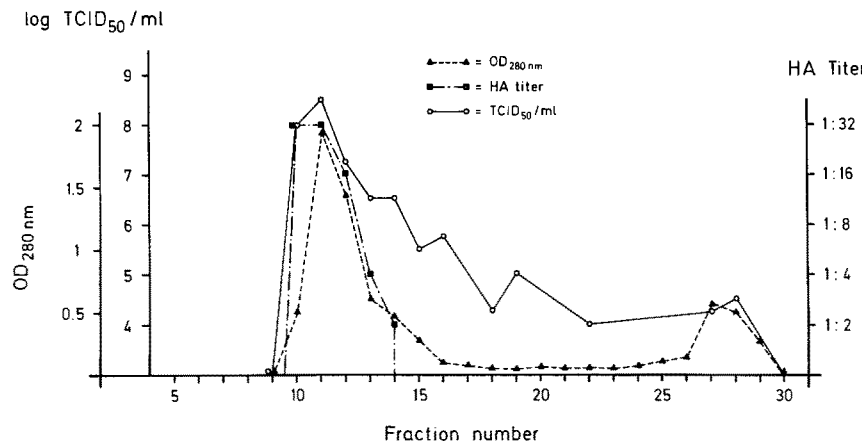


Fig. 1. Gel-filtration (Seph. 4B) of BTV. The virus was only Freon-extracted

In order to elucidate the chemical nature of the BTV hemagglutinin, purified virus preparations were treated with 0.01 M $KJO_4$, 0.4 per cent trypsin or 0.17 U RDE as indicated in Materials and Methods. Trypsin- and $KJO_4$-treatment removed the HA-activity completely whereas RDE treatment caused no alteration of the HA-titer. Infectivity titrations which were performed in parallel with the same preparations showed that the trypsin treatment led to a decline of 3 $\log_{10}$ $KID_{50}$/ml whereas $KJO_4$ addition caused a complete loss of infecitvity.

*Association of Hemagglutinin with Virus Infectivity*

Results obtained after passing BTV over a Sepharose 4B column indicate that the BTV hemagglutinin (Fig. 1) is closely associated with the virus particle. The correlation between infectivity and hemagglutination titer is further substantiated by the results obtained with density gradient centrifugation of purified BTV where the peak infectivity fractions yielded the highest HA-titers (Results not shown). The results presented in Table 1 further allow to calculate the ratio of infectious particles to HA-units being approximately $10^6$ PFU/ml to 1 HA-unit using guinea pig RBC.

*Hemagglutination Inhibition of BTV*

Nonspecific inhibitors were found in all sera tested. As can be seen in Table 4, these inhibitors were effectively removed by treatment with $KJO_4$. Similarly, trypsin- and RDE-treatment lead to a partial removal of nonspecific inhibitors.

Table 4. *Removal of nonspecific serum inhibitors*

| Treatment | Nonspecific serum inhibition titer | | |
|---|---|---|---|
| | Calf serum | Sheep serum | Rabbit serum |
| None[b] | > 1:4096[a] | 1:256 | 1:512 |
| M/90 $KJO_4$ | < 1:10 | < 1:10 | 1:10 |
| Trypsin 0.4% | n. d.[c] | 1:40 | n. d. |
| Neuraminidase | n. d. | 1:40 | n. d. |
| Chloroform (20%) | > 1:4096 | 1:256 | 1:16 |
| M/90 $KJO_4$ + Chloroform (20%) | < 1:10 | < 1:10 | 1:320 |

[a] 4 HAU BTV
[b] All sera were inactivated 56° C, 30 minutes
[c] Not done

Table 5. *HA inhibition titers of various sheep and calf sera with BTV types 3 V, 5 V and 10 V*

| | neg. calf serum | neg. sheep serum | ref.T3 serum | ref.T5 serum | ref.T10 serum | sheep540 (20d.p.i.) | sheep540 (60d.p.i.) | sheep541 (60d.p.i.) | calf 33/37 (128d.p.i.) |
|---|---|---|---|---|---|---|---|---|---|
| Type 3V | —[a] | — | 1:320 | — | — | 1:640 | 1:160 | — | — |
| Type 5V | — | — | — | 1:640 | — | — | — | 1:160 | — |
| Type 10V | — | — | — | — | 1:80 | — | — | — | 1:80 |

[a] HAI titer < 1:10 S 540 experimentally infected with type 3; S 541 experimentally infected with type 5

Table 6. *HI-titer of a sheep experimentally infected with BTV type 3*

| 0 | 10[a] | 28 | 42 | 54 | 68 | 82 | 90 | 112 | neg. sheep | Ref. T10 |
|---|---|---|---|---|---|---|---|---|---|---|
| —[b] | 1:40 | 1:640 | 1:640 | 1:160 | 1:320 | 1:160 | 1:160 | 1:160 | — | — |

[a] Days post infection
[b] < 1:10

HI-antibodies to BTV hemagglutinin proved to be type specific as illustrated in Table 5. HI antibodies of one sheep infected artificially were followed over a period of 3 months (Table 6). HI was first detected 10 days after infection, reaching a peak titer (1:640) between 20 and 40 days post infection followed by a HI-titer of 1:160 for the remaining observation period. Considerably higher HI-titers were obtained from hyperimmunized rabbits where HI-titers reach values of up to 1:40960.

## Discussion

BTV does agglutinate RBC from different species provided that the antigen has been adequately purified. Increased purification extended the range of species, the RBC of which were agglutinated. The failure to obtain hemagglutination even with relatively high titered tissue culture fluid virus must therefore be ascribed to the known affinity of BTV to cellular material (13) whereby the HA reactive sites on the virion are masked. Additionally, the failure of VERWOERD *et al.* (13) to demonstrate hemagglutination by BTV must be due to the exclusive study of the attenuated strain of serotype 10. In fact, when we tested avirulent strain of BTV 10, only a slight hemagglutination solely with sheep erythrocytes was observed, for which in addition considerably more virus as measured by the infectivity titer had to be used as compared to wild type virus. Possibly, avirulent BTV strains possess a hemagglutinin which is different from that of the virulent strains. As hemagglutination by the avirulent strain is confined to sheep RBC, one has to assume that during continuous egg passage structures essential for the wide scale hemagglutination are altered.

The fact that HA occurred to same titers at 4° C, 20° C, and 37° C with the exception of chicken RBC indicates that the BTV affinity for the latter is weaker than for the other 2 sorts of RBC tested. This is further substantiated by the partial elution of BTV from chicken RBC within 24 hours, an observation not made with the other RBC, where the HA-titer remained stable for 24 hours.

Incubation of BTV with trypsin and $KJO_4$ removed viral reactive sites for HA and decreased infectivity whereas RDE did not alter the HA-titer nor the infectivity of BTV. This may suggest that the BTV protein active in the red cell attachment similar to reoviruses (6) may be a glycoprotein. The relationship of HA-titer and infectivity as observed in gel filtration studies further indicates that BTV hemagglutinin is closely associated with the virus particle. Considering the strain specificity of BTV as exposed in the hemagglutination inhibition test it is further possible to allocate the HA reactive site to protein 2 and 5 on the outer shell of BTV, as the latter have been shown to be responsible for the type specificity of BTV, which would correspond to the L-type particles as described by MARTIN and ZWEERINK (7). Further work is in progress to elucidate the structural differences of these 2 proteins in avirulent and virulent strains.

Antibodies to BTV have been demonstrated by complement fixation, neutralisation, immunodiffusion (2) and ELISA-tests (HÜBSCHLE, in preparation). When testing for HI-activity during the present studies we found high titers in sera from cattle, sheep and hyperimmunized rabbits, in the latter of up to 1:40000. HI antibodies seem to follow the same pattern as has been described for serum

neutralisation titers (10). No study directly comparing neutralizing and HI-titers has been published, however. The advantage of the described hemagglutination inhibition test lies probably in the availability of a new diagnostic tool for the rapid differentiation of BTV serotypes.

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