Onderstepoort Journal of Veterinary Research,
Volume 28, Number 3, September, 1960.
The Government Printer, Pretoria.

A PRELIMINARY ANTIGENIC CLASSIFICATION OF STRAINS OF BLUETONGUE VIRUS

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In an immunological study of ten bluetongue virus isolates by *in vivo* cross immunity tests, the antigenic plurality of strains was demonstrated by Neitz (1948). From the results of these tests it was not possible to group any of the strains on the basis of antigenic structure.

In the past, extensive use has been made of the cross protection test in sheep, both for diagnostic purposes and for the immunogenic classification of bluetongue virus isolates. This procedure has not proved completely satisfactory, and the need for a reliable *in vitro* neutralisation test has become an urgent necessity. Haig, McKercher & Alexander (1956) demonstrated the development of specific cytopathic changes, and their inhibition by homologous antibody, as a result of the multiplication of bluetongue virus in cultures of lamb kidney cells. A technique thus became available for *in vitro* immunological studies based upon the virus-antibody neutralisation reaction, using the inhibition of cytopathic changes in monolayer tissue cultures as the index.

Materials and Methods

Virus strains.—During the past 50 years numerous specimens from outbreaks of bluetongue have been received by this laboratory for investigation. These specimens have been stored as infective blood in Edington's O.C.G.* at 4° C. In this preliminary study 22 isolates were selected. These included some of the original strains used by Neitz, more recent material collected during the 1953 and 1958 bluetongue seasons, as well as two specimens received from outside the borders of the Union of South Africa.

The origin of a number of these isolates has been described in detail previously, Neitz (1948), Haig, McKercher & Alexander (1956). Relevant data in respect of all the strains used in this study are given in Table 1.

* 500 ml. glycerine; 5 ml. carbolic acid; 5 gm. potassium oxalate; and 500 ml. water.

Received for publication on 9 December, 1959.—Editor.

TABLE 1

Origin and passage level of bluetongue virus isolates

Original Donor	Isolate	Date	Passage Level in Sheep	Origin
Ovine............	Theiler*...............	1900	Unknown	Cape Province
Bovine...........	Bekker................	1933	Unknown	Standerton
Ovine............	Camp*................	1937	Unknown	Onderstepoort
Ovine............	Mimosa Park*.........	—	26	East London
Bovine...........	Byenespoort*..........	1941	8	Pretoria
Ovine............	University Farm*.......	1942	2	Pretoria
Ovine............	Nelspoort*............	1944	2	Beaufort West
Ovine............	Cyprus*..............	1944	11	Cyprus
Bovine...........	Jansen................	1950	1	Rustenburg
Ovine............	Schoeman.............	1951	3	Utrecht
Ovine............	Bloukop...............	1952	3	Ermelo
Ovine............	Mossop...............	1953	2	Machadodorp
Ovine............	Zaaiplaats.............	1953	2	Middelburg, Tvl.
Ovine............	Vlakfontein†...........	1953	2	Jagersfontein
Ovine............	Onderstepoort..........	1953	3	Onderstepoort
Ovine............	Utrecht................	1955	3	Utrecht
Ovine............	Portugal...............	1956	3	Portugal
Ovine............	Biggarsberg............	1958	2	Vryheid
Ovine............	Vryheid................	1958	1	Vryheid
Ovine............	Middelburg............	1958	2	Middelburg, C.P.
Ovine............	Lydenburg.............	1958	2	Pilgrims Rest
Ovine............	Strathene..............	1958	1	Vryheid

* Neitz, 1948.
† Haig, McKercher, Alexander, 1956.

Cell cultures.—Kidneys from lambs between the ages of one to six weeks were obtained immediately after slaughter and prepared by Trypsin-dispersion, following the method of Youngner (1954). Roller tubes were seeded with 1 ml. of a cell suspension containing $1 \cdot 5 \times 10^6$ cells per millilitre in a modified Banks buffered salt solution (B.S.S.) containing 0·6 grams calcium chloride per litre (Weiss), 10 per cent inactivated bovine serum, 0·5 per cent lactalbumin hydrolysate and 0·01 per cent yeastolate.

Cultures were used in this study when cellular outgrowths were confluent, usually between the fifth and seventh days.

Antigens.—The susceptibility to bluetongue of a group of selected sheep was confirmed by a negative complement fixation test (Shone, Haig & McKercher, 1956). Sheep were infected by the intravenous administration of 2 ml. of infective blood. The temperature of each animal was recorded twice daily, and at the height of the febrile reaction blood was collected in O.C.G. and stored at 4° C. It was observed previously by Haig, McKercher & Alexander (1956) that the ability of bluetongue virus to multiply in sheep kidney cells in culture, is facilitated by prior propagation of the virus in the fertile hen's egg. The stored virulent virus from those sheep which recovered from infection was adapted to propagation in eggs, as described by Alexander (1947). Infected embryos of the third serial passage were emulsified, and a 10 per cent tissue suspension prepared in a buffered phosphate saline containing 1 per cent lactose and 0·5 per cent peptone, pH 7·4. After distribution in ampoules the material was freeze-dried and stored at — 20° C.

TABLE 2

Neutralisation of bluetongue tissue culture

Antigen											
Isolate	Titre	Theiler	Bekker	Camp	Mimosa Park	Byenes-poort	University Farm	Nels-poort	Cyprus	Jansen	Schoeman
Theiler..........	†4·5	*31,620	100	10	.	10	10	.	.	10	10
Bekker..........	5·5	100	316,200	.	.	.	.	.	.	.	.
Camp...........	5·5	.	.	31,620	.	.	.	.	.	.	.
Mimosa Park....	5·0	.	.	.	10,000	.	.	.	100,000	1,000	.
Byenespoort......	5·0	.	.	.	.	100,000	.	.	.	.	.
University Farm..	6·0	.	.	.	.	.	100,000	.	.	.	.
Nelspoort........	6·0	.	.	.	.	.	.	100,000	.	.	.
Cyprus..........	4·0	.	.	.	10,000	.	.	.	10,000	10,000	.
Jansen...........	5·0	10	10	.	316,200	10	.	.	316,200	316,200	.
Schoeman........	3·5	.	.	.	.	.	10	.	.	10	3,162
Bloukop.........	5·5	.	.	.	.	.	.	.	.	.	316,200
Mossop..........	5·5	.	10	.	31	.	.	.	31	10	.
Zaaiplaats.......	4·0	10,000	316	.	.	.	.	.	.	.	.
Vlakfontein......	6·0	.	.	.	.	.	.	31,620	.	.	.
Onderstepoort....	6·0	.	.	.	1,000,000	.	.	10	1,000,000	1,000,000	.
Utrecht..........	4·5	.	.	10	10	10	10	10	10	10	.
Portugal.........	5·0	.	.	.	.	.	.	.	.	.	.
Biggarsberg......	4·0	.	.	.	.	.	.	.	.	.	10,000
Vryheid..........	6·0	.	.	.	.	.	.	.	.	.	.
Middelburg......	6·0	1,000,000	1,000	.	.	.	.	.	.	.	.
Lydenburg.......	4·5	.	.	.	.	.	.	.	.	.	.
Strathene........	4·5	.	.	.	.	.	.	.	.	.	.

* Results expressed as neutralising in

† Logarithmic expression of the recip

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p359-360b →

Table 2 cont.

antigens by convalescent antisera

Antisera											
Bloukop	Mossop	Zaai-plaats	Vlak-fontein	Onderste-poort	Utrecht	Portugal	Biggars-berg	Vryheid	Middel-burg	Lyden-burg	Strathene
.	.	31,620	.	10	.	.	.	.	31,620	.	.
.	.	3,162	.	.	.	.	.	.	100	.	.
.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	100,000	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	100,000	.	.	.	.	.	.	.	.
.	.	.	.	10,000	.	.	.	.	.	.	.
.	10	10	.	316,200	.	10	.	10	.	10	10
3,162	.	.	.	.	.	.	3,162	.	10	.	10
31,620	.	.	31	.	.	.	316,200	.	.	.	.
10	316,200	10	10	10	.	.	10	10	.	.	10
.	.	10,000	.	.	.	.	.	.	10,000	.	.
.	.	.	1,000,000	.	.	.	.	.	.	.	.
.	10	.	.	1,000,000	.	.	.	.	.	.	.
.	.	.	.	10	31,620	.	.	.	.	.	.
.	.	.	.	.	.	316,200	.	.	.	.	.
10,000	.	.	.	.	.	.	10,000	.	.	.	.
.	.	.	.	.	.	.	.	31,620	.	31,620	.
.	.	1,000,000	.	.	.	.	.	.	1,000,000	.	.
.	.	.	.	.	.	.	.	31,620	.	31,620	.
.	.	.	.	.	.	.	.	.	.	.	31,620

dices.
rocal of the end point dilution.

p359-360b

← p359-360a

Virus multiplication in tissue culture was initiated by the inoculation of two tubes of cultures with 0·2 ml. of a reconstituted ampoule of freeze-dried embryo suspension, after the growth medium had been changed to the modified medium without bovine serum. The infected cultures were returned to the incubator at 37° C and rolled for one-and-a-half to two hours, after which they were washed twice before adding 1 ml. of serum-free media. Three serial passages were made, and antigens for neutralisation tests prepared from the pooled culture fluids harvested when the monolayer of the third tissue culture passage showed 75 per cent specific degeneration. These antigens were stored in rubber stoppered tubes at 4° C until required.

Sera.—Convalescent sera were collected from each recovered animal between the 30 to 45th day. Following separation of the clot, each serum was Seitz filtered and stored at −20°C. All sera were inactivated at 56° for 30 minutes. As a result of the limited number of animals surviving infection with unmodified virus, it was not possible to prepare pools of immune convalescent sera.

Neutralisation tests.—A series of preliminary investigations showed the titre of antibodies in the sera of these sheep to be low, as determined by tissue culture methods. In this study it was therefore considered desirable to determine to what extent serial dilutions of an antigen would be neutralised by undiluted antisera. The test was made by adding to each of a row of six tubes 0·3 ml. of the serum being examined. An equal volume of serial ten-fold dilutions of the antigen under investigation was then added to each tube. The mixtures, held in an ice water bath during the manipulations, were shaken and then maintained for a period of one-and-a-half hours at 4°C. Finally two roller tube cultures were seeded with 0·2 ml. of each serum virus mixture. Cultures were incubated at 37°C and the development of the specific cytopathic change was recorded daily from observations made under an optical system giving 84-fold magnification. Concurrently, the virus titre of the antigen was determined by serial ten-fold dilution in undiluted sheep serum, known to be free from specific antibodies. The 50 per cent end-point of the antigen neutralised n the presence of each antiserum was calculated by the method of Reed & Muench (1937), at that stage of development when the cultures inoculated with the highest infective dilution of the antigen titration showed 50 per cent cell destruction.

Experimental Results

The results of the neutralisation tests are given in Table 2. Twelve distinct antigenic groups were demonstrated amongst the 22 strains examined, as indicated in Table 3. Although a random selection of material was used in this study, the existence of stable antigenic types is illustrated by the strains of Group 3, which have a wide global origin, or by the strains of Group 4, where a period of over 50 years separates the isolation of the first and last recorded strains. The reciprocal cross-neutralisation amongst isolates of these groups further substantiates this assumption. With the exception of Group 7, the authenticity of these results has been confirmed beyond doubt, by the identification and classification of numerous subsequent isolates, as part of a separate study which will be reported in due course.

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TABLE 3

Antigenic grouping of strains examined

Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9	Group 10	Group 11	Group 12
Biggarsberg Bloukop Schoeman	Vryheid Lydenburg	Cyprus Mimosa Park Jansen Onderstepoort	Theiler Zaaiplaats Middelburg Bekker	Mossop	Strathene	Utrecht	Camp	University Farm	Portugal	Nelspoort Vlakfontein	Byenespoort

Discussion

The development of the roller tube tissue culture technique has provided a valuable indicator system for the *in vitro* serum-virus neutralisation test, in the study of many viruses. This is particularly true of the bluetongue virus, as it is now possible to differentiate unequivocally the main antigenic types.

It is apparent from these results that the antigenic response of sheep to infection by virulent virus is sufficiently type specific to permit the use of convalescent sera for the identification of isolates. The methods employed and the limited number of strains involved in this study do not permit an evaluation of the extent to which variation occurs within an antigenic type, although the results with the Bekker strain suggest a slight heterogenesis.

At present very little is known of the relationship between vector and reservoir and the associated factors which determine the incidence and severity of an epizootic. Prophylactic immunisation appears, therefore, to be the only rational method of controlling the disease. In the Union of South Africa, in spite of the extensive use of a quadrivalent egg-adapted vaccine, frequent reports are received of clinical symptoms of bluetongue in immunised flocks. These breakdowns may be of such frequency and severity as to constitute a significant economic loss. It is therefore of the utmost importance that the components of any vaccine must be sufficiently comprehensive to cover the wide variety of antigenic types now known to exist. Only by the identification of the types involved in these outbreaks, as well as of those strains recently introduced into countries previously free of the disease, will it be possible to maintain a close correlation between the predominant strains and those selected for prophylactic purposes.

Summary

As a preliminary investigation the existence of 12 distinct antigenic types of bluetongue virus was established. Where the facilities are available, the methods employed provide a rapid and accurate procedure for the antigenic identification of field specimens.

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