THE APPLICATION OF IMPROVED TECHNIQUES TO THE IDENTIFICATION OF STRAINS OF BLUETONGUE VIRUS

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ABSTRACT

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Previously the isolation and identification of strains of bluetongue virus has been tedious, so that the introduction of techniques to shorten the delay was considered highly desirable.

Neutralization tests were conducted on the principle of the inhibition of plaque development by serum diffusing through an agarose overlay. Fish spine beads filled with serum were placed on the overlying cells. The utilization of serum mixtures further provided a saving of materials. These techniques when applied to a group of field specimens were found to give reliable results. Similarly homologous antibody in the convalescent serum of recovered donor sheep could be demonstrated by this technique and served to confirm the immunological classification of the samples.

INTRODUCTION

The clinical diagnosis of bluetongue in sheep is often difficult, due to the marked variations in the severity of the symptoms and lesions. During the course of an epizootic, animals within a flock are encountered in various stages of the disease and symptoms may vary from a mild transient hyperaemia to a severe inflammatory process, involving the mouth, respiratory tract, muscles and hooves. These variations may be due to the intensity of solar radiation (Neitz & Riemerschmid, 1944), differences in breed susceptibility or the virulence of the particular strain of virus involved (Neitz, 1948). In view of the multiplicity of antigenic types of virus which exist (Howell, 1960) it is important to confirm the diagnosis by isolation of the responsible virus and to determine the antigenic identity of the strains as early as possible, particularly in those regions where the disease has not been previously diagnosed. The speed with which the virus can be isolated and identified is dependent upon the techniques employed.

In a recent report (Goldsmit & Barzilai, 1965) it was shown that the intravenous inoculation of embryonated chicken eggs reduced the incubation period and increased the sensitivity of this host, when used for the primary isolation of bluetongue virus from field samples.

This paper describes the application of further improved techniques to the isolation and identification of the virus from field specimens received from natural outbreaks of the disease during the 1965-66 season.

MATERIALS AND METHODS

Field samples

Samples of whole blood, collected in an equal volume of Edington's OCG* were submitted from suspected cases of the disease. Wherever possible and after receipt, these samples were stored at 4°C.

Fertile eggs

Fertile hens' eggs were obtained from a closed flock of White Leghorns maintained at this Institute. After 7 days preincubation the eggs were inoculated onto the chorio-allantoic membrane according to the method of Gorham (1957). The eggs were then returned to an incubator, operating at 33.5°C and examined daily by transillumination. From the 2nd day onwards all embryos of a particular specimen, dying on the same day, were harvested and macerated separately in tissue homogenizers. The pulped embryo tissue was suspended in 0.066M phosphate buffer pH 7.4, which contained in addition, 1 per cent neutral peptone and 5 per cent lactose. For each embryo harvested, 5 ml of diluent was added. Finally a pool was prepared from each specimen by adding together in equal volume the stored embryo suspension from each day's harvest.

Tissue cultures (a) *Primary lamb kidney monolayers:* Roller tube cultures of primary lamb kidney cells were prepared by trypsin dispersion according to the method of Youngner (1954). For attachment and growth, cell clusters were suspended in a balanced salt solution, without trace elements, prepared according to the formula of Rappaport (1956), to which 0.5 per cent lactalbumin and 10 per cent bovine serum were added. Before seeding with virus, cultures were washed twice with phosphate buffered saline (PBS) (Dulbecco & Vogt, 1954). In the maintenance medium, foetal calf serum at a final concentration of 2 per cent replaced the bovine serum.

(b) *Mouse connective tissue cells:* Petri plate cultures were prepared for plaque assay from cells derived from the cell line NCTC Strain L, clone 929 (Sanford, Earle & Likely, 1948) as previously described (Howell, Verwoerd & Oellermann, 1967). Where the recovery of virus from individual plaques was desired, an additional 8 ml of agarose overlay containing 1 in 20,000 neutral red was added 18 to 24 hours before the plates were examined.

Preliminary treatment of samples

As a result of low virus titres and frequent bacterial contamination of previously examined samples, reinfection of bluetongue susceptible Merino sheep was found to be advantageous. For this purpose 2 ml of each blood sample was inoculated into 2 sheep by the intravenous route. These animals were housed in stables and their rectal temperatures recorded daily. During the febrile reaction, blood was withdrawn and added to an equal volume of 0.066M phosphate buffer containing 1 per cent peptone and 25 IU per ml of heparin. These samples were then stored at 4°C until required for further examination. One of each pair of sheep was injected on the eighth and tenth days after infection, with 500 mg of chlortetracycline.†

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*Oxalate-Carbol-Glycerine

| Carbolic acid | 5 g | Potassium oxalate | 5 g |
| Glycerine | 500 ml | Distilled H₂O | 500 ml |

†Aureomycin injectable, Cyanamid

For the inoculation of fertile eggs or tissue cultures, a 1 in 5 dilution of blood from one of the reacting sheep was prepared in maintenance medium. Embryonated eggs were each inoculated with 0.2 ml of diluted blood while 1.0 ml was used for the inoculation of each type of tissue culture. Adsorption of the virus was allowed to proceed in infected tissue cultures at 28°C for 3 hours, after which they were washed twice with prewarmed PBS. One and a half millilitres of maintenance medium was then added to each roller tube culture. Petri plate cultures were finally overlayed with 8.0 ml of Eagle's medium containing 0.5 per cent agarose and incubated at either 33.5°C or 37°C as indicated (Table 4).

Neutralization tests

(a) *Antisera*: (i) *Convalescent serum samples*. All experimental animals were bled at the commencement of the investigation and 35 days after injection. After separation of the clot, the serum was stored at —20°C until required. (ii) *Type antisera*. Type specific antisera to each of 16 antigenic types of virus were prepared in young adult guinea pigs. Groups of 20 guinea pigs were injected individually by the intraperitoneal route with 5 ml of tissue culture propagated virus. Two injections were given at 10 day intervals and the serum collected 30 days after the last injection. The serum from all guinea pigs within a group was pooled and stored at —20°C. Before use the sera were inactivated at 56°C for 30 minutes.

(b) *Test procedure*: (i) *Identification and serological typing of virus strains*. Samples of virus for identification were obtained from roller tube cultures of lamb kidney cells showing advanced cytopathic effect (CPE). These tubes had been infected previously with plaques selected from petri plate cultures seeded with suspensions of infective chick embryo tissue.

A 1 in 10 and 1 in 100 dilution of each virus sample were seeded onto duplicate petri plate cultures. After adsorption, 8 ml of overlay was added and allowed to gel. Eight fish spine beads each containing 0.02 ml of a mixture of antisera, prepared according to the scheme set out in Table 1, were then placed in strict sequence onto the surface of the overlay. After 3 days incubation the plates were stained with a 1 in 20,000 dilution of neutral red in PBS. Areas of plaque inhibition could then be read on the same day or if necessary on succeeding days if plaque development was slow. By reference to the latin square arrangement of the antisera in Table 1 the two mixtures giving zones of plaque inhibition as a result of the presence of a common antiserum in both, were used to determine the antigenic identity of the plaque-forming virus.

TABLE 1 *Composition of antiserum mixtures*

Mixture No.	1	2	3	4
5	1*	5	9	13
6	2	6	10	14
7	3	7	11	15
8	4	8	12	16

*Identity of type specific guinea pig antiserum

(ii) *Detection of neutralizing antibody in the sera of donor sheep*. The preinfection and convalescent sera from the experimental sheep, which had reacted and survived the injection of the field samples, were tested for neutralizing antibodies to each of the 16 immunological types of virus. These tests were performed by seeding sufficient petri plate cultures with a suitable dilution of each of the type strain antigens. After adsorption and the addition of the overlay, fish spine beads

filled with each serum sample were placed in strict sequence onto the surface of the overlay. After staining on the 3rd or 4th day of the test, areas of plaque inhibition were recorded.

RESULTS

Clinical response of experimental sheep to infection with field samples

An evaluation of the severity of the symptoms and the course of the disease following artificial infection of the experimental sheep with the various samples, confirmed the earlier observations on the marked variation in the clinical response of affected sheep (Table 2). The incubation period varied from 3 to 8 days with a mean of 5.7 days. The mean duration of the febrile reaction exceeding 39°C was only 3.6 days with a variation of 2 to 7 days.

It will be observed that the highest temperature recorded during the febrile reaction bore little relation to either the severity of the clinical symptoms or the final outcome of the disease. The climax of the febrile reaction was both irregular and unpredictable.

Sheep with barely detectable lesions succumbed, whereas other animals inoculated with the same strain of virus, passed through a severe reaction with pronounced symptoms and recovered.

The role of secondary bacterial complications in determining the outcome of disease is generally recognised, but difficult to evaluate. In this group of sheep, the higher incidence of recovery amongst the treated animals appears to be significant, in comparison with those animals which received no antibiotic therapy.

Primary isolation of virus in fertile hens' eggs

The results of the recovery of virus from the 1 in 5 dilution of blood inoculated into the 7 day-old fertile hens' eggs are summarized in Table 3. Embryo mortality was considered specific if it took place after 48 hours of incubation and the harvested embryos appeared cherry red. Death of the embryos was confirmed as being due to the multiplication of bluetongue virus by the demonstration of plaques in the petri plate cultures of mouse connective tissue cells seeded with dilutions of the supernatant fluid of the harvested embryo pools.

After 7 days incubation all the plated samples obtained from the reacting sheep produced plaques with titres indicating infectivity of the chicken embryo tissue suspensions of $10^{-2.9}$ to $10^{-6.7}$ plaque forming units (PFU) per ml.

In comparison, the recovery of virus from the blood samples seeded onto the particular tissue culture systems employed was disappointing (Table 4). In lamb kidney roller tubes only one sample produced specific CPE when incubated at 37°C. Similarly the recovery of virus by direct plaque assay was also poor, in so far as only ten of the 17 samples were positive. A lower temperature of incubation did not appear to improve the susceptibility of either tissue culture system.

It could therefore be concluded from these results that the 7 day-old chicken embryo incubated at 33.5°C was the most susceptible system for the primary isolation of virulent bluetongue virus. Once the virus had multiplied in chicken embryo tissue there was no difficulty adapting the virus to mouse connective tissue cells and obtaining plaques under agarose.

Immunological classification of strains of virus isolated in fertile hen's eggs

In order to obtain some degree of uniformity, strains of virus were identified which had undergone a similar process of isolation and adaptation. Virus samples were obtained from plaques selected from plates, which had

TABLE 2 *Clinical response of experimental sheep to the injection of field samples*

Sample No.	Identification of experimental sheep	Incubation period (Days)	Highest Temperature °C	Febrile reaction in excess of 39°C		Clinical severity of disease	Outcome
				Duration	Days after injection		
26/66	22151	6	41.5	3	6–8	+++1	Died
	22155T	7	42	4	7–10	++	Recovered
27/66	21341	5	41	2	5–6	+++	Died
	22140T	6	42	4	6–9	++	Recovered
28/66	20175	3	42	7	3–9	++++	Recovered
	22150T	7	41.5	3	7–9	+++	Recovered
29/66	21351	6	41.5	2	6–7	++	Recovered
	22149T	6	42	3	6–9	++	Recovered
30/66	21364	5	42	2	5–6	+	Recovered
	22157T	7	41	2	7–8	+	Recovered
31/66	20149	8	40.5	3	8–10	++	Died
	22165T	6	40	5	6–10	+++	Recovered
32/66	20147	5	41.5	7	5–11	+++	Died
	22163T	6	41	4	6–9	++++	Died
34/66	20183	7	42	2	7–8	—	Died
	22139T	4	41	4	4–7	++	Recovered
35/66	20124	5	42	5	5–9	+	Died
	22170T	5	41.5	4	5–8	+++	Died
38/66	21316	4	43	3	4–6	+	Recovered
	22148T	6	41.5	5	6–10	++	Recovered
39/66	21310	4	41.5	4	4–7	+++	Died
	22158T	7	42	4	7–10	+++	Recovered
47/66	20185	4	41	4	4–7	+++	Died
	22156T	5	41.5	5	5–8	+++	Recovered
51/66	21352	6	41	2	6–7	++	Recovered
	22142T	5	41	3	5–7	++	Recovered
53/66	20178	5	42	3	5–7	—	Died
	33143T	4	41.5	5	4–8	+++	Recovered
54/66	20174	8	41.5	2	8–9	+	Recovered
	22125T	6	41	4	6–8	++	Recovered
73/66	21577	6	41.5	3	6–8	++	Died
	22147T	8	40	3	8–10	++	Recovered
76/66	21598	5	42	4	5–8	+++	Died
	22160T	6	41.5	3	6–8	+++	Recovered

T = Animal treated - see text
(1) = The symbols +, ++, +++ and ++++ indicate the extent of involvement of the susceptible tissues and the increasing degrees of inflammatory reaction.

been previously infected with the limiting dilution of the pools of infective chicken embryo tissue. At least five plaques were selected from each sample. Each of these plaques was seeded directly into a roller tube culture of primary lamb kidney cells. The recovery of virus from these plaques, as indicated by the development of progressive CPE in roller tubes is given in Table 5. The time taken for each particular plaque to infect and produce 75 to 100 per cent CPE in roller tube cultures was found to be irregular and varied from one plaque to another, even when selected from the same strain. This would suggest that the concentration of virus in the localized areas of virus multiplication in the petri plate cultures also varied to the extent that 30 of the 85 plaques selected, yielded insufficient virus to establish infection in roller tube cultures.

Among this group of samples, strains of virus representing nine serological types were identified. The inhibition of plaque formation by the diffusion of the mixtures of antiserum through the agarose onto the infected monolayer was highly type specific and no doubt was ever experienced as to the identity of the strains of virus. These results confirmed earlier unpublished observations on the application of this test to the study of bluetongue virus homologous antibody.

An illustration of the pattern of plaque inhibition produced by the antiserum mixtures on plates infected with samples representing Types 1, 4 and 12 respectively is shown in Plate 1. The complete lack of inhibition of plaque formation by any of the other antiserum combinations is clearly indicated.

Immunological response of donor sheep to the injection of field samples

The development of specific neutralizing antibodies to the particular strain of virus recovered from the donor sheep is indicated in Table 6. All the sera which were
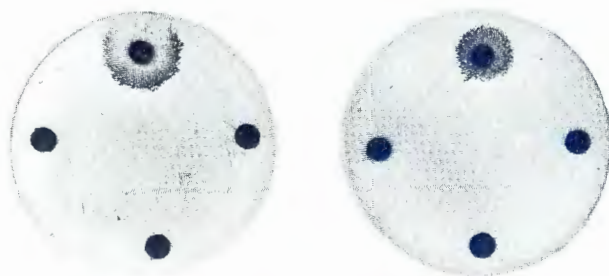
Sample 38/66 (Type 1)
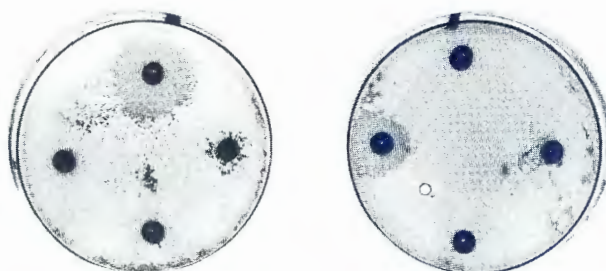
Sample 29/66 (Type 4)

Sample 30/66 (Type 12)

Key to position of antiserum mixtures

PLATE 1   Plaque inhibition by mixtures of antiserum contained in fish spine beads

TABLE 3 *Isolation of virus from field samples in fertile hens' eggs*

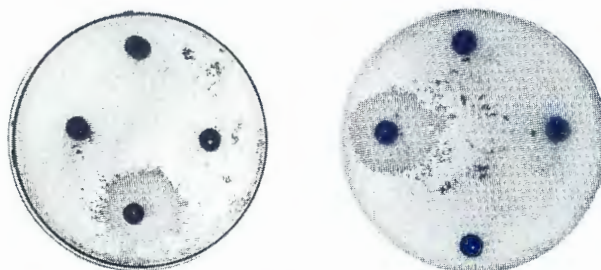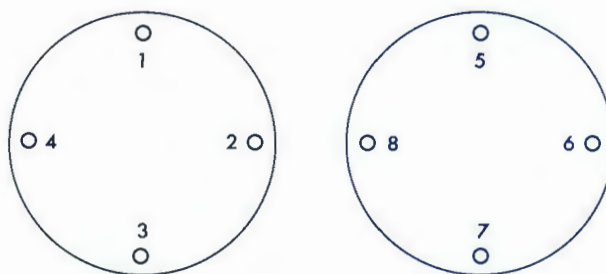| Identification of sample producing clinical reaction | Experimental sheep | Embryo mortality at 33.5°C | | | | | | | Infectivity of pooled embryo tissue suspensions PFU per ml |
| | | Days after inoculation | | | | | | | |
		1	2	3	4	5	6	7	
26/66	22151	0/6	0/6	0/6	0/6	0/6	1/5	0/5	8×10^2
27/66	21341	0/6	0/6	1/6*	0/5	0/5	1/4	2/2	8×10^2
28/66	20175	0/6	0/6	0/6	0/6	1/5	2/3	0/3	8×10^3
29/66	21351	0/6	0/6	0/6	0/6	1/5	1/4	0/4	8×10^4
30/66	21364	0/6	0/6	0/6	0/6	1/5	1/4	0/4	8×10^3
31/66	20149	0/6	0/6	1/5	0/5	1/4	3/1	0/1	1×10^4
32/66	20147	0/6	0/6	0/6	0/6	1/5	0/5	1/4	6×10^6
34/66	20183	0/6	0/6	0/6	0/6	2/4	0/4	0/4	3×10^5
35/66	20124	0/6	0/6	0/6	0/6	1/5	1/4	0/4	2×10^5
38/66	21316	1/5	0/5	0/5	0/5	4/1	0/1	0/1	1×10^4
39/66	21310	1/5	0/5	0/5	0/5	3/2	1/1	0/1	2×10^5
47/66	20185	0/6	0/6	0/6	0/6	4/2	1/1	0/1	5×10^5
51/66	21352	1/5	0/5	0/5	0/5	2/3	0/3	1/2	3×10^4
53/66	20178	0/6	0/6	0/6	0/6	2/4	2/2	0/2	1×10^6
54/66	20174	0/6	0/6	1/5	0/5	1/4	2/2	0/2	5×10^3
73/66	21577	1/5	0/5	0/5	0/5	1/4	0/4	0/4	2×10^6
76/66	21598	1/5	0/5	0/5	0/5	1/4	0/4	0/4	3×10^3

*1/6 Ratio of embryos found dead to embryos still alive

TABLE 4 *Isolation of virus from field samples in tissue cultures*

Identification of sample producing clinical reaction	Experimental sheep	Multiplication in tissue culture			
		Lamb kidney monolayers		NCTC strain L clone 929 derived cells	
		CPE observed at incubation temperature		PFU per ml at incubation temperature	
		37°	33.5°	37°	33.5°
26/66	22151	None	None	0	0
27/66	21341	,,	,,	0	0
28/66	20175	,,	,,	50	0
29/66	21351	,,	,,	25	0
30/66	21364	,,	,,	0	0
31/66	20149	,,	,,	270	0
32/66	20147	,,	,,	0	0
34/66	20183	,,	,,	55	0
35/66	20124	,,	,,	300	0
38/66	21316	,,	,,	0	0
39/66	21310	,,	,,	30	0
47/66	20185	,,	,,	100	0
51/66	21352	,,	,,	0	0
53/66	20178	,,	,,	30	0
54/66	20174	,,	,,	0	0
73/66	21577	Positive	,,	60	0
76/66	21598	None	,,	50	0

examined at a dilution of 1 in 5 gave areas of plaque inhibition exceeding 18 mm in diameter, which when measured against the dose of virus used in these tests ($10^{-3.5}$ to $10^{-4.0}$ PFU per ml) represents a significant rise in antibody titre. These results confirmed the serological typing of the strains of virus isolated from the samples in so far as only homologous neutralizing antibody was detected in the convalescent antisera. Furthermore the absence of neutralizing antibody to any other immunological type of virus confirmed the homogenous nature of this particular group of samples collected from the naturally infected sheep.

DISCUSSION

Diseases caused by viruses with a multiple antigenic structure complicate the procedures required during the course of diagnostic work. The classification of the responsible strain of virus into its particular serological group is an important factor in determining the epizootiology of the disease, as well as the evaluation of the efficacy of prophylactic immunization. This is particu-

larly true of bluetongue, where to date 16 immunologically distinct types of virus have been identified.

It has been observed that the success with which primary isolation can be achieved in the recognised laboratory host systems is often unpredictable. Fernandes (1959) experienced no difficulty in the adaptation and propagation of a single virulent strain of bluetongue virus in monolayer cultures of primary lamb kidney cells. During the past few years the routine examination of numerous specimens in this laboratory has not been rewarded with the same degree of success. These failures could possibly be accounted for by variations in the susceptibility of different batches of primary lamb kidney cultures further complicated by the relatively low titre of virus in certain of the blood samples. Alternatively, certain intrinsic characteristics of the particular strains, or of the immunological group to which they belong, may have been responsible.

The results presented in this report suggest that the fertile hen's egg, incubated at 33.5°C, was the most susceptible host system, although the concentration of virus in the blood samples was invariably of low infecti-

TABLE 5 *Identification of plaques selected from cultures seeded with chick embryo virus*

Sample No.	Plaque identification	Incubation period (Days)	Serological type	Sample No.	Plaque identification	Incubation period (Days)	Serological type
26/66	a	4*	2	38/66	a	Neg.	—
	b	2	2		b	9	1
	c	4	2		c	7	1
	d	2	2		d	4	1
	e	2	2		e	Neg.	—
27/66	a	6	2	39/66	a	4	1
	b	7	2		b	3	1
	c	2	2		c	Neg.	—
	d	3	2		d	3	1
	e	Neg.	—		e	Neg.	—
28/66	a	3	5	47/66	a	3	3
	b	2	5		b	4	3
	c	3	5		c	5	3
	d	Neg.	—		d	3	3
	e	8	5		e	4	3
29/66	a	3	4	51/66	a	Neg.	—
	b	Neg.	—		b	4	8
	c	Neg.	—		c	Neg.	—
	d	Neg.	—		d	4	8
	e	Neg.	—		e	5	8
30/66	a	2	12	53/66	a	4	6
	b	3	12		b	9	6
	c	Neg.	—		c	6	6
	d	Neg.	—		d	3	6
	e	Neg.	—		e	8	6
31/66	a	6	4	54/66	a	Neg.	—
	b	7	4		b	Neg.	—
	c	4	4		c	Neg.	—
	d	7	4		d	8	11
	e	6	4		e	Neg.	—
32/66	a	4	2	73/66	a	4	12
	b	5	2		b	Neg.	—
	c	3	2		c	Neg.	—
	d	3	2		d	Neg.	—
	e	Neg.	—		e	Neg.	—
34/66	a	10	3	76/66	a	4	3
	b	3	3		b	Neg.	—
	c	4	—		c	7	3
	d	5	3		d	Neg.	—
	e	6	3		e	Neg.	—
35/66	a	Neg. 3	—				
	b	3	3				
	c	4	3				
	d	Neg.	—				
	e	6	3				

*Period taken for cells in roller tube cultures to develop more than 75 per cent CPE

vity. While plaque assay with mouse fibroblast cells appears to be a more sensitive method of detecting virus than direct isolation in primary lamb kidney monolayers, a more sensitive cell type is still required.

The plaque inhibition test with fish spine beads, as described by Porterfield (1960), has been used in the study of a number of the arthropod-borne viruses (Porterfield, 1961; Williams, Woodall & Porterfield, 1962). It has been found that this test can be applied equally well to demonstrate specific neutralization of bluetongue virus by homologous antibody. In this study the technique was applied to the identification of strains of virus, as well as to the detection of neutralizing antibody in the convalescent serum of sheep.

The identification of nine immunological types amongst the group of specimens is typical of the results obtained in previous seasons. It is apparent that the known immunological types of bluetongue virus are widely and randomly distributed within the main enzootic regions. The simultaneous isolation of numerous antigenic types of virus from a single flock of infected sheep suggests that a similar distribution of virus strains must occur within the insect vector population. The simultaneous infection of a sheep by more than one strain of virus through the feeding of the abundantly prevalent insect vectors therefore appears to be a distinct possibility. Although in this study the number of clones identified from each specimen was small, each sample nevertheless appeared to be composed of a homogenous virus population. This was confirmed beyond doubt by the development of homologous antibody to only a single antigenic type of virus in the convalescent serum of each sheep.

The economy and ease with which the routine typing procedure can be carried out, together with the incorporation of a technique which permits cloning of the virus population, make the examination of a large number of clones from a potentially mixed population a practical proposition. The introduction of this procedure has considerably shortened the time required for

TABLE 6 *Development of virus neutralizing antibodies in the convalescent sera of sheep injected with field samples*

Sample No.	Virus type isolated	Identification of donor	Serum sample	Plaque inhibition of type strain antigen															
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
26/66	2	22155	Pre	—*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			Post	—	+†	—	—	—	—	—	—	—	—	—	—	—	—	—	—
27/66	2	22140	Pre	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			Post	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
28/66	5	20175	Pre	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			Post	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—
		22150	Pre	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			Post	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—
29/66	4	21351	Pre	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			Post	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—
		22149	Pre	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			Post	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—
30/66	12	21364	Pre	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			Post	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—
		22157	Pre	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			Post	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—
31/66	4	22165	Pre	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			Post	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—
34/66	3	22139	Pre	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			Post	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
38/66	1	21316	Pre	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			Post	+	—	—	—	—	—	—	—	—	•	—	—	—	—	—	—
		22148	Pre	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			Post	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
39/66	1	22158	Pre	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			Post	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
47/66	3	22156	Pre	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			Post	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
51/66	8	21352	Pre	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			Post	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—
		22142	Pre	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			Post	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—
53/66	6	22143	Pre	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			Post	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—
54/66	11	20174	Pre	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			Post	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—	—
		22125	Pre	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			Post	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—	—
73/66	12	22147	Pre	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			Post	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—
76/66	3	22160	Pre	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			Post	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—

* — No inhibition   † + Inhibition

the isolation and typing of a sample of the virus. Based on the sum of the mean incubation periods, suitably collected and preserved blood samples could be typed within 15 days.

SUMMARY

Strains of bluetongue virus, isolated from a group of field samples were subjected to cloning and typed by the use of a plaque inhibition test. This procedure was found to give reliable results and led to a considerable saving of both time and materials.

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