Studies on Bluetongue. VI. Animal Transmission Studies

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ABSTRACT

The Cyprus strain of bluetongue virus was successfully transmitted through six passages and the Station strain through one passage in calves. Although the animals developed no visible evidence of infection, viremia as shown by both passage and fluorescent antibody examination of infected foetal bovine kidney culture, and by serological conversion was nevertheless demonstrated. No enhancement of virulence for calves or sheep was shown following bovine passage. A ewe inoculated in late pregnancy with blood drawn from a calf 59 days after its infection, gave birth to a lamb from whose blood the virus was isolated. Significant complement-fixation titres persisted for at least 200 days.

RESUME

La fièvre catarrhale ovine a été transmise à des veaux pendant six passages utilisant la souche de "Cyprus" et pour un passage avec la souche "Station". Bien que les animaux n'aient développé aucun signe clinique d'infection, néanmoins une virémie, mise en évidence par l'examen à l'immunofluorescence et suivie par une conversion sérologique, a été démontrée. Les passages successifs du virus chez les bovins n'ont pas montré d'accroissement de la virulence pour les veaux ou les moutons. En plus on a isolé le virus du sang d'un agnelet né d'une brebis inoculée tard durant la gestation avec du sang prélevé d'un veau 59 jours après son infection. Des taux significatifs de la fixation du complément ont persisté chez ces animaux pendant au moins 200 jours.

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Introduction

While bluetongue is generally regarded at present as being a disease of sheep, it has long been known that the virus is capable of infecting cattle (1). There has, in fact, been the suggestion that the bovine species is the definitive host (2). Other species including goats and wild ruminants can become naturally infected and may be of great importance in the ecology of the disease (3).

Studies of infection of bovines with bluetongue virus have been conducted mainly in Africa, and from these investigations a number of general facts have emerged. With the exception of the report by Bekker *et al* (1), who may have been dealing with a complicated condition, these studies agree that in cattle, the reaction is minimal, being limited to a short thermal response. Other work has shown that virus can be isolated from cattle over a four to five month asymptomatic course, that periodic viremia occurs, and repeated reinfection with heterologous strains is commonplace (2, 4).

Work on various aspects of bluetongue infection has been conducted in our laboratory for some time (5, 6, 7, 8, 9). Where this has involved experimental infection of cattle, the mildness of the disease in this species has been confirmed. Furthermore, the modified direct complement fixation test and the immunofluorescence technique applied to infected tissue cultures have been found to be satisfactory means for demonstrating the presence of this infection.

The present study was undertaken to investigate several possibilities: firstly, whether serial passage through cattle

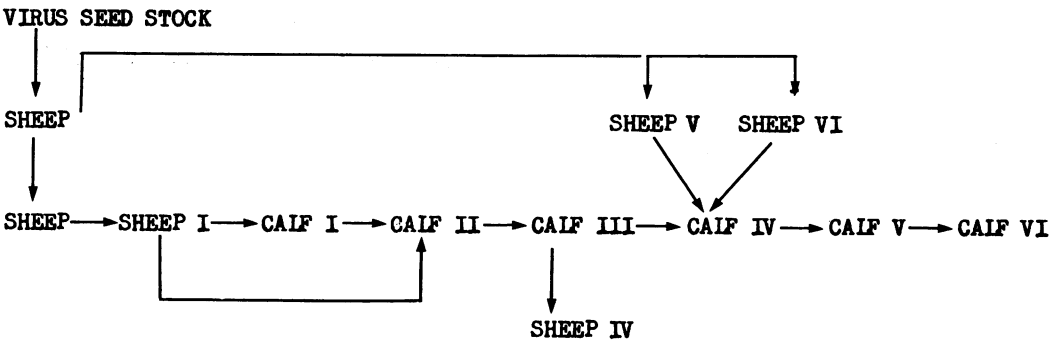

Fig. 1. Diagrammatic representation of bovine and ovine passages of Cyprus strain of Bluetongue virus.

might enhance the virulence of bluetongue virus for this species or for sheep, and secondly, whether infection with a single strain of virus would bring about a periodic viremia which might be reflected in a fluctuating complement-fixation titre.

Materials and Methods

Infectious Agents

Two strains of bluetongue virus (BT) were used in this study. The Cyprus (Type 3) strain was obtained in 1960 from the Division of Veterinary Services, Onderstepoort, Republic of South Africa, as a freeze-dried ten percent suspension of chick embryo tissues of the third egg-passage. It had received an additional egg passage via the chorioallantoic membrane, followed by inoculation into sheep.

The Texas Station strain was obtained in 1965 from Dr. C. W. Livingston, College of Veterinary Medicine, Texas A. and M. University. This material was received as second-passage, ovine whole blood in O.C.G.[1] solution, and served to initiate further sheep passages by its inoculation into Sheep II.

Experimental Animals

Cattle

All cattle used were animals of mixed breeding, from 4 to 12 months of age, obtained from local herds. All were prebled and held in isolation quarters for variable observation periods, during which all remained clinically normal.

Calf I was inoculated intravenously (i/v) and subcutaneously (s/c) with 10.0 ml. by each route of defibrinated blood drawn from Sheep I at the height of its febrile response.

Calf II received i/v 30.0 ml. of a pool composed of heparinized blood from Calf I drawn on the 7th post-inoculation day (dpi) and defibrinated blood from Sheep I This calf was bled daily for ten days, the defibrinated blood samples pooled, and 100.0 ml. of this pool inoculated i/v into Calf III. From the 35th dpi until the 343rd dpi Calf II was bled at approximate weekly intervals for serological testing and/or virus isolation attempts.

Calf III, following exposure, was bled on ten consecutive days. A 10.0 ml. aliquot of the defibrinated blood obtained on the 7th dpi was divided and 5.0 ml. were inoculated i/v and s/c respectively into Calf IV. Following this, Calf III was bled weekly from the 21st to the 336th dpi.

Calf IV was bled daily for the first nine days after inoculation. A pool of the bleedings of the 5th, 6th and 8th dpi was inoculated i/v and s/c, 10.0 ml. by each route, into Calf V. From the 21st dpi Calf IV was bled weekly for serum and/or virus isolation until the 315th dpi. In addition, this calf was challenged by an inoculation i/v on the 57th dpi and s/c on the 58th dpi with 10.0 ml. of defibrinated blood drawn from Sheep V at the height of its febrile response. On the 59th dpi, it was inoculated s/c with 10.0 ml. of defibrinated blood similarly drawn from Sheep VI.

Blood samples were taken daily from Calf V until the 10th dpi and weekly thereafter through the 242nd day. Defibrinated blood taken from this calf on the 7th dpi was inoculated (5.0 ml. i/v and 5.0 ml. s/c) into Calf VI.

[1]Potassium oxalate 5 gm
Carbolic acid 5 ml.
Glycerine 500 ml.
Distilled water 500 ml.

Calf VI was bled daily for nine days and weekly thereafter through the 205th dpi.

Calf VII was inoculated (5.0 ml. i/v and 5.0 ml. s/c) with defibrinated blood drawn from Sheep III at the peak of its febrile response on the 8th dpi. This calf was bled daily for ten days and then weekly through the 316th dpi.

Sheep

Adult sheep of mixed breeding, obtained from local flocks were used in this study. All were adjudged clinically normal during a pre-experimental observation period.

Sheep I was inoculated with virulent blood of the second sheep passage of the Cyprus strain, and responded with a temperature rise to 106.8°F. on the 7th dpi. Blood obtained at this time provided the inoculum used for Calf I.

Sheep IV, also exposed to the Cyprus strain, was inoculated (10.0 ml. i/v and 10.0 ml. s/c) with freshly defibrinated blood drawn from Calf III on the 59th dpi. It was bled weekly from the 19th through the 287th dpi.

Sheep II received 5.0 ml. i/v and s/c of the blood in O.C.G. received from Texas and containing the Station strain. Defibrinated blood was harvested at the peak of febrile response (7th dpi) and similarly inoculated into Sheep III. Defibrinated blood from this latter sheep served as inoculum for Calf VII. Subsequently, Sheep III was bled weekly for serological and/or virological examination through a 330-day observation period.

Sheep V was inoculated with virulent blood from the first sheep passage of the Cyprus strain, and underwent a febrile response which reached a maximum of 106°F. on the 6th dpi.

Sheep VI received the same inoculum as Sheep V, reacting to 105.2°F. on the 7th dpi. These latter two animals supplied the blood used to challenge Calf IV. These blood samples were taken from each animal at the height of its thermal response.

The inoculations of calves and sheep described above are diagrammatically represented in Figs. 1 and 2.

Tissue cultures

Primary bovine foetal kidney (BFK) cells, prepared by a method previously described (8) were used for virus isolation attempts and for immunofluorescence staining. For this latter procedure, cells were grown as coverslip cultures in Leighton tubes.

VIRUS SEED STOCK → SHEEP II → SHEEP III → CALF VII

Fig. 2. Diagrammatic representation of bovine and ovine passages of Station Strain of Bluetongue virus.

Complement-fixation tests

The modified direct complement-fixation test (C.F.), as described in another publication (7) was used.

Fluorescent antibody technique (F.A.T.)

Coverslip cultures were examined by the technique described elsewhere (9). Examination of infected cultures was conducted three to five days after inoculation.

Results

Cyprus strain

With the exception of Calf III and Calf IV, inoculation of the Cyprus strain of BT virus, which had produced a typical response in Sheep I, failed to elicit either a febrile response or any other clinical manifestation of infection throughout the entire observation period. Calf III responded on the 7th dpi with a temperature elevation to 104.6°F. and remained above 103°F. for an additional four days. No other clinical abnormality was noted in this animal. Calf IV underwent a transitory elevation of temperature during the 9th dpi, with a return to normal by the following morning. Slight nasal catarrh, coupled with mild congestion of the conjunctivae and buccal mucosa, anorrexia and ruminal atony were noted on the 14th dpi, and persisted for two days.

The attempted demonstration of the BT agent by the F.A.T. was negative when applied to tissue cultures inoculated with the serial bleedings of Calf II. With Calf III, positive results were obtained with samples taken from the 6th to 9th dpi inclusive. Calf IV gave a suspicious reaction on the 4th dpi, positive results on days 5, 6, 8 and 9, and negative on day 7. Positive results were obtained with the blood samples taken from Calf V on the 4th through 7th dpi, Calf VI gave suspicious reactions on the 6th and 8th dpi, was negative on the 7th and positive on the 9th dpi. The above results are summarized in Table 1.

Serum of none of the calves inoculated

TABLE I. Temperature response and F.A.T. results obtained with calves inoculated with The Cyprus strain of BT virus

Calf	Days																				Observed to (dpi)	Maximum T after 10th dpi
	1		2		3		4		5		6		7		8		9		10			
	T	FAT	T	FAT	T	FAT	T	FAT	T	FAT	T	FAT	T	FAT	T	FAT	T	FAT	T	FAT		
I	X	X	102.4	X	101.2	X	101.2	X	101.6	X	101.4	X	101.6	–	101.2	X	101.6	X	101.2	X	51	102.2
II	101.4	–	101.0	–	102.0	–	101.0	–	101.2	–	102.0	–	101.4	?	101.8	–	101.6	–	101.4	–	114	102.6
III	102.2	X	102.0	–	102.4	–	102.5	–	102.4	–	102.0	+	104.6	+	103.6	++	104.0	++	103.4	–	101	103.0
IV	102.0	–	102.0	–	101.8	–	102.1	?	101.6	++	100.4	++	101.4	–	102.4	++	103.0	++	100.5	X	269	104.0
V	101.8	–	102.0	–	102.0	–	101.6	+	102.0	++	102.4	++	102.4	+	102.0	–	102.0	–	102.2	–	196	103.0
VI	101.2	–	102.4	–	102.2	–	102.4	–	101.6	–	102.4	?	X	–	101.0	?	101.6	+	102.0	X	157	104.0

X = Not done
– = Negative
+ = Positive
? = Suspicious
T = Temperature in °F.

with the Cyprus strain of BT virus showed any reaction in our C.F. test prior to infection. Calf I, which was tested on several occasions following injection, failed to develop a detectable serum titre. Calf II, however, responded with a serum titre of 1:10 by the 35th dpi, which reached its maximum level of 1:20 by the 63rd dpi, and slowly decreased in a fluctuating manner until it had disappeared by the 336th dpi. A serum titre of 1:10 was the highest level exhibited by Calf III over an observation period longer than 350 days. Calf IV first revealed a detectable serum titre on the 28th dpi, which reached its maximum of 1:80 by the 49th dpi and persisted throughout a 318 day observation period. Serum of Calf V reacted at a 1:10 dilution on the 21st dpi and reached its maximum level of 1:40 by the 35th dpi. A level of C.F. antibody persisted in its serum throughout the 245 day observation period. A serum titre of 1:5 was exhibited by Calf VI on the 28th dpi, and the maximum level of 1:20 was attained on the 42nd dpi. A level of 1:10 was retained until this animal was killed at 202 days. These data are presented in Table II.

Sheep IV, following inoculation with the 59th dpi blood of Calf III failed to show any clinical abnormality. On the 18th day following its inoculation, this sheep gave birth to a weak lamb which died the following day. Both ewe and lamb were bled as soon as possible after parturition. The serum was harvested and the clot from each was separately inoculated into BFK cultures for attempted virus isolation and F.A.T. examination. Serum from the ewe was tested for C.F. antibody, but proved to be negative at this time. However, it reacted to a titre of 1:5 on the 28th dpi and attained a maximum level of 1:40 on the 91st dpi. F.A.T. examination of BFK cultures inoculated with the clot from the ewe's blood sample taken at parturition failed to reveal the presence of BT virus. Similar examination of the clot from blood taken from the lamb at that time, however, gave a positive result. Table III records the CF titres obtained by testing the serum of Sheep IV periodically throughout the observation period.

Texas Station Strain

Sheep II responded to inoculation with the Station strain by developing a febrile

TABLE II. Complement-fixation titres exhibited by calves inoculated with the Cyprus strain of bluetongue virus

Day after inoculation	Calf I	II	III	IV	V	VI
0	–	–	–	–	–	–
7	–	–	–	–	–	–
14	–	–	–	–	–	–
21	–	–	–	–	1:10	–
28	–	–	–	1:10	1:20	1:5
35	–	1:10	–	1:40	1:40	1:10
42	–	1:5	–	1:40	1:20	1:20
49	–	–	1:5	1:80	1:20	1:10
56	Killed	–	1:5	1:80	1:20	1:20
63		1:20	X	1:40	1:20	1:10
70		1:20	1:5	1:40	1:40	1:10
77		1:20	1:5	1:40	1:40	1:10
84		1:20	1:10	1:20	1:10	1:5
91		–	1:10	1:40	1:10	–
98		1:20	–	1:10	1:10	1:5
105		1:20	–	1:10	1:10	1:10
112		–	1:10	1:10	1:10	1:5
119		1:20	–	1:20	1:20	1:10
126		1:10	1:5	1:20	–	1:10
133		1:20	1:5	1:20	1:5	1:10
140		1:10	1:5	1:20	1:10	1:10
147		1:10	–	1:20	1:20	1:10
154		–	1:5	1:20	1:10	1:10
161		1:5	1:5	1:40	1:40	1:10
168		–	1:5	1:40	1:20	–
175		1:10	1:10	1:20	1:40	1:10
182		1:10	1:10	1:20	1:20	1:10
189		1:10	1:10	1:20	1:20	1:10
196		1:10	1:10	1:10	1:10	1:10
203		1:10	1:10	1:5	1:20	1:10
210		1:10	–	1:20	1:10	
217		1:10	1:10	1:40	1:20	
224		1:10	1:10	1:10	1:20	
231		1:10	1:5	1:40	1:10	
238		1:10	–	1:20	1:40	
245		–	1:10	1:40	1:20	
252		1:5	1:10	1:20		
259		1:10	–	1:10		
266		1:5	1:10	1:10		
273		1:10	1:10	1:20		
280		–	1:10	1:20		
287		1:40	1:10	1:20		
294		1:10	1:10	1:10		
301		1:5	1:10	1:10		
308		–	–	1:20		
315		1:5	1:10	1:10		
322		1:10	1:5			
329		1:5	1:10			
336		–	1:10			
343		–	X			

– = Negative
X = Not done

response to 105.0°F. on the 6th dpi, which reached a maximum level of 105.8°F. on the following day. Blood taken at this time proved infective as indicated by positive results when inoculated BFK cultures were examined by the F.A.T. Another aliquot of this blood served as inoculum for Sheep III. Sheep II died on the 13th dpi, after showing clinical symptoms consistent with a diagnosis of bluetongue.

TABLE III. Complement-fixation titres obtained with the serum of Sheep IV following inoculation with blood taken from Calf III 59 days after inoculation with Cyprus bluetongue virus.

Day after inoculation	C. F. Titre
0	X
7	X
14	X
21	–
28	1:5
35	1:10
42	1:5
49	1:10
56	–
63	–
70	–
77	1:20
84	1:10
91	1:40
98	1:40
105	1:20
114	1:40
119	1:40
126	1:20
133	1:40
140	1:20
147	1:20
154	1:20
161	1:20
168	1:5
175	1:5
182	1:20
189	1:20
196	1:20
203	1:20
210	1:20
217	1:10
224	1:20
231	1:20
238	1:20
245	1:20
252	1:10
259	1:5
266	1:10
273	1:10
280	1:5
287	1:20

– = Negative
X = Not done

Sheep III, serologically negative when tested prior to inoculation, reacted by development of a thermal response to 105.6°F. on the 8th dpi. Blood collected at this time was used to inoculate Calf VII. A pool was made from the blood samples drawn from Sheep III on the first through eighth days after inoculation, and positive results were obtained when BFK cultures inoculated with an aliquot of this pool were examined by F.A.T. When first tested on the 49th dpi, Sheep III showed a CF serum titre of

TABLE IV. Results of Complement-fixation tests conducted on a Sheep and Calf inoculated with the Texas Station strain of bluetongue virus

Day after inoculation	Sheep III	Calf VII	Day after inoculation	Sheep III	Calf VII
0	X	X	168	1:20	1:20
7	X	X	175	1:10	1:20
14	X	X	182	1:10	1:20
21	X	X	189	1:5	1:5
28	X	1:5	196	1:5	1:5
35	X	1:10	203	1:5	1:5
42	X	1:40	210	1:5	1:10
49	1:80	1:40	217	1:5	1:20
56	1:80	1:80	224	–	1:10
63	1:160	1:20	231	1:5	1:20
70	X	1:20	238	1:10	1:10
77	1:80	1:20	245	1:5	1:10
84	1:80	1:40	252	1:10	1:20
91	1:80	1:40	259	1:10	1:10
98	1:40	1:10	266	1:10	1:10
105	1:40	1:10	273	1:10	1:10
112	1:20	1:5	280	1:10	–
119	1:40	1:10	287	1:10	1:10
126	1:10	1:10	294	1:10	1:10
133	1:10	1:10	301	1:10	1:10
140	1:20	1:20	308	1:10	1:10
147	1:10	1:40	315	1:10	
154	1:10	1:20	322	1:20	
161	1:10	1:20			

X = Not done
– = Negative

1:80. This titre increased to a peak of 1:160 by the 63rd dpi.

Calf VII, also serologically negative before use, showed a transient temperature reaction on the 9th and 10th dpi which could not be associated with other clinical signs. F.A.T. examination of cultures inoculated with a pool of blood drawn on days 2 through 9 yielded negative findings. However, a CF serum titre of 1:5 was present on the 28th dpi which reached a maximum level of 1:80 on the 56th dpi. Both Sheep III and Calf VII maintained variable CF serum titres throughout 331 and 316 day observation periods respectively. Results of CF tests conducted on these animals are presented in Table IV.

Discussion

Serial passage of the agent through calves was achieved, as shown by the results of CF titration of sera and virus isolation from blood, presented in Tables I, II, and IV. Throughout the duration of the experiment, no clinical signs, other than transient thermal reactions in two calves, were observed in animals inoculated with either the Cyprus or Station strains. Therefore, rapid, serial bovine passage failed to enhance the virulence for this host. Furthermore, blood taken from the third calf passage, when inoculated into a susceptible sheep did not elicit a clinical response more pronounced than that observed with virus passed through sheep only. On the 19th day after inoculation, this sheep gave birth to a lamb with bluetongue, as demonstrated by the presence of the agent in blood drawn on the day of parturition. The ewe, asymptomatic throughout, nevertheless became serologically positive, as shown in Table III. The failure of this ewe to exhibit a clinical response could be due either to a low virus content of the particular inoculum used, since this consisted of blood drawn from Calf III as late as the 59th dpi, or to some alteration in the virus itself. This latter possibility is in accord with the view expressed by Howell (3) that considerably prolonged viremia can occur in animals that react with a febrile response only, as opposed to those showing more severe clinical disease. However, the fact that the virus persisted this long in this calf, as attested to by infection of the lamb *in utero*, demonstrates that it can survive in this host for a relatively prolonged period. The ability to persist through at least six serial bovine passages, without producing

marked clinical signs, has important implications in the host-parasite relationship of this agent. It could be interpreted as lending support to the hypothesis of Du Toit (2) that cattle may be the definitive host of bluetongue virus and that sheep serve only as tangential hosts. Certainly it strongly supports the view that bovines are important in the epidemiology of the disease. This is of very real practical significance to areas of the world free from bluetongue.

The level of CF reactivity in the serum of inoculated calves, although showing minor fluctuation, persisted in most animals throughout the observation period. The challenge of Calf IV with virulent sheep-passaged virus of a homologous strain on the 57th, 58th and 59th dpi did not cause any alteration in its CF titre. In addition, no febrile reaction or other clinical response occurred following challenge.

It has previously been observed (10, 11, 12) that congenital abnormalities can occur in lambs born to ewes that have been inoculated between the 4th and 8th weeks of gestation with a modified live bluetongue virus vaccine. Also, Richards and Cordy (13) have reported that lesions caused by bluetongue virus infection in the central nervous system of sheep and mice vary with the age of the host, and appears to be influenced by the stage of immunological maturity of the infected animal. Unfortunately, post mortem examination of the lamb born to Sheep IV was not conducted when it died the day after birth, making it impossible to compare their interesting observations with those of a lamb infected much later in gestation. The presence of the virus in the newborn lamb but not in the blood of its dam collected at the same time may be a reflection of a higher susceptibility of the foetus than the adult to infection with this virus.

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