Bluetongue virus does not persist in naturally infected cattle

L.F. Melville, N.T. Hunt, S.S. Davis & R.P. Weir

Department of Business Industry and Resource Development, Darwin, NT 0800, Australia

Summary

Studies were designed to test if observations by Takamatsu *et al.* in 2003 were applicable to natural infection of cattle with bluetongue virus (BTV). These observations suggested that ovine gamma delta T-cells could become persistently infected and subsequent midge feeding could induce virus replication. Skin biopsies and blood were collected from 28 cattle naturally infected with BTV-1. Blood samples were processed for virus isolation by embryonated chicken egg inoculation and for serology by BTV competitive enzyme-linked immunosorbent assay and BTV-1 virus neutralisation. BTV-1 was isolated from the blood of all animals and serology confirmed infection with BTV-1. A total of 288 skin biopsies were collected and cultured in the presence of interleukin 2 and epidermal growth factor. Sampling commenced as soon as either serology or virus isolation indicated infection with BTV and continued at weekly intervals for at least eight weeks then monthly for another two months. The natural viraemias in this experiment ranged from one to five weeks. BTV-1 was isolated from only one skin biopsy sample. This sample was collected during the week in which the animal was viraemic. These findings provide compelling evidence that BTV does not persist in gamma delta T-cells in the skin of naturally infected cattle.

Keywords

Australia – Bluetongue virus – Bovine skin fibroblasts – Cattle – Natural infection – Persistence.

Introduction

Recent studies in the United Kingdom have reported that infectious bluetongue virus (BTV) could be recovered from ovine skin biopsies more than nine weeks post infection (9). This experimental work was conducted using laboratory-adapted BTV-1.

Adaptation of field isolates of BTV to growth in tissue culture is known to alter the biological properties of the virus (1). Experiments were therefore designed to assess if the observations reported by Takamatsu *et al.* (9) were applicable to natural BTV infection of cattle. These experiments were conducted at a site of known high arbovirus activity where cattle are continuously exposed to insect attack. Regular monitoring at this site also allows periods of natural BTV activity to be identified.

Materials and methods

Sampling collection and frequency

Two sentinel cattle herds at Beatrice Hill Farm (12°39′S, 131°20′E), approximately 50 km south-east of Darwin, Northern Territory, Australia were sampled weekly. Group 1 was sampled from 6 September 2002 until 10 April 2003 and Group 2 was sampled from 10 April 2003 until 7 August 2003. Each group contained 24 animals.

Ten ml of blood for serum and 10 ml of lithium heparin anticoagulant treated blood were collected weekly from the sentinel cattle for the period of the experiment. This included all days on which skin biopsy samples were collected. Skin biopsy samples were collected from a total of 28 animals beginning as soon as either serology or virus isolation indicated possible infection with BTV. Biopsy sampling continued weekly for eight weeks from initial sampling and then monthly for another two months.

Skin biopsy collection

Duplicate 6 mm skin biopsies were taken weekly from the backline of each selected sentinel animal for the duration of the experiment. The site was clipped, then washed with 70% ethanol and the biopsy taken. The biopsies were placed in 5 ml of heart brain infusion broth containing antibiotics. The samples were kept at 4°C for 2 h before processing.

Cattle skin fibroblast cultures

The skin biopsies were processed for culture using a method adapted from Takamatsu and Jeggo (8). The outer hair and skin layer were removed and discarded and the samples were cut into small sections, treated with trypsin ethylene-diaminetetraacetic acid (EDTA) for 45 min at room temperature and stirred continuously. The processed cells were recovered by centrifugation and washing and each sample cultured in a single well of a 24-well plate. The medium used was minimum essential medium (MEM) supplemented with 20% foetal bovine serum, 1 mM/ml of sodium pyruvate, 10 ng/ml epidermal growth factor, 10 IU/ml of recombinant human interleukin 2 and antibiotics.

The cultures were maintained at 37°C/5% CO_2 and the cultures harvested when they reached >80% confluency, usually 8 to 11 days post seeding. Cells were removed by scraping and combined with the culture supernatant and stored at –70°C until processed for virus isolation.

Co-culture of peripheral blood mononuclear cells and skin fibroblast culture

Five animals from Group 2 were selected for peripheral blood mononuclear cells (PBMC) skin fibroblast culture. A primary bovine skin fibroblast cell line was developed from a healthy non-infected animal. Using the method described above and then weekly passage, the cell line could be maintained for at least 20 passages. The cell line was shown to be able to be infected with BTV-1 (data not shown). PBMC were isolated from anticoagulant treated blood by gradient centrifugation (Nycoprep, Norway) and co-cultured with confluent bovine skin fibroblast cultures (medium as described above) for 7 days. The co-cultures were then harvested and stored at –70°C until processed for virus isolation.

Virus isolation

The anticoagulant treated blood samples and skin fibroblast culture samples were processed for virus isolation through embryonated chicken eggs (ECE) as described by Gard *et al.* (2), with the final cell passages through microtitre plates rather than cell culture tubes. The microtitre cell culture plate method for virus isolation is adapted from Lindsay *et al.* (5). In this method, duplicate wells of a 96-well microtitre plate containing C6/36 mosquito cell cultures were used as a first passage. The second passage used C6/36, BSR (7) and porcine stable equine kidney (PSEK) mammalian cell cultures, followed by a third passage that comprised BSR (a clone of baby hamster kidney cell line) and PSEK cell cultures.

The ECE homogenates were inoculated for first passage in C6/36 mosquito cell cultures and the plates were incubated at room temperature (25°C) in a humidified container for 7 days. The first passage supernatant was then inoculated to identical wells of three plates containing C6/36, BSR or PSEK cells. The plates were examined for the presence of CPE from day three post inoculation. The type and extent of CPE was recorded and at 80-100% CPE, the supernatant was aseptically removed from the CPE-positive wells and inoculated to 25 cm2 BSR tissue culture flasks for the production of seed stock virus for identification. Inoculation of the third cell culture passage used the C6/36 second passage plates. The second passage C6/36 supernatant was inoculated to identical wells of two plates containing either BSR or PSEK cells and examined as described above.

Virus identification

Virus isolates were identified by a combination of BTV antigen capture enzyme-linked immunosorbent assay (ELISA) (4) and virus neutralisation (VN) (3).

Serology

Bluetongue group and serotype-specific antibody was monitored by competitive ELISA (c-ELISA) (6) and VN (3).

Results

During the observation period, 14 cattle in each group of 24 were naturally infected with BTV-1. Infection was confirmed by virus isolation from blood samples and by serology. The periods of viraemia varied from one to five weeks.

Duplicate skin biopsy samples were taken from individual animals for between 16 and 24 weeks post initial detection of viraemia. Between 7 and 13 biopsies were taken from each animal. A total of 288 skin biopsies were examined from the two groups of cattle. Results are shown in Tables I and II.

Only one skin biopsy yielded BTV-1 following fibroblast culture and virus isolation. This biopsy, from animal number 80, was taken in the week when the animal was viraemic. Infectious BTV was not isolated from any the remaining 287 skin biopsies.

Table I
Blood, serum and skin biopsies processed from cattle infected with BTV-1: Group 1

Animal	Test	Weeks from first isolation of BTV-1																	
		0	1	2	3	4	5	6	7	8	9	10	11	12	16	21			
31	Virus blood	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–			
	Virus skin	NT	–	–	–	NT	–	–	–	–	–	–	–	–	–	–			
	Antibody*	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
		0	1	2	3	4	5	6	7	8	12	17							
32	Virus blood	+	+	–	–	–	–	–	–	–	–	–							
	Virus skin	NT	NT	–	–	–	–	–	–	–	–	–							
	Antibody*	–	+	+	+	+	+	+	+	+	+	+							
		0	1	2	3	4	5	6	7	11	16								
33	Virus blood	+	+	+	+	–	–	–	–	–	–								
	Virus skin	NT	NT	–	–	–	–	–	–	–	–								
	Antibody*	–	+	+	+	+	+	+	+	+	+								
		0	1	2	3	4	5	6	7	8	9	13	18						
37	Virus blood	+	+	+	–	–	–	–	–	–	–	–	–						
	Virus skin	NT	–	–	–	–	–	–	–	–	–	–	–						
	Antibody*	–	+	+	+	+	+	+	+	+	+	+	+						
		0	1	2	3	4	5	6	7	8	9	10	11	15	20				
39	Virus blood	+	+	–	–	–	–	–	–	–	–	–	–	–	–				
	Virus skin	NT	–	–	NT	–	–	–	–	–	–	–	–	–	–				
	Antibody*	–	+	+	+	+	+	+	+	+	+	+	+	+	+				
		0	1	2	3	4	5	6	7	8	9	13	18						
40	Virus blood	+	+	+	+	+	–	–	–	–	–	–	–						
	Virus skin	NT	NT	NT	–	–	–	–	–	–	–	–	–						
	Antibody*	–	+	+	+	+	+	+	+	+	+	+	+						
		0	1	2	3	4	5	6	7	8	12	17							
41	Virus blood	+	+	+	–	–	–	–	–	–	–	–							
	Virus skin	NT	–	–	–	–	–	–	–	–	–	–							
	Antibody*	–	+	–	+	+	+	+	+	+	+	+							
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	19	24
44	Virus blood	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
	Virus skin	NT	NT	NT	NT	–	–	–	NT	–	–	–	–	–	–	–	–	–	–
	Antibody*	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		0	1	2	3	4	5	6	7	8	9	13	18						
46	Virus blood	+	+	–	–	–	–	–	–	–	–	–	–						
	Virus skin	NT	NT	NT	–	–	–	–	–	–	–	–	–						
	Antibody*	–	+	+	+	+	+	+	+	+	+	+	+						
		0	1	2	3	4	5	6	7	8	9	10	14	19					
47	Virus blood	+	+	+	–	–	–	–	–	–	–	–	–	–					
	Virus skin	NT	–	NT	–	–	–	–	–	–	–	–	–	–					
	Antibody*	–	+	+	+	+	+	+	+	+	+	+	+	+					
		0	1	2	3	4	5	6	7	8	9	10	11	12	16	21			
48	Virus blood	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–			
	Virus skin	NT	NT	–	–	NT	–	–	–	–	–	–	–	–	–	–			
	Antibody*	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	17	22		
51	Virus blood	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–		
	Virus skin	NT	NT	–	–	–	NT	NT	–	–	–	–	–	–	–	–	–		
	Antibody*	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
		0	1	2	3	4	5	6	7	8	9	10	11	15	20				
52	Virus blood	+	+	+	–	–	–	–	–	–	–	–	–	–	–				
	Virus skin	NT	NT	–	NT	–	–	–	–	–	–	–	–	–	–				
	Antibody*	–	+	+	+	+	+	+	+	+	+	+	+	+	+				
		0	1	2	3	4	5	6	7	8	9	10	11	12	16	21			
53	Virus blood	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–			
	Virus skin	NT	–	–	–	NT	–	–	–	–	–	–	–	–	–	–			
	Antibody*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			

NT not tested * antibody c-ELISA or BTV-1 neutralising antibody

Table II
Blood, serum and skin biopsies processed from cattle infected with BTV-1: Group 2

Animal	Test	Weeks from first isolation of BTV-1															
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	19	23
61	Virus blood	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
	Virus skin	NT	NT	NT	NT	NT	–	–	–	–	–	–	–	–	–	–	
	Antibody*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		0	1	2	3	4	5	6	7	8	9	10	11	16	20		
62	Virus blood	+	+	–	–	–	–	–	–	–	–	–	–	–			
	Virus skin	NT	NT	NT	NT	NT	–	–	–	–	–	–	–	–			
	Antibody*	–	–	–	–	+	+	+	+	+	+	+	+	+			
		0	1	2	3	4	5	6	7	8	9	10	11	16	20		
	Virus blood	+	+	–	–	–	–	–	–	–	–	–	–	–			
	Virus skin	NT	–	–	–	–	–	–	–	–	–	–	–	–			
	Antibody*	–	+	+	+	+	+	+	+	+	+	+	+	+			
		0	1	2	3	4	5	6	7	8	9	10	11	16	20		
66	Virus blood	+	–	–	–	–	–	–	–	–	–	–	–	–			
	Virus skin	NT	NT	NT	–	–	–	–	–	–	–	–	–	–			
	Antibody*	–	+	+	+	+	+	+	+	+	+	+	+	+			
		0	1	2	3	4	5	6	7	8	9	10	18	22			
68	Virus blood	+	+	+	+	–	–	–	–	–	–	–	–				
	Virus skin	NT	NT	–	–	–	–	–	–	–	–	–	–				
	Antibody*	–	+	+	+	+	+	+	+	+	+	+	+				
		0	1	2	3	4	5	6	7	8	9	17	21				
69	Virus blood	+	+	+	–	–	–	–	–	–	–	–					
	Virus skin	NT	–	–	–	–	–	–	–	–	–	–					
	Antibody*	–	–	+	+	+	+	+	+	+	+	+					
		0	1	2	3	4	5	6	7	8	9	17	21				
73	Virus blood	+	+	–	–	–	–	–	–	–	–	–					
	Virus skin	NT	–	–	–	–	–	–	–	–	–	–					
	Antibody*	–	+	+	+	+	+	+	+	+	+	+					
		0	1	2	3	4	5	6	7	8	9	10	11	19	23		
74	Virus blood	+	+	–	–	–	–	–	–	–	–	–	–	–			
	Virus skin	NT	NT	NT	–	–	–	–	–	–	–	–	–	–			
	Antibody*	–	+	+	+	+	+	+	+	+	+	+	+	+			
		0	1	2	3	4	5	6	7	8	9	10	11	17	21		
77	Virus blood	+	+	–	–	–	–	–	–	–	–	–	–	–			
	Virus skin	NT	NT	–	–	–	–	–	–	–	–	–	–	–			
	Antibody*	–	+	+	+	+	+	+	+	+	+	+	+	+			
		0	1	2	3	4	5	6	7	8	9	17	21				
78	Virus blood	+	+	–	–	–	–	–	–	–	–	–					
	Virus skin	NT	–	–	–	–	–	–	–	–	–	–					
	Antibody*	–	+	+	+	+	+	+	+	+	+	+					
		0	1	2	3	4	5	6	7	8	9	10	11				
79	Virus blood	+	+	–	–	–	–	–	–	–	–	–	–				
	Virus skin	NT	NT	NT	–	–	–	–	–	–	–	–	–				
	Antibody*	–	+	+	+	+	+	+	+	+	+	+	+				
		0	1	2	3	4	7	12	16								
80	Virus blood	+	–	–	–	–	–	–									
	Virus skin	+	–	–	–	–	–	–									
	Antibody*	–	+	+	+	+	+	+									
		0	1	2	3	4	5	6	7	8	9	10	11	12	15	19	
83	Virus blood	+	+	–	–	–	–	–	–	–	–	–	–	–	–		
	Virus skin	NT	NT	NT	NT	NT	–	–	–	–	–	–	–	–	–		
	Antibody*	–	+	+	+	+	+	+	+	+	+	+	+	+	+		
		0	1	2	3	4	5	6	7	8	9	10	11	12	15	19	
84	Virus blood	+	+	–	–	–	–	–	–	–	–	–	–	–	–		
	Virus skin	NT	NT	NT	NT	NT	–	–	–	–	–	–	–	–	–		
	Antibody*	–	–	–	–	+	+	+	+	+	+	+	+	+	+		

NT not tested * antibody c-ELISA or BTV-1 neutralising antibody

Lymphocytes were harvested from blood samples collected up to 7 weeks after the first isolation of BTV-1 from five of the infected cattle in Group 2. In three of these animals, sampling commenced during viraemia. In the remaining two animals, sampling commenced two weeks after the first isolation of BTV-1 when the animals were no longer viraemic. A total of 30 PBMC skin fibroblast co-cultures were performed. Infectious BTV-1 was not isolated from any of these cultures (Table III).

Table III
Peripheral blood mononuclear cells co-cultured with skin fibroblast

Animal	Test	Weeks from first isolation of BTV-1					
		1	2	3	4	5	6
68	Virus blood	+	+	+	–	–	–
	Virus PBMC:fibroblast	–	–	–	–	–	–
		0	1	2	3	4	5
73	Virus blood	+	+	–	–	–	–
	Virus PBMC:fibroblast	–	–	–	–	–	–
		2	3	4	5	6	7
74	Virus blood	–	–	–	–	–	–
	Virus PBMC:fibroblast	–	–	–	–	–	–
		0	1	2	3	4	5
78	Virus blood	+	+	–	–	–	–
	Virus PBMC:fibroblast	–	–	–	–	–	–
		2	3	4	5	6	7
79	Virus blood	–	–	–	–	–	–
	Virus PBMC:fibroblast	–	–	–	–	–	–

PBMC peripheral blood mononuclear cells

Additional tests were used to confirm the presence of lymphocytes in the skin fibroblast cultures. A skin biopsy from each animal was taken in formalin for histopathology. Examination of haematoxylin and eosin stained sections confirmed the presence of lymphocytes in the skin biopsies. Smears were also made of centrifuged deposits from the skin fibroblast cultures. These were stained with 'Diff Quik' (Lab Aids Pty Ltd) and examined for the presence of lymphocytes. Lymphocytes were identified in these smears.

A primary skin fibroblast cell line was developed from a healthy non-infected bovine. This cell line was maintained for at least 20 passages at weekly intervals. The ability of this cell line to support the growth of BTV was confirmed by inoculation of known BTV-1. The cell line showed cytopathic effect three days after inoculation and the identification of BTV-1 was confirmed by VN (3).

Exposure of the cattle to insect attack was confirmed using a mechanical aspirator. Six animals were used for midge collection and six collections each of 5 min were made. The total area of skin over which collections were made was approximately 3.5 m^2. Collections were made each week during the period of observation and *Culicoides* vector species sorted and counted. *Culicoides actoni*, *C. brevitarsis* and *C. fulvus* were all collected from the cattle with *C. actoni* being the dominant species. An average of 863 midge vector species were collected during a total collection time of 30 min between 5.15 pm and 7.45 pm.

Discussion

Takamatsu *et al.* (9) suggested that BTV could persist in γδ T-cells in the skin of infected sheep. Subsequent midge feeding could then induce virus replication. This finding was proposed as a mechanism for 'overwintering' survival of BTV. It does however also raise concerns about the movement of sheep and cattle with antibodies to BTV and the possible reintroduction of international restrictions on movement of such animals.

The experimental work conducted by Takamatsu *et al.* used laboratory-adapted virus. Adaptation of field isolates of BTV to growth in tissue culture has been shown to alter the biological properties of BTV (1). The studies described in this paper were conducted to determine if natural BTV infections in cattle could result in persistent infection of γδ T-cells and if infectious BTV could be recovered from skin biopsies from these cattle. The cattle were exposed to biting midges throughout the observation period.

The work was performed over 11 months and involved 288 individual skin biopsies from cattle naturally infected with BTV-1. Only one skin sample yielded infectious BTV-1 and that was taken when the animal was viraemic. As many of the skin biopsies were blood contaminated it is probable this was virus contamination from blood at the peak of viraemia. Lymphocyte co-cultures with a skin fibroblast cell line also failed to yield any infectious BTV. This work also showed a maximum viraemia of five weeks, confirming that viraemia with BTV-1 in cattle is limited. Isolation of BTV-1 from the blood of these cattle and from one cultured skin biopsy indicated that the systems used were sensitive enough to detect virus if present. These findings provide compelling evidence that BTV does not persist in γδ T-cells in the skin of naturally infected cattle.

Experimental work is currently underway at two locations in Australia to see if wild or laboratory-adapted virus can persist in sheep. Confirmation of

BTV persistence in the skin of sheep should be obtained before this finding is accepted as the mechanism for 'overwintering'. This work confirms there is no need to restrict the movement of seropositive cattle post viraemia, as such animals are not persistently infected.

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