Cultured skin fibroblast cells derived from bluetongue virus-inoculated sheep and field-infected cattle are not a source of late and protracted recoverable virus

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INTRODUCTION

The clinical significance of bluetongue disease has been reemphasized by recent disease incursions into previously unaffected parts of Europe causing more than 250 000 sheep deaths and associated emergency control measures (Baylis, 2002). The importance of the disease in trade considerations is manifest by the OIE Terrestrial Code (OIE, 2004). This details a series of requirements for surveillance, quarantine and certification prior to movement of animals and certain animal products. Article 2.2.13.1 indicates that the infective period for Bluetongue virus (BTV) is 60 days and this is the length of time applied to quarantine periods. Characteristics of the host animal and the infecting virus influence the length of the infective state and the period of quarantine is based on previous studies reporting virus recovery periods from infected sheep and cattle. Viraemic periods in experimentally infected sheep have ranged from less than 20 days post-infection (p.i.) (Bonneau et al., 2002; Jeggo et al., 1987) to between 30 and 54 days in other studies (Koumbati et al., 1999; Richards et al., 1988). Hence, the quarantine period of 60 days imposed by OIE regulations is in accordance with these results. However, more recent experimental data indicated that infectious virus was recoverable at 9 weeks p.i. from 100 % of a group of experimentally infected sheep, a period of 5 weeks after the end of detectable viraemia (Takamatsu et al., 2003). These authors not only reported the recovery of virus from cultured sheep skin fibroblasts (SFs) 9 weeks p.i., but also suggested that exposure of sheep to Culicoides sp. prior to collection of skin biopsies for culture is a significant but not critical factor that may enhance the likelihood of virus recovery. The purpose of the current study was to examine the possibility that BTV persists not only in sheep, as suggested by Takamatsu et al. (2003), but also, because of their role as a natural host of BTV, in cattle. We also investigated the effect of additional exposures of the sheep to Culicoides sp. on the recovery of virus.

METHODS

Animals. Independent experiments were conducted at two laboratory sites, the Australian Animal Health Laboratory (AAHL) and the Berrimah Veterinary Laboratories (BVL). All animal work was approved by the relevant institutional animal ethics committees and carried out in accordance with approved guidelines (National Health and Medical Research Council, Australia, 2004).
**Sheep.** BVL and AAHL used ten merino and ten merino crossbreed sheep (all aged between 1 and 2 years), respectively, for experimental infection, housed at both laboratories in insect-proof accommodation.

**Cattle.** Forty-eight Brahman and Brahman-cross cattle, aged 6–12 months, were located at Beatrice Hill Farm (12° 39'5 131° 20' E) approximately 50 km southeast of Darwin, Northern Territory, Australia, from September 2002 to August 2003. The Beatrice Hill site is a monitoring point for BTV activity in northern Australia. BTV-seronegative animals were introduced to this site and monitored weekly by virus isolation and monthly by serology for evidence of BTV activity. Seroconversion can occur in any month but is most common during the wet summer months (January–May).

**Insect collections.** Insect collections at Beatrice Hill were made to confirm the exposure of cattle to attack by midges and to provide live insects for experimental exposure of sheep. Crepuscular collections of midges were made by mechanical aspiration from cattle (Melville et al., 2005) that received unrestricted *Culicoides* sp. exposure at their field location. 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 m². This yielded a mean of 863 midges of known vector species collected over a 30 min period between 5:15 pm and 7:45 pm (sunset at 6:25–6:41 pm over the study period). Collections were made each week during the period of observation and *Culicoides* sp. were sorted and counted. *Culicoides actoni* and *Culicoides marksi* were the dominant species collected. Collected insects were used for subsequent exposure of sheep at both laboratory sites.

Collections were sorted and unfed nulliparous *C. marksi* or *C. actoni* were selected for feeding on sheep at either the BVL or the AAHL site. Insects were air-freighted overnight to AAHL, located 3133 km southeast of the collection point. These shipments were timed to permit commencement of sheep exposures to *Culicoides* sp. promptly after arrival of the insects at the laboratory. Insect collections were inspected on arrival and estimates of viability were made. Only collections with an estimated viability of >80% were used for subsequent work.

**Virus and inoculation.** Preparations of BTV serotype 1 (BTV-1) and sheep inoculation schedules are shown in Table 1. Laboratory-housed sheep were inoculated with virus produced either by passage of isolates in cell culture (CSIRO156) or by serial animal passage of BTV-infected sheep blood (DPP559), or derived directly from field bovine blood (V5745). Sheep at both AAHL and BVL were assigned to two groups of five; four animals in each group were inoculated with virus (adapted or non-adapted) and one control animal was uninoculated (Table 2). Blood samples were collected from sheep prior to inoculation and on the days after inoculation shown in Table 2.

Field-sited cattle were infected with BTV through natural exposure. Animals were monitored weekly for BTV by sampling of blood for virus isolation and serology.

**Exposure of sheep to insect feeding.** Exposure of sheep to insects occurred in two phases: a primary exposure, prior to inoculation with BTV and a series of three p.i. exposures, commencing at around 8 weeks p.i. and prior to the taking of skin biopsy samples for growth in vitro and virus analysis (Table 2). Sheep were exposed experimentally to *Culicoides* sp. pools by apposition of an enclosed housing affixed by adhesive to the inside thigh of each animal. Midge were immobilized using CO₂ gas and then placed into the housing. After exposure for approximately 6 h, the midges were removed by flooding with alcohol and aspiration into a container. Alcohol-fixed midges were examined under low-power magnification to confirm that some feeding had occurred, and stored for future testing if required.

**Collection of biopsies.** Biopsies were collected from sheep in association with exposures to *Culicoides* sp. made in the BTV pre- and post-inoculation phases. Biopsies were taken 8 h after exposure to insects. A 6 mm biopsy punch was used to collect two full-thickness skin biopsies from the site of exposure and also from a similar site on the opposite unexposed leg. In addition, biopsies were collected at various times in the interval between BTV inoculation and the first post-inoculation *Culicoides* sp. exposure (Table 2).

Cattle biopsies were collected as duplicate 6 mm skin samples from the mid-back of a total of 28 animals, beginning as soon as either serology or virus isolation indicated possible infection with BTV. Biopsy sampling continued weekly up to 11 weeks after the initial biopsy and then at longer intervals up to 24 weeks.

**SF and leukocyte cultures.** SF cultures were prepared and maintained as described previously (Takamatsu et al., 2003). In brief, these were prepared by treating minced skin biopsies with trypsin and culturing the cells from the resultant suspension. Murine submaxillary gland epidermal growth factor (Sigma) and human recombinant interleukin 2 (IL-2) (lymphocyte growth factor; Sigma) were added as supplements to the culture medium. Cultures were maintained until confluent and subcultured once if required for maintenance. At the end of this period, culture media were assessed by BTV antigen-detection ELISA (Hawkes et al., 2000; Stanislawek et al., 1996) and selectively by real-time PCR at AAHL or by embryonated chicken egg inoculation at BVL.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Isolate</th>
<th>Growth in cell culture</th>
<th>Inoculum</th>
<th>Titre (ml⁻¹)</th>
<th>Inoculation regime</th>
<th>No. sheep inoculated</th>
<th>No. uninoculated sheep</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTV-1</td>
<td>CSIRO156</td>
<td>Adapted</td>
<td>Culture medium</td>
<td>1 × 10⁶⁰⁷ TCID₅₀</td>
<td>1 ml SC</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>BTV-1</td>
<td>DPP559</td>
<td>Non-adapted</td>
<td>Culture medium</td>
<td>1 × 10⁶⁰⁷ EID₅₀</td>
<td>1 ml SC</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>BTV-1</td>
<td>CSIRO156</td>
<td>Adapted</td>
<td>Culture medium</td>
<td>1 × 10⁶⁻⁸ TCID₅₀</td>
<td>0-1 ml ID, 2-0 ml IV</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>BTV-1</td>
<td>V5745</td>
<td>Non-adapted</td>
<td>Bovine blood</td>
<td>1 × 10⁶⁻⁵ EID₅₀</td>
<td>0-1 ml ID, 2-5 ml IV</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

TCID₅₀ 50% embryo infectious dose; SC, subcutaneously; ID, intradermally; IV, intravenously.
Absence of protracted BTV recovery in SF cultures

Table 2. Intervention schedule for sheep held at the AAHL and BVL sites

Numbers in bold indicate blood-sampling days. Shaded boxes indicate biopsy-collection days.

<table>
<thead>
<tr>
<th>Schedule</th>
<th>Time (days) relative to inoculation day</th>
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<tbody>
<tr>
<td></td>
<td>AAHL</td>
</tr>
<tr>
<td></td>
<td>Culture-adapted virus</td>
</tr>
<tr>
<td>Pre-inoculation Culicoides exposure</td>
<td>-21</td>
</tr>
<tr>
<td>Inoculation (day 0)</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
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<tr>
<td>11</td>
<td>12</td>
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<td>14</td>
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<td>19</td>
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<td>26</td>
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<tr>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>First p.i. Culicoides exposure</td>
<td>47</td>
</tr>
<tr>
<td>Second p.i. Culicoides exposure</td>
<td>61</td>
</tr>
<tr>
<td>Third p.i. Culicoides exposure</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>96</td>
</tr>
</tbody>
</table>

Leukocyte cultures were prepared at AAHL by separating the mononuclear cell component from whole unclotted blood on Lymphoprep (Axis-Shield) and culturing the cells in the presence of IL-2 (Takamatsu et al., 2003). Cultures were maintained for 2–4 weeks, with cell removal to prevent overgrowth and medium supplementation during this time. At the end of this period, a suspension of BHK cells was added to the cultures to act as a source of BTV-susceptible cells. Antigen-detection ELISA for BTV was carried out 1 week after addition of the BHK cell suspension.

Assays

Antibody. Specific antibody responses to BTV in the serum of sheep and cattle were measured by competitive ELISA using a monoclonal antibody with binding specificity for the virus serogroup antigen (VP7) (Lunt et al., 1988; Martyn et al., 1991).

Virus isolation and antigen detection. Virus isolation from blood and culture supernatants was carried out by intravenous inoculation of 11-day-old embryonated chicken eggs (Goldsmit & Barzilai, 1968). At AAHL, bloods collected on days 4, 7, 11, 14, 18, 21 and 28 p.i. were used for virus recovery attempts. At AAHL, virus isolation was only attempted on bloods collected on days 5 and 7 p.i. Liver/spleen homogenates harvested from inoculated eggs and SF and leukocyte culture supernatants were tested for the presence of BTV antigen as described previously (Hawkes et al., 2000; Stanislawek et al., 1996). The assay could detect VP7 antigen in infected-culture supernatant fluid diluted to approximately 10⁶ TCID₅₀ ml⁻¹.

PCR. Nucleic acids were extracted from BTV-infected cells and experimental samples using either an RNAsy mini kit (Qiagen) or a total nucleic acid extraction kit in a MagNA Pure system (Roche) according to the manufacturer’s protocol. Synthesis of cDNA was carried out with MultiScribe reverse transcriptase (Applied Biosystems) using random primers as described in the protocol. The reverse transcriptase (RT) mix contained 2 µl 10 × TaqMan RT buffer, 4-4 µl 25 mM MgCl₂, 4 µl dNTPs (2-5 mM each), 0-4 µl RNase inhibitor (20 U µl⁻¹), 0-5 µl MultiScribe RT (50 µU µl⁻¹), 1 µl 50 µM random hexanucleotide primers and 5-7 µl RNase-free distilled water per sample. Two microlitres of RNA was added to give a final volume of 20 µl, which was then incubated at room temperature for 10 min, 48 °C for 30 min and 95 °C for 5 min.

The real-time primers and TaqMan MGB (Applied Biosystems) probes were designed from the BTV segment 3 sequences AY277929 and AY277934 (Pritchard et al., 1995) using Primer Express version 1.5 (Applied Biosystems). The BTV primers were BTVR3-1357F’ (5’-GAGAACCCGCCCTACGT-3’) and BTVR3-1456R (5’-TCGCG- ATCAGTGCACA-3’). The fluorogenic 5’ nucleoside MGB probes were BTVNSW R3 (5’-FAM-ATGGTCACCGTACATAC-MGBFQ-3’) and BTJVAC R3 (5’-FAM-ATGCCACGTATATAC-MGBFQ-3’). A 2 µl aliquot of cDNA was used in the PCR (final volume of 25 µl) in each well of a 96-well MicroAmp optical reaction plate (Applied Biosystems) containing 12-5 µl Taqman universal PCR master mix, 6-4 µl distilled water, 0-4 µl 18 M BTVR3-1396F and 1-3 µl 18 µM BTVR3-1475R primers and 1-3 µl 5 µM BTNV NSW R3 or BTJVAC R3 6-carboxyfluorescein (FAM)-labelled MGB probe. Where appropriate, 18S rRNA was used as an extraction control and housekeeping gene (Applied Biosystems) as described in the protocol. The cDNA samples were amplified in a GeneAmp 7900 Sequence Detection System (Applied Biosystems) using the standard PCR program of 50 °C for 2 min, 95 °C for 10 min and 45 cycles of 95 °C for 15 s and 60 °C for 60 s. Ct values < 39 were read as positive for BTV genetic material and values > 39 were considered to be negative. The real-time PCR (TaqMan) assays were developed and standardized against the BTV isolates DP559 and CSIRO156 and used to assess levels of the target nucleic acid in blood and primary SF culture samples. Detection level limits by PCR for both culture-adapted and non-adapted BTV corresponded to 10⁹–10¹⁰ infectious units ml⁻¹.

RESULTS

Confirmation of infection in sheep

Sixteen sheep were experimentally inoculated with BTV (Table 1) and infection was confirmed by isolation of the virus from the blood of 13 animals. No overt clinical signs...
were observed. At BVL, where viraemia in both groups of inoculated sheep was monitored over 4 weeks, viraemia was not detected beyond 14 days p.i. The laboratory-adapted CSIRO156 isolate resulted in only low levels of viraemia and virus was not recovered from the blood of three out of eight sheep. Three of these sheep were all located at AAHL. However, there were other indices of infection for these and the other inoculated animals. Viral RNA was detected in the blood of all sheep in samples collected variously between days 5 and 14 p.i. Viral RNA was less uniformly detected at later stages p.i. By day 40 p.i. at AAHL, only three sheep (all inoculated with DPP559) were positive by PCR and by day 56 p.i., viral RNA was detected in seven of the eight sheep at BVL. All inoculated sheep had seroconverted to BTV-1 when assessed at 28 days p.i. and remained seropositive until the final blood collection. Uninoculated control sheep in each of the four groups remained seronegative throughout the experiment.

**Recovery of virus from cultured leukocytes**

Attempts (at AAHL only) to recover BTV from leukocyte cultures collected over the course of the experiment yielded virus only from samples collected in the first week p.i. The cell-adapted virus CSIRO156 was isolated from one leukocyte culture established from a day 7 p.i. blood collection. Three cultures of leukocytes derived from sheep inoculated with non-adapted DPP559 virus yielded virus from a single blood sample collected on day 4 p.i. and two collections made on day 7 p.i. No cytopathic effect was evident in leukocyte cultures and in vitro cultures could be maintained for several weeks prior to addition of BHK cells. However, no productive infections resulted from leukocyte cultures established from blood collected after day 7 p.i.

**Experimental exposure of sheep to Culicoides sp.**

The number of insects used in exposures was dependent on the availability of Culicoides sp. obtained by aspiration from cattle. As the number collected varied greatly, numbers of insects applied to sheep in each of the three p.i. exposures ranged from several dozen to several hundred. Identification of blood-meal-fed insects collected after the exposure process confirmed the interaction between insect and sheep. The levels of successful feedings were also variable. After one feeding, seven out of ten recovered pools had some blood-engorged insects, although generally 5% or fewer insects were involved. Recovery and identification of blood-engorged insects confirmed that insects had fed on the majority of sheep in at least one of the three p.i. exposure events.

**Detection of virus in sheep SF cultures**

A total of 94 (64 at AAHL and 30 at BVL) SF cultures was established in the post-BTV-inoculation phase of the experiment without re-exposure to Culicoides sp.; of these, only three were shown to contain virus. The three positive cultures originated from biopsies taken 4 and 5 days p.i. with non-adapted DPP559 virus and cell-adapted CSIRO156 virus, respectively. Two SF isolations of CSIRO156 were made from sheep for which there had been no recovery of virus from blood. A similarly established culture from a day 4 p.i. biopsy of a sheep inoculated with the non-adapted DPP559 virus also produced virus. Presence of the virus was generally indicated by focal degeneration of the culture and positive results were confirmed by antigen-detection ELISA and real-time PCR.

One hundred (40 at AAHL and 60 at BVL) SF cultures were also established using skin biopsies collected on the three Culicoides sp. exposure days (Table 2) from the BTV-inoculated sheep. No virus was isolated from 60 SF cultures by inoculation of embryonated eggs (BVL only) or detected by antigen ELISA and targeted PCR assessment of 40 culture supernatants (AAHL only). The results are summarized in Table 3.

**Attempts to recover BTV from skin biopsies of naturally infected cattle**

Exposure of cattle to Culicoides sp. was confirmed by mechanical aspiration and examination of collected pools. During the observation period, 28 of the 48 cattle were naturally infected with BTV-1. Infection was confirmed by virus isolation from blood samples and by serology. The periods of viraemia varied from 1 to 5 weeks.

Duplicate skin biopsy samples were taken from each animal commencing when viraemia was indicated by serology or virus isolation and continuing weekly for between 4 and 11 weeks after the initial biopsy. In some animals, sampling continued at longer intervals up to 24 weeks. Between seven and 13 biopsies were taken from each animal. A total of 288 skin biopsies were examined from the cattle. Only one skin biopsy yielded BTV-1 following SF culture and virus isolation. This biopsy was taken in the period during

### Table 3. Recovery of BTV from SF cultures established from sheep and cattle

<table>
<thead>
<tr>
<th>Animal group</th>
<th>No. biopsies collected</th>
<th>Period of biopsy collection</th>
<th>Time/origin of BTV-positive SF culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory-infected sheep</td>
<td>194</td>
<td>Up to day 96 p.i.</td>
<td>Day 4 p.i./one sheep inoculated with DPP559</td>
</tr>
<tr>
<td>Field-infected cattle</td>
<td>288</td>
<td>Up to 150 days after detection of viraemia</td>
<td>Day 5 p.i./two sheep inoculated with CSIRO156 In viraemic period/one cow</td>
</tr>
</tbody>
</table>
which the animal was viraemic. BTV was not isolated from any of the remaining 287 skin biopsies.

**DISCUSSION**

The investigations described here were designed to ascertain whether BTV persisted in the skin not only of sheep, as suggested by Takamatsu et al. (2003), but also of cattle. The experiments were carried out at two laboratories and both non-adapted and culture-adapted BTV were used to test the hypothesis. Even low-passage cell culture may alter the characteristics of a BTV stock (Flanagan & Johnson, 1995) and the experiments of Takamatsu et al. (2003) utilized BTV-1 and BTV-3 that had been passaged in cell culture several times.

A series of independent detection systems was used to reveal the presence of BTV in SF cultures. Attempted isolation of virus from culture supernatants was supplemented with direct detection of antigen by ELISA and amplification of viral nucleic acid by PCR. We considered the application of PCR in this study to be an important investigative tool with the potential to confirm, with considerable sensitivity, the presence of BTV in test samples. In the event that BTV was isolated from infected sheep in the post-viraemic phase, PCR would have been used to test skin biopsy material directly and sequencing would have been undertaken to establish the relationship between the detected genome sequence of inoculated and recovered viruses. The inclusion of molecular techniques in our study adds an element of investigation that was not utilized in the work by Takamatsu et al. (2003).

Whilst positive PCR and seroconversion of all 16 inoculated sheep confirmed infection of all inoculated animals, three sheep inoculated with the laboratory-adapted strain CSIRO156 were not confirmed to be viraemic by egg inoculation. It is presumed that this virus replicates to only low titres, suggested also by the negative PCR result at day 40 p.i. for these animals at AAHL. It is also significant that the method of detecting viremia (as an indicator of infection) was separate and independent from the virus persistence recovery techniques, which used SF cultures.

BTV was recovered from SF cultures of both sheep (at AAHL) and cattle (at BVL) established from biopsies collected several days after inoculation or natural infection. Such observations acted as positive controls, demonstrating that the processes undertaken were capable of recovering virus from infected SF cultures. After the viraemic period, no virus was isolated from SF cultures derived from experimentally infected sheep or from naturally infected cattle. As temporal fluctuations in recoverability of BTV (Takamatsu et al., 2003) might influence outcomes, multiple samplings were made over a protracted observation period in order to increase confidence that virus might be detected if persistent in the animals and recoverable in this manner. The absence of infectious virus in these samples was supported by supplementary testing using molecular and immunological techniques.

Blood leukocyte cultures were established to investigate possible persistent BTV infection of γδ T lymphocytes. It has been proposed that recruitment of chronically infected γδ T lymphocytes to the skin could lead to infection of feeding insects, thus providing a mechanism for re-emergence of the same virus serotype in the absence of an otherwise continuous vector–host cycle, as occurs in the winter period (Takamatsu et al., 2003). In our study, cultures of blood leukocytes could be maintained for several weeks and BTV could then be recovered by the addition of BHK cells. However, virus was not detected from leukocytes collected for culture beyond 7 days p.i., indicating that persistently infected circulating leukocytes are an unlikely source of BTV recrudescence.

The clear discrepancy between our results and those of Takamatsu et al. (2003) warrants further comment. The published data reported late-stage recovery of virus from the six sheep that were infected; our results from 16 sheep and 28 cattle were 100% negative at a similar time after infection. Of 24 samplings over a period of 32 h, Takamatsu et al. (2003) reported 16 isolations of BTV from SF cultures. In our study, there were 100 ovine SF cultures established after Culicoides sp. exposure and 94 ovine SF cultures collected before post-inoculation Culicoides sp. exposure, from which no positives were obtained beyond the first week p.i. In a similar pattern, only one of 288 SF cultures established from field-located cattle was positive for virus and this had been collected during the viraemic phase.

Replication of pathogenesis experiments with BTV can be difficult, so the significance of the results by Takamatsu et al. (2003) remains unresolved, although there are now observations to the contrary. Many factors such as virus strain and passage history, breed of sheep, conditions of culture and isolation, nature of the insect exposure and sampling from the animal are all potential sources of variation. Where complete standardization between laboratories is impractical, it is the consensus of experimental results that may support or challenge a hypothesis. Our decision to investigate the possible persistence of BTV using Australian isolates, rather than a South African strain, was justifiable on the basis of greater relevancy in an Australian context.

The data presented here are not consistent with the hypothesis proposed by Takamatsu et al. (2003) that an overwintering mechanism for BTV is the late or protracted emergence of sequestered virus in previously infected sheep at dermal sites exposed to feeding midges. Based on the evidence presented herein, we believe that there is currently insufficient evidence to support a general role for this proposal in the epidemiology of BTV.

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