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Investigating bluetongue virus persistance in sheep

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TABLE OF CONTENTS

1. ABSTRACT

This project was conducted to verify recent results published by United Kingdom researchers suggesting that bluetongue virus (BTV) could persist in the skin of infected sheep and subsequent midge feeding could then induce virus replication. This finding was proposed as a mechanism for the "over wintering" survival of BTV. These results raised concerns about the movement of sheep and cattle with antibodies to BTV and the possible reintroduction of international restrictions on movement of such animals.

Sheep were inoculated with wild type and laboratory-adapted bluetongue virus serotype 1 (BTV1). Infection was confirmed by virus isolation and serology. Skin biopsies were taken at 14, 28, 42, 55, 56, 69, 70, 83 and 84 days post inoculation. Midges were fed on the sheep 14 days before inoculation and again prior to biopsy on days 56, 70 and 83. BTV1 was not detected in any cultured skin biopsy. This work provided unequivocal evidence that BTV does not persist in the skin of previously infected sheep and is not reactivated by midge feeding.

Acceptance that BTV does not persist in the skin of animals should allow the movement of seropositive animals to bluetongue sensitive markets provided the animals are located in an area without current bluetongue activity for an accepted period prior to export.

2. EXECUTIVE SUMMARY

Early in 2003 a paper by Takematsu et al. (2003) describing BTV persistence in sheep was published in the Journal of General Virology. Takematsu et al. suggested that BTV might persist in $\chi\delta$T cells in the skin of infected sheep even in the presence of neutralising antibodies and that virus replication was "induced" by midge feeding. The finding of BTV in the skin of sheep infected 9 weeks previously recalled the regulatory problems that were caused by previous reports describing persistence of the virus in a "persistently infected" bull. These reports were ultimately retracted. Such reports could lead to the reintroduction by regulatory agencies of international regulations banning movement of sheep and cattle with antibodies to BTV.

Experiments were designed to determine the veracity of these observations in sheep using both a laboratory-adapted strain of BTV1, as described by Takamatsu et al., and a wild type isolate of the same serotype. This comparison was necessary, as adaptation of field isolates of BTV to growth in tissue culture is known to alter the biological properties of the virus.

Sheep were inoculated with wild type and laboratory-adapted BTV1. Infection with BTV1 was confirmed by virus isolation in all inoculated sheep. The virus was isolated from blood for up to 14 days post inoculation. All inoculated sheep had seroconverted to BTV1 by 28 days post inoculation and remained seropositive until the final blood collection. Uninoculated control sheep remained seronegative throughout the experiment. Skin biopsies were taken at 14, 28, 42, 55, 56, 69, 70, 83 and 84 days post inoculation. Midges were fed on the sheep 14 days before inoculation and again prior to biopsy on days 56, 70 and 83. Biopsies were cultured for 7 days and the supernatant examined for the presence of BTV1 by virus isolation and BTV antigen capture enzyme-linked immunosorbent assay (BACE). A selected number were also tested by polymerase chain reaction (PCR). BTV1 was not identified in any sample. This work provided unequivocal evidence that bluetongue virus does not persist in the skin of previously infected sheep and is not reactivated by midge feeding.

Widespread international support for the concept of bluetongue virus persistence in animals could have a significant medium and long-term adverse impact on the Australian livestock industry. Regulatory authorities in bluetongue sensitive countries could respond by banning the movement of bluetongue susceptible animals with antibodies to BTV. The current negotiating strategy would become obsolete, trade gains made could be threatened and export opportunities could be severely affected. These export opportunities lie principally with bluetongue sensitive markets, as the majority of existing markets do not have bluetongue requirements.

Providing strong evidence that BTV does not persist in previously infected animals is essential to counteract published work suggesting that persistence does occur experimentally in sheep. Acceptance that BTV does not persist in animals should allow the movement of seropositive animals to bluetongue sensitive markets provided the animals are located in an area without current bluetongue activity for an accepted period prior to export.

3. RESEARCH REPORT

3.1 Background and Industry Context

During the last decade, Australian efforts to reduce bluetongue virus (BTV) trade restrictions have been extensive. Drawing on the knowledge and experience of Australian scientists, Biosecurity Australia have developed and refined a strategy for the easing of BTV restrictions. The strategy is based on zoning of BTV in Australia and evidence that BTV does not persist in animals beyond a known viraemic period. This strategy was challenged by recent work in the United Kingdom that suggested that BTV could persist in the skin of infected sheep.

Early in 2003 a paper by Takematsu et al. (2003) describing BTV persistence in sheep was published in the Journal of General Virology. Takematsu et al. suggested that BTV might persist in $\chi\delta$T cells in the skin of infected sheep even in the presence of neutralising antibodies and virus replication was "induced" by midge feeding. Four sheep were exposed to midges 18 days prior to intradermal infection with BTV. Nine weeks post infection they were subjected to a further round of midge feeding. In these sheep virus was detected in the supernatant of cells cultured from skin biopsies taken before and 8 hours after the second midge feeding. Two sheep that had not been sensitised to midges 18 days previously, were also infected with BTV and subject to a single round of midge feeding 9 weeks post infection. In these sheep virus was found in the medium of cultured skin cells derived from biopsies taken 8 hours after feeding. No virus was detected prior to midge feeding.

The finding of BTV in the skin of sheep infected 9 weeks previously recalled the regulatory problems that were caused by previous reports describing persistence of virus in a "persistently infected" bull. These reports were ultimately retracted. Such reports cause concern because acceptance by regulatory agencies could lead to reintroduction of international regulations banning movement of sheep and cattle with antibodies to BTV.

Australian experiments were designed to determine the veracity of the observations of Takamatsu et al. in sheep using both a laboratory-adapted strain of BTV1, as described by Takamatsu et al., and a wild type isolate of the same serotype. Adaptation of field isolates of BTV to growth in tissue culture is known to alter the biological properties of the virus. For example, unlike its wild type progenitor, an attenuated, laboratory-passaged strain of BTV-23, which did not produce disease in adult sheep, was a potent cause of early foetal death and to a much lesser extent, foetal malformation (Flanagan and Johnson 1995).

3.2 Project Objectives

1. To determine if wild type or laboratory adapted BTV is present in skin biopsies taken from sheep at regular intervals from the time of viraemia to at least 12 weeks post infection and cultured for one week *in vitro*.

2. To determine if midge feeding at biopsy sites at 8, 10 and 12 weeks post infection has any effect on either the presence or the titre of virus found in the medium of cultured skin cells.

3.3 Materials and Methods

3.3.1 Sheep

Ten sheep were housed in a single room in the Berrimah (outside Darwin) animal house. Sheep were "sensitised" by an initial feeding with wild caught nulliparous unfed *C.actoni* or *C.marksi.* Fourteen days later four sheep were infected by intradermal and intravenous inoculation of blood containing wild type BTV1 currently circulating at Beatrice Hill, 60 km south east of Darwin. Four sheep were inoculated with a laboratory adapted strain of the same virus. The remaining two animals were uninoculated controls. Sheep were bled prior to inoculation and then on days 4,7,11, 14, 18, 21 28,56 and 84 post inoculation. Skin biopsies were taken at 14, 28, 42, 55, 56, 69, 70, 83, and 84 days post inoculation. On days 56, 70 and 84 post inoculation *C. actoni* or *C.marksi* were fed on the sheep prior to skin biopsies being taken.

3.3.2 Skin biopsy collection

Skin biopsies of 6 mm diameter were taken from the inside thigh of each animal. The site was 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 4^0C for two hours before processing.

3.3.3 Sheep skin fibroblast cultures

The skin biopsies were processed for culture using a method adapted from Takamatsu and Jeggo (1989). The samples were cut into small sections and treated with trypsin ethylene-diaminetetraacetic acid (EDTA) for 30 minutes at 37^0C. After 5 minutes of incubation the samples were drawn up and expelled several times through an 18 gauge needle and 5 mL syringe. Samples were shaken after 10 and 20 minutes incubation. 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 u/mL of recombinant human interleukin 2 and antibiotics.

The cultures were maintained at 37^0C/5% CO_2 and the cultures harvested when they reached >90% confluency at 7 days. Cells were removed by scraping and combined with the culture supernatant and stored at -70^0C until processed for virus isolation or BACE (Hawkes et al. 2000). Selected samples were also examined for viral genetic material by PCR (McColl and Gould 1991).

3.3.4 Virus isolation and quantification

BTV in sheep blood and in the medium of cultured skin biopsies was assayed by intravenous inoculation into 10 day-old embryonated chicken eggs. The presence of BTV in embryos was confirmed by BACE on homogenised livers harvested from the embryos. Selected blood samples were also examined for viral genetic material by PCR.

3.3.5 Serology

Bluetongue group and serotype specific antibody was monitored by competitive ELISA (White et al. 1991) and virus neutralisation.

3.3.6 Insect collection and utilisation

Crepuscular collections of midges were made by aspiration from cattle. Collections were sorted and unfed nulliparous *C.marksi* or *C.actoni* selected for feeding on sheep. Housings designed to enable the midges to feed on the sheep were glued to the inside thigh of each animal. Midges were introduced to the housing following knocking down with CO_2. After about six hours exposure the midges were removed by flooding with alcohol and aspiration to a container. Alcohol fixed midges were examined to confirm that some feeding had occurred and stored for future testing if required.

3.4 Results

3.4.1 Virus inoculation and confirmation of infection

Four sheep received 0.1ml by intradermal inoculation and 2ml by intravenous inoculation of laboratory culture-adapted BTV1.The titre of this virus was 1X $10^{5.8}$ $TCID_{50}$ in 1ml. Four sheep received 0.1ml by intradermal and 2.5ml by intravenous inoculation of cattle blood containing BTV1. This blood was collected from a bovine inoculated with blood known to contain BTV1. The titre of virus in the blood was 1X $10^{4.3}$ EID_{50} in 1ml.

Infection with BTV1 was confirmed by virus isolation in all inoculated sheep. Virus was isolated from blood for up to 14 days post inoculation as shown in Table 1. The maximum titre of virus in blood was $1X10^{5.3}$ EID_{50} in 1ml. Individual titres are recorded in Table 1. All inoculated sheep had seroconverted to BTV1 by 28 days post inoculation and remained seropositive until the final blood collection. Uninoculated control sheep remained seronegative throughout the experiment.

The presence of BTV1 viral RNA was detected using PCR on a selected number of blood samples. All inoculated sheep were positive at 14 days post inoculation and 7/8 were still positive at 56 days post inoculation as shown in Table 1.

Table 1 Sheep Virus Isolation, PCR and Serology

Animal No.	Day 0	Day 4		Day 7		Day 11		Day 14			Day 18	Day 21	Day 28		Day 56		Day 84
	Serol*	V Isol#	Titre	V Isol	Titre	V Isol	Titre	V Isol	Titre	PCR	V Isol	V Isol	Serol	V Isol	Serol	PCR	Serol
3-01 (control)	-	-		-		-		-		-	-	-	-	-	-	-	-
3-02	-	+	4.1	+	4.1	+	4.3	+	2.7	+	-	-	+	-	+	-	+
3-03	-	+	4.3	+	4.3	+	4.5	+	3.7	+	-	-	+	-	+	+	+
3-04	-	+	4.3	+	4.5	+	3.1	+	2.7	+	-	-	+	-	+	+	+
3-05	-	+	3.7	+	5.3	+	3.7	-		+	-	-	+	-	+	+	+
3-06 (control)	-	-		-		-		-		-	-	-	-	-	-	-	-
3-07	-	+	4.5	+	3.9	+	4.1	-		+	-	-	+	-	+	+	+
3-08	-	+	4.5	+	4.3	+	4.1	+	3.9	+	-	-	+	-	+	+	+
3-09	-	+	4.3	+	4.3	+	4.3	+	2.7	+	-	-	+	-	+	+	+
3-10	-	+	4.5	+	4.1	+	3.9	+	2.7	+	-	-	+	-	+	+	+

Serology* both BTV c ELISA and BTV1 VNT

V Isol# Isol E on sheep blood and BACE on egg homogenates

Titre $log_{10}EID_{50}$/ml

PCR@ Realtime(Taqman) PCR (both Java C and Aus A) conducted at AAHL

3.4.2 Skin fibroblast cultures

Skin biopsy cultures were taken at days 14, 28 and 42 post inoculation and prior to any post sensitisation midge exposure. On day 55 post inoculation a skin biopsy was taken prior to exposure to biting midges on day 56. The midges were attached to the opposite leg to that from which the biopsy was taken. Following 6 hours of exposure the midges were removed and a second biopsy taken. This sample was collected from the area on which the midges had been feeding. The same procedure was repeated on days 69 and 70 and again on days 83 and 84 post inoculation. All biopsy supernatants were examined for the presence of BTV1 by virus isolation and BACE. A selected number were also tested by PCR. BTV1 was not identified in any sample as shown in Table 2.

Table 2 Sheep Skin Biopsy Virus Isolation and PCR

Animal No.	Day 14			Day 28		Day 42		Day 55		Day 56			Day 69		Day 70		Day 83		Day 84	
	BACE*	V Isol#	PCR@	BACE*	V Isol	BACE*	V Isol	BACE*	V Isol	BACE*	V Isol	PCR	BACE*	V Isol	BACE*	V Isol	BACE*	V Isol	BACE*	V Isol
3-01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-04	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-06	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-07	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-08	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-09	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

BACE* BTV antigen capture ELISA performed on skin fibroblast cultures

V Isol# Isol E on skin fibroblast cultures and BACE on egg liver homogenates

PCR@ Realtime(Taqman) PCR (both Java C and Aus A) conducted at AAHL

3.5 Discussion

These experiments were conducted to test aspects of the hypothesis of Takematsu et al. (2003) indicating that infectious BTV could be recovered 81 days after infection by culturing skin fibroblasts, and that exposure to biting midges enhanced the recovery.

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 the virus. Claims of virus persistence and reactivation were therefore investigated using Australian isolates of BTV1 as both culture-adapted and unadapted virus. This experiment showed viraemia in sheep was limited to 14 days and there was no evidence of BTV in skin samples collected after the period of viraemia. This period of viraemia is shorter than that found in previous work with naturally infected cattle (Melville 2000) and indicates that current protocols requiring 60 days quarantine in a bluetongue free zone provide a large safety margin for safe export of live animals. PCR on selected blood samples detected BTV1 viral RNA at 56 days post inoculation, indicating that this technique is very sensitive. It is important to remember however that detection of viral RNA does not mean that viable infectious virus is present. Skin samples collected at the same time were negative by PCR, indicating viral RNA could not be detected even by this very sensitive technique.

Exposure to biting midges failed to reactivate BTV1 in the skin of animals previously infected with either wild or laboratory strains of the virus. Additional sensitivity was provided in this work by using PCR on selected samples and this technique confirmed the negative findings of virus isolation and antigen ELISA on all skin fibroblast samples taken eight hours after exposure to biting midges. These results are clearly different from the United Kingdom results, where similar cultures were all positive by virus isolation.

While the hypothesis proposed in the Takematsu paper is both interesting and intriguing, it is perhaps unfortunate that the authors did not deal with issues of significance, reproducibility and the implications of results more critically. A weight of evidence to the contrary may ultimately be required to demonstrate that these observations may be peculiar to the conditions under which those experiments were conducted.

3.6 Achievement of Objectives

3.6.1 Objective 1

To determine if wild type or laboratory adapted BTV is present in skin biopsies taken from sheep at regular intervals from the time of viraemia to at least 12 weeks post infection and cultured for one week *in vitro*. Sheep were inoculated with both wild type and laboratory adapted BTV1. Skin biopsy samples were taken at 2,4,6,8,10 and 12 weeks post inoculation. These samples were cultured for 7 days and examined for the presence of BTV by three different methods. No evidence of BTV was found in any skin sample.

3.6.2 Objective 2

To determine if midge feeding at biopsy sites at 8, 10 and 12 weeks post infection has any effect on either the presence or the titre of virus found in the medium of cultured skin cells. Wild caught midges were fed on the skin of the sheep prior to collection of skin biopsy samples at weeks 8, 10 and 12 weeks post inoculation. Midge feeding failed to reactivate BTV1 in the skin of previously infected sheep and all skin biopsy samples were negative when cultured and examined for virus.

The achievement of these objectives clearly demonstrated that BTV does not persist in the skin of sheep following infection. These results, combined with similar experimental results from work at the Australian Animal Health Laboratory (AAHL)(Lunt 2004) and monitoring of naturally infected cattle in Darwin (Melville et al. 2003), provide a body of information which clearly show BTV does not persist in infected animals.

3.7 Impact on Meat and Livestock Industry

Widespread international support for bluetongue virus persistence in animals could have a significant medium and long-term adverse impact on the Australian livestock industry. Regulatory authorities in bluetongue sensitive countries could respond by banning the movement of bluetongue susceptible animals with antibodies to BTV. The current negotiating strategy would become obsolete, trade gains made could be threatened and export opportunities could be severely affected. These export opportunities lie principally with bluetongue sensitive markets, as the majority of existing markets do not have bluetongue requirements.

Providing strong evidence that BTV does not persist in previously infected animals is essential to counteract published work suggesting that persistence does occur experimentally in sheep. Acceptance that BTV does not persist in animals should allow the movement of seropositive animals to bluetongue sensitive markets provided the animals are located in an area without current bluetongue activity for an accepted period prior to export.

3.8 Conclusions and Recommendations

These experiments clearly showed that BTV does not persist in the skin of previously infected animals. Biosecurity Australia can use this information in trade protocol development and to protect previous agreements based on a limited viraemia with BTV. Acceptance by trading partners of the lack of persistence will be assessed in the coming months and any need to provide additional evidence will be identified by Biosecurity Australia.

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