Forty years continous monitoring for bluetongue virus at an Australian site of high arbovirus activity. What have we achieved?

Lorna Melville*, Richard Weir, Neville Hunt, Steven Davis & Susan Walsh

Department of Primary Industry and Fisheries, GPO Box 3000, Darwin NT 0801, Australia.

* *Corresponding author at: Department of Primary Industry and Fisheries, GPO Box 3000, Darwin NT 0801, Australia. Tel.: +61 889992251, e-mail: lorna.melville@nt.gov.au.*

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Summary
Beatrice Hill Farm (BHF) near Darwin, Australia was identified in the early 1970's as a site of high arbovirus activity. The first isolation of Bluetongue virus (BTV) in Australia was made on BHF in 1975. Since then, there has been continuous monitoring for BTV at BHF, the virus has been isolated on a yearly basis, with the only exception of 1990. All 10 serotypes known in Australia have been isolated at this site and an assessment of their biological behaviour made. Over the years, the methods and intensity of monitoring have been changed. In recent years molecular techniques have permitted more detailed examination of the origins of the viruses and their natural behaviour in field situations. Data collected at BHF have allowed modelling to detect likely origins of the BTVs that regularly enter Australia through wind borne infected *Culicoides* from South East Asia. Concurrent vector monitoring led to assess the *Culicoides* species more likely to be involved with transmission of these viruses.

Monitoraggio del virus della Bluetongue (BTV) in un sito ad alta attività di arbovirus in Australia

Parole chiave
Analisi,
Australia,
Culicoides,
Monitoraggio,
Sorveglianza,
Virus della Bluetongue.

Riassunto
Nei primi anni '70, Beatrice Hill Farm (BHF) nei pressi di Darwin, in Australia, è stata identificata come sito ad alta attività di arbovirus. Il primo isolamento del virus della Bluetongue (BTV) in Australia è stato rilevato proprio in quest'area nel 1975. Da allora, la zona è stata soggetta ad un monitoraggio continuo. Il virus è stato costantemente isolato con la sola eccezione del 1990. Tutti i 10 sierotipi noti in Australia sono stati isolati in questo sito. È stato anche valutato il loro comportamento. Negli anni, sono cambiati i metodi e la frequenza del monitoraggio. Negli ultimi anni le tecniche molecolari ci hanno dato la possibilità di avere informazioni più precise sulle origini e sul comportamento del virus. I risultati ottenuti ci hanno permesso di individuare le origini dei ceppi di BTV che entrano regolarmente in Australia attraverso *Culicoides* infetti provenienti dal Sud-Est asiatico. Il monitoraggio di questo vettore ha permesso di determinare le specie di *Culicoides* più coinvolte nella trasmissione di BTV.

Introduction

Beatrice Hill Farm (BHF) is a Northern Territory of Australia government research farm located about 50 km South-East of Darwin at 12°39'S 131°20'E. This area of Australia lies in the semi-arid tropics and experiences hot, wet Summers and warm, dry Winters. It is within the Northern Territory of Bluetongue virus (BTV) endemic zone, which is located between approximately 11-17° South. This site was identified as an area of high arbovirus activity in the early 1970's, when sentinel herds and associated entomology studies were first established in Australia for monitoring cattle viruses. As a result of the intense entomology work at BHF, the first

isolation of BTV in Australia was made from a mixed pool of *Culicoides* collected in 1975 (St George *et al.* 1978). Since then, there has been continuous monitoring for BTV at this site. Monitoring cattle for BTV is supported by regular light trap collections of *Culicoides*. This work is largely funded by the National Arbovirus Monitoring Program (NAMP), which started in 1993 and is jointly funded by the livestock industries and state and federal governments.

Materials and methods

BHF sentinel herd

The BHF sentinel herd consists of 24 young bovines sourced from the BTV free zone and replaced each year when there is minimal arbovirus activity. Lithium heparin and EDTA blood samples are collected each week from all animals, serum samples are collected on a monthly basis. This work is conducted under animal ethics approval number A11033 Charles Darwin University Animal Ethics Committee.

Virus isolation

Virus isolation is conducted in real time using 2 parallel systems. Lithium heparin blood samples are inoculated to embryonated chicken eggs (ECE) as described by Melville and colleagues (Melville *et al.* 2005). The ECE homogenates are passaged onto mosquito cell cultures (*Aedes albopictus* C6/36). The second passage uses C6/36 and Baby Hampster Kidney (BSR) (BHK21 clone) mammalian cell cultures. A third passage uses BSR cell cultures to detect any cytopathology due to virus replication.

The ethylenediaminetetra-acetic acid (EDTA) blood samples are centrifuged and the white cell fraction inoculated to C6/36 cells. The second and third passage uses BSR cells as described by Melville and colleagues (Melville *et al.* 2005). Viruses are identified by a combination of immunofluorescent antibody, real time polymerase chain reaction (qRT-PCR) and virus neutralisation (VN) tests.

Serology

Serum samples are tested for BTV antibodies by competitive ELISA and if positive by VN tests (Melville *et al.* 2005).

Molecular analysis

With the co-operation of the Australian Animal Health Laboratory, a selection of BTV isolates has been subjected to genetic analysis each year since 1992. This has identified a number of different genotypes circulating at BHF and enabled tracking of their movements (Pritchard *et al.* 2004). A detailed analysis of BTV-1 isolates has also been conducted to study the evolution of this virus over 30 years (Boyle *et al.* 2014).

Entomology

A light trap for insect collections is operated for 3 consecutive nights each month. Insects are collected into alcohol and the *Culicoides* species sorted and identified. Improvements to light trap design has seen replacement of the incandescent globe with a green LED. This in turn has required modifications to cope with the increased size of collections. Equipment was designed to enable accurate splitting of the collections (Hunt *et al.* 2009) and screening of the light traps was trialled to exclude large by catches. Molecular analysis of collections has also been used to identify the presence of BTV.

Data management

All virus isolation, serology and entomology data are entered onto a web-based national database. This enables national zones to be developed for trade purposes (Cameron 2001).

Dispersal modelling

Atmospheric dispersal modelling was used to identify a putative source site following the introduction of new BTV serotypes in 2007 and 2008 (Eagles *et al.* 2014).

Results

Virus isolation and characterisation

Eight serotypes of BTV have been isolated at BHF (serotypes 1, 3, 9, 15, 16, 20, 21, and 23) since the beginning of the program until 1986. Although the introduction of the ECE isolation system in 1986 resulted in a large increase in the number of BTVs isolated, no new serotypes were isolated for 20 years, until BTV-7 was found in 2007 and BTV-2 the following year. Table I summarises the isolation results until 2014. BTV-1 is the most commonly isolated serotype, being found in most years. The frequency of occurrence of other serotypes is highly variable. Characteristics of natural infections from 1992-2003 have been reported in a previous study (Melville 2004). Table II shows the annual variation in infection rates and activity between 2004 and 2014.

Table I. *Bluetongue virus serotypes at Beatrice Hill Farm.*

Year	Serotypes
1975-1991	1, 3, 9, 15, 16, 20, 21, 23
1992	16, 20
1993	1
1994	1, 21
1995	20, 21
1996-2000	1, 20
2001	1, 9, 16
2002-2004	1, 21
2005	1, 20, 21
2006	1, 20
2007	7
2008	1, 2
2009	2, 3
2010	1, 7
2011	1, 20
2012	1, 20
2013	1, 7
2014	1, 20

Table II. *Characteristics of natural Bluetongue virus infections, 2004-2014.*

Year	Bluetongue serotype	Sentinels infected	Detectable period of viremia (weeks)	
			Min.	Max.
2004	1	15/24	1	4
	21	6/24	1	4
2005	1	2/24	1	3
	20	18/24	1	7
	21	2/24	2	2
2006	1	23/23	1	4
	20	16/23	1	3
2007	7	4/24	2	3
2008	1	20/24	1	6
	2	15/24	1	7
2009	2	22/24	2	8
	3	19/24	1	6
2010	1	18/23	1	6
	7	4/23	1	1
2011	1	15/24	1	3
	20	17/24	1	4
	2	3/24	1	2
2012	1	23/24	1	5
	20	2/24	1	2
2013	1	19/24	1	3
	7	11/24	1	6
	20	3/24	1	2
2014	1	9/24	1	3
	20	11/24	1	5

Table III. *Bluetongue virus genotypes at Beatrice Hill Farm.*

Year	Genotype
1975-1991	Australia A
1992	Australia A/Java A
1993	Australia A
1994	Australia A/Java C
1995	Java C/Malaysia A
1996-2000	Java C
2001	Australia A/Java C/Malaysia A
2002-2004	Java C
2005	Java A/Java C
2006	Java C
2007	Australia A
2008	Java C/Malaysia A
2009	Malaysia A
2010	Malaysia A
2011	Australia A/ Malaysia A
2012	Malaysia A/Java C
2013	Related to Western topotype/Malaysia A
2014	Australia A/Malaysia A

Molecular analysis of BTV isolates from BHF has shown significant genetic variability on a yearly basis, as described in Table III. A retrospective study of the earliest isolates of BTV from BHF indicated that only 1 genotype was circulating during the period 1975-1991. In 1992, a genotype previously found only in South East Asia was detected for the first time. Since that time, there have been regular incursions of previously exotic genotypes and evidence that reassortment of gene segments has occurred among different viruses circulating simultaneously. A detailed genetic analysis of BTV-1 isolates has shown 4 major periods of BTV-1 evolution. Each was marked by a period of relative stability of evolving genome segments followed by replacement and extinction by the introduction of novel genome segments (Boyle *et al.* 2014).

Entomology

Five species of *Culicoides* are currently recognised as proven vectors in Australia. These species are *Culicoides brevitarsis*, *Culicoides actoni*, *Culicoides fulvus*, *Culicoides wadai*, and *Culicoides dumdumi*. All such species have been identified at BHF. Attempts to improve surveillance in remote areas have shown that BTV can be identified in alcohol collections of *Culicoides* (Melville *et al.* 2008). Although this system is less sensitive than detection in the mammalian host, it does have application in remote areas of Northern Australia where it is impossible to maintain cattle herds.

Dispersal modelling

For the introduction of BTV-7 in March 2007 a potential dispersal event occurred approximately 2 weeks earlier from sites across West Timor and Timor-Leste, as well as 2 months prior, from the same source region. For the introduction of BTV-2 in January 2008, the modelling indicated that dispersal could have occurred 1 week earlier from a site in West Timor (Eagles *et al.* 2014).

Discussion

Over the past 40 years, the surveillance program at BHF has identified all the BTV serotypes present in Australia and documented their annual occurrence. The system is sensitive and robust for the detection of new incursions into Australia. In both 2007, when BTV-7 was first detected, and 2008 when BTV-2 was first detected, retrospective serology showed no evidence of these viruses in previous years. Similarly, serology associated with virus isolation shows that most infections are detected by weekly virus isolation.

In addition to detection of new serotypes in 2007 and 2008, genetic analysis of isolates has demonstrated incursions of viruses of South East Asian origin in 1992, 1994, 1995, 2001, 2009, 2010, and 2013. These new genotypes have become established in the BHF environment for a limited number of years, before being replaced by a different genotype. Reassortant viruses have been detected on a number of occasions. A detailed analysis of BTV-1 isolates over 30 years has shown that the dominant mechanism prompting genetic diversity at BHF was the introduction of new viruses and reassortment of genome segments with existing viruses (Boyle *et al.* 2014).

The development of qRT-PCR tests for BTV group and serotypes has provided a mechanism for rapid detection of BTV in both cattle blood and insects, showing the potential to improve surveillance in Northern Australia. These techniques are being introduced as part of the routine testing of the NAMP. However, they will not replace routine virus isolation at BHF, which is designed as a surveillance system for commonly isolated arboviruses, not just BTV.

Dispersal modelling has confirmed the likely origins of BTV and vector incursions into Australia. The use of an atmospheric dispersal model has confirmed the value of the BHF site as a location for intensive surveillance activities. Such modelling can contribute to the strategic use of limited surveillance resources (Eagles *et al.* 2014), enabling selection of the most appropriate site across the northern coastline.

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