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ORIGINAL ARTICLE

The potential for bluetongue virus serotype 16 to cause disease in sheep in New South Wales, Australia

S Gestier,[a,b] DS Finlaison,[a] K Parrish[a] and PD Kirkland[a]*

Bluetongue virus serotype 16 detection in NSW In coastal New South Wales (NSW), bluetongue virus (BTV) serotypes 1 and 21 are endemic and transmitted in most years without evidence of disease. However, serotype 16 (BTV-16) infection was detected for the first time in NSW in November 2016 in cattle undergoing testing for export. Retrospective testing of blood samples collected from sentinel cattle as part of the National Arbovirus Monitoring Program (NAMP) established that the first detected transmission of BTV-16 in NSW occurred in April 2016 in sentinel cattle on the NSW North Coast. Subsequently, until 2022, BTV-16 has been transmitted in most years and was the predominant serotype in the 2018–2019 transmission season. The data available suggests that BTV-16 may have become endemic in NSW.

Experimental studies During experimental infection studies with BTV-16, all sheep were febrile, with the peak of viremia occurring 6–10 days after inoculation. There was nasal and oral hyperaemia in most sheep with several animals developing a nasal discharge and nasal oedema. All sheep developed coronitis of varying severity, with most also developing haemorrhages along the coronary band. There was a high incidence of haemorrhage in the pulmonary artery, epicardial petechiae, extensive pericardial haemorrhages and moderate body cavity effusions including pericardial effusions.

Conclusion Overall, experimental pathogenicity findings suggest moderate disease may occur in sheep in the field. These findings, when combined with climatic variability that could result in an expansion of the range of *Culicoides brevitarsis* into major sheep-producing areas of the state, suggest that there is an increasing risk of bluetongue disease in NSW.

Keywords bluetongue disease; bluetongue virus; cattle; *Culicoides*; National Arbovirus Monitoring Program; pathogenicity; sheep

Abbreviations BTV, Bluetongue virus; Ct, cycle threshold; DPI, Department of Primary Industries; dpi, days postinoculation; NSW, New South Wales; qRT-PCR, real-time quantitative reverse transcription PCR; NAMP, National Arbovirus Monitoring Program; Qld, Queensland; RBC, red blood cells

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Bluetongue is a disease mainly of small ruminants, caused by an arthropod-borne virus of the same name (bluetongue virus [BTV]; genus *Orbivirus*, family *Sedoreoviridae*).[1] Globally, there are currently more than 30 known serotypes with 1–24 listed as notifiable by the World Organisation for Animal Health.[2,3] Sheep are the species most affected with clinical signs of varying severity including swelling of the lips and tongue, oral and nasal haemorrhage and ulceration, lameness with hyperaemia and haemorrhage of the coronary band, emaciation, weakness and death.[4–6] Surviving sheep can have breaks in the wool and prolonged convalescence.[4,7] Postmortem lesions include characteristic haemorrhages at the base of the pulmonary artery, haemorrhages in lymph nodes and other tissues, pulmonary oedema and effusions in body cavities.[4,6] BTV strains vary considerably in their virulence both between and within serotypes; other factors including breed, age, UV exposure, high temperature, rough terrain and stress also contribute to disease expression.[4,6,8,9]

Thirteen serotypes of BTV have been detected in Australia (1–5, 7, 9, 12, 15, 16, 20, 21 and 23).[10–19] Surveillance using sentinel cattle is carried out annually by the National Arbovirus Monitoring Program (NAMP) to define annual limits of BTV transmission for zoning to support international trade.[20] Most serotypes of BTV have only been isolated from sentinel cattle located in Northern Australia[21,22] where there is thought to be an ongoing introduction of BTV serotypes and genotypes via wind-dispersal of infected vectors from South East Asia.[23–25] Clinical BTV is rare in sheep in Australia and has yet to be observed in commercial flocks, in part because there has been little geographical overlap between major sheep-producing areas and the distribution of the main insect vector, *Culicoides brevitarsis*.[21]

The distribution of BTVs is vector-dependent and in south-eastern Australia extends seasonally from north to south along the east coast of New South Wales (NSW) in most years with conditions favourable to the insect vector. Before 2016, only two serotypes (BTV-1 and BTV-21) had been detected in NSW.[25] These are also the predominant serotypes circulating in Queensland (Qld), although there has been some previous evidence of limited transmission of BTV-2 and possibly BTV-23 in northern QLD.[15,26,27] More recently, the transmission of BTV-15 has also been observed in Qld.[28]

*Corresponding author.
[a]Virology Laboratory, Elizabeth Macarthur Agriculture, Institute Department of Primary Industries, Menangle, New South Wales, Australia; peter.kirkland@dpi.nsw.gov.au
[b]Biosecurity Sciences Laboratory, Department of Agriculture and Fisheries, Brisbane, Queensland, Australia

BTV-16 was first detected serologically in Australia in the Northern Territory (NT) in 1984 and was isolated in 1986,[14] then isolated again sporadically over the next 16 years.[21] In 2001, disease due to BTV-16 infection was observed in a small group of sheep introduced to the NT, resulting in a 30% mortality. Subsequently, restrictions were placed on further introductions of sheep to the NT.[29] Between 2002 and 2012, BTV-16 was detected infrequently and only in sentinel herds in northern Australia.[19] BTV-16 was then detected in 2013 in the QLD Western Downs Region (Chinchilla)[27]; unpublished NAMP data recorded further detections in northern Qld at Innisfail in 2014 (B. Hill, personal communication) followed by Cooktown in 2016 (B. Hill, personal communication). There were no BTV-16 detections in the 2014–2015 monitoring season.

In 2016, serological testing for the export of animals quarantined in South Australia suggested that young cattle from NSW had been infected with BTV-16, based on the results of serotype-specific serology performed at the Australian Centre for Disease Preparedness (ACDP), Geelong, Victoria. Movement tracing indicated that the animals were sourced from the NSW north coast in April 2016. Based on the age of the animals and no history of movement interstate where there may have been potential for exposure to BTV, it was concluded that infection had occurred in NSW between February and June 2016.

This article reports the investigation into the first detection and the ongoing transmission of BTV-16 in NSW in the subsequent four transmission seasons. Given the previous association of BTV-16 in Australia with disease in sheep and this detection close to commercial sheep-producing areas, experimental infections of sheep were undertaken to determine the virulence of a NSW field strain of BTV-16.

Materials and methods

BTV serology

For serological surveillance of BTV infection in the field and experimentally infected sheep, serum samples were tested for BTV antibodies using a blocking ELISA (bELISA) directed at conserved epitopes on the viral core protein VP7.[30] The published method varied by producing antigen in HmLu-1 cell cultures inoculated with BTV-23 and using an alternative mouse monoclonal antibody (PK-4, J. White, ACDP, unpublished). Results were expressed as percentage inhibition (PI) with a cut-off of <50% for negative results. Samples with PI values between 50% and 65% were inconclusive and >65% positive. Seroconversion was classified as a change in bELISA results for successive serum samples from negative to positive.

BTV real-time quantitative reverse transcription PCR

Red blood cells (RBCs) from EDTA-treated or clotted blood samples were tested in a pan-BTV-reactive real-time quantitative reverse transcription PCR (qRT-PCR) assay targeting segment 10 (NS3).[31] Total nucleic acid was extracted from 25 µL of packed RBC using a magnetic bead-based kit and a magnetic particle handling system,[32] with purified nucleic acids eluted in a 50 µL volume and denatured by heating at 95°C for 5 min. Samples were tested as described previously[32] with an exogenous internal control included in the extraction buffer with each sample. The result was classified as negative if no amplification was observed after 45 cycles.

NAMP surveillance

The NAMP has operated annually in NSW since 1993.[21] In NSW, monitoring of sentinel herds occurs from October to June/July to cover the transmission period for BTVs that are transmitted mainly by *C. brevitarsis*. Clotted blood samples (and EDTA-treated blood after the detection of BTV seroconversions) were collected at regular intervals from NAMP sentinel herds comprising 10–15 young cattle per herd at up to 40 locations across NSW.[20] These sites were chosen to provide coverage of the area of NSW in which *C. brevitarsis* may be detected and also the *C. brevitarsis*-free areas in southern and western NSW.[19] Sentinel cattle were aged 5–6 months when first sampled and were born on the property where sampled. Blood samples were collected between 2- and 8-fold times during the monitoring season, with the more frequent collections occurring in endemic coastal areas. The sampling of animals in NAMP herds was approved by the Animal Ethics Committee of the Elizabeth Macarthur Agricultural Institute, AEC Reference No. M13/04, M16/10 and M19/08.

All samples collected as part of the NAMP were initially tested in the BTV bELISA. Where seroconversions were detected in sentinel cattle, the blood sample collected at the time of seroconversion, the previous sample and all subsequent samples were tested by pan-BTV qRT-PCR. Samples that were positive in the pan-BTV qRT-PCR were tested using BTV-1, 16 and 21 serotype-specific qRT-PCR-assays[33] for the 2015–2016 to 2019–2020 monitoring seasons.

Surveillance of NSW sheep in BTV endemic zone

After the detection of BTV-16 infection in cattle in NSW, sheep were tested on farms within the NSW endemic BTV zone at Paterson ($n = 55$) and Scone ($n = 30$) in 2017 and at Paterson ($n = 30$) in 2019. Serum samples were initially tested for BTV antibodies by bELISA, and, when infection of sheep was detected, screening for viraemia was initially undertaken with a pan-BTV qRT-PCR followed by BTV-1, BTV-16 and BTV-21 qRT-PCR specific assays.

BTV-16 sheep virulence study

Sheep. Merino sheep aged approximately 4 years were sourced from the bluetongue-free zone of NSW. The BTV-free status of these animals was also confirmed by negative results in the BTV bELISA and the pan-BTV qRT-PCR. The sheep were held in temperature-controlled (approximately 23°C) insect-proof biosecure containment facilities. These animal studies were approved by the Animal Ethics Committee of the Elizabeth Macarthur Agricultural Institute, AEC Reference No. M17/05.

Inoculum. The primary source of inoculum was clotted blood collected in May 2017 from a naturally infected sentinel calf located near Scone in the NSW Hunter Valley. This blood sample had a Ct value of 28.51 in the pan-BTV qRT-PCR and 32.2 in the BTV-16 specific qRT-PCR. The virus was amplified by intravenous inoculation of 10- or 11-day-old embryonated chicken eggs. The infected chicken livers were collected after 3 days, a 10% homogenate was prepared and was confirmed to contain BTV-16 (type-specific qRT-PCR Ct value = 22.2). For *in vivo* amplification, a single adult merino sheep was inoculated subcutaneously with 4 mL of

Table 1. Assignment of clinical scores to experimental bluetongue disease in sheep (reproduced with permission from Li[34])

Component	Score	Clinical signs
Nose	0.5	Suspect, slight clear watery discharge.
	1	Slight reddening of the nasal mucosa and clear watery discharge.
	1.5	Clear watery discharge and slight oedema of the nostrils.
	2	Hyperaemia of the nasal mucosa, oedema of the nostrils and formation of discharge that forms crusts around nostrils.
	2.5	Pronounced oedema of the muzzle and nostrils and formation of discharge that forms crusts around nostrils.
	3	Pronounced oedema of the muzzle and nostrils, haemorrhages of mucosa and pronounced nasal discharge that forms crusts with blood stains around nostrils.
	3.5	Pronounced haemorrhages of mucous membranes and oedema of muzzle and nostrils, blood-stained discharge.
	4	Ulceration and necrosis of muzzle and nasal mucous membranes, pronounced nasal discharge and crust formation with blood stain around nostrils.
Mouth	0.5	Suspect, very slight reddening of mucous membranes.
	1	Reddening of mucous membranes.
	1.5	Reddening of mucous membranes and slight oedema of the lips.
	2	Pronounced hyperaemia of mucous membranes and oedema of the lips.
	2.5	Pronounced oedema of lips and cyanosis of mucous membranes.
	3	Pronounced hyperaemia and cyanosis of the mucous membranes, variable-sized haemorrhages of the mucous membranes or the dental pad, pronounced oedema of lips and face.
	3.5	Petechial haemorrhages with ulceration and necrosis of mucous membranes.
	4	Intense petechial haemorrhages with ulceration and necrosis of mucous membranes, hyperaemia and cyanosis of the tongue.
Feet	0.5	Suspect, very slight reddening of the inner skin of the interdigital cleft.
	1	Petechial reddening of the inner skin of the interdigital cleft.
	1.5	Very slight hyperaemia of the bulb and reddening of the inner skin of the interdigital cleft.
	2	Slight hyperaemia of coronary band and bulb, pronounced hyperaemia and reddening of the inner skin of the interdigital cleft.
	2.5	Petechial haemorrhages of the inner skin of the interdigital cleft and hyperaemia of the coronary band.
	3	Hyperaemia of the coronary band and bulb, intense haemorrhages of the inner skin of the interdigital cleft or haemorrhages of the coronary band.
	3.5	Intense haemorrhages of the inner skin of the interdigital cleft and pronounced haemorrhages of coronary band.
	4	Wide haemorrhages of coronary band and intense haemorrhages of the inner skin of the interdigital cleft.
Lethargy/depression	1	Sheep occasionally sit down.
	2	Sheep quite often sit down due to weakness.
	3	Sheep always sit down, forced to stand up with a stiff stance.
	4	Sheep cannot stand up and are very weak.
Death/euthanasia	8	Sheep died due to bluetongue infection or euthanased due to severe clinical signs of bluetongue in consideration of its welfare.

phosphate-buffered gelatin saline (PBGS) containing 100 µL of the liver homogenate. Blood was collected daily to monitor BTV viremia by qRT-PCR. On day 7 at peak viremia (pan-BTV qRT-PCR Ct 20.98, BTV-16 qPCR Ct 23.61), 100 mL of EDTA-treated blood was collected and shown by titration in mosquito cells (*Aedes albopictus*, clone C6/36) to have a virus titre of 4.6 $\log_{10}$/mL. As cytopathology is inconsistently observed in C6/36 cells when infected with BTV, 50 µL of the culture supernatant from the primary C6/36 cultures was passaged 'well to well' to microplate cultures of BHK_{21} cells and observed for evidence of cytopathology for 5 days.

Challenge study. Sheep ($n = 8$) included in the pathogenicity study received 1 mL subcutaneous injections of the BTV-positive blood at two locations on the side of the neck. The negative control sheep ($n = 1$) received 2 × 1 mL subcutaneous injections of blood from an uninfected, seronegative merino sheep.

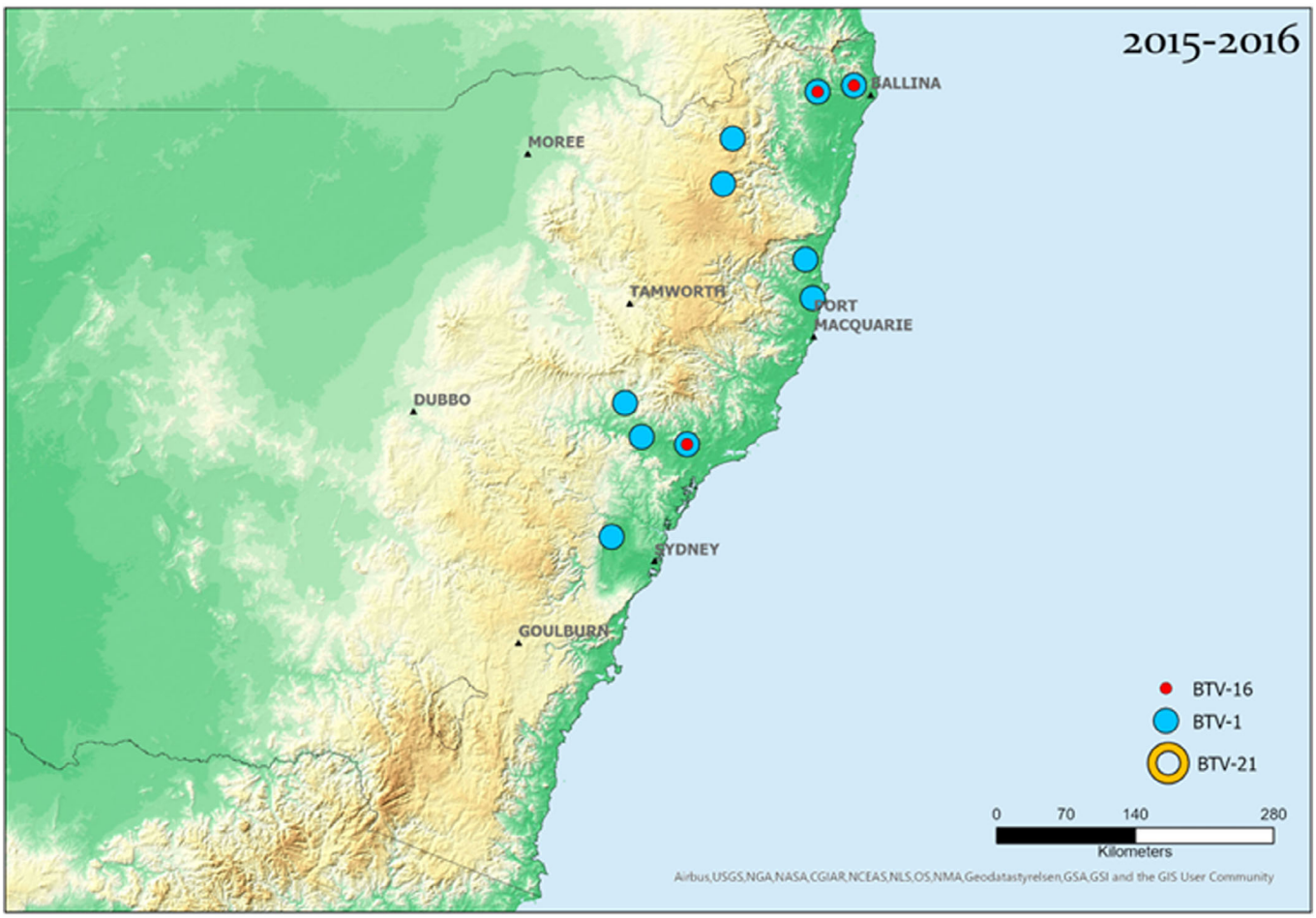

Figure 1. **Geographical distribution of detections of BTV-1 and BTV-16 in NSW NAMP sentinel cattle by serotype-specific qRT-PCR during the 2015–2016 monitoring season.**

Clinical scoring system. The clinical scoring system described by Li[34] was used to assess each sheep for clinical signs consistent with bluetongue, with separate component scores allocated for clinical signs relating to the nose, mouth, feet, lethargy/depression and euthanasia (Table 1). Sheep were scored by these clinical criteria before inoculation on day 0 to establish individual baseline scores then daily for the duration of the study.

Blood sampling and temperature monitoring. Clotted and EDTA-treated blood was collected at 0, 3–10 and 14 days post-inoculation (dpi) and finally, again the day before euthanasia (19 or 20 dpi) to monitor the occurrence of BTV infection by qRT-PCR and seroconversion by BTV bELISA. Rectal temperatures were measured each day before the clinical scoring and collection of blood samples.

Necropsy. Sheep were humanely euthanased at 20 or 21 dpi using intravenous pentobarbitone sodium (Lethobarb: Virbac, Milperra, NSW, Australia). Macroscopic lesions observed at postmortem examination were recorded.

Results

Serological evidence of BTV-16 in NSW during 2016

NAMP surveillance. Routine NAMP surveillance during the 2015–2016 monitoring season had identified BTV transmission on the NSW north coast from late January 2016, with only BTV-1 detected. There was no evidence of BTV-2 and BTV-21 using type-specific qRT-PCR. Subsequent to the serological detection of BTV-16 in the cattle being prepared for export, strategic retrospective testing was performed with the BTV-16-specific qRT-PCR on all NSW sentinel cattle samples where BTV seroconversions had been detected. Through this retrospective testing, BTV-16 was detected in sentinel cattle at Lismore (3/10 samples positive) and Casino (5/10 positive) in April 2016 and at Paterson (1/10 positive) in June 2016 (Figure 1).

During the NAMP 2016–2017 season, BTV-16 was detected in sentinel cattle at three sites in the Hunter Valley (Paterson, Singleton and Scone) of NSW (Figure 2) and was undetected elsewhere between the Hunter Valley and the southern-most point of detection in Qld at Allora. BTV-16 infection was detected concurrently with BTV-1

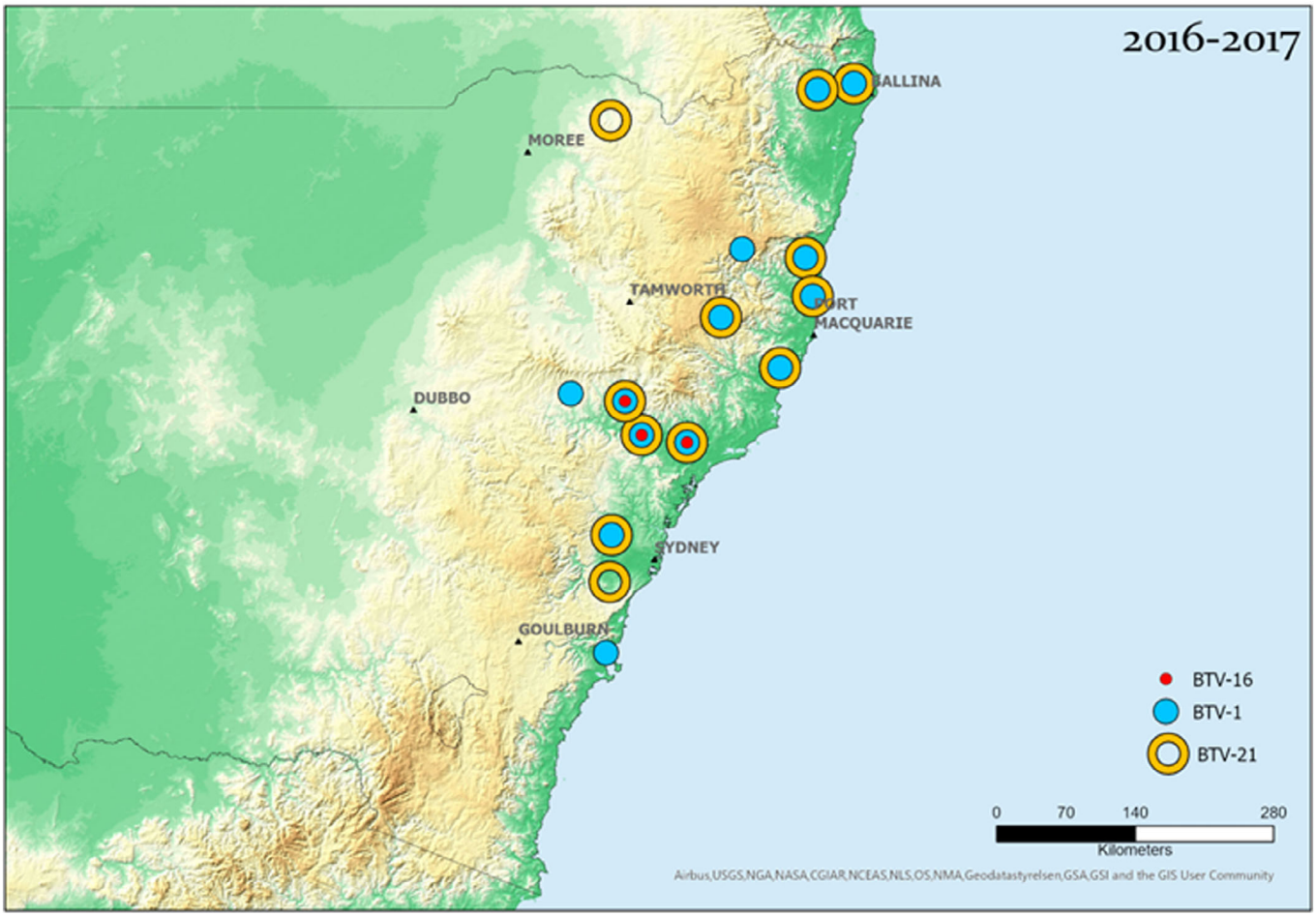

Figure 2. **Geographical distribution of BTV-1, BTV-16 and BTV-21 detections by serotype-specific qRT-PCR in NSW sentinel cattle during the 2016–2017 monitoring season.**

and BTV-21 at Paterson and Singleton from April 2017 and at Scone from May 2017.

While BTV viraemia was detected in 23 of 55 sheep at Paterson in 2017, BTV-16 was not detected in any of the sheep sampled. BTV-1 was detected in 9 sheep, BTV-21 in 11 sheep, BTV-1 and BTV-21 in 2 animals and the serotype was not determined in 1 animal, which had a Ct value close to the limit of sensitivity of the pan-reactive qRT-PCR assay. At Scone, two viraemic sheep were detected; one infected with BTV-1 and the other with BTV-21.

BTV-16 was not detected in NSW sentinel herds during the NAMP 2017–2018 monitoring season (Figure 3).

During the 2018–2019 monitoring season, BTV-16 was detected at 13 sites across NSW. BTV-16 was the sole serotype detected at 12 of these sites (Figure 4). BTV-16 transmission was first detected on the far north coast at Casino in January 2019 and as far south as Camden on the coastal plain by early June. BTV-16 transmission was also detected on the northern tablelands at Glen Innes and in the north-west slopes region at Warialda, Gravesend and Coolatai. BTV-1 was also detected in a single animal in the herd at Warialda in conjunction with BTV-16 and in a single animal at Narrabri in the absence of BTV-16.

Testing of 30 sheep at Paterson in late June 2019 detected six seropositive animals, with BTV-16 infection confirmed by type-specific qRT-PCR. No disease was observed in this flock.

BTV transmission in NSW during the 2019–2020 transmission season was extensive despite the severe drought affecting the state until January 2020 (Figure 5). In contrast to the previous year, BTV-1 was the predominant serotype detected, with BTV-16 only detected at Lismore and Paterson. In 2020, BTV-1 was first detected in Lismore in February, whereas BTV-16 was not detected until April 2020. Conversely, BTV-16 was detected at Paterson in March 2020, with BTV-1 first detected in the next month.

Finally, in 2021, there was BTV-16 transmission on the NSW North Coast followed, in 2022, by extensive transmission throughout the range of *C. brevitarsis* in NSW.

BTV-16 sheep pathogenicity study

Viral RNA loads in blood. All inoculated sheep became infected, with viraemia first detected by pan-reactive qRT-PCR between 3 and 5 dpi (mean time to first detection 3.75 dpi). Peak viraemia occurred between 6 and 10 dpi (mean 7.75 dpi) with a mean Ct value of

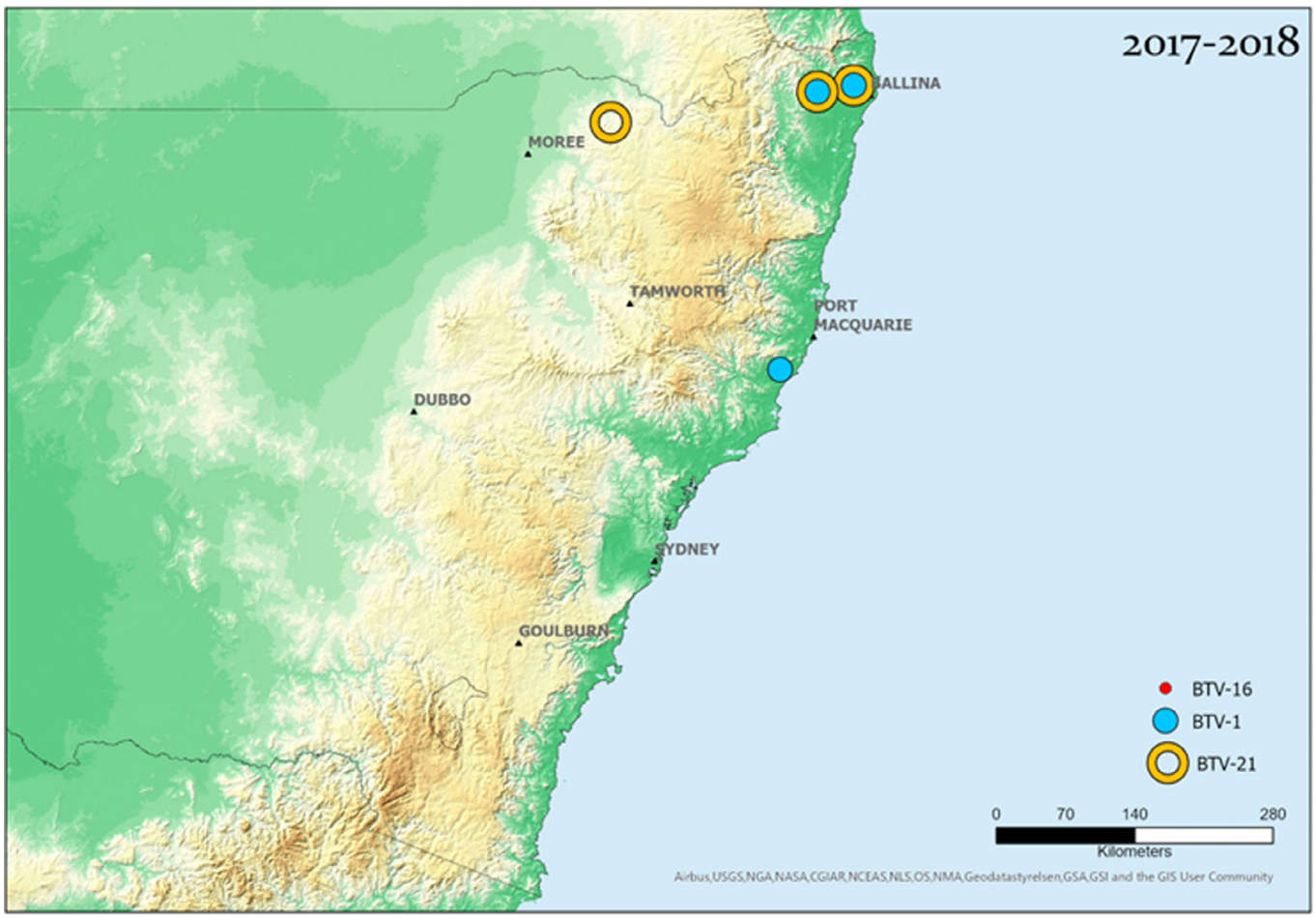

Figure 3. **Geographical distribution of BTV-1 and BTV-21 RT-qPCR positive NSW NAMP sentinel cattle herds during the 2017–2018 monitoring season.**

22.1 (range 19.02–24.68; Table 2). BTV RNA was detected in all challenged sheep until termination of the trial at 20 or 21 dpi. BTV RNA was not detected in the negative control sheep by qRT-PCR at any time.

BTV serology. Two animals seroconverted at 7 dpi and the remainder of the challenged sheep seroconverted between 9 and 14 dpi (Table 2). The mean time to seroconversion was 10.5 days. The negative control sheep remained seronegative.

Clinical signs. Infected animals initially developed clinical signs affecting the nose and mouth, with later involvement of the feet (see Appendix 1). Beginning at 6 dpi, 6/8 infected animals showed hyperaemia of the nasal mucosa, sometimes with nasal discharge (Figure 6A) and concurrent oral hyperaemia (Figure 6B). Nasal lesions observed in three animals progressed to a minimum score of 2 (hyperemic nasal mucosa, oedema of nostrils and crusting discharge around nostrils) at 8–10 dpi. Hyperaemia of the oral mucus membranes was observed in all animals between 6 and 19 dpi, with one animal also developing oedema of the lips at 9 dpi. Pyrexia (>40.0°C) was detected in 6/8 challenged animals between 8 and 10 dpi. From 10 dpi, all animals developed coronitis of variable severity (Figure 6C), which typically progressed after initially presenting with hyperaemia of the interdigital epithelium. From 14 dpi, 6/8 infected animals also developed focal or petechial haemorrhage of the coronary band, claw or bulb (Figure 6C,D).

Necropsy findings. Multifocal haemorrhages were observed within the wall of the pulmonary artery (Figure 7A) in five of the eight challenged sheep. Two sheep had multifocal myocardial petechiae. Five sheep had pericardial effusions (defined as >10 mL), with the largest recorded volume of 44 mL with a mean of 21.2 mL; three of these sheep had concurrent abdominal effusion. In addition to haemorrhages in the pulmonary artery or myocardium, four sheep also had haemorrhages within the pericardium (Figure 7B), epicardium (Figure 7C) and omental adipose tissue. There were no significant necropsy findings in the negative control animal.

Discussion

BTV surveillance

BTV-16 was first detected in blood samples from NSW NAMP sentinel cattle herds collected in 2016 after retrospective testing. Despite BTV-1 detection in these animals earlier in the transmission season,

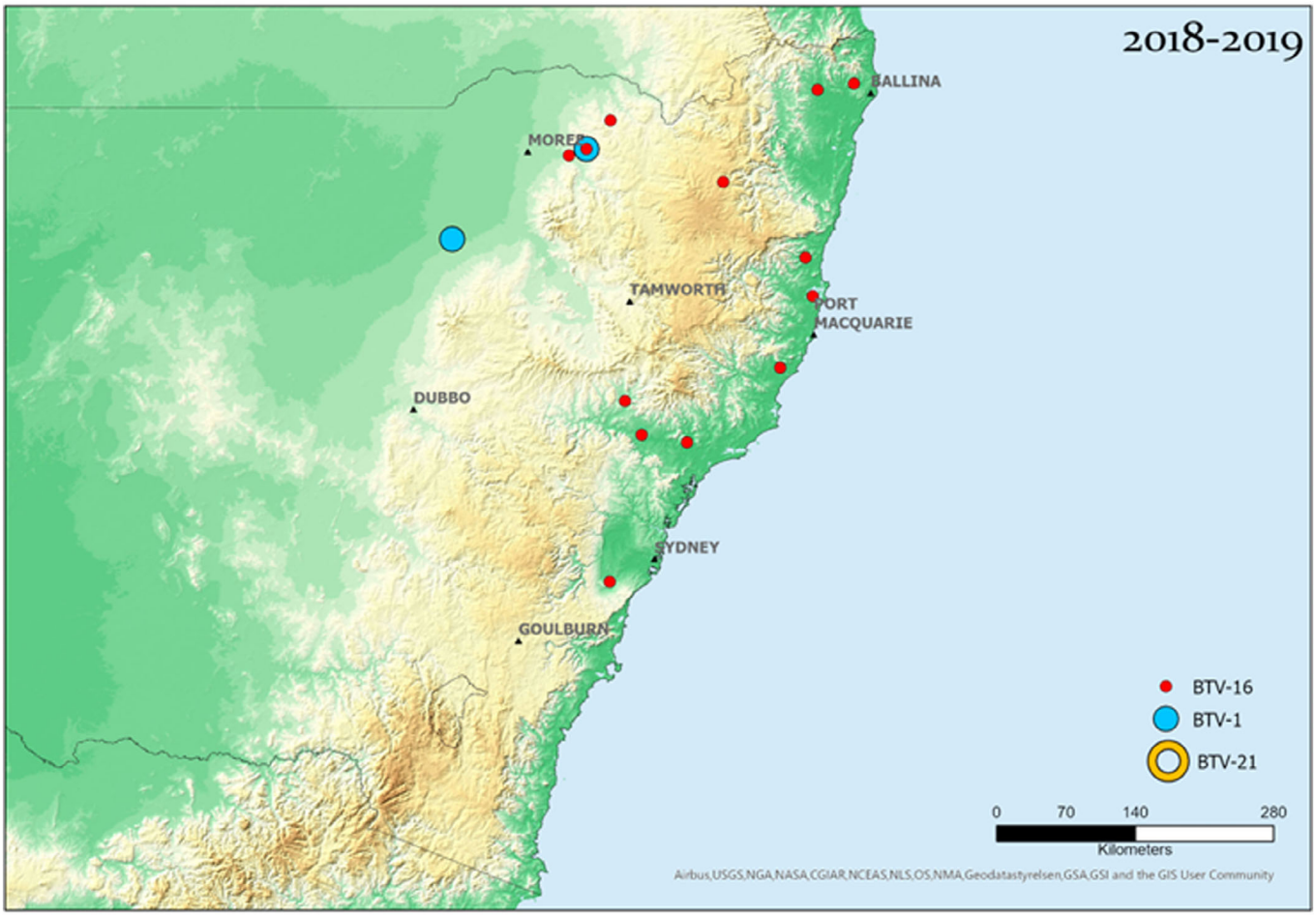

Figure 4. **Geographical distribution of BTV-1 and BTV-16 detections by serotype-specific qRT-PCR in NSW NAMP sentinel cattle during the 2018–2019 monitoring season.**

the detection of BTV-16 was a timely reminder that animals may be infected with multiple BTV serotypes even at the southern range of transmission in Australia. Until the 2012–2013 monitoring season, the determination of circulating BTV serotypes had been achieved serologically by virus neutralisation test (VNT) using serum samples collected at the end of the season. The introduction of the first of a suite of serotype-specific BTV qRT-PCRs during the 2012–2013 season provided 'real-time' identification of circulating BTV serotypes and the capability to undertake the rapid, highly sensitive testing as was applied retrospectively to confirm the first incursion of BTV-16 into NSW. Initially in NSW, serotype-specific qRT-PCR assays were only used to detect BTV-1, BTV-2 and BTV-21 but in the 2016–2017 season, the BTV serotype-specific qRT-PCR panel was expanded to detect BTV-1, BTV-2, BTV-16 and BTV-21, and from the 2017–2018 season, a panel of 3 serotype-specific multiplex qRT-PCR assays have been used to detect each of the 12 serotypes of BTV that had been previously detected in Australia.[33]

The origins of the BTV-16 strain that was first detected in NSW in 2016 remain obscure. Before 2016, the BTV serotypes selected for monitoring by NAMP in NSW were those known to circulate in this state and those detected in southern Qld, which in the 2015–2016 season were BTV-1, -2 and -21.[15,18] BTV-16 had been only infrequently detected in northern Qld before this, but, perhaps, in hindsight one notable result (but ultimately of uncertain significance) was the detection of antibodies to BTV-16 by VNT at Chinchilla (south-eastern Qld) in 2013.[27] Apart from this transient detection of possible BTV-16 infection in southern Qld, there was no observable geographical trend of movement of BTV-16 south towards the NSW border as the two subsequent BTV-16 detections both occurred in northern Qld, first at Innisfail in 2014 (B. Hill, personal communication) by VNT and then at Cooktown in May 2016 (B. Hill, personal communication) when BTV-16 was isolated. BTV-16 was next detected in south-eastern Qld in September 2016,[35] and during the remainder of the 2016–2017 season, BTV-16 infections became almost state-wide in Qld. However, as is now known, BTV-16 had already circulated in NSW during the previous 2015–2016 season. It is of interest to note that the distances between the Innisfail and Cooktown sites are approximately 1450 and 1700 km, respectively, to the north of the far north coast of NSW (Lismore and Casino), and the lower Hunter Valley (Paterson) is a further 460 km south. Perhaps the detections of BTV-16 at Cooktown in May 2016 and south-eastern Qld later in 2016 are indicative of low levels of transmission that were not detected in sentinel

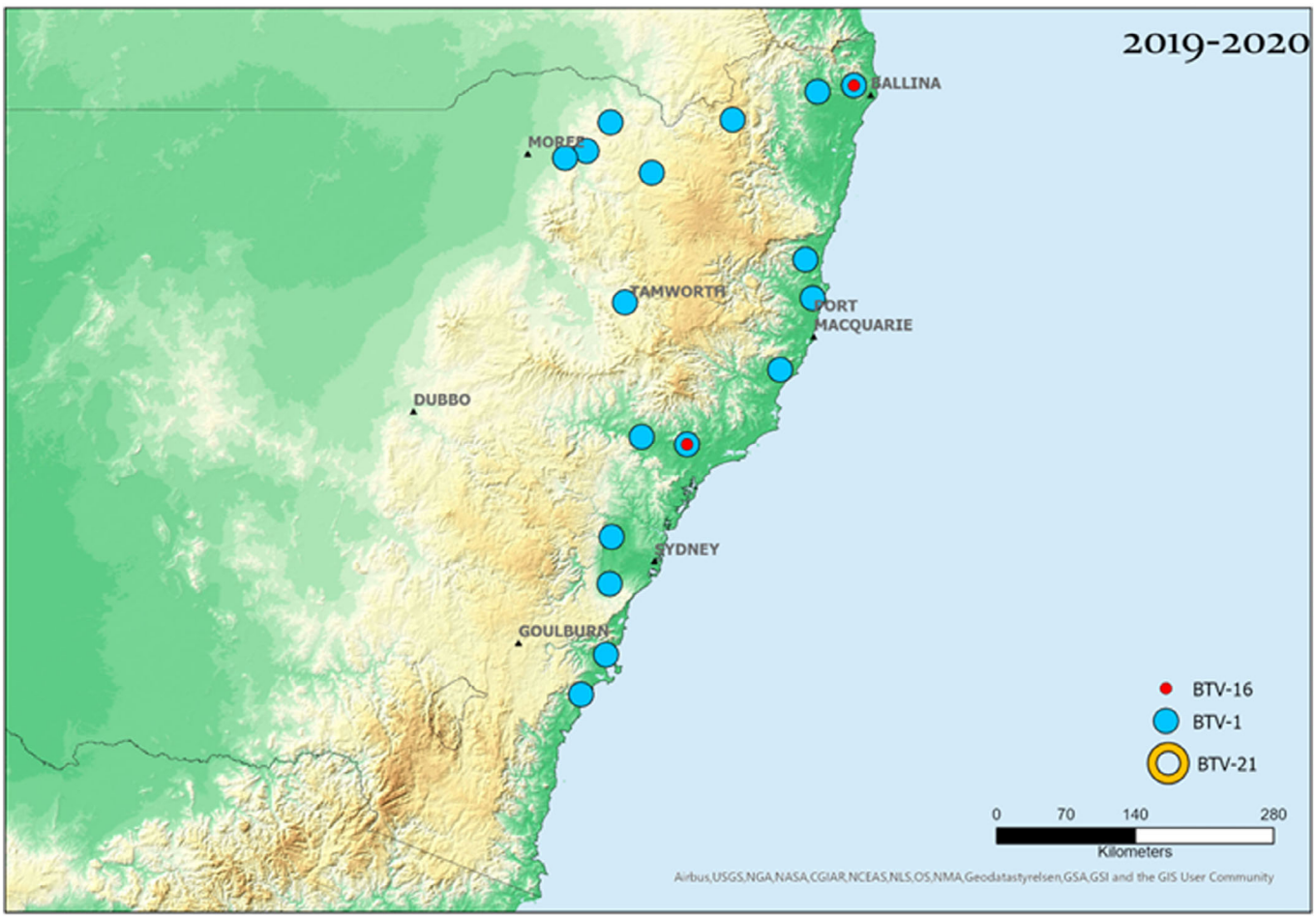

Figure 5. Geographical distribution of BTV-1 and BTV-16 detections by serotype-specific RT-qPCR in NSW NAMP sentinel cattle during the 2019–2020 monitoring season.

Table 2. BTV pan-reactive qRT-PCR Ct values for experimentally infected sheep for 21 days postinoculation

	Days postinoculation										
Animal	0	3	4	5	6	7	8	9	10	14	19/20
1	-	36.87	30.99	27.52	22.00	[b]**19.02**	20.18	20.21	[c]20.39	21.41	25.46
2	-	-	-	37.08	31.90	28.35	25.63	24.91	[b]**24.68**	[c]26.71	27.74
3	-	22.31	21.57	22.71	[b]**20.80**	[c]20.97	20.96	21.58	23.15	26.29	27.29
4	-	38.39	29.85	25.35	20.90	20.69	[b]**20.41**	[c]21.32	21.30	22.85	24.46
5	-	-	-	35.77	32.90	26.99	24.69	[b]**24.32**	24.59	[c]26.84	29.31
6	-	29.38	23.47	22.35	[b]**20.80**	[c]20.87	21.92	22.64	23.80	26.91	26.72
[a]7	-	-	-	-	-	-	-	-	-	-	-
8	-	32.35	28.39	25.34	23.20	[b]**21.62**	21.64	[c]21.64	21.79	23.38	24.76
9	-	-	-	35.69	31.00	25.46	[b]**24.86**	24.87	25.25	[c]27.55	29.04

A dash (–) indicates no BTV RNA was detected.
[a] Unchallenged control animal.
[b] Bold value is lowest Ct (reflecting highest virus load) for each animal.
[c] Indicates the day of BTV seroconversion for each infected animal.

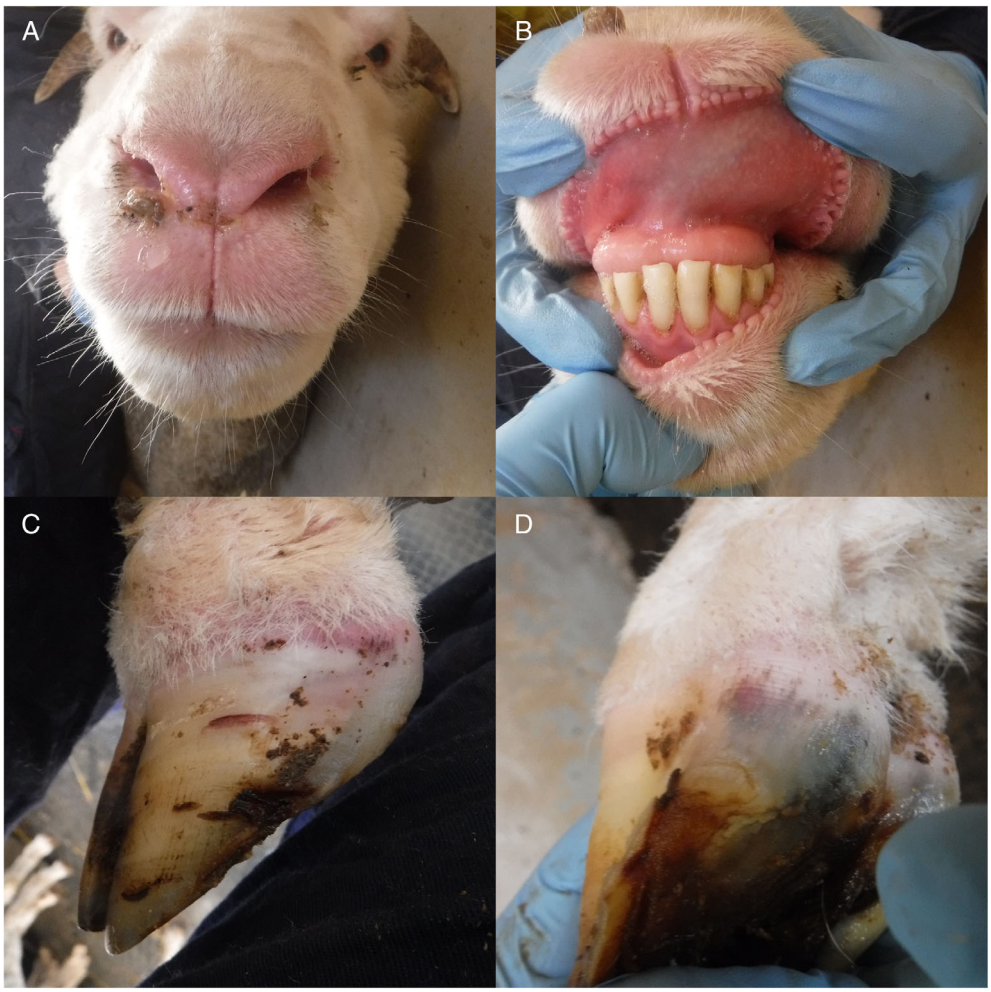

Figure 6. Clinical observations after BTV-16 experimental infection in adult merino sheep. (A) Nose and lips of Animal 4, 9 days postinoculation. There is oedema and hyperaemia of the nostrils and lips, with clear watery nasal discharge that forms crusts around the nostrils. (B) Same animal as shown in (A). There is hyperaemia in the oral mucus membranes. (C) Hoof of Animal 4, 17 days postinoculation. There is locally extensive hyperaemia of the coronary band and bulb and focal ecchymotic haemorrhages in the hoof wall. (D) Hoof of Animal 3, 17 days postinoculation showing haemorrhage of the heel bulb.

herds until 2016. If this was the case, BTV-16 may have been circulating in southern Qld from early 2016 but was, by chance, first detected on the far north coast of NSW due to more intensive routine monitoring of these herds. Both animal movements and long-distance dispersal of vectors may also have played a role in these events. Molecular epidemiology involving whole genome sequencing may help to understand this relationship further.

Notably, after the single BTV-16 detection at Paterson in the Hunter Valley in 2016, the only transmission of BTV-16 in 2017 was in the Hunter Valley despite widespread transmission of BTV-1 and BTV-21 in other NSW locations (Figure 2). While the reason for this localised transmission is unclear, we speculate that it may be due to virus overwintering[36] in conjunction with the highest ever recorded vector numbers in the region in the month before the first detection,[35] animal movements or long-distance dispersal of infected vectors.

After limited BTV transmission in NSW in 2017–2018, during the 2018–2019 season, BTV-16 for the first time became the predominant serotype in NSW, with a high rate of transmission throughout the range of *C. brevitarsis*, the main vector, despite widespread and enduring drought conditions state-wide and in neighbouring Qld.[37]

For the first time in 2019, BTV-16 was detected as far south as Camden on the coastal plain, on the northern tablelands near Glen Innes and extending west into the north-west slopes region, the latter two of which have significant commercial sheep production.

The detection of BTV-16 in six of seven successive transmission seasons clearly indicates that, along with BTV-1 and BTV-21, this serotype should now be considered endemic in NSW.

BTV-16 sheep pathogenicity study

The detection of BTV-16 in NSW and its transmission in regions where there are large sheep flocks raises a heightened awareness of the potential for bluetongue disease to occur in Australia. Although BTV virulence is not directly linked to serotype,[6] previous experimental transmission studies[38] with an Australian isolate of BTV-16, together with a disease outbreak in a small hobby flock that was moved into the endemic bluetongue zone in northern Australia, have demonstrated the capacity of this serotype to be a significant pathogen of sheep.[29] The present studies, although only involving a small number of animals, have also shown that a strain of BTV-16 detected in southern Australia has pathogenic potential and should not be ignored. Overall, the clinical findings of this study are similar

to the mild to moderate disease observed in sheep experimentally infected with various Australian serotypes of BTV.[39–42] In the current study, all experimentally BTV-16 infected sheep developed pyrexia of >40°C by 8–10 dpi and from 10 dpi developed coronitis towards the end of the febrile period, consistent with natural BTV infection in sheep.[4,43] Lesions in most animals with coronitis progressed to coronary haemorrhages from 14 dpi. These findings and their progression are comparable to previous experimental BTV-16 infections undertaken in Qld 30 years ago.[38] Involvement of the nose and mouth was common in the current study but was rare in the previous BTV-16 experimental infection study in which only one animal exhibited mild facial oedema and nasal exudate.[38] The mild to moderate clinical signs and lesions in both studies may have been due to the indoor conditions under which the sheep were housed. Collectively, the lesions observed in these sheep would most likely be exacerbated if these animals were outdoors in a grazing environment in summer or early autumn when natural BTV infection is most likely to occur in NSW. In addition to climatic conditions, other factors influencing disease severity include sheep breed, age, exposure to sunlight, walking on rough ground and stress.[29] The detrimental effects of bluetongue on the mobility and grazing capacity of free-range sheep are well-known[4,6,44,45] but, despite mouth and/or foot lesions, anorexia and lameness were not observed in the current experimental study and were often absent or occurred inconsistently in other Australian experimental transmissions where sheep have been housed indoors in a temperature-controlled environment.[39–41] A predominance of pericardial effusions as seen in the current study could, in a disease outbreak, contribute to reduced exercise tolerance and exacerbate overall reduced mobility seen with naturally occurring bluetongue disease. In experimental pathogenicity studies, the time at which sheep are euthanased can influence the range and severity of lesions observed. In pathogenicity studies of Australian BTV-1, BTV-15, BTV-21 and BTV-23, sheep were necropsied mostly between 12 and 24 days; however, if left until 20–29 days, haemorrhages were often milder or absent – suggesting resolution of these lesions.[41] Similarly, on necropsy at 21–22 dpi, another experimental infection study of Australian BTV-1, BTV-3, BTV-9 and BTV-16 noted evidence of previous haemorrhage appearing as black discolouration of the pulmonary arterial wall.[42] Subsequent experiments showed pulmonary artery haemorrhage, and other gross lesions were present at 7 and 10 dpi but absent at 14 dpi.[42] This may have implications for the current pathogenicity study findings; while there was significant cardiovascular disease, the haemorrhages observed may have been more severe or abundant if necropsies had been performed earlier in the disease time course.

Potential for BTV-16 to cause bluetongue in sheep in NSW

Outbreaks of bluetongue disease occur when either susceptible sheep are introduced into an area where virulent strains of BTV are endemic or when a virulent strain of BTV is transmitted by a vector when its range has expanded beyond its normal range under favourable environmental conditions.[46]

Given that strains of BTV-16 have been shown to have the potential to cause moderate clinical disease and a mortality rate of up to 30%,[29] even under experimental conditions, it is likely that disease

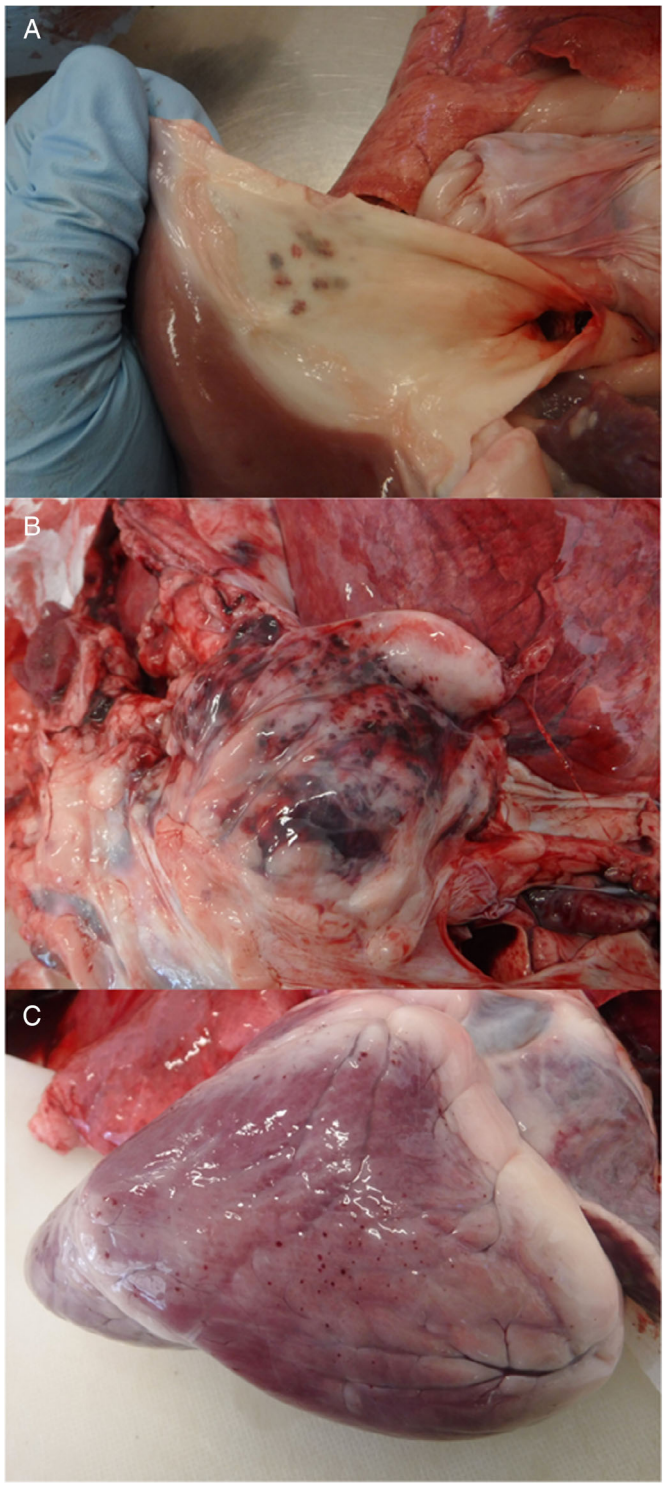

Figure 7. Gross pathology findings after BTV-16 experimental infection in adult merino sheep. (A) Endothelial surface of pulmonary artery of Animal 1, 20 days postinoculation. Within the arterial wall, there is multifocal haemorrhage. (B) Pericardium, Animal 5, 20 days postinoculation. Within the pericardium, there is multifocal to locally extensive haemorrhage. (C) Epicardium, Animal 5, 20 days postinoculation. The epicardium has multifocal petechial haemorrhage.

could be observed if there is extensive transmission of BTV-16 in major sheep-raising areas. The main vector of BTV in NSW is *C. brevitarsis*, which has a distribution that extends southwards from the Qld border through the coastal river regions and the Hunter Valley and south as far as Camden, with intermittent detections further south as far as Meringo (10 km south of Moruya). There is intermittent presence of this midge on the northern tablelands with extensions on the north-west slopes and plains west to Lightning Ridge and south-west approaching Coonamble. While few sheep are in the coastal regions, there are large sheep populations in the northern tablelands and north-west slopes regions.[47] Under this range of *C. brevitarsis*, approximately three million sheep could be at risk of developing bluetongue disease.[47] Currently, sheep on the northern tablelands and slopes are at greatest risk but with increasing climatic variability and warmer temperatures, it is likely that the proportion of the NSW flock at risk will increase significantly. While BTV is not usually spread as efficiently or widely as Akabane and the other related Simbu viruses that are endemic in NSW, the distribution of these orthobunyaviruses does provide a guide to the potential scale of the sheep population at risk.

In favourable seasons in the past (and particularly after a drought), Akabane virus and BTV (and hence competent midge populations) have been detected throughout the central west region and onto the far south coast and southern tablelands as far as the Victorian border.[48,49] It is well recognised that the distribution and abundance of *C. brevitarsis* are sensitive to changes in temperature.[50] Modelling has shown that a temperature rise of 2°C could result in this midge expanding its range to cover most of NSW and a large portion of Victoria.[51] With such a change in distribution, it is estimated that at least an additional six million sheep could become at risk.[47]

In conclusion, there appears to be an increased risk of bluetongue disease in south-eastern Australia, particularly near the current margins of the principal vector, *C. brevitarsis*. The NAMP monitors the distribution of BTVs and vectors in Australia and provides alerts to the changing distribution of BTV and vectors in NSW, providing an early warning system for the risk of a disease outbreak. At present, sentinel cattle herds and vector traps are located throughout and at the margins of the current distribution of BTV and insect vectors. However, there may in future be a need to consider adjustments to the location and frequency of BTV and vector sampling to optimise this 'early warning' function, especially in a period of climatic volatility. Further, the possibility that tropical midge species that are competent vectors of BTV could move south and become established should not be overlooked, given the extreme rainfall events that have occurred in 2022. Collectively, these scenarios have the potential to expose more sheep to BTV infection as well as introducing additional viruses and vectors to sheep-raising regions, each of which could lead to an outbreak of bluetongue disease.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site: http://onlinelibrary.wiley.com/doi/10.1111/avj.13288/suppinfo.

Appendix 1. Clinical severity scores for sheep experimentally infected with BTV-16. Values for the nose and mouth are shaded orange; feet scores are shaded blue for the eight challenged sheep (animals 1–6, 8,9) and one unchallenged negative control sheep (animal 7). Animals were scored daily for up to 20 days postinoculation. The numerical scores and colour shading densities increase with increased severity of clinical signs. Blanks indicate a clinical score of 0. Grey fill indicates that the observation was not taken due to previous euthanasia.

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