Characterisation and monitoring of neutralisation-resistant VP2 phenotypes in BTV-1 isolates from northern Australia collected over a twenty-year period

J.R. White[(1)], V. Boyd[(1)], J.K. Brangwyn[(1)], C.J. Duch[(1)], L.I. Pritchard[(1)], L.F. Melville[(2)], T.D. St George[(3)] & B.T. Eaton[(1)]

(1) Australian Animal Health Laboratory, CSIRO, Division of Livestock Industries, Private Mail Bag 24, Geelong, VA 3220, Australia
(2) Department of Primary Industry and Fisheries, Berrimah Veterinary Laboratory, Post Office Box 990, Darwin, NT 0801, Australia
(3) 16 Tamarix Street, Chapel Hill, QLD 4069, Australia

Summary

Phenotypic profiles of the VP2 protein of isolates of bluetongue virus serotype 1 (BTV-1) collected from Queensland and the Northern Territory, Australia, between 1979 and 1986 were analysed using neutralising monoclonal antibodies (MAbs) raised to the prototype isolate of Australian BTV-1 collected in the Northern Territory in 1979. Two distinct profiles were found. Northern Territory isolates exhibited the prototype profile, yet those from Queensland had a significantly different ('resistant') profile. Nucleotide sequencing of gene segment 2 from both groups of isolates was undertaken. When the nucleotide sequences of isolates from a later period in each State were analysed (1997-2001), all exhibited the 'resistant' profile. Thus, a novel VP2 phenotype, already in existence in Queensland, had supplanted a pre-existing VP2 phenotype in the Northern Territory between the two periods. Furthermore, amino acid differences between the resistant and prototype VP2 proteins were analogous to amino acid substitutions known to be associated with neutralisation resistance. The host immune response may therefore have contributed to selection of the 'resistant ' phenotype.

Keywords

Australia – Bluetongue – Monoclonal antibody – Neutralisation – Nucleotide – Phenotype – Profile – Resistance – Sequence – Selection – Viral protein 2.

Introduction

Earlier cross-neutralisation data (10, 14) and subsequent phylogenetic analysis of the gene segment 2 sequence of various bluetongue viruses (BTV) has provided a good understanding of the level of genetic relatedness between different serotypes of BTV and between isolates of the same serotype from well separated geographic regions (9, 15, 18, 19). To determine whether isolates of the same BTV serotype from within a restricted geographic region show significant phenotypic variation, neutralising monoclonal antibodies (MAbs) were used to demonstrate the co-existence of different VP2 antigenic profiles (1, 12, 13, 16). Phylogenetic analysis has further confirmed the co-existence of multiple VP2 phenotypes in geographically restricted field locations for both BTV (3, 4) and a related orbivirus (11).

We were interested to investigate the extent of phenotypic variation in the VP2 protein of field isolates of Australian BTV-1 from several isolated geographic locations in Northern Australia collected over a substantial time period. Of particular interest was the potential for changes in phenotypic expression over time and whether any associations could be made between those amino acid sites found to differ between isolates and sites known to be related to virus neutralisation (8, 17).

Materials and methods

Virus isolation and propagation

Field isolates of BTV-1 were initially propagated in chick embryos from infected bovine blood and then plaque-picked and passaged in cell culture (Vero and/or BHK-21 cells). In addition to the prototype isolate CSIRO156 (CS156) initially isolated from Beatrice Hill in the Northern Territory, the following isolates were used in this study: CS786, CS787, CS1564, CS1566, V1240 and V1241 (Queensland) and CS160, CS418, CS420, CS421, CS445, V930, V4045, V4234, V4907, V4942, V4959, V4961, V4962, V4968 and V4977 (Northern Territory). The attenuated vaccine strain of South African BTV-1 was obtained from CSL, Melbourne, Australia. Attenuation was achieved by prior extensive passage in chick embryos at the Onderstepoort Veterinary Research Laboratory in South Africa.

Monoclonal antibodies

Neutralising murine MAbs E3/F4, D8/A1, J3/G6, A2/D2, E6/A4, E10/A11 and D8/A12 were raised to a purified, infected cell lysate of CS156 as previously described (7, 17).

Serum neutralisation test

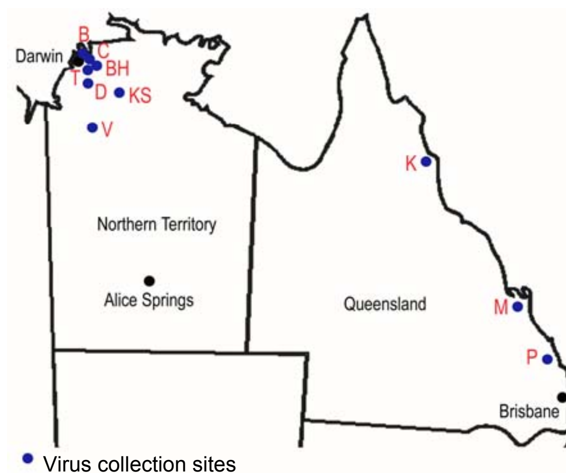
Approximately 200 $TCID_{50}$ units of virus in 25 µl of Eagle's minimum essential medium (EMEM) were mixed in triplicate within the wells of a 96-well microtitre tray with MAb appropriately diluted in EMEM. The plate was incubated for 1 h at 37°C, then 200 µl of EMEM maintenance medium (with 2% [v/v] foetal calf serum) containing approximately 10^5 Vero cells was added to each well and the plate incubated for 4 days at 37°C under 4.5% CO_2. Following incubation, neutralisation titres were estimated by determining the highest dilution of MAb that prevented cytopathic effect (CPE) in at least 50% of the monolayer. Neutralisation titres were determined by the addition of 0.75 mg/ml of 3-(4,5, dimethylthiazol-2-*yl*)-2,5-diphenyltetrazolium bromide (MTT) to the growth medium followed by dissolving the resultant dye in 100 µl of *iso*-propanol/well and determination of the absorbance at 560 nm.

Nucleotide sequencing

Viral RNA was isolated from clarified, virus-infected cell culture supernatant using the QiAamp viral RNA mini kit (Qiagen, Australia). Following denaturation of dsRNA with deionised formamide, reverse transcriptase (RT) and polymerase chain reaction (PCR) amplification was achieved using a Qiagen one step RT-PCR kit incorporating the appropriate primer sets. Eighteen to 22-mer primer sets were designed to amplify five separate 600-800 nucleotide regions along the gene 2 segment. Amplified DNA was purified using Qiaquick (Qiagen) then transferred to a sequencing reaction using the Abi Prism BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster, California). Product sequence was read on an automated DNA sequencer (Abi Prism model 377). Sequence homologies were determined using the Lasergene software package (Dnastar Inc., Madison, Wisconsin).

Results

Six isolates of BTV-1 from four collection sites in the Northern Territory (Beatrice Hill, Tortilla Flat Coastal Plains and Berrimah) and four isolates from two well separated sites in Queensland (Kairi and Peachester) (Fig. 1) were tested for their susceptibility to neutralisation by a panel of seven VP2-specific MAbs raised to the prototype isolate of BTV-1 Australia (CS156) (Table I). All five of the Northern Territory isolates collected from 1979 to 1980 from Beatrice Hill, Tortilla Flats and Coastal Plains (CS160, CS418, CS420, CS421, CS445) essentially displayed the same susceptibility to neutralisation as the prototype virus. The single Northern Territory isolate from Berrimah in 1986 (V930) showed resistance to neutralisation by two of the MAb panel. However all four of the isolates from Queensland (CS786, CS787, CS1564, CS1566) collected from 1981 to 1983, showed substantial



B Berrimah
C Coastal Plains Research Station
BH Beatrice Hill
D Douglas Daly Research Farm
T Tortilla Flats
KS Katherine Research Station
V Victoria River Research Station
K Kairi
M Maryborough
P Peachester

Figure 1
Location of virus collection sites in the Northern Territory and Queensland, Australia

resistance to up to five MAbs, indicating that significant phenotypic variation existed in the VP2 protein of BTV-1 populations in each State. To assess the extent and nature of this variation, gene segment 2 of all ten isolates was completely sequenced and the degree of homology between the isolates determined. All six Northern Territory isolates were essentially identical to the prototype virus (>99.7% protein sequence homology), whereas the four Queensland isolates all exhibited a second phenotypic profile which varied from 2.6% to 2.9% from the prototype virus, depending on the particular isolates compared. It was presumed the individual amino acid differences between the two phenotypes may, at least in part, relate to the ability of the relevant isolates to resist neutralisation by particular MAbs.

When gene segment 2 of nine Northern Territory isolates (from Berrimah, Katherine, Douglas Daly and Victoria River) and two Queensland isolates (from Maryborough) (Fig. 1) collected between 1997 and 2001 was fully sequenced, all isolates were found to have essentially the same genotypic profile as the neutralisation resistant phenotype present in Queensland in the earlier collection period (between 99.3% and 99.9% protein sequence homology). These results indicated a unique VP2 phenotype, present in Queensland, had been introduced into the Northern Territory at some time prior to 1997 and became the dominant form due to some selective advantage.

To investigate whether the immune response of host animals represented a potential for selective pressure on the VP2 phenotypes, we examined the individual amino acid differences between the prototype and resistant forms, isolated in each of the two collection periods (Table II). In all, 25 amino acid differences existed between the prototype phenotype and all isolates displaying the resistant phenotype from the first collection period. Fourteen of these involved significant amino acid changes. The substitution at aa219 (Ser-Gly) was only seen in isolate CS787 and the substitution at aa586 (Gly-Glu) only occurred in isolates CS786 and CS1566. In addition to the 14 shared aa substitutions, three further significant amino acid differences existed between the resistant phenotype from the earlier collection period and that from the later period. Of the total of 19 significant changes identified, 10 sites were identical to amino acid differences existing between the prototype sequence and an attenuated vaccine strain of an isolate of South African BTV-1 (Table II). However, despite these similarities, the overall amino acid homology between the resistant phenotype and the vaccine strain was the same as that previously observed between the later sequence and the prototype strain (CS156) (81%) (8). Eight sites showed analogous changes to those amino acid substitutions induced in variant viruses by selection with individual neutralising MAbs. Five of these sites were included in the 10 sites of significant difference present in the vaccine strain. The vaccine strain strongly resisted neutralisation by five MAbs of the total panel (8, 17) (Table I). All variant viruses and the vaccine strain had previously been shown to be resistant to neutralisation by a bovine reference antiserum to CS156, indicating the relevance of these amino acid sites to the immune response of a common host animal for BTV in Australia (17).

Table I
The susceptibility of the prototype isolate, other field isolates and the South African vaccine strain of bluetongue virus serotype 1 to neutralisation by a panel of monoclonal antibodies

BTV-1 isolate	Neutralising monoclonal antibody (reciprocal titre)						
	E3/F4	D8/A1	J3/G6	A2/D2	E6/G4	E10/A11	D8/A12
CS156	1024	512	256	256	2048	128	512
CS160	4096	256	256	128	2048	512	4096
CS418	1024	512	256	128	2048	256	512
CS420	512	512	256	512	256	128	1024
CS421	4096	1024	512	128	2048	256	4096
CS445	1024	256	256	256	2048	512	4096
V930	2048	512	256	256	2048	8	–
CS786	1024	256	64	8	–	–	–
CS787	2048	32	–	32	–	–	–
CS1564	1024	256	64	–	–	–	–
Vaccine strain	8192	–	–	256	–	32	–

Table II
Significant amino acid differences observed between the prototype VP2 sequence of bluetongue virus serotype 1 (BTV-1) (Aust.) and the sequences of the resistant phenotype, neutralisation-escape variants and the vaccine strain of BTV-1 (South Africa)

Amino acid substitutions		
Resistant phenotype	**Neutralisation-escape variants**	**Vaccine strain**
55 (Gly-Asn)		55 (Gly-Ser)
125 (Gln-Arg)		
209 (Arg-Gln)[c]	211 (Gly-Asp)[a]	
219 (Ser-Gly)[d]	218 (Phe-Cys)[b] 219 (Ser-Gly)[a]	219 (Ser-Gly)
267 (Arg-His)	267 (Arg-Cys)[a] [b]	267 (Arg-Gln)
270 (Tyr-His)	270 (Tyr-His)[b]	270 (Tyr-His)
303 (Asn-Asp)[e]	308 (Phe-Val)[b]	
384 (Leu-Ser)		384 (Leu-Ser)
395 (Isoleu-Thr)		395 (Isoleu-Thr)
435 (Isoleu-Thr)		435 (Isoleu-Thr)
470 (Asn-Ser)		
490 (Ser-Pro)[e]	491 (Gln-Leu)[a]	490 (Ser-Thr)
512 (Ala-Val)		
539 (Gln-Glu)	539 (Gln-His)[a]	
586 (Gly-Glu)[f]	586 (Gly-Val)[b]	586 (Gly-Ala)
593 (Glu-Lys)		
750 (Asn-Asp)		750 (Asn-Asp)
760 (Ser-Leu)[e]		
892 (Leu-Phe)		

a) Gould and Eaton (8)
b) Unpublished data
c) Arg-Pro in CS786
d) Not seen in CS786, CS1564 and CS1566
e) Only in isolates collected after 1997
f) Only in CS786 and CS1566

The Northern Territory isolate (V930) from the first collection period (isolated in 1986) which exhibited some resistance to MAb neutralisation possessed three significant amino acid changes compared to the prototype virus, i.e. aa158 (Ser-Pro), aa314 (Asn-Asp) and aa628 (Arg-Cys).

Discussion

It is likely that phenotypic variants of individual BTV-specified proteins are constantly generated in the field (2, 5, 6) such that at any given time and possibly despite the selection pressure of a host immune system (6, 13), a particular viral protein within a BTV population actually exists as a heterologous collection, comprising major stable protein phenotypes along with a series of minor phenotypic variants (2, 3). The unique characteristics of the VP2 sequence of the Northern Territory isolate V930, analysed in this study, might represent a manifestation of this phenomenon. The selection pressures arising from vector/host interactions and the immune response of host animals are likely factors in specific phenotypes being maintained or 'fitter' phenotypes becoming established (2, 6).

In this study, by producing MAb-defined antigenic profiles and sequence data from BTV-1 isolates collected over a significant time period, we have demonstrated that at some point within the last two decades at least two distinct and stable phenotypic forms of the VP2 protein co-existed in Northern Australia. The maintenance of separate phenotypes in relatively close, yet distinct geographic regions has been observed for a related orbivirus (11). At some point in the approximately twenty-year period between virus isolations, a VP2 phenotype already in existence in Queensland, was introduced into the Northern Territory and became dominant at all collection sites. A temporal involvement in the establishment of new and dominant phenotypic forms of VP2 has been documented by others (4). We have also shown that a strong association exists between the significant amino acid changes present in the dominant neutralisation resistant phenotype (compared to the prototype VP2 sequence) and changes known to be associated with experimentally generated neutralisation-escape variants and from a highly resistant isolate of BTV-1 from a geographic region remote from Australia (Table II). The data generated in this study thus suggest the incursion into the Northern Territory and eventual dominance of the 'resistant' phenotype may be due to the combined effects of time and selection pressure imposed by the immune response of host animals.

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