Prevalence of varicella-zoster virus genotypes in Australia characterized by high-resolution melt analysis and ORF22 gene analyses

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DNA sequence variation analysis has divided varicella-zoster virus (VZV; *Human herpesvirus 3*) into distinct geographical clades: European, Asian, African and Japanese. These genotypes are becoming increasingly prevalent within regions atypical to their original source and there has been the suggestion of recombination between genotypes. Seventy-eight clinical isolates from hospitalized patients with varicella were collected in New South Wales, the Northern Territory, Western Australia and Victoria from 2006 to 2009. The wild-type strains and the vaccine strain (vOka) were differentiated by single nucleotide polymorphism detection using high-resolution melt analysis of five target genes (ORF1, -21, -37, -60 and -62), and by DNA sequence analysis of a 484 bp region of ORF22. Phylogenetic analysis showed that 46 % (36/78) of the clinical isolates were European clade 1 (C/E1) strains, 21 % (16/78) were European clade 3 (B/E2) strains, 12 % (9/78) were Asian/African clade 5 (A/M1) strains, 10 % (8/78) were clade 4 (J2/M2), 6 % (5/78) were clade 2 (J/J) and 5 % (4/78) belonged to the novel clade VI. No significant association was shown between VZV genotype and region, age or gender. Although European strains were most common, the results suggest an increase in African/Asian, Japanese and clade VI genotypes circulating in Australia.

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INTRODUCTION

Chickenpox (varicella) is a childhood disease caused by the dsDNA varicella-zoster virus (VZV; *Human herpesvirus 3*). After primary infection, the virus becomes latent in sensory ganglia and has the potential to reactivate to produce herpes zoster (shingles), especially in older adults and immunocompromised people (Hope-Simpson, 1965). Global vaccination has reduced the disease burden (Vazquez *et al.*, 2001). In Australia, a varicella vaccine was licensed for use in 1999, but the vaccination programme was only publicly funded in 2005, so the impact of varicella vaccination on disease incidence has not yet been fully established because of limited data. However, surveillance studies have indicated that mass vaccination is associated with an increasing mean age of infection and absolute increases in varicella incidence among older groups (Cohen & Lipsitch, 2008), especially among unvaccinated adults (Brisson *et al.*, 2000; Edmunds & Brisson, 2002). This could be attributed to a decrease in exogenous boosting through lowered exposure to children with varicella and a waning cell-mediated immunity to VZV. Reports show that recurrent herpes zoster can be caused by genetically distinct genotypes (Taha *et al.*, 2006).

This potentially presents a higher burden of disease. It is uncertain whether specific VZV genotypes are more virulent and possess an increased potential to cause severe complications. A single amino acid change in VZV isolated from patients with primary varicella has been shown to enhance the ability of the virus to spread both *in vitro* and in human skin implants in SCID-hu mice (Grose *et al.*, 2004).

Genotypic data show that VZV is specific to geographical location (Barrett-Muir *et al.*, 2002, 2003; Quinlivan *et al.*, 2002) and can be classified into European, African, Asian and Japanese genotypes. More recently, an increasing number of mixed strains have been identified, suggesting genetic recombination (Loparev *et al.*, 2004; Norberg *et al.*, 2006; Peters *et al.*, 2006). The nomenclature of VZV genotypes has been based on different molecular typing methods, and only recently has a new universal nomenclature been introduced, separating the genotypes into five major clades (1–5) with two novel clades, VI and VII, still to be confirmed (Breuer *et al.*, 2010; Sengupta *et al.*, 2009).

Data on Australian VZV genotypes in Australia are limited (LaRussa *et al.*, 1998; Loparev *et al.*, 2007). Moreover, the information is limited on which circulating genotypes are associated with severe complications. Using two popular genotyping strategies, the scattered single-nucleotide polymorphism (SNP) method (Parker *et al.*, 2006) and

Abbreviations: HRMA, high-resolution melt analysis; SNP, single nucleotide polymorphism.

sequence analysis of ORF22 (Loparev *et al.*, 2004, 2007) targeting four nucleic acid mutations, this investigation reports on VZV genotypes circulating within Australia from 2006 to 2009 and the possible impact of the newly introduced varicella vaccine on VZV lineages. For simplicity, this study classified VZV genotypes using the agreed universal nomenclature, with reference to the classification by Barrett-Muir *et al.* (2003) and Loparev *et al.* (2004).

METHODS

VZV clinical samples. A total of 78 clinical isolates were collected from 2006 to 2009 from individuals presenting with varicella complications requiring hospitalization. Varicella infection was diagnosed clinically by the onset of a vesicular skin rash. Patients were from the Royal Darwin Hospital in the Northern Territory ($n=31$), the Princess Margaret Hospital ($n=28$) in Western Australia, the Royal Children's Hospital, Victoria ($n=2$), and Westmead Hospital ($n=15$), Concord Hospital ($n=1$) and Nepean Hospital ($n=1$) in New South Wales. Forty-eight patients were male and 30 were female with ages ranging from 4 months to 83 years.

DNA was extracted from vesicular swabs, blood or cerebrospinal fluid using a PureLink Genomic DNA Mini kit (Invitrogen). The DNA concentration was measured at A_{260}/A_{280} using UV spectrophotometry (Beckman DU Series 500) and the samples were diluted to a final concentration of $8 \text{ ng } \mu\text{l}^{-1}$.

Laboratory controls comprising two preparations of the Oka/Merck (Varivax, CSL Biotherapies; Varilrix, GlaxoSmithKline) strains of the live, attenuated VZV (vOka) were propagated in MRC-5 cells and harvested at 8 days post-infection. A low-passage VZV clinical isolate, designated strain Schenke (VZV-S; Centre for Virus Research, Westmead Millennium Institute, NSW, Australia), was included as a wild-type laboratory reference.

Multi-SNP analysis. VZV strains were genotyped by real-time PCR amplification, followed by high-resolution melt analysis (HRMA) of SNPs using a method described previously (Toi & Dwyer, 2008). Five ORFs were selected: 1 (SNP 685), 21 (SNP 33725), 37 (SNP 66288), 60 (SNP 101464) and 62 (SNP 106262). The gene targets, ranging between 65 and 103 bp in size, were amplified in separate reactions using published primers (Parker *et al.*, 2006).

Briefly, a Rotor-Gene 6000 (Qiagen) was used for real-time PCR amplification and HRMA of the five ORFs. The PCR was performed in a total volume of $20 \text{ } \mu\text{l}$ containing 40 ng DNA, 200 nM dNTPs, 300 nM each primer, $2 \text{ } \mu\text{l}$ $10 \times$ PCR buffer (Sigma), 2.5 mM MgCl_2 , $1 \text{ } \mu\text{l}$ $20 \times$ EvaGreen (Biotium), 1 U JumpStart *Taq* DNA polymerase (Sigma) and RNase-/DNase-free water. The thermal cycling reactions consisted of initial denaturation for 1 min at 94°C , followed by 40 cycles of denaturation at 94°C for 10 s, annealing at 61°C for 20 s and extension at 72°C for 20 s.

Amplification was followed immediately by HRMA, commencing with a pre-hold cycle (30 s at 50°C). Ramping conditions for the five gene targets were as follows: ORF 62 ($83\text{--}93^\circ\text{C}$), ORF 60 ($80\text{--}90^\circ\text{C}$) and ORF 37 ($72\text{--}82^\circ\text{C}$), and ORF 21 and 1 ($73\text{--}83^\circ\text{C}$) with fluorescence data acquisition set at 0.1°C increments. All amplicons were electrophoresed on a 2.5% agarose gel as a check for non-specific products. Inclusion of the wild-type strain VZV-S and vaccine strains Varivax and Varilrix provided a reference point in melting-curve shifts for distinguishing between wild-type strains, and between wild-type and vaccine strains. VZV-S is genetically similar to reference strain Dumas in ORF1, -21, -37, -60 and -62.

DNA sequence analysis of ORF22. The method of Loparev *et al.* (2004) was used to genotype ORF22, with some modifications. Forward primer $5'\text{-AACAAACGACTCGGGTTTGTG-3'}$ and reverse primer $5'\text{-AGCCAATGCGGGTATTGTGA-3'}$ spanning the region nt 37825–38309 of the gene target were designed with Primer-BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). This 484 bp fragment contains five SNPs capable of resolving five of the recognized VZV genotypes. The PCR was performed on a Veriti thermal cycler (Applied Biosystems) in a total volume of $25 \text{ } \mu\text{l}$ containing $100\text{--}300 \text{ ng}$ DNA, 200 nM dNTPs, 200 nM each primer, $2 \text{ } \mu\text{l}$ $10 \times$ PCR buffer (Sigma), 2.5 mM MgCl_2 , 1 U JumpStart *Taq* DNA polymerase (Sigma) and RNase-/DNase-free water. The thermal cycling reactions consisted of initial denaturation for 1 min at 94°C , followed by 10 cycles of 94°C for 20 s, 57°C for 20 s and 72°C for 30 s, and 30 cycles of 94°C for 20 s, 57°C for 20 s and 72°C for 30 s with an added 5 s per cycle. A final extension was carried out at 72°C for 5 min.

The five nucleotide mismatches (SNP nt positions 37902, 38036, 38055, 38081, 38097) were identified by DNA sequence analysis. PCR amplicons underwent enzymic pre-sequencing treatment with 1–2 μl PCR product, $1 \text{ } \mu\text{l}$ each exonuclease I ($10 \text{ U } \mu\text{l}^{-1}$) and shrimp alkaline phosphatase ($2 \text{ U } \mu\text{l}^{-1}$; USB Corporation) and $33 \text{ } \mu\text{l}$ sterile distilled water. The mixture was treated for 15 min at 37°C , inactivated for 15 min at 80°C and held at 4°C in a thermal cycler. A total volume of $12 \text{ } \mu\text{l}$ containing $11 \text{ } \mu\text{l}$ of the clean PCR product and $1 \text{ } \mu\text{l}$ of either forward or reverse primer (3.3 pM) were sequenced on a 3730 DNA Sequencer (Applied Biosystems) using ABI BigDye Terminator chemistry version 3.1.

Bioinformatics. The test samples were compared against prototypes in GenBank: strain Dumas (GenBank accession no. X04370), vaccine strains (vOka) Varivax (DQ008355) and Varilrix (DQ008354), CA123 (DQ457052) and VZV subtypes pOka (AB097933), HJO (AJ871403) and M2DR (DQ452050). DNA sequences were checked in the BioEdit version 7.0.4.1 (Hall, 1999) sequencing editing program. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura *et al.*, 2007). The non-protein coding sequence of SNPs at positions 685, 33725, 66288, 101464, 106262, 37902, 38036, 38055, 38081 and 38097 of each test sample was compared. The number of differences model was used to assess the support for the major clades and phylogenetic trees constructed using the neighbour-joining method (Saitou & Nei, 1987) with bootstrap resampling (1000 repeats) (Efron *et al.*, 1996; Felsenstein, 1985). The association of VZV specific genotypes with age, gender and region was measured by a two-way analysis of variance. Results are reported using the new VZV universal nomenclature shown in Table 1.

RESULTS AND DISCUSSION

A combination of SNP analysis using an HRMA of amplicons from ORF1, -21, -37, -60 and -62 and sequence analysis of ORF22 encompassing five SNPs was important for sufficient discrimination between VZV genotypes in this study. The limitation of applying each method alone was that analysis of ORF22 SNPs only does not differentiate European clade 1 from clade 3 strains, instead showing a uniform European genotype (Loparev *et al.*, 2004; Sergeev *et al.*, 2006). Equally, the HRMA of ORF1, -21, -37, -60 and -62 did not subdivide clade 2 and clade 4 Japanese strains into their distinctive subtypes. SNP 106262 (ORF62) discriminates only between VZV wild-type strains and the vaccine strain.

Table 1. Universal VZV nomenclature (Breuer *et al.*, 2010) and strains used in the phylogenetic analysis

New universal nomenclature clade	Reference strain	Scattered SNP*	ORF22 sequence analysis†	Geographical distribution
1	Dumas	C	E1	Europe
2	pOka	J	J	Far East
3	HJO	B	E2	Europe
4	M2DR	J2	M2	Central and South America, Asia
5	CA123	A	M1	Asia, Africa
VI‡		–	M4	
VII‡		–	M3	

*Barrett-Muir *et al.* (2002, 2003).

†Loparev *et al.* (2004, 2007).

‡Clades yet to be confirmed.

Phylogenetics of VZV genotypes

A phylogram presenting the evolutionary relationship of the VZV genotypes is shown in Fig. 1. Based on the theory that the evolutionary origin of VZV is by co-evolution with humankind (Grose, 2006), clades representative of different global regions were evident, with some of our isolates matching the profiles of more than one global region.

Comparison of test isolates with the selected prototype reference strains showed that European clade 1 and Japanese clade 2 isolates were 100 % similar to the reference strains Dumas and pOka, respectively. As expected, the vaccine strain (vOka) was most closely related to the Japanese clade 2 cluster, sharing a common ancestor (pOka) from which the live-attenuated VZV vaccine preparation is derived.

Eleven of the test isolates in clade 3 (European) were most similar to HJO, with the exception of four samples (NSW2, NSWra, WA18 and WA21), which shared both clade 1 (SNP 33725) and African/Asian clade 5 (SNP 101464) markers. Only one sample (WA9) grouped in the African/Asian clade 5 had similarity to both clade 3 (SNP 66288) and clade 5. Clade 4 samples ($n=8$) appeared to be the most genetically varied, with some sharing African, Asian and Japanese markers. For instance, NT6 and NT25 had similar SNPs to clade 2/clade 5 (SNP 101464) and clade 1/clade 2 markers. Genetic variation strongly suggests recombination events with a common ancestry to shared genotypes. Four clinical isolates only were grouped into the novel clade VI, and appear to be genetically closer to European and Asian/African genotypes in the phylogram. A small number of clade VI strains associated with African immigrants has also been reported in Europe (Loparev *et al.*, 2007).

Prevalence of VZV genotypes

The prevalence and distribution of VZV genotypes by region are shown in Table 2. The European genotypes

(clades 1 and 3) predominated, with the majority of isolates distributed in Western Australia ($n=17$) and the Northern Territory ($n=24$). Twelve per cent (9/78) of the test isolates were African/Asian strains (clade 5) located mainly in Western Australia. Eight of the test isolates were grouped into clade 4, which is common to Central America, South America and Asia, and were for the most part isolated from patients in the Northern Territories (5/8). Five Japanese strains (clade 2) similar to pOka were found in New South Wales, Western Australia and the Northern Territory.

The prevalence of European clade 1 (46 %) and clade 3 (21 %) strains in Australia was lower compared with the USA (80 %) (Loparev *et al.*, 2004), northern and southern Europe (65 %) (Loparev *et al.*, 2009) and Finland (Koskinen *et al.*, 2007). A higher diversity of VZV genotypes in New South Wales was evident when compared with Western Australia and the Northern Territories, with no single genotype presenting an advantage. This may possibly be attributed to a more transient population in New South Wales compared with other states and territories. Two-way analysis of variance showed a significant difference ($P=0.02$; $F=4.01$) between VZV genotypes alone, with no association between VZV genotype and region. Bonferroni post-tests indicated that clade 1 genotypes significantly predominated in the Northern Territories.

African/Asian strains (clade 5) were second in prevalence to European VZV strains, indicative of an increasing number of these genotypes. Loparev *et al.* (2007) reported a 7.87 % prevalence of clade 5 genotypes in eastern Australia from specimens collected during 2003 and 2004. This study showed a majority of this circulating strain in Western Australia (6/9) from this more recent collection of isolates. Geographically, clade 5 is widespread in Central Africa and Indochina (Loparev *et al.*, 2000; Quinlivan *et al.*, 2002) and its increasing numbers in Australia are possibly due to the migration of people from

Fig. 1. Phylogenetic tree (neighbour-joining method) of VZV genotypes and GenBank reference strains (●, DUMAS; ■, HJO; ▲, CA123; ◆, M2DR; ▼, pOka and ▽, vOka) based on single SNP differences in ORF1, -21, -37, -60 and -62 and multi-SNPs in a 484 bp region of ORF22. The percentage of 1000 bootstrap replicates and the regions from which specimens were obtained are indicated. NSW, New South Wales; NT, Northern Territory; WA, Western Australia; VIC, Victoria. Bar, 0.5 substitutions per site.

these tropical regions. The distribution and relatedness of strains in Australia demonstrates that the spread of VZV following major human migrations has made a strong contribution to its geographical epidemiology. In future, larger groups should be studied so that inferences can be made on whether the distribution of the different VZV clades is representative of the state and territory.

The age of hospitalized patients ranged from 4 months to 83 years with a median age of 13 years. The range and median age by genotype is shown in Table 2. Interestingly, all of the type VI strains were isolated from children below the age of 7 years. The identification of recombinant strains, especially in young children, suggests previous exposure to a mixture of genotypes. However, no significant relationship

was shown between VZV genotype and age of the individual. The epidemiology of VZV infection is different in temperate climates, where most unimmunized children have primary VZV infection in their first decade, compared with tropical regions, where the population may remain susceptible to VZV infection well into adulthood (Barrett-Muir *et al.*, 2003; Loparev *et al.*, 2004; Quinlivan *et al.*, 2002).

This study showed a high diversity of VZV genotypes circulating within Australia, with an emergence of recombinant strains. Loparev *et al.* (2007) previously showed the range of VZV strains in eastern Australia to be broader than in any other region, including Europe, Africa and North America. Moreover, Japanese strains (clade 2) were

Table 2. Prevalence and distribution of VZV genotypes by age and state/territory

NSW, New South Wales; WA, Western Australia; NT, Northern Territories; VIC, Victoria.

Clade	Age		n (%)				
	Range	Median	NSW	WA	NT	VIC	Total
1	1–83	10	4 (11)	13 (36)	17 (47)	2 (6)	36 (46)
2	1–71	8	2 (40)	2 (40)	1 (20)	0 (0)	5 (6)
3	0.4–63	5	5 (31)	4 (25)	7 (44)	0 (0)	16 (21)
4	1–73	17	2 (25)	1 (13)	5 (63)	0 (0)	8 (10)
5	2–76	12	2 (22)	6 (67)	1 (11)	0 (0)	9 (12)
VI	0.5–7	5	2 (50)	2 (50)	0 (0)	0 (0)	4 (5)
Total			17	28	31	2	78 (100)

identified, which are absent in Europe (Loparev *et al.*, 2009). The proximity of Australia to the Asia-Pacific regions may in part explain the isolation of Japanese wild-type VZV strains. In this regard, ongoing surveillance of VZV genotypes is important, especially for monitoring future varicella adverse events, as sequences sampled at different times can provide an estimated rate of evolution (Pybus, 2006). The genotypes most associated with hospitalized varicella patients remain unclear, and genotype virulence may be influenced more by the level of host response and immunity than by specific genotype. However, this study does provide preliminary evidence that no individual clades are more likely to be associated with disease requiring hospitalization, although there is a possibility that individual strains will have heightened pathogenicity associated with specific mutations.

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