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Distribution of varicella-zoster virus (VZV) wild-type genotypes in northern and southern Europe: Evidence for high conservation of circulating genotypes☆

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ABSTRACT

Phylogenetic analysis of 19 complete VZV genomic sequences resolves wild-type strains into 5 genotypes (E1, E2, J, M1, and M2). Complete sequences for M3 and M4 strains are unavailable, but targeted analyses of representative strains suggest they are stable, circulating VZV genotypes. Sequence analysis of VZV isolates identified both shared and specific markers for every genotype and validated a unified VZV genotyping strategy. Despite high genotype diversity no evidence for intra-genotypic recombination was observed. Five of seven VZV genotypes were reliably discriminated using only four single nucleotide polymorphisms (SNP) present in ORF22, and the E1 and E2 genotypes were resolved using SNP located in ORF21, ORF22 or ORF50. Sequence analysis of 342 clinical varicella and zoster specimens from 18 European countries identified the following distribution of VZV genotypes: E1, 221 (65%); E2, 87 (25%); M1, 20 (6%); M2, 3 (1%); M4, 11 (3%). No M3 or J strains were observed.

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Introduction

Varicella-zoster virus (VZV) is a highly contagious human pathogen that causes varicella (chickenpox) on primary infection, during which a lifelong latent infection is established (Arvin, 1996). Latent virus can reactivate, usually later in life, to cause zoster (shingles), a painful rash illness. VZV can be transmitted to susceptible persons from either disease condition, although zoster carries a significantly lower risk of transmission; varicella cases transmitted from zoster patients may reintroduce strains that circulated decades earlier, which probably contributes to the unusually high conservation of the VZV genome. For reasons that remain unclear the epidemiology of primary VZV infection varies geographically; in temperate climates, VZV infects >90% of children during the first decade of life, whereas in tropical countries primary infection is often delayed until adulthood (Lee, 1998; Lolekha et al., 2001). Several studies have demonstrated a distinctive geographic distribution of the major VZV genotypes aligning with temperate versus tropical regions of the globe (Barett-Muir et al., 2003; Dayan et al., 2004; Lolekha et al., 2001; Loparev et al., 2007a; Quinlivan et al., 2002; Waganaar et al., 2003). The regional dominance of specific genotypes might have been established by climate and/or other

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factors, such as host–virus interactions or introduction of strains to naïve populations through immigration or travel. Modern genotyping strategies may help to resolve these questions. Our approach has established the regions in which various VZV genotypes predominate, and these data, combined with historical human migration patterns, identified the likeliest points of origin for these genotypes. Thus far geographic dominance has been observed for E1 and E2 (Europe), M1 (Africa) and J (Japan) genotypes. (Loparev et al., 2004, 2007b; Muir et al., 2004; Sergeev et al., 2006). The dominance of E genotype strains in North America and Australia was likely established during European colonization in the 16th and 17th centuries. Relatively recent changes in immigration and/or international travel patterns for certain geographic regions (notably Europe, Australia, and the United States) are now characterized by co-circulation of a variety of VZV genotypes. These countries are potential "melting pots" for VZV evolution due to the enhanced opportunity for dual infection followed by inter-strain recombination (Koskiniemi et al., 2007; Loparev et al., 2007a, 2004; Norberg et al., 2006; Quinlivan et al., 2002; Sengupta et al., 2007).

Earlier studies indicate that as many as seven distinct VZV genotypes are currently circulating, which we designate as E1, E2, J, M1, M2, M3, and M4 (Loparev et al., 2007a, 2007b, 2004; Sauerbrei et al., 2004; Sergeev et al., 2006). At least 5 of these same genotypes have also been detected using other approaches, although different nomenclatures are applied by each group of investigators (Barrett-Muir et al., 2003; Campsall et al., 2004; Carr et al., 2004; Muir et al., 2004; Parker et al., 2006; Peters et al., 2006; Wagenaar et al., 2003). These studies have consistently observed distinctive geographic distributions of VZV genotypes. We recently published a two-amplicon method (Loparev et al., 2007b) that identifies all of the phylogenetic groups identified by every method, and verifies the presence of 5 confirmed and 2 provisional genotypes M3 (Sergeev et al., 2006) and M4 (Loparev et al., 2007a). Essentially, we identified SNP that discriminate E1-genotype from E2 in ORF21, ORF22 [region 2], and ORF50. Analysis of SNP in any one of these regions is sufficient to discriminate E1 strains from other European strains. The combination of ORF22 [region 1]-based genotyping (Loparev et al., 2004) with analysis of any one of the E1-discriminating SNP thus permits the identification of every described VZV genotype.

Two additional positions in ORF38 (69349) and ORF54 (95241), originally used to confirm Oka vaccine adverse events, also provide useful information for genotyping. These two markers are not Oka vaccine-specific, but serve rather to distinguish some J, M1, M2, and M4 strains from other genotypes. A subset of J genotype isolates (e.g., pOka, which was the source virus for Oka vaccine) contain a PstI restriction site in ORF38, and the ORF54 sequence includes a BglI restriction site common to J, M1, M2, and M4 strains. The identification of SNP by using restriction enzyme hydrolysis has been supplanted by amplicon sequencing, but it remains convenient to refer to these markers by their restriction enzyme specificities. In addition to the J strains common in Japan, Australia, and some east Asian countries (Loparev et al., 2007a, 2007b, 2004; Sauerbrei et al., 2004; Sergeev et al., 2006), Bgl+ strains are also commonly isolated in tropical climates (e.g., equatorial Africa, India, Bangladesh, southern China, Central America, and northern Australia). In contrast, Bgl− viruses are common in temperate climates (Loparev et al., 2007b; Norberg et al., 2006). Two Pst−Bgl− isolates have also been observed in Australia (Campsall et al., 2004) and Germany (Sauerbrei and Wutzler, 2007).

Several alternative methods for genotyping VZV strains have been published, each of which resolves four or five of the seven genotypes identified using the two-amplicon genotyping method (ORF22 [region 1] plus either ORF21, ORF22 [region 2] or ORF50). Two-amplicon sequence analysis is the only method in current use that identifies all of 7 VZV genotypes (Loparev et al., 2007a, 2007b, 2004; Sauerbrei et al., 2004; Sergeev et al., 2006).

In this report we describe the distribution of VZV genotypes of clinical isolates obtained from 18 European nations. We observed that VZV isolates of genotypes E1, E2, M1, M2, and M4 are circulating in Europe, but did not detect either J or M3 isolates. In addition, we identify a set of reference SNP, identical in each particular VZV genotype, that should be useful for monitoring the occurrence of new genotypes or recombinant viruses. The dominant genotypes observed in this study were E1 and E2, which occurred in variable proportion in northern and southern Europe. We also show a close phylogenetic relationship for E1, E2, and M4 genotypes, the latter of which was recently described in southern Europe (Spain, France, and Italy).

Results

Identification and phylogenetic analysis for new M4 genotype

Phylogenetic analysis of 18 completely sequenced wild-type VZV strains segregated those strains into 5 distinct genotypes (Fig. 1A). Based on this analysis, the recently sequenced Russia 1999 strain was placed into the E1 genotype. The members within each genotype displayed between 99.90 and 99.97 identity at the nucleotide level, corresponding to 33 to 47 single base-pair differences. Representatives of different genotypes had between 140 to 210 single base-pair differences. Representative E1 and E2 genotypes isolated in Europe and in North America displayed a high level of internal stability.

Targeted sequence analysis of a short region in ORF22 [region 1], coupled with analysis of variable SNP in ORF21, ORF22 [region 2] or ORF50, robustly identified the same 5 genotypes, and furthermore differentiated two provisional genotypes (M3 and M4) for which no complete genome sequences are available.

We performed targeted sequence analysis of 3 representative M4 strains Spain_4242, France_4415, and Italy_4053 in 30 different genomic regions (Table 1) selected on the basis of SNP variability with respect to each putative genotype. 90 SNP were identified in the amplified regions, representing about half of the total SNPs identified comparing whole genome sequences of E1, E2, M1, M2 and J strains (Norberg et al., 2006; Peters et al., 2006). The sequence data from the 3 representative M4 strains, in addition to data from an M1 isolate (Congo_24), was aligned with published sequence data from the 19 completely sequenced strains, and phylogenetic analysis was performed. The results of that examination are presented in Fig. 1B, which reveals that the 3 M4 isolates cluster as a distinct genotype. All 6 genotypes were unambiguously separated from one another, with high bootstrap values (data not shown). The Russia 1999 and Congo_24 isolates were allocated to E1 and M1 genotypes based on their respective reference SNP (Fig. 1B, Table 1). These data reinforce the allocation of strains represented by France 4415, Spain 4242, and Italy 4053 to a separate genotype, M4.

VZV SNPs useful for strain genotyping

At least two approaches were taken for genotyping VZV: the analysis of 2 short amplicons (two-amplicon method), or the identification of individual unique SNP in 5–7 amplicons, each of which identifies a different genotype. To demonstrate proof of concept, two-amplicon testing was performed in this study analyzing three different regions containing SNP that differentiate E1 strains from E2 strains (ORF21, ORF22 [region 2], and ORF50); in actual practice, only one of those regions would be evaluated in addition to ORF22 [region 1].

The first strategy (the two-amplicon method) provides the broadest range of genotype recognition short of performing whole genome sequence analysis. The E group (E1+E2), M1, M2, M3, M4, and J genotypes can be differentiated by analyzing the combination of 4 SNP located in ORF22 [region 1] (Table 1). The E group viruses can be differentiated into E1 and E2 by examining variable SNP from any one of the following positions: 2596, 2643 (ORF3), 5827 (ORF6), 12284 (ORF10), 14390, 14402 (ORF11), 18054 (ORF12), 23294, 23429, 24092

(ORF16), 25478, 24654 (ORF17), 33725, 33728 (ORF21), 39394, 39530 (ORF22 [region 2]), 68172, 68254 (ORF37), 75103 (ORF40), 78385, 78545 (ORF43), 87841 (ORF50) and 113243 (ORF66) (Table 1). E1, E2 and M4 genotypes are all Bgl− in ORF54, while M1, M2, M3 and J strain are all Bgl+.

The other approach takes advantage of SNP specifically associated with individual genotypes SNPs. We identified a number of genotype-specific SNP in the course of this study.

E1 isolates included the reference strain VZV-Dumas, and 9 other strains. As shown in Table 1, E1-specific SNP were located at positions

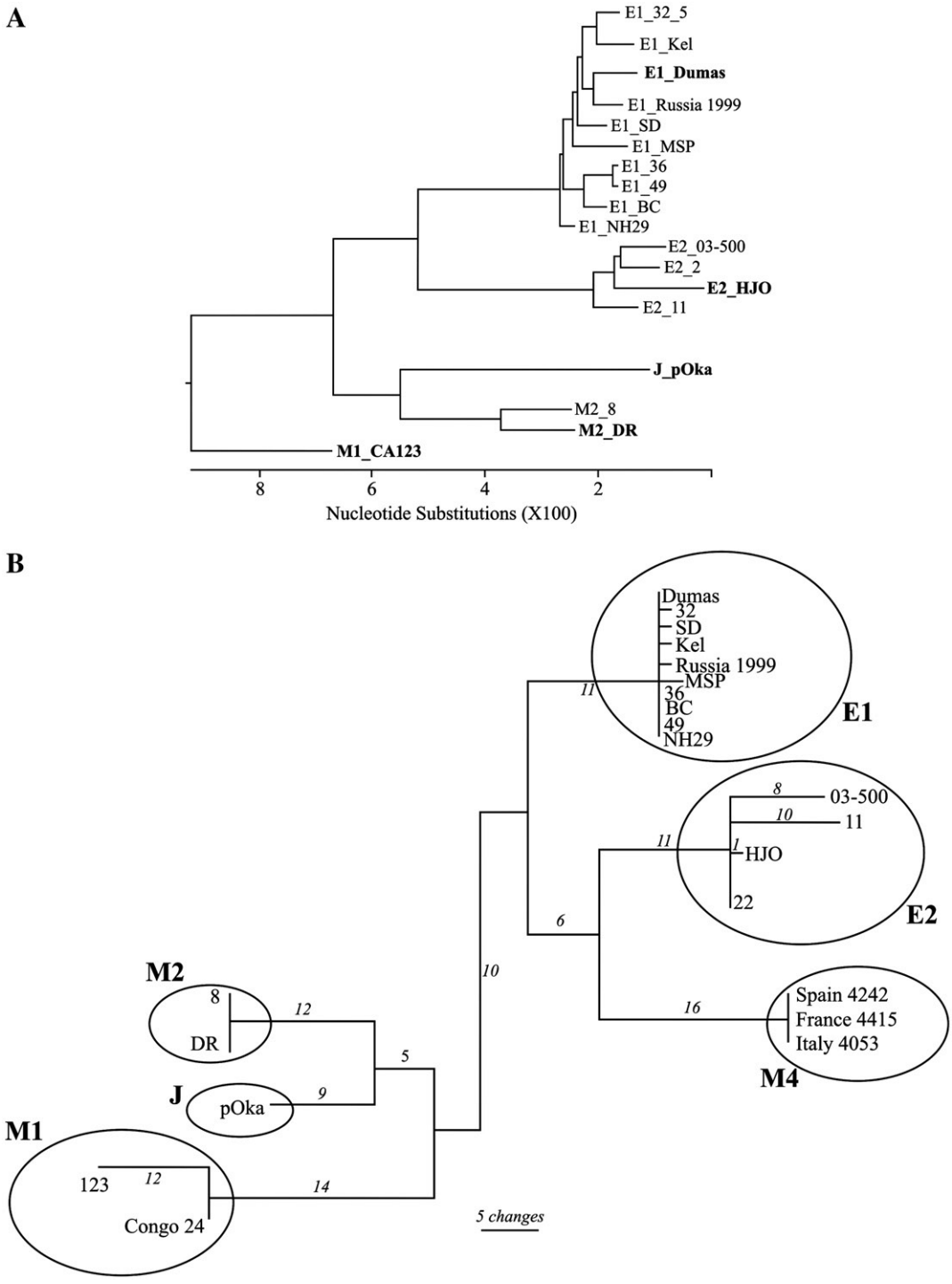

Fig. 1. CLUSTALW v1.83 phylogenetic analysis based on the complete VZV genomes (A). Number of nucleotide substitutions supporting each genotype are shown. Reference strains marked in bold in each genotype. This phylogenetic analysis included complete sequences for the 5 variable regions (R1–5) and the origin of replication. (B) PAUP phylogenetic analyses of aligned sequences of variable regions in 26 VZV ORF. The numbers on the connectors indicate the number of nucleotide differences. CLUSTALW v1.83 phylogenetic analysis based on the complete VZV genomes (A). Number of nucleotide substitutions supporting each genotype are shown. Reference strains marked in bold in each genotype. This phylogenetic analysis included complete sequences for the 5 variable regions (R1–5) and the origin of replication. (B) PAUP phylogenetic analyses of aligned sequences of variable regions in 26 VZV ORF. The numbers on the connectors indicate the number of nucleotide differences.

Table 1
Analysis of genomic variation using data from multiple VZV open reading frames: a key to the VZV genotype nomenclatures in current use[a]

ORF	position	strain	MSP	BC	DUMAS	NH29	49	36	Sveta	32	Kel	SD	11	22	03-500	HJO	MA4*	CA123	CN	DR	8	pOka	
		genotype	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E2	E2	E2	E2	M4	M1	M1	M2	M2	J	
1	508		C	C	C	C	C	C	C	C	C	C	C	C	C	C	T	C	C	C	C	C	†
	685		G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	A	A	A		†
	789		T	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	C	C	C		†
	790		T	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	C	C	C		†
	791		T	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	C	C	C		
3	2591		T	T	T	T	T	T	T	T	T	T	T	T	T	T	G	T	T	T	T		
	2596		T	T	T	T	T	T	T	T	T	C	C	C	C	C	T	T	T	T	T		
	2643		G	G	G	G	G	G	G	G	G	G	A	A	A	A	A	G	G	G	G		
6	5827		C	C	C	C	C	C	C	C	C	A	A	A	A	A	C	C	C	C	C		
	6022		A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	A	A	A	A		
	6850		G	G	G	G	G	G	G	G	G	G	G	G	G	G	A	A	A	A	G		‡
	6853		G	G	G	G	G	G	G	G	G	G	G	G	G	G	T	T	T	T	T		
	7091		C	C	C	C	C	C	C	C	C	C	C	C	C	C	A	A	A	A	A		
8	9081		G	G	G	G	G	G	G	G	G	G	G	G	G	G	A	A	A	G	G		
9	11317		G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	A	A	G		‡
	11449		A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	A	A	A	A		
10	12284		T	T	T	T	T	T	T	T	T	C	T	C	C	C	C	C	C	C	V		
	12285		T	T	T	T	T	T	T	T	T	C	T	C	C	C	C	C	C	C	V		
11	13846		G	G	G	G	G	G	G	G	G	G	G	G	G	C	G	G	G	G	G		
	13870		C	C	C	C	C	T	T	T	T	C	C	C	C	C	C	C	C	C	C		
	13961		G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G		
	13997		G	G	G	G	G	G	G	G	G	A	A	A	A	G	A	A	G	G	G		
	14390		T	T	T	T	T	T	T	T	T	C	C	C	C	C	C	C	C	C	C		
	14402		T	T	T	T	T	T	T	T	T	G	G	G	G	T	T	T	T	T	T		
12	17834		C	C	C	C	C	C	C	C	C	C	C	C	C	T	C	C	T	T	T		
	18052		T	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C		
	18054		T	T	T	T	T	T	T	T	T	C	C	C	C	T	C	C	T	T	T		
	18082		C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	T		‡
16	23294		A	A	A	A	A	A	A	A	A	G	G	G	G	G	G	G	A	A	G		‡
	23429		A	A	A	A	A	A	A	A	A	G	G	G	G	G	G	G	A	A	A		
	23665		A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G		
	24092		T	T	T	T	T	T	T	T	T	C	C	C	C	T	C	C	T	T	T		
17	24533		A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A		
	24578		A	A	A	A	A	A	A	A	A	G	G	G	G	G	G	G	A	A	G		‡
	24654		C	C	C	C	C	C	C	C	C	T	T	T	T	T	T	T	C	C	T		‡
	24656		A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A		
21	33725		T	T	T	T	T	T	T	T	T	C	C	C	C	C	C	C	C	C	C		§†
	33728		T	T	T	T	T	T	T	T	T	C	C	C	C	C	C	C	C	C	C		§†
22	37902		A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G		¶§†
	38055		T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	C		¶†
	38081		A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	C	C	C		¶†
	38177		G	G	G	G	G	G	G	G	G	G	G	G	G	G	A	G	G	A	A		¶†
	39263		G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	A		†
	39394		G	G	G	G	G	G	G	G	G	A	A	A	A	A	A	A	G	G	A		§†
	39530		A	A	A	A	A	A	A	A	A	G	G	G	G	G	G	G	A	A	G		§†

ORF	position	strain	MSP	BC	DUMAS	NH29	49	36	Sveta	32	Kel	SD	11	22	03-500	HJO	MA4*	CA123	CN	DR	8	pOka	
		genotype	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E2	E2	E2	E2	M4	M1	M1	M2	M2	J	
28	51168		C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	T	
	51200		C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	T	T	C	‡
	51762		C	C	C	C	C	C	C	C	C	C	T	T	C	C	C	C	C	C	C	C	
	51840		G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	A	A	G	‡
35	64259		T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	C	‡
	64375		T	T	T	T	T	T	T	T	T	T	T	C	C	C	C	C	C	C	C		
	64522		C	C	C	C	C	C	C	C	C	C	C	C	C	C	T	T	C	C	C		
	64529		G	G	G	G	G	G	G	G	G	G	A	G	G	G	G	G	G	G	G		
	64703		G	G	G	G	G	G	G	G	G	G	G	G	G	G	A	A	A	A	G		‡
	64740		C	C	C	C	C	C	C	C	C	C	C	C	C	C	T	T	T	T	C		
37	68101		T	T	T	T	T	T	T	T	T	C	C	C	C	T	T	T	T	T	T		‡
	68172		G	G	G	G	G	G	G	G	G	A	A	A	A	A	A	A	A	A	A		‡
	68254		C	C	C	C	C	C	C	C	C	C	T	T	T	C	C	C	C	C	C		
40	74699		T	T	T	T	T	T	T	T	T	T	T	T	T	G	T	T	T	T	T		
	75073		C	C	C	C	C	C	C	C	C	C	C	C	C	A	C	C	C	C	C		
	75103		A	A	A	A	A	A	A	A	A	G	G	G	G	A	A	A	A	A	A		
	75255		C	C	C	C	C	C	C	C	C	C	C	C	C	T	T	C	C	C	C		
	75256		C	C	C	C	C	C	C	C	C	C	C	C	C	T	T	C	C	C	C		
	75259		C	C	C	C	C	C	C	C	C	C	C	C	C	T	T	C	C	C	C		
43	78385		C	C	C	C	C	C	C	C	C	C	T	T	T	T	C	C	C	C	C		
	78526		C	C	C	C	C	C	C	C	C	C	C	C	C	C	A	C	C	C	C		
	78545		G	G	G	G	G	G	G	G	G	A	A	A	A	G	G	G	G	G	G		
48	84983		G	G	G	G	G	G	G	G	G	G	G	G	G	G	A	A	A	A	A		
	85041		A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	G	G	G	A		‡
50	87841		C	C	C	C	C	C	C	C	C	T	T	T	T	T	T	T	T	T	T		§†
54	95241*		T	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	C	C	C		†
	95300		C	C	C	C	C	C	C	C	C	C	C	C	C	C	T	C	C	C	C		
	95546		G	G	G	G	G	G	G	G	G	G	G	G	G	G	A	A	A	A	A		
	95601		T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	G		‡
	95610		T	T	T	T	T	T	T	T	T	T	T	T	T	T	G	T	T	T	T		
55	98437		T	T	T	T	T	T	T	T	T	T	T	T	T	C	T	T	C	C	C		
56	98659		C	C	C	C	C	C	C	C	C	C	C	C	C	T	C	C	T	T	C		‡
	98765		T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	C		
	98807		A	A	A	A	A	A	A	A	A	A	A	A	A	C	A	A	A	A	C		
	98825		T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	C	C	C	T		‡
58	99981		C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	T		‡
	100114		T	T	T	T	T	T	T	T	T	T	T	T	T	A	A	A	A	A	A		
	100123		T	G	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T		
	100151		T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	G		‡
60	101464		C	C	C	C	C	C	C	C	C	C	C	C	C	C	A	A	C	C	C		
66	112962		T	T	T	T	T	T	T	T	T	T	C	C	C	C	C	C	T	T	T		
	113084		C	C	C	C	C	C	C	C	C	C	C	A	A	A	C	C	C				
	113169		G	G	G	G	G	G	G	G	G	G	G	G	G	T	G	G	G				
	113256		G	G	G	G	G	G	G	G	G	G	G	G	T	G	G	G	G				
	113243		A	A	A	A	A	A	A	A	A	C	C	C	C	C	C	A	A				

Prototype E Strain Marker
Prototype J Strain Marker
E1-Speciofic Marker
E2-Speciofic Marker
M4-Speciofic Marker
M1-Speciofic Marker
M2-Speciofic Marker
Shared Marker E1, M2
Shared Marker E2, M1
Shared Marker M4, M1
Shared Marker E2, M4
Shared Marker M1, M4
Shared Marker M2, J
Shared Marker M2, J
Shared Marker E1, E2, M4
Shared Marker M1, M2, J
Shared Marker E1, M4, M1
Shared Marker E2, M4, M1, J
Shared Marker M4, M1, M2
Subset of E1
Subset of E2
Strain-Specific Marker

n.e. = no equivalent genotype detected. *This is a provisional VZV genotype. No complete reference sequences are currently available. †Markers used to determine phylogenetic tree in Fig. 1B, in order to permit evaluation of M4 isolates. ‡Markers used to definitively distinguish M2 and J strains. ¶Markers used for original ORF22-based method (14). §Additional markers used to perform two-amplicon method (only ORF21 or ORF50 markers are needed, not both).

[a] Comparison data used all available completely sequenced VZV reference strains and partially sequenced clinical specimens of M4 isolated in Spain, France and Italy.

12284, 12285 (ORF10), 14390 (ORF 11), 33725, 33728 (ORF21), 68172 (ORF37) and 87841 (ORF50). There are E2-specific SNP at positions 14402 (ORF11), 68101 (ORF37), and 75103 (ORF40). Four genotype-specific SNP were found for the discrimination of M1 strains: 24533, 24656 (ORF17), 64522 (ORF35), and 101464 (ORF60).

Genotype M2 is represented in Table 1 by strains DR and VZV-8. M2-specific SNPs were identified at position 11317 in ORF9, and at positions 51200 and 51840 in ORF28. Our analysis of M4 isolates demonstrated the consistent presence of genotype-specific SNPs at positions 508 (ORF1), 75073 (ORF 40), and 113256 (ORF66).

Unique markers for J genotype strains were observed at position 18082 (ORF12), 37902, 39263 (ORF22), 51168 (ORF28), 64259 (ORF35), 95601 (ORF54), 98765 (ORF56), and 100151 (ORF58). No J genotype strains were detected among the isolates analyzed in this study. Although J strains have been found to be circulating at low levels in the UK (Sengupta et al., 2007), it should be noted that authors used a method that is unable to differentiate between M2 and J genotype based on SNP located at position 685. This is an important consideration for interpreting observations using SNP that differentiate M2 and J strains, since the M2 strains are sometimes isolated in Europe (Loparev et al., 2007a, 2004; Sengupta et al., 2007). J and M2 strains share mutations at positions 685, 789, 790, 791, 6853, 7091 and several others (Table 1). Since the J-genotype-specific SNP at position 37902 is included in the ORF22 [region 1] amplicon we reported previously, that genotype can be unambiguously identified using ORF22 [region 1] alone.

M4-specific SNP were identified at positions 508 (ORF1), 75073 (ORF40) and 113256 (ORF 66). M4 is closely related to genotypes E1 and E2 based on phylogenetic analysis (Fig. 1B); these strains have identity with E2 strains at positions 2596, 2642, 5827, 68254, 78385, 78545 and share SNPs with both E1 and E2 in at least 20 different loci (Table 1).

Evaluation of the two-amplicon ORF22-based genotyping scheme using clinical specimens

We used an enhanced two-amplicon genotyping method (ORF22 [region 1] plus E1-specific SNP in ORF21, ORF22 [region 2] and ORF50) to genotype wild-type isolates collected in Europe. These data are presented in Table 2. Overall, 308 (90.1%) isolates were identified as either E1 or E2 viruses. There were 221 (65%) E1 and 87 (25%) E2 VZV strains identified. In addition there were 20 (6%) M1 isolates, 3 (1%) M2 isolates, and 11 (3%) M4 isolates found. No J or M3 isolates were identified in this study.

Evaluation of the stability of globally circulating VZV genotypes using a targeted subset of SNP

The stability of 6 of the 7 prospective genotypes was examined for all 342 VZV isolates collected from Europe by sequencing targeted regions of the genome in ORF 1, ORF21, ORF22, ORF38, ORF50, and ORF54. The specific SNP evaluated are indicated in the right hand column of Table 1 by the symbol †. The variability of wild-type isolates at these 5 loci, which comprise a total of 14 SNP, was compared to the completely sequenced reference strains Dumas (E1), HJO (E2), CA123 (M1), DR (M2), and P-Oka (J) and with M4 isolates identified using multiple SNP analysis. Representative M4 isolates were also evaluated at these regions. All E1, E2, M1, and M4 strains consistently have the nucleotide guanine at position 685 (ORF1); all E1, E2, and M4 strains have identical SNP profiles at positions 789, 790, and 791 (ORF1) 6853, 7091 ORF6, 84983 ORF48, 95241 and 95546 (ORF 54). E2, M1, M2, M4 and J strains were identical at positions 14390 (ORF11), 33725, 33728 (ORF21), 68172 ORF37), and 87841 (ORF50). All 308 E1, E2 and M4 strains were PstI+ (ORF38) BglI− (ORF54), and the genotype M1, M2 strains identified in this study were PstI+BglI+ (Table 2).

Additional evidence indicating stable differences between J and M2 isolates

Only 3 M2 isolates were detected among the viruses we obtained from Europe; no J isolates were found. We analyzed 4 additional M2 strains (Australia) and 10 wild-type J strains (Japan and Australia) to evaluate the consistency of SNP that differentiate M2 and J genotype strains. Altogether, 18 SNP were evaluated that were useful for discriminating these two genotypes, at positions 6850 (ORF6), 11317 (ORF9), 18082 (ORF12), 23294 (ORF16), 24578, 24654 ORF17), 37902 (ORF22), 51200, 51840 (ORF28), 64259, 64703, 64740 (ORF35), 85041 (ORF48), 95601 (ORF54), 98659, 98825 (ORF56), 99981 and 100151 (ORF58). All 7 M2 strains had SNP profiles identical to both fully sequenced M2 strains (DR and 8) at the loci identified above, which are indicated in the right hand column of Table 1 with the symbol ‡. The 10 J viruses had SNP profiles identical to the reference wild-type strain pOka at the loci identified above. As such, care must be taken to discriminate them to avoid mistyping of M2 European isolates as J strains.

Evaluation of varicella vaccine-associated SNP among European wild-type VZV isolates

We analyzed the 342 European isolates for their SNP profiles at loci that are used to discriminate Oka varicella vaccine from wild-type VZV strains. Three vaccine-specific SNP at positions 106262, 107136, and

Table 2
Sample collection and genotyping data for clinical samples from Europe using a panel of SNP in ORF 22 [region 1 and 2], ORF 21, ORF 38, ORF50, and ORF 62

Country of origin	# Isolates	E1	E2	M1	M2	M3	M4	J	Clinical Isolate	Tissue Culture	Varicella	Zoster
Iceland	17	1	15	0	1	0	0	0	0	17	5	12
Finland	28	10	18	0	0	0	0	0	28	0	3	25
Estonia	3	3	0	0	0	0	0	0	0	3	3	0
Lithuania	5	4	1	0	0	0	0	0	0	5	5	0
Latvia	9	8	1	0	0	0	0	0	0	9	9	0
Belarus	15	12	3	0	0	0	0	0	15	0	0	15
Germany	51	20	21	9	1	0	0	0	41	10	41	10
Greece	3	3	0	0	0	0	0	0	3	0	3	0
Russia (Europe)	57	55	2	0	0	0	0	0	57	0	57	0
Poland	4	2	2	0	0	0	0	0	0	4	4	0
Ukraine	6	5	1	0	0	0	0	0	6	0	6	0
Czech Republic	15	7	8	0	0	0	0	0	15	0	6	5
Romania	4	2	2	0	0	0	0	0	4	0	4	0
France	19	12	2	2	1	0	2	0	0	19	4	15
Italy	17	11	4	0	0	0	2	0	17	0	17	0
Spain	31	15	0	9	0	0	7	0	31	0	31	0
Bulgaria	38	34	4	0	0	0	0	0	38	0	Unk	Unk
Albania	20	17	3	0	0	0	0	0	20	0	19	1
Total	342	221	87	20	3	0	11	0	275	67	217	83
ORF38 BglI+		0	0	20	3	0	0	0				
ORF54 PstI−		0	0	0	0	0	0	0				
ORF62 Small+		0	0	0	0	0	0	0				
ORF62 Small+		0	0	0	0	0	0	0				

107252 (ORF62) were evaluated, as well as the two SNP at ORF38 and ORF54 that are not vaccine-specific, but help to discriminate vaccine J genotype from other wild-type genotypes. None of the European VZV isolates studied here carried any of the vaccine-specific VZV markers in ORF62, and as such all represented wild-type strains (Loparev et al., 2007a, 2007b, 2004). Among the European isolates, 319 were PstI+BglI− (E1, E2 and M4), and 23 were PstI+BglI+ (M1, M2, M3 and Pst+ J strains). No PstI−BglI+ (the pOka J genotype carried by the vaccine) strains were identified. No BglI−PstI− isolates were identified in this study.

Discussion

The VZV genome is highly conserved, averaging about one SNP for every 1000 bp of sequence (Loparev et al., 2004; Norberg et al., 2006; Peters et al., 2006). Recent studies suggest that recombination has likely played a role in the emergence of currently circulating genotypes of VZV (Norberg et al., 2006; Peters et al., 2006; Quinlivan et al., 2002). These factors complicate strategies both for performing VZV genotypic analysis and strain surveillance. Despite these obstacles, extensive study of VZV genetic variation by several groups and, more recently, the availability of nearly two dozen genomic sequences for diverse wild-type and vaccine VZV isolates, has provided persuasive evidence for at least 7 distinct, stably circulating genotypes of VZV (Faga et al., 2001; Barrett-Muir et al., 2003; Campsall et al., 2004; Carr et al., 2004; Dayan et al., 2004; Koskiniemi et al., 2007; Loparev et al., 2007a, 2007b, 2004; Muir et al., 2004; Norberg et al., 2006; Parker et al., 2006; Peters et al., 2006; Quinlivan et al., 2002; Sauerbrei and Wutzler, 2007; Schmidt-Chanisit et al., 2007; Sengupta et al., 2007; Sergeev et al., 2006; Tyler et al., 2007; Wagenaar et al., 2003). These observations provided the impetus for this study, with the specific aim of unifying and expanding on all of the genotyping strategies currently in use.

M3 genotype virus has been isolated in the US (Sergeev et al., 2006), but it is not a subject of this study and it will not be discussed further in this paper.

Five groups of reference strains with complete genome sequences (Dumas, HJO, CA123, DR, and pOka) laid the foundation for a general method of VZV genotyping. In addition, sequence information from 31 amplicons representing 25 open reading frames and 90 individual SNP was used to demonstrate that the 11 southern European isolates belonging to the provisional genotype M4 clearly segregate into their own phylogenetic cluster. Discovery of a new M4 genotype demonstrates that ORF 22 sequence evaluation is not only useful for the surveillance of all currently recognized genotypes, but can also be useful for the discovery of new VZV genotypes.

In addition, examination of sequence variation at 9 reference SNP in ORF 21, 22, 38, 54, and 62, demonstrated that the characteristic SNP signatures for each of the 6 genotypes studied here are stably maintained among circulating VZV strains, indicating their utility as an epidemiological tool.

Intra-genotypic variation

Excluding varicella vaccine sequences (all derived from the pOka strain), complete genomic sequences have now been determined for 19 VZV wild-type strains. This includes a complete sequence for the strain, Russia 1999, reported here — a Dumas-like E1 genotype virus isolated approximately 3 decades after the Dumas strain was isolated from a case of varicella in the Netherlands. The two viruses are strikingly similar, with only 24 single base differences between them (0.02% variation at the DNA level). These differences included four mutations at positions 1758, 9488, 59981, and 101425 that have not been detected in any other isolates of VZV regardless of genotype. Russia 1999 shared SNP with strains Kel and 32 at position 15870; with Kel and SD at positions 51920 and 53523; with strain 32 at positions 74071, 76377 and 92535; with strains 32, Kel and SD at position 102403; and with Dumas at positions 104898 and 109044. Similar SNP analysis of complete genomic sequence for four E2 strains and two M2 strains revealed a comparable level of genotype stability.

Categories of VZV genomic variation

This investigation confirms previous observations (Loparev et al., 2007b; Norberg et al., 2006) that three types of genomic variation (excluding repeat regions and ORIs) are observed among VZV strains: 1) genotype-specific mutations, 2) mutations shared by some but not all genotypes, and 3) strain-specific individual mutations. Examples of genotype-specific SNP are observed at positions 35725 (E1), 68101 (E2), 101464 (M1), and 11317 (M2); of shared markers at positions 625, 789, 790, 791, 2596, and 6850; and of strain-specific markers at position 100123, which has thus far been observed only in the E1 strain, BC. Several methods have relied on SNP analysis to resolve VZV genotypes (Carr et al., 2004; Loparev et al., 2004, 2007b; Muir et al., 2004; Parker et al., 2006; Sauerbrei et al., 2004). However, one of those methods (Carr et al., 2004; Muir et al., 2004; Parker et al., 2006) fails to discriminate between J genotype and M2 and the single-amplicon ORF22 [region 1] method (Loparev et al., 2004; Sauerbrei et al., 2004) discerns E1 and E2 genotypes collectively as E group viruses. The method described by Muir et al. (2004) is also unable to distinguish between M4 and E2 genotypes, as we recently demonstrated (Loparev et al., 2007b). It is possible to resolve the limitations of any of these approaches by adding SNP to the analysis that expand the capacity to resolve genotypes, as recently employed for the ORF22 [region 1]-based genotyping method (Loparev et al., 2007b). For example, incorporating a genotype-specific J marker (e.g., position 37902) and an M2 marker (e.g., position 51200) into the method (Carr et al., 2004; Barrett-Muir et al., 2003; Parker et al., 2006) would render it capable of discriminating at least 5 genotypes. Genotype-specific SNP could be used exclusively in a method aimed at identifying all of the recognized genotypes, but this approach would require having at least 6 specific SNP for the genotypes examined in this study (a 7th would be required to identify the provisional genotype M3). This approach assumes the absolute stability of the selected targets and would fail to account for minor variability among strains of the same genotype. Limiting a method to a handful of genotype-specific SNP would likely preclude the detection of novel genotypes and cause the misdiagnosis of genotypes or genome variants formed through recombination. This limitation was illustrated here with the potential for misdiagnosing J and M2 genotypes. A more versatile approach would take advantage of genotype-specific and markers shared among subsets of genotypes, the strategy we have generally relied on.

Recombination in VZV

There is a growing body of evidence suggesting that recombination occurs among wild-type strains of VZV (Loparev et al., 2004; Norberg et al., 2006; Peters et al., 2006; Sengupta et al., 2007), and that some VZV genotypes have signatures of recombination events (Norberg et al., 2006). We initially used the term *mosaic* to characterize several closely related phylogenetic groupings of strains that consisted of variable alternating patchworks of E-genotype and J-genotype genetic markers (Loparev et al., 2004). Later, complete genome sequences for M1 (CA123 and Congo 24) and M2 (DR and 8) viruses confirmed this prediction and, combined with analysis of complete sequence data from strains of other genotypes, revealed that in fact 3 more recent VZV genotypes (M3 has not been sufficiently characterized) are probably derived from VZV ancestral genotypes. Initial studies suggest that M1, E1 and J are the likeliest progenitor genotypes, but a more definitive insight into this question awaits complete genomic sequences for all 7 genotypes. Genotype J and M2 viruses share the genotypic marker at position 685: M1 and M2 viruses at position

6850, E2 and M4 viruses at positions 2596, 2643, and 5827, and a stretch of SNP at positions 789, 790, and 791 differentiate M1, M2, and J viruses from E1, E2, and M4 viruses. From this perspective, a genotyping strategy based on ORF22, ORF21 and ORF50 SNPs differentiated the 5 well-established VZV genotypes, and facilitated the identification of the M3 and M4. At the same time we demonstrated that the genomic signatures for all 6 of the genotypes examined here are quite stable, with almost no evidence of multiple recombination intermediates. Thus, the evolution of the currently circulating genotypes is likely to have occurred over a rather long period of time — certainly more than a century, based on the level of stability among isolates from recent cases of varicella and herpes zoster.

Characteristics of E2 strains

We observed unexpected irregular SNP in E2 strains propagated in tissue culture (positions 12284, 12285, and 113243). This is a potentially important point since, based on the mosaic pattern of E2 strains, multiple recombination points, a close phylogenetic relationship of E2 viruses with E1 and M4 strains can be postulated. At the same time, E2 viruses share genomic markers with other M and J viruses, for example at positions 12284, 12285, 24578, and 39394.

Although HJO and related viruses have a mosaic structure, they were designated as E2 viruses since the first representative strains for this genotype were identified in the UK. Moreover, the reference strain HJO was isolated in Germany (accession number AJ871403). This also seems consistent with our observation that E2 strains were the second most abundant genotype in Europe (87/342; 25.4%). In addition, E1 and E2 viruses are the only genotypes that are consistently negative for the BglI restriction site in ORF54, and approximately two-thirds of their genome is identical, including the SNP located in ORF22. E2 viruses are, however, broadly distributed. They are common or dominant in northern European countries such as Iceland (15/17, 88.2%), Finland (18/28, 64.2%), and Germany (21/51, 41.2%). E2 strains have also been isolated in the US, Canada, and Australia, and have been isolated from tropical regions of the globe.

The characteristics of M4 strains

Three European M4 genotype isolates obtained for this study had complete identity at the 90 SNP we evaluated. These isolates were acquired separately, that is, they were not obtained from a single outbreak or nation. Although these strains have a close phylogenetic relationship with E1 and E2 strains, they are regarded as an imported genotype based on their low prevalence in Europe and their association with African immigrants to European countries (Loparev et al., 2007b). M4 strains have a unique mosaic SNP pattern in ORF22 [region 1] (Sergeev et al., 2006) and can be discriminated from the other genotypes using that sequence alone.

The distribution of VZV genotypes in Europe

The number of E1 and E2 isolates identified among 342 European isolates (90%) was consistent with the strain distribution (80%) observed in the United Kingdom (Sengupta et al., 2007). A significant fraction of E1 and E2 isolates (38%) were obtained from cases of zoster, which occurs predominantly in persons over age 50. As such, these represented older infections and reinforce the viewpoint that the E genotypes emerged first as the predominant genotypes in Europe. E1 and E2 strains isolated from the 18 European countries described in this study are essentially identical to those obtained from

Table 3
Primers for analysis of genomic variations in clinical specimens

ORF	Forward	Sequence 5′→3″	Position	Reverse	Sequence 5′→3″	Position	Amplicon size (bp)	Reference
1	v3	aac agt ctt acg att gcc tag a	378–399	v4	agt agt ttc atg tag ttg agt t	548–569	191	
1	vzv1f	tca gct ggc ttt tct aag aat tcg	427–450	vzv1r	tat ttt tgg tat ccg caa tgt c	911–932	505	(Arvin, 1996; Carr et al., 2004)
3	v10	acg gct gta ggt caa cat aca	2166–2183	v11	acg ccc gtc cat acg gta aat	2812–2832	666	
6	v16	tgt gct gtc gac aaa ggc ct	5656–5675	v17	tcc cta tgc gtt agt ggg gtc t	6130–6151	495	
6	v18	tct cgt agt gaa gcg gca aca	6710–6739	v19	tcc ata tgg atg gtc ctc cac t	7144–7165	455	
8	v22	gcc atc tta tga aga ggt gcg t	8660–8681	v23	aga taa ttg tgt cgc ctc cga	9201–9221	561	
9	v26	aat ggc atc ttc cga cgg t	11008–11026	v27	tcg gca gcc ttt tgt gc	11585–11601	593	
10	v28	tgt gga cgc att tga tga atc	12231–12251	v29	aca gaa gac gcg cca aac tt	12831–12850	619	
11	v31	tcg gat gat ttt ccg gga ta	13800–13819	v29	tcg gta ctg taa ctt att tcc ggg t	14302–14326	526	
11	v33	taa tac gaa ttg ccg cgt cta a	14910–14931	v34	ttg cac acg aat taa ccg ga	15499–15518	608	
12	v38	agc gaa ctt ctc gcg ttt ta	17766–17785	v39	aag tct ccc atg gta ccg gta a	18430–18451	685	
16	v46	acg gca gaa cgg aat cca ctg	22980–23000	v47	cag tat aat tgg act ggg ccg	23530–23551	571	
21	vzv21f	taa tga att gag gcg cgg ttt a	33497–33518	vzv21r	cac gtg tag ctc caa aaa ac tag g	33976–33999	502	
22	ORF22R1f	ggg ttt tgt atg agc gtt gg	37837–37856	ORF21R1r	ccc ccg agg tcc gta ata tc	38264–38283	446	(Arvin, 1996; Carr et al., 2004)
22	v72	tac gtg gga cga tgc atg ga	39068–39087	v73	tcg aag tgg aac ccc atc agt	39699–39719	651	
28	v91	cag aag act gtg aca gtg ccc a	50869–50890	v92	acc ctc tcc gat aga cct gtg a	51498–51519	650	
35	v112	acg tct tcg ctc tct aac gga c	64163–64184	v113	acc ctc tcc gat aga cct gtg a	51498–51519	650	
37	v117	atc cat gtg tac ggc agc tca	67684–67704	v118	gag tca tcc ccg tgc atg tct	68377–68397	713	
38	PstA	ttg aac aat cac gaa ccg tt	69250–69269	PstB	cgg gtg aac cgt att ctg ag	69580–69599	349	(Grose et al., 2004; LaRussa et al., 1992)
40	v127	tga tgg cat atc agg cgt acg a	74412–74422	v128	agc agg acg tag tcc gcc ga	75093–75112	700	
40	v129	tcg gcg gac tac gtc ctg ct	75093–75112	v130	aag tcg gcc gag cgt atc ca	75093–75112	700	
43	v133	aca ggt caa tgc gaa ccg tgg tgt	78353–78376	v134	tcg gat ctg tgt tgg aag ttt gcg ct	78991–79017	664	
48	v143	tgg cac gat cgg gat tgg ata gga t	84668–84692	v144	tga tca gga gta cgg ata tca gcc gga	85269–85295	627	
50	vzv50f	cgc acc caa agt gaa cat cat	87736–87756	vzv50r	tct cgg atg tca aat atg tta cga	88227–88250	514	(Arvin, 1996; Carr et al., 2004)
54	Nla	gaa acc cct gca cca tta aa	95109–95128	Fok	tcc ctt cat gcc cgt tac at	95311–95330	221	(Grose et al., 2004, LaRussa et al., 1992)
54	v160	atc tgg gac agt tct gcc gct tg	95275–95295	v161	tga cca ggg ctt gaa tgg aag taa c	95888–95910	635	
56/57	v165	tgg gtt tgt gtc cat cgt aaa cgc t	98302–98326	v166	tgg gtc aga acc gac cca gga	99021–99041	739	
58	v167	cgt tct cgt acg tcc atg acc gct a	98613–98634	v168	ttc cgt acc gac ggc att gct t	100231–100252	1639	
60	62f	tct taa cca cga ttc ccg ata gc	101421–101443	62r	gta tat gag gcg tgg gac tat gc	101474–101496	75	(Loparev et al., 2007c)
66	V62-12	tgg cgc cat ctc aac atc a	113087–113105	v62-13	agg ttg cgc ttt gca gct aga	113549–113569	482	

Table 4
Key to wild-type VZV strains used in the complete genome alignment and genotyping studies

VZV strain	Genotype */**/***	Case type[¶]	Year of isolation	Country of isolation	Accession[#]	Reference
Dumas[‡]	E1/C/A	V	1970s	Netherlands	X04370	Davidson and Scott, 1986
BC	E1/C/A	Z	1999	Canada	AY548171	Grose et al., 2004
MSP	E1/C/A	V	1995	US	AY548170	Grose et al., 2004
SD	E1/C/A	N/A	1980	US	DQ479953	Peters et al., 2006
KEL	E1/C/A	Z	2002	US	DQ479954	Peters et al., 2006
36	E1/C/A	V	1998	Canada	DQ479958	Peters et al., 2006
49	E1/C/A	V	1999	Canada	DQ479959	Peters et al., 2006
32 p5	E1/C/A	V	1976	US	DQ479961	Peters et al., 2006
Russia	E1/C/A	Z	1999	Russian Fed.	EU154348	This study
NH293	E1/C/A	V	2000	US	DQ674250	This study
HJO	E2/B/D	Z	1990s	Germany	AJ871403	
11	E2/B/D	Z	1996	Canada	DQ479955	Peters et al., 2006
22	E2/B/D	Z	1998	Canada	DQ479956	Peters et al., 2006
03-500	E2/B/D	N/A	2003	Canada	DQ479957	Peters et al., 2006
CA123	M1/A/–	V	1990s	US	DQ457052	Norberg et al., 2006
Cng24	M1/A/–	V	1996	DRC[†]		This study
DR	M2/J/C	Z	2000	US	DQ452050	Norberg et al., 2006
8	M2/J/C	Z	1995	Canada	DQ479960	Peters et al., 2006
02-1-40	M3/–/–	V	2002	US		Sergeev et al., 2006
Sp4242	M4/–/–	V	2003	Spain		Loparev et al. 2007a
Fr4415	M4/–/–	V	2002	France		Loparev et al. 2007a
It4053	M4/–/–	V	2005	Italy		This study
pOka	J/J/B	V	1970	Japan		Gomi et al., 2002

Genotype nomenclature: *Loparev et al., 2004; **Barrett-Muir et al., 2003; ***Peters et al., 2006; – indicates no equivalent genotype detected. [¶]V=varicella, Z=herpes zoster, N/A=not available. [‡]Bold type indicates reference strain for this genotype. [†]Democratic Republic of Congo.

North America, Australia, and New Zealand (Loparev et al., 2004; Norberg et al., 2006; Peters et al., 2006). Isolates representing five VZV genotypes (E1, E2, M1, M2 and M4) were identified from cases of herpes zoster in this survey, indicating that all of these genotypes have been circulating in Europe for between five and eight decades. E1 strains are more common than E2 strains in Europe, North America, and Australia.

On the basis of limited SNP analysis reported here, E1 and E2 strains are closely related to M4 strains, with the closest similarity between E2 and M4. Since M4 strains appear to be of African origin (Loparev et al., 2007b), it would be useful to determine complete genomic sequence for one or more M4 viruses to clarify the evolution and natural history of these genotypes. For example, such information might provide evidence for the speculation that E2 and M4 viruses may have arisen in remote European colonies through recombination events. In those environments the recombinant strains might have effectively out-competed the original local strains and imported E1 strains, establishing themselves in the population after importation to Europe. Alternatively, E2 and M4 strains might have arisen in Europe in countries with E1 and M strains co-circulating, spreading afterwards to colonies; or recombinant variants of E2 could have been selected by temperate climates to which increasing numbers of people migrated from regions with warmer climates. Earlier studies indicated that VZV genotypes preferentially distribute based on warm versus cool climates (Loparev et al., 2004). We noted that M4 strains are more common than E2 strains in southern Europe.

Another important observation in this study was the complete absence of J strains in Europe. J genotype strains have been reported in the UK (Parker et al., 2006; Sengupta et al., 2007), but their protocol would not have distinguished between J and M2 isolates. J genotype strains do, however, appear to be entering into populations with a history of Asian immigration, such as western North America and Australia (Loparev et al., 2004, 2007b, Wagenaar et al., 2003), and J genotype may be competing with E1 for dominance in Australia (Loparev et al., 2007b). It appeared initially that J genotype strains were literally restricted to Japan, but more recently isolates have been identified in other Eastern Asian countries (unpublished data) and Australia (Loparev et al., 2007b), and isolates that were probably J genotype have been observed in Thailand (Wagenaar et al., 2003). In this respect, the distribution of wild-type J strains has important implications for future varicella vaccine adverse event surveillance in different regions of the globe.

Summary

These data illustrate the relationships between VZV genotypes that were circulating in Europe prior to the use of Oka varicella vaccine (genotype J), and may provide insights useful for interpreting events that could occur following the broad future introduction of the vaccine virus in regions originally free of J viruses. It also underscores the utility of this approach to VZV genotyping for the comprehensive identification of recognized genotypes, the detection of new genotypes, and for VZV vaccine strain surveillance.

Table 5
Comparison of recently reported genotyping methods for VZV strains

Genotyping method	Genotype designation							Reference
31-SNP	E1	E2	J	M1	M2	M3*	M4*	This report[¶]
Multi-SNP (MSNP)	C	B	J	A	J	n.e.	n.e.	Barrett-Muir et al., 2003; Campsall et al., 2004; Carr et al., 2004; Muir et al., 2004; Quinlivan et al., 2002
ORF22 region 1 (22R1)	E	E	J	M1	M2	M3	M4	Dayan et al., 2004; Loparev et al., 2000a, 2004; Sergeev et al., 2006
Whole Genome Canada (WGCan)	A	D	B	n.e.[†]	C	n.e.[‡]	n.e.[‡]	Peters et al., 2006
Whole Genome CDC (WGCDC)	E1	E2	J	M1	M2	n.e.[‡]	n.e.[‡]	Norberg et al., 2006; Loparev et al., 2007b

*Provisional genotypes based on targeted SNP analysis. No complete sequences are available at this time. †CA123 sequence (M1 strain) was published subsequent to the publication of this method. ‡ Cannot be determined by this method since complete sequences are unavailable. ¶Determined using the two-amplicon method.

Genotypic analysis is further advanced for VZV than for any other human herpesvirus, with the possible exception of HHV-8. Studies of VZV genomic diversity have led to the identification of multiple, stably circulating genotypes, to the establishment of the distinctive global distribution of genotypes, and to the observation that this distribution is in flux in countries that encourage immigration. In this study, we also demonstrate that methods used for genotyping the highly conserved VZV need to strike a reasonable balance between whole genome sequencing (impractical as a routine laboratory tool) and targeted sequence analysis. Since all of the herpesviruses have relatively stable DNA genomes, it is likely that some of the obstacles overcome for effectively genotyping VZV will be extendable to other members of the herpesvirus family.

Materials and methods

Patients

342 specimens from 217 cases of varicella and 83 cases of zoster were collected between 1998 to 2006 from patients seen in local hospitals in Albania, Belarus, Bulgaria, Czech Republic, Estonia, Finland, France, Germany, Greece, Iceland, Italy, Latvia, Lithuania, Poland, Romania, Russian Federation (European region), Spain, and Ukraine. The origin of several strains was described in a previous publication (Loparev et al., 2004). Thirty-eight of the isolates had no available data on clinical presentation. DNA from 67 of the isolates was obtained from cells infected with VZV recovered from vesicular swabs.

Molecular epidemiology

VZV DNA positive specimens were placed on Whatman FTA filters and stored at room temperature. DNA samples obtained from Iceland, Estonia, Lithuania, Latvia, Germany, Poland, and France were extracted from 70 µl of cell suspension infected with single passage VZV from the isolates. Specimens were applied to FTA cards (Whatman, Inc., Florham Park, NJ) to inactivate virus, and were shipped to CDC for VZV genotyping. One 3-mm-diameter punch from FTA cards was prepared for PCR according to the manufacturer's instructions. In several instances filters were washed with Whatman FTA washing reagent and reused in several PCR reactions.

VZV Oka vaccine virus was discriminated from wild-type viruses by evaluating the SNP at positions ORF62 (106262, 107252, and 107136) using real-time FRET (fluorescent resonance energy transfer)-based PCR as previously described (Loparev et al., 2000b, 2007c; Sauerbrei et al., 2004). We also performed quantitative real-time PCR with the Minor Groove Binding (MGB) Eclipse probe System for Allelic Discrimination (Epoch Biosciences, Inc.) to evaluate VZV Oka vaccine-specific SNP, including the MspI/NaeI restriction site at position 107252, using an approach similar to that described by Campsall et al. (2004). Briefly, modified MGB Eclipse primer mixes contained forward and reverse primers for amplification 5′-GCC CAA AAA CAC TTT ATC CTA C and 5′-GTT GTT GGA GAA GGG TGA; MGB Dark Quencher Eclipse probe Mix — GCC TTT GCC AGC-(FAM) wt probe and (Dark Quencher CPG) 5′-GAG CCT TTG CCG G-(TET) mutant probe were used for detection. To ensure adequate DNA to perform multiple (10–20) amplification procedures, only samples with at least 1×10^6 VZV genome copies were used for procedures requiring extensive SNP analysis. The PstI and BglI restriction sites in ORF 38 and 54 were evaluated using fluorescent probes as previously described (Dayan et al; 2004). For ORF22 genotyping, the forward and reverse primers (p22R1f and p22R1r) were designed to amplify a 447-bp fragment (positions 37837 to 38264) that contains 4 SNP capable of resolving most of the recognized genotypes of VZV (Loparev et al., 2004; Sengupta et al., 2007). Multi-locus DNA sequence analysis at ORF 1, 3, 6, 8, 9, 10–12, 16, 17, 21, 22, 28, 35, 37, 40, 43, 48, 50, 54–56, 58, 60, 62, 66 was performed on amplicons produced using the primers listed in Table 3. DNA sequence was determined on an ABI Prism 3100 DNA sequencer (Perkin-Elmer, Foster City, CA.) using the BigDye terminator method as previously described (Loparev et al., 2004). All genomic loci refer to the published sequence for the Dumas strain (GenBank accession #9625875). The published wild-type genomic strain sequences used in these analyses are identified in Table 4. The Russia_1999 strain was isolated in 1999 from an adult with herpes zoster in the Russian Federation. The complete sequence for this strain has been deposited in GenBank (accession number EU154348). For ease of comparison with other reports in the literature, we have included a key to all nomenclature systems for VZV in current use (Table 5).

Bioinformatics

The CLUSTALW v1.83 and BioEdit Sequence Alignment Editor for Windows 95/98/NT/XP www.mbio.ncsu.edu/BioEdit/bioedit.html) programs were used to analyze all of the complete VZV genomic sequences currently available, and specifically to generate sequence alignments and phylogenetic trees. Each alignment was inspected and optimized to remove the 5 repeat regions (R1 to R5) and length variability in the origin of replication. Bayesian posterior probability inference of phylogeny used MrBayes version 3.1. MrBayes settings for the best-fit model (GTR+I+G) were selected by hLRT in MrModeltest 2.2. Phylogenetic trees were generated using TREEVIEW. Nucleotide sequences obtained from targeted sequencing of three M4 isolates from France, Spain and Italy were analyzed and aligned with the Sequencher program version 4.6 (Gene Codes Corp., Ann Arbor, MI, USA). Each alignment was inspected and Genetics Computer Group Wisconsin Package, version 10.3 (Accelrys, San Diego, CA, US) phylogenetic analyses were performed by using PAUP version 4.01 (Sinauer Associates, Sunderland, MA, US). All phenograms were drawn as unrooted trees.

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