Toward Universal Varicella-Zoster Virus (VZV) Genotyping: Diversity of VZV Strains from France and Spain[▽]

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Thirty-one isolates from France and Spain were genotyped using a published method analyzing DNA sequence variation in open reading frame (ORF) 22, together with an evaluation of three well-characterized single nucleotide polymorphisms (SNP) in ORF 38, 54, and 62. Nineteen were allocated to the European (E) genotype, six were mosaic-1 (M1), and two were mosaic-2 (M2). Four strains were assigned to a new genotype, mosaic-4 (M4). All isolates were wild type, with no Oka vaccine-associated markers. No isolates of the mosaic-3 (M3) or Japanese (J) genotype were observed. We also evaluated 13 selected isolates of E, J, M1, and M2 strains (9 of the 31 described above) using an alternative genotyping method based on the assessment of multiple SNP located in ORF 1, 9, 10, 21, 31, 50, 54, 62, and 68. This method assigns wild-type varicella-zoster virus (VZV) strains to seven genotypes: A1, A2, J1, B1, B2, C, and C1. VZV isolates identified as E (ORF22 method) had the genetic signature of genotype C VZV strains, M1 strains were A1, and M2 were A2. No J strains were detected, but parental Oka and vaccine Oka (genotype J) corresponded to genotype J1. M4 isolates (B) share the SNP array observed for M1 and E viruses, and probably represent recombinants between African-Asian (M1) and European (E) viruses. The two genotyping methods, using entirely different genomic targets, produced identical clusters for the strains examined, suggesting robust phylogenetic linkages among VZV strains circulating in Europe.

Varicella-zoster virus (VZV) is a highly infectious, nearly ubiquitous pathogen that affects all human populations. However, the epidemiology of VZV infection varies geographically (2, 10, 13, 14). In countries with a temperate climate, most children have a primary VZV infection at school age, with marked seasonal increases in varicella cases in the springtime, and <1 to 3% of people remain susceptible to infection past the age of 20. In contrast, in tropical countries, particularly rural populations, >10 to 20% of the population may remain susceptible to VZV infection well into adulthood (3, 14). Several studies have demonstrated a distinctive geographic distribution of the major VZV genotypes aligning with cool versus warm climate regions of the globe (4, 16, 19). It is unclear whether the strain distribution is actually driven by climate or other factors, such as immigration patterns.

The implementation of routine universal varicella vaccination in the United States created a need to distinguish wild-type infections from vaccine injuries, and as United States varicella incidence declines to near zero, individual VZV strain surveillance will become essential to continued monitoring of vaccine impact. VZV has only a single recognized serotype, and the viral genome

is highly conserved (9, 16, 19); as such, conventional phylogenetic approaches to viral strain identification are not readily applied to VZV. Nonetheless, several methods for VZV strain identification and genotyping have been independently developed and are currently in use (4, 6, 11, 12, 16).

While interstrain genomic variation for VZV is limited to about 0.1% and consists almost entirely of single-nucleotide changes dispersed evenly across the genome, nucleic acid variation that facilitates the genotyping of wild-type VZV isolates has been identified (4–7, 10–12, 16, 19, 21, 22). Restriction fragment length polymorphism has been used to characterize VZV wild-type isolates and distinguish them from the Oka vaccine strain, taking advantage of single nucleotide polymorphisms (SNP) in open reading frame 38 (ORF38) (+ or – for a PstI site) and ORF54 (+ or – for a BglII site). The vaccine is PstI[–] BglII⁺ at these loci (11, 12). All Japanese VZV isolates characterized thus far are either PstI⁺ BglII⁺ or PstI[–] BglII⁺, while the majority of U.S. isolates have a PstI⁺ BglII[–] profile (11, 12, 15). The PstI⁺ BglII⁺ profile also predominates in Europe and eastern Australia (12, 16). Thus, most of the time the detection of an ORF54 BglII restriction site in VZV strains taken from the United States, Europe, or eastern Australia is a reliable marker for Oka vaccine. However, BglII⁺ strains are common not only in Japan but in most tropical regions (10, 12, 16, 19), and BglII⁺ wild-type strains have been isolated in North America and Australia. As such, it is by no means an infallible marker for the vaccine strain outside Japan. BglII[–] strains (with the exception of Japan) are predominant in temperate climates

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TABLE 1. Country of primary varicella infection (in zoster patients) based on patient recall

Specimen no. and country of specimen collection	Disease (country where primary infection occurred)	Patient age (yr)	Patient type ^a	Department	VZV genotype
1, France	Zoster (France)	27	H	Internal medicine	M1
2, France	Zoster (Algeria)	37	A	Infectious diseases	M4
3, France	Zoster (Italy)	61	H	Internal medicine	E
4, France	Zoster (France)	78	A	Stomatology	E
5, France	Zoster (Portugal)	43	H	Internal medicine	E
6, France	Zoster (France)	37	A	Infectious diseases	E
7, France	Zoster (Portugal)	57	H	Hematology	E
8, France	Zoster (France)	26	H	Hematology	E
9, France	Zoster (France)	37	A	Infectious diseases	E
10, France	Zoster (France)	31	H	Infectious diseases	E
11, France	Zoster (France)	32	H	Hematology	E
12, France	Zoster (Cameroon)	40	H	Infectious diseases	M1
13, France	Varicella	27	H	Obstetrics	E
14, France	Zoster (Portugal)	55	A	Internal medicine	E
15, France	Varicella	26	H	Infectious diseases	M4
16, France	Zoster (Algeria)	60	A	Internal medicine	M2
17, France	Zoster (Columbia)	37	A	Infectious diseases	E
18, France	Varicella	37	A	Infectious diseases	E
19, France	Varicella	10	H	Hematology	E
1, Spain	Varicella	0.2	A	Pediatric emergency	M1
2, Spain	Varicella	2.7	A	Pediatric emergency	E
3, Spain	Varicella	3.6	A	Pediatric emergency	M4
4, Spain	Varicella	1.1	A	Pediatric emergency	E
5, Spain	Varicella	3.5	A	Pediatric emergency	E
6, Spain	Varicella	2.3	A	Pediatric emergency	E
7, Spain	Varicella	0.4	A	Pediatric emergency	E
8, Spain	Varicella	7.1	A	Pediatric emergency	E
9, Spain	Varicella	2.7	A	Pediatric emergency	E
10, Spain	Varicella	7.3	A	Pediatric emergency	E
11, Spain	Varicella	3.5	A	Pediatric emergency	E
12, Spain	Varicella	2.1	A	Pediatric emergency	M1

^a A, ambulatory; H, hospitalized.

all over the world (16), and the site has served as an important marker for Asian, African, and Japanese strains (18).

A heteroduplex mobility assay (HMA) was recently used to characterize VZV genomic variation among strains circulating in the United Kingdom and elsewhere (3, 4), and SNP were identified that are useful for VZV genotyping. HMA evaluates a combination of selected SNP in ORF 1, 9, 10, 21, 31, 50, 54, 62, and 68. The same laboratory attempted to improve this method, employing a broader panel of SNP, but the modified HMA led to essentially equivalent results (4, 18). Four major clades were distinguished using HMA (A, B, C, and J) of VZV isolates collected worldwide (4). VZV genotypic variations were associated with the geographical region in which infection was acquired: clade A strains had an African-Asian distribution; clades B and C were mainly found in Europe. J clade strains were associated with Japan (18, 19). Evidence for recombination between A and C strains was also observed, and these strains were assigned to a separate VZV clade B. Clade B has never been observed in the United States (20) and shares the ORF 54 Bgl⁺ marker with C strains. Another group examined clinical isolates of VZV collected in the Republic of Ireland for genetic variation by SNP analysis using HMA. VZV strains representing same 4 genotypes (A, B, C, and J) were also identified in this country (6).

We developed a novel strategy for VZV genotyping based on sequencing of a short region in ORF22 (447 bp) using DNA amplified directly from clinical samples without virus isolation (16). Using this method, more than 500 VZV strains isolated in

the United States and other countries around the world were sorted into three discrete geographically distributed genotypes: E (European), J (Japanese), and M (mosaic) (16, 20). Mosaic strains, which are Bgl⁺, predominated in tropical latitudes. M genotype strains carry assortment of E- and J-like SNP in ORF22. This method also resolves a number of distinct subgenotypes M type viruses, designated M1, M2, M3, and in this report, M4 (16).

In this study we compared two VZV genotyping methods: ORF22-based genotyping (16) and HMA (4, 6). Genotypes assigned to 31 strains obtained from recent cases of varicella and zoster in France and Spain indicated a robust correlation between the two approaches to genotyping. This was true despite the lack of any overlap between the genomic targets used by these methods. We also identified and characterized a new Bgl⁺ strain using the ORF22 method, designated M4, that corresponds to the strain B viruses identified using HMA.

MATERIALS AND METHODS

Patients and specimens. Patient information is summarized in Table 1. Nineteen specimens were obtained from cases of varicella or zoster in France from 1998 through 2004 (age range, 10 to 78 years; mean age, 42 years); 11 patients were ambulatory, and 8 were hospitalized. All specimens from France were frozen infected MRC5 cells that had been inoculated with material from vesicular swabs. For zoster patients, the country in which primary varicella infection occurred was recorded. Twelve vesicular swab specimens were obtained from cases of pediatric varicella in Spain (age range, 3 months to 7.3 years; mean age, 3 years). All were seen at the emergency room or for outpatient visits to Hospital Universitari Germans Trias i Pujol in Badalona during the spring of 2004. A sterile swab was used to collect

TABLE 2. Comparison of genotyping results by ORF22 and HMA methods^a

Strain identification	No. of isolates	ORF54 BglI site	ORF38 PstI site	ORF62 SmaI site	Genotype result by method:	
					ORF22	Multi locus
Dumas	1	—	+	—	E	C
France 2004	14	—	+	—	E	C
Spain 2004	8	—	+	—	E	C
France 2004	2	+	+	—	M1	A1
Spain 2004	2	+	+	—	M1	A1
France 2004	1	+	+	—	M2	A2
France 2004	2	—	+	—	M4	B
Spain 2004	2	—	+	—	M4	B
Oka vaccine	1	+	—	+	J	J1
Parental Oka	1	+	—	—	J	J1

^a Although restriction enzyme sites are indicated, SNP were detected using fluorescent resonance energy transfer-based melt curve analysis. Oka vaccine viral DNA was prepared from a commercial lot of varivax (Merck and Co.). Dumas is the laboratory European reference strain; parental Oka is the laboratory Japanese reference strain.

virus from the base of a single unroofed vesicular lesion for patients recruited in Spain. In the case of samples from France, 70 µl of suspended cells infected with single-passage VZV isolate was tested. Specimens were applied to FTA cards (Whatman, Inc., Florham Park, NJ) to inactivate virus for shipment to the CDC for VZV genotyping. A subset of 9 isolates (of 31, representing genotypes E, M1, M2, and M4) were also evaluated using HMA (18). Two U.S. isolates (1 M1 isolate, 1 M2 isolate) and the Oka parental strain (genotype J) were also evaluated by HMA.

Molecular epidemiology. One to three 1-mm-diameter punches from FTA cards were prepared for PCR according to the manufacturer's instructions. In several instances, FTA filters were washed after use with Whatman FTA washing reagent and reused for additional PCRs. PCR-based VZV diagnostic assays were performed as described previously (4, 16).

To distinguish the VZV Oka vaccine strain from wild-type viruses, the ORF 62 SNP at position 106262 was determined using fluorescent resonance energy transfer-based PCR performed on a LightCycler (Roche, Pleasanton, CA) as previously described (17). Vaccine SNP in ORF 38 (PstI) and ORF 54 (BglI) were also evaluated using the same technology (8, 16).

The PCR forward and reverse primers (p22R1f and p22R1r) were designed to amplify a 447-bp fragment (positions 37837 to 38264) of VZV ORF22 as described in reference 16. Sequence variation observed between the Dumas strain (E genotype, GenBank accession no. 9625875) and Oka parental strain (J genotype, GenBank accession no. 26665422) at 4 polymorphic loci in the amplicon was used to assign genotype (16). J genotype strains have identity at all four single-base polymorphisms in the ORF22 fragment (corresponding to the bases displayed in the pOka reference J strain), as do E genotype strains (corresponding to the bases displayed by the Dumas reference E strain). The M genotype is more heterogeneous and probably comprises multiple genotypes distributed preferentially in subtropical and tropical regions as well as strains imported into temperate countries. M genotype strains carry a combination of E and J markers in the ORF22 fragment. HMA sequence analyses at ORF 1 (positions 560, 561, 685, 703, 750, 763, 766, 789, 790, 791, 829, and 892), ORF 21 (positions 33646, 33647, 33722, 33725, and 33728), ORF 31 (positions 57224, 57301, 57397, and 57955), ORF 37 (positions 66646 and 68142), ORF 50 (position 87841), ORF 54 (positions 95108, 95118, 95241, 95262, 95300, 95333, and 95339), and ORF68 (positions 116255, 116320, 116467, and 116762) were performed as described by Carr et al. and Muir et al. (6, 18).

RESULTS

Characterization of VZV isolates using vaccine markers and ORF22-based genotyping. We analyzed genetic variation among VZV strains obtained from varicella and zoster patients in France and Spain. The 31 specimens were first confirmed as PCR-positive, wild-type VZV using PCR targeting SNP in ORF 38, 54, and 62. None of the VZV strains included in the study carried the SmaI restriction site in ORF62, position 106262 (Table 2) and, as such, were wild-type strains (1, 15,

17). Then 31 strains were genotyped through sequence analysis of the ORF22 amplicon (Table 2). Using this approach, 22 isolates were genotyped as E, 4 as Mosaic variant 1 (M1), and 1 as M2. Finally, 2 strains from France and 2 from Spain displayed a novel combination of mutations at four loci (A, position 37902; T, position 38055; C, position 38081; A, position 38177) and were assigned to a new mosaic variant, M4 (Fig. 1). In addition, all 19 E strains were PstI⁺ BglI[−], and M1

ORF22 Method		Genotype E					M1		M2		M4		J	
Multi-locus		Genotype C					A1		A2		B		J1	
ORF	Position (Dumas ref)	Dumas	France 1	France 2	Spain 1	Spain 2	France	Spain	US Isolate	France	US Isolate	France	Spain	pOka
	236	A	A	A	A	A	A	A	A	G	G	ND	ND	G
1	560	T	T	T	T	T	T	T	T	T	T	T	T	T
	561	G	G	G	G	G	G	G	G	G	G	G	G	G
	685	G	G	G	G	G	G	G	A	A	G	G	A	A
	703	T	T	T	T	T	T	T	T	T	T	T	T	T
	750	G	G	G	G	G	G	G	G	G	G	G	G	G
	763	T	T	T	T	T	T	T	T	T	T	T	T	T
	766	A	A	A	A	A	A	A	A	A	A	A	A	A
	789	T	T	T	T	T	C	C	C	C	C	T	T	C
	790	T	T	T	T	T	C	C	C	C	C	T	T	C
	791	T	T	T	T	T	C	C	C	C	C	T	T	C
	829	T	T	T	T	T	T	T	T	T	T	T	T	T
	892	C	C	C	C	C	C	C	C	C	C	C	C	C
9	11906	A	A	A	A	A	G	G	G	G	ND	ND	G	
10	11285	T	T	T	T	T	C	C	C	C	C	ND	ND	C
	11286	T	T	T	T	T	C	C	C	C	C	ND	ND	C
21	33646	G	G	G	G	G	G	G	G	G	G	G	G	G
	33647	A	A	A	A	A	A	A	A	A	A	A	A	A
	33722	T	C	C	C	C	C	C	C	C	C	C	C	C
	33725	T	T	T	T	T	C	C	C	C	C	C	C	C
22	33728	T	T	T	T	T	C	C	C	C	C	C	C	C
	37902	A	A	A	A	A	A	A	A	A	A	A	A	G
	38055	T	T	T	T	T	T	T	T	C	C	T	T	C
	38081	A	A	A	A	A	C	C	C	C	C	C	C	C
31	38177	G	G	G	G	G	G	G	A	A	A	A	A	A
	57224	A	A	A	A	A	A	A	A	A	A	ND	ND	G
	57301	A	A	A	A	A	C	C	C	C	C	ND	ND	C
	57397	G	G	G	G	G	G	G	G	G	G	ND	ND	T
50	87841	C	C	C	C	C	T	T	T	T	T	T	T	T
54	95108	C	C	C	C	C	C	C	C	C	C	C	C	C
	95118	G	G	G	G	G	G	G	G	G	G	G	G	G
	95241 (Bgl)	T	T	T	T	T	C	C	C	C	C	T	T	C
	95262	G	G	G	G	G	G	G	G	G	G	G	G	G
	95300	C	C	C	C	C	T	T	T	C	C	C	C	C
	95333	T	T	T	T	T	T	T	T	T	T	T	T	T
62	95339	C	C	C	C	C	C	C	C	C	C	C	C	C
	107165	C	C	C	C	C	T	T	T	T	T	ND	ND	T
	107307	T	T	T	T	T	C	C	C	C	C	ND	ND	C
	108747	A	A	A	A	A	G	G	G	G	G	ND	ND	G
68	108951	G	G	G	G	G	A	A	A	A	A	ND	ND	A
	116255	G	G	G	G	G	G	G	G	G	G	G	G	G
	116320	C	C	C	C	C	C	C	C	C	C	C	C	C
	116467	T	T	T	T	T	C	C	C	T	T	C	C	T
	116869	T	T	T	T	T	G	G	G	T	T	ND	ND	T

FIG. 1. pOka is the parental Oka strain from which varicella vaccine was derived. Sequence positions are based on the published genomic sequence for the Dumas strain. Green cells indicate European genotype (E) markers; yellow cells are Japanese genotype (J) markers, rose cells are markers unique to various M genotype variants, and uncolored cells reflect markers that are consistent across genotypes. ND, not determined.

and M2 mosaic strains were PstI⁺ BglI⁺, in contrast with the Oka parental control, which carried the PstI⁻ BglI⁺ profile typical of J strains (Table 2).

Comparison of VZV genotyping methods. Results obtained by HMA (together with the ORF22 results) are presented in Fig. 1. Targeted sequences in ORF1, 9, 10, 21, 31, 50, 54, 62, and 68 revealed nearly perfect identity of all E strains with the reference E strain Dumas. Only a single difference at position 33722 was detected and only in the Dumas laboratory strain. All French and Spanish VZV isolates identified as E by our method belonged to genotype C using HMA. Similarly, isolates that were M1 and M2 by the ORF22 method were A1 and A2, respectively, by HMA. No J genotype strains were collected from these European countries, but control strain parental Oka vaccine virus (Fig. 1) as well as 3 other Japanese wild-type viruses (data not shown) corresponded to J1 genotype using HMA. Four specimens (2 each from Spain and France) belong to genotype B using HMA. These strains were identical to each other at all tested loci and were assigned to a new variant genotype (M4) using the ORF22 method, based on single-amplimer sequencing. This variant has common J-, M1-, M2-, and E-associated SNP and could represent a recombinant genotype. M4 strains also carry the PstI⁺ BglI⁻ marker profile characteristic of E genotype isolates, distinguishing them from M1, M2, and M3 variants, which are PstI⁺ BglI⁺ at ORF 38 and 54. At the same time, SNP in position 116467 for the M4 strain (Fig. 1) were identical to 25 tested M1 isolates from Central Africa, India, and Bangladesh (unpublished observation).

DISCUSSION

The VZV genome is very highly conserved, complicating strain genotyping and surveillance; nonetheless, there is evidence from several studies that the genotypes identified by several recent approaches are robust and have distinctive geographic distributions (16, 18, 19). Previous studies have shown that in Europe and other temperate climates, E genotype VZV strains (C genotype by HMA) predominate (16). In the current study, analysis of 44 SNP in open reading frames (ORF 1, 9, 10, 21, 22, 31, 38, 50, 55, 54, 62, and 68) identified considerable homology among genotype E strains collected from varicella and zoster patients from different regions of France and Spain. Among E genotype strains, only a single difference at position 33722 was observed and only in the Dumas laboratory strain. Given that this variation may be associated with prolonged cultivation in tissue culture, it may be advisable to exclude this mutation from genotyping protocols. Twenty-two of 31 isolates (71%) were E genotype; the remaining 9 isolates were of various M genotypes, 3 of which were collected from patients who experienced their primary varicella infection while living in a climate where M strains predominate. A restriction fragment length polymorphism-based method modified for use with fluorescent probes showed all 22 E isolates from France and Spain had a PstI⁺ BglI⁻ profile similar to strains circulating in North America and Argentina (8, 12). Using HMA (18), the results were identical for all of the targeted SNP; all 22 E isolates were C genotype using this approach. This was not surprising, given that 98% of the population in France and Spain were European residents. However travel from Africa to

Europe is common, and accordingly, mosaic strains M1 and M2 (probably imported from tropical countries) were also identified among the isolates. In countries with tropical climates (Guinea Bissau, Zambia, Bangladesh, and southern India), circulating VZV strains usually have a BglI⁺ marker in ORF54 and have characteristic M SNP (15, 19). We identified 4 belonging to a new mosaic variant, M4, which is BglI⁻ and has an SNP profile consistent with recombination between M and E genotypes. This genotype has never been observed in the United States. We propose that M4 could have evolved recently from a tropical M strain(s) of African or Asian origin that was less able to compete with E strains in temperate climate regions or from an E strain in Europe. The recombinant M4 variant may have been selected by temperate climates to which increasing numbers of people migrated from tropical regions. The migration of persons native to tropical regions has been an ongoing and increasingly common occurrence in France and Spain for a number of decades. Of the five M strains isolated in France in this study, 3 came from zoster patients who developed varicella while living in Africa (2 in Algeria, 1 in Cameroon). The two remaining M isolates came from patients who were infected in France but were of Algerian origin and likely were living with persons native to Algeria. In contrast, none of the E isolates were obtained from patients with an African history. The M4 strains circulating in Spain, France, Great Britain, and Ireland are identical at the SNPs examined here. No J strains were detected in France or Spain during this study; J strains have been detected thus far only in Japan, Korea, and rarely, in countries with substantial immigration of persons native to those countries, notably the United States, Canada, and Australia (16, 19, 20). Thus, if a recombination event was responsible for the establishment of the M4 variant, it was unlikely to involve a J genotype strain.

Although certain genotypes of VZV predominate in specific geographic regions, generally associating with climate, strain diversity has been generally observed among VZV isolates in the United Kingdom, Brazil, and the United States that is attributed to the increased mobility of human populations in recent history (4, 12, 16, 19). Varicella strain diversity in countries with active immigration has modulated molecular epidemiology patterns that remain more distinctly defined in regions where immigration is uncommon or discouraged (10, 19). Thus, the epidemiological pattern of currently circulating VZV strains is apparently in flux, with an interruption of strain distribution patterns that were previously established by climatic conditions and/or the relative isolation of human populations. The current study reveals a pronounced identity of phylogenetic links between VZV strains circulating in France and Spain, as evidenced by the complete concordance of two distinct genotyping methods with nonoverlapping genomic targets, and tends to support this hypothesis (16). The data presented here demonstrate the utility and comparability of both VZV genotyping strategies, which sorted wild-type VZV strains into identical phylogenetic clusters. The availability of technically accessible, reliable methods for genotyping VZV strains will serve a critical function in countries with broad varicella vaccination policies, since tracking individual strains and identifying probable sources of infection is needed to effectively monitor vaccine impact. In addition, the study of global VZV genotype patterns will likely lead to a better un-

derstanding of global transmission patterns both before and after vaccination and of the evolutionary trends of this nearly ubiquitous virus.

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