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Varicella-zoster Virus Clades Circulating in Spain over two decades

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Abbreviations: Varicella-zoster virus (VZV), Cerebral Spinal Fluid (CSF), Open reading frame (ORF), vaccine-OKA (vOKA), base pair (bp), years old (y.o.), Single Nucleotide Polymorphisms (SNPs).

ABSTRACT

Background: Despite childhood universal VZV immunization was introduced in 2015, there are no data on VZV clade distribution in Spain.

Objectives: To characterize the varicella-zoster virus strains circulating in Spain between 1997 and 2016.

*Study Design***:** In this retrospective study, we determined the VZV clades in 294 patients with different pathologies (mainly encephalitis, zoster and varicella) by sequencing three fragments within ORF 22, ORF 21 and ORF 50 and, subsequently analyzing 7 relevant SNPs.

*Results***:** Among these 294 patients, 132(44.9%) patients were infected by clade 1, 42(14.3%) patients by clade 3, 19(6.5%) by clade 5, 29(9.9%) by clade VI and 3(1%) by clade 4. Four patients (1.4%) were infected by clade 2 vOKA strains, who received one dose of live-attenuated varicella vaccine. Putative recombinant clade 1/3 was identified in 6 cases (2.0%). Results obtained from partial sequences were assigned to clade 1 or 3 in 56(19%) patients and clade 5 or VI in 3(1.0%) patients. In the multivariate analysis, encephalitis was independently associated with clades 1 and 3 and age >14y.o. (P=0.035 and P=0.021, respectively). Additionally, Madrid had significant fewer cases of encephalitis compared with the rest of regions analyzed (P=0.001).

*Conclusions***:** Higher prevalence of clades 1 and 3 and their relation with encephalitis and age >14y.o. suggest earlier introduction of this clades in Spain. Putative interclade 1 and 3 recombinants are circulating in patients with encephalitis, herpes zoster and varicella. Several cases were related to vOKA vaccination but vaccine strains do not seem to circulate in the general population.

1. Background

Varicella-zoster virus (Human herpesvirus 3) is the etiologic agent of chickenpox (varicella) following primary infection and shingles (herpes zoster), which results from reactivation of latent virus [1]. In Spain, varicella vaccination was recommended in susceptible children between 10 and 14 y.o. since 2005 [2]. From 2005 to 2011, the incidence of VZV and the rate of hospitalized children decreased 16% and 64%, respectively [3]. Childhood universal immunization at the age of 15 months was introduced in 2015, although some regions initiated this schedule earlier. The attenuated vaccine virus consists of heterogeneous Oka strain derived from a parental OKA clinical isolate, and it has been commercialized under the names of VarilRix and VariVax.

Vaccination policies should rely on epidemiological surveillance, including the molecular characterization of circulating viruses producing, not only varicella, but also herpes zoster or neurological disease, in order to evaluate the impact of vaccine programs on the incidence and the pattern of viral circulation. Moreover, since recombination events are frequent in VZV [4], surveillance studies are also relevant for evaluating the emergence of new vaccine/wildtype or wildtype/wildtype recombinants that can result from increased rates of vaccination or migration, respectively.

Different genotyping SNP-based methodologies have been published [5, 6]. The CDC located a 447 bp fragment upstream of the C-terminal coding region of the ORF 22 that was highly polymorphic and allowed the identification of four genotypes [7]. Following this initial publication, SNPs from ORF 21 or ORF 50 were added to those used originally in the ORF 22 genotyping scheme [8, 9]. Using this strategy, up to seven stable genotypes were identified (E1, E2, M1, M2, M3, M4 and J) within three genogroups (E, M and J). At the international VZV Nomenclature Meeting in 2008, a common nomenclature based on phylogenetic clades was agreed upon and seven clades were established: Clade I (E1), Clade 2 (J), Clade 3 (E2), Clade 4 (M2); Clade 5 (M1), Clade VI (M4) and Clade VII (M3) [10].

Clade 1 and 3 are the most prevalent in Western Europe and in areas of the world predominantly settled by Europeans, while clade 2 was predominant in Japan and the surrounding countries. Clades 4 and 5 are mostly prevalent in Asian and African countries [9, 11, 12, 13]. However, more recently several groups observed an increased circulation of viruses from clade 5 [14, 15].

In Spain, there are no data on VZV clade distribution, except for the 31 patients from Catalonia diagnosed with varicella that were included in the study of Loparev et al. [16].

2. Objectives

The aim of this study was to characterize VZV clade distribution using clinical samples from patients with different pathologies that were received at the National Center of Microbiology in Spain from 1997 to 2016.

3. Study Design

3.1. ***Patients and Specimens:*** Clinical specimens were received at the CNM from 78 hospitals from different geographical areas of Spain for virological diagnosis between 1997 and 2016. Clinical and demographic data were available and included sex, age, type of infection and whether or not it was a vaccine suspected case. Sequences from 7 VZV strains were used as reference control for sequence alignment: Strain Dumas, clade 1 (accession number X04370), Strain pOKA, clade 2 (accession AB097933); Strain HJO, clade 3 (accession number AJ871403); Strain DR, accession DQ452050, clade 4; Strain CA123, accession DQ457052, clade 5; **Strain Sp4242, clade VI [9];** Strain **02-1-40, clade VII [10].** This project was approved by the Ethics Committee of the "Instituto de Salud Carlos III" (CEI PI 68_2017-v2).

3.2. ***DNA extraction, PCR and sequencing*****:** DNA was extracted using the Virus Pathogen Midi Kit in the Qiasymphony robot platform, according to the manufacturer's instructions (Qiagen). In order to increase the number of sequence data, a novel nested PCR was designed to amplify regions of

three different ORFs (ORF 21, ORF 22 and ORF 50), primer sequences are shown in Table 1. The first PCR amplification round was performed as previously described [9] and for the second round the following conditions were used: a denaturalization step at 95ºC for 5 min, followed by 45 cycles of 94ºC 30sec, 55ºC, 58ºC for ORF 22 and ORF21/50, respectively for 30 sec and 72ºC 45 sec, eventually an elongation step at 72ºC for 15 min. All reactions were carried out using the Platinum SuperFi DNA Polymerase reaction kit (Invitrogen). PCR products were processed for Sanger dideoxy sequencing with BigDye v. 3.1 (Applied Biosystems) in a ABI PRISM 3100 sequencer (Applied Biosystems, California, USA).

3.3. ***DNA sequence analysis, clade determination and characterization of wild-type vs. vaccine VZV strain:*** DNA sequence analysis was carried out using the Lasergene SeqMan software. Aligments of raw sequence data were performed against expected amplicons using the Dumas strain as a reference sequence (GenBank accession number X04370). Determination of the clades was based on the genotyping strategy proposed by Loparev et al. [8] and adapted to the new clade nomenclature [10] that consisted of sequencing a fragment of ORF22 containing 4 SNPs and either ORF21/50 containing 2 SNPs and 1 SNPs, respectively.The three amplicons and all relevant SNPs positions at 37902, 38055, 38081, and 38177 for ORF22, positions 33725 and 33728 for ORF21 and position 87841 for ORF50 were sequenced and analyzed. Clade 2 VZV strains were further investigated to determine whether they were vaccine-related VZV by analyzing the three vaccine-specific SNPs located in the ORF62 at position 106262, 107136, and 107252 [17].

3.4. ***Statistical analysis:*** A descriptive analysis of the variables was performed. Categorical variables were analyzed by the Chi-square test or Fisher exact test. Multivariate logistic regression model was used to evaluate the possible confounding factors of having genogroup E and the development of encephalitis. Variables included in the model were those that had significance in the bivariate analysis (such as genogroup and region) or those that had clinical relevance (such as age and year

of detection). In all statistical analysis, conditions of application have been checked to be applied in each test. Results were analyzed using SPSS version 15.0 software (SPSS, Chicago, IL). Association was expressed by odds ratio (OR) and the 95% confidence interval (95% CI). Differences were considered statistically significant when *P* values were below 0.05.

4. Results

4.1. ***Study population*****:** Table 2 summarizes baseline characteristics of the patients included in the study. Detailed information for each patient regarding age, year of detection, pathology and particular SNPs is shown in table 1 of supplementary material. Clinical samples used in this study were residual samples available after performing the PCR test for diagnosis. Thus, a total of 294 VZV PCR-positive clinical samples from 294 patients which yielded sequence DNA data for reliable analysis were available for this study. Neurological disease and other related complications were overrepresented because the CNM is a national reference laboratory for this type of disease. There was no reported epidemiological relationship between patients. The type of sample collected was as follows: 164 (55.8%) were CSF, 92 (31.3%) vesicle fluid, 3 (1.0%) esophagus biopsy, 2 (0.7%) gastric biopsy, 1 (0.3%) bowel biopsy, 2 (0.7%) cervix brushed, , 4 (1.4%) humor vitreous, 3 (1.0%) bronco alveolar wash, 2 (0.7%) amniotic fluid, 1 (0.3%) blood and 11(3.7%) serum.

4.2. ***Sequence analysis*****:** Complete sequences of DNA fragments containing relevant SNPs were obtained in 235 (79.9%) of the samples. The remaining 59 (20.1%) samples were partially sequenced. The distribution of clades obtained is described in Table 3. In the results from complete sequences, clade 1 was the most predominant in 132 (44.9%) of the samples analyzed, followed by clade 3 in 42 (14.3%) of the samples. Results obtained from partial sequences were assigned to clade 1 or 3 in 56 (19%) samples and clade 5 or VI in 3 (1.0%) samples. All 4 (1.4%) cases of clade 2 identified (3 children with herpes zoster and 1 immunocompromised adult patient with encephalitis and retinitis) were VZV vaccine related since vOKA specific SNPs located in the ORF62 at position 106262, 107136, and 107252 were found. These results confirmed clinical suspicion of vaccine-related cases. All 4 cases of clade 2 VZV received only one dose of the VZV vaccine, and one of them developed severe neurological disease. In addition, six out of the 235

VZV with complete sequences were identified as putative recombinants between clade 1 and clade 3. The identified SNPs are as follows: ORF 22: A, T, A, G for positions 37902, 38055, 38081, and 38177, respectively; ORF 21: C, C for positions 33725 and 33728, respectively; ORF 50: C for position 87841. Of the putative interclade recombinants found, 4 were detected from CSF in patients with encephalitis, 1 with zoster and 1 with varicella.

4.3. ***Analysis of clade distribution:*** We first analyzed the association between developing a specific clinical pathology and having an infection with VZV of different clades (Figure 1). Of the three most frequent pathologies, encephalitis tended to be associated more frequently with the clades 1 (64; 41.8%) and 3 (26; 17%), zoster with clades 1 (45; 50.6%) and 3 (13; 14.6%) and varicella with the clade 1 (9; 42.8%), clade 5 (3; 14.3%) and VI (2; 9.5%).We next analyzed the association of the clades with a specific pathology and geographical distribution (Table 4.a). A statistically significant association was found between clades and pathology, where encephalitis was more frequently associated with clades 1 and 3 than clades 4, 5 and VI (56.4% vs 37%; P=0.01). In addition, bivariate analysis revealed that clades 4, 5 and VI were significantly more frequent in Madrid than in the other regions of Spain studied (22.7% vs 11.8%; P=0.023). In order to find significant association between variables, we aggregated the related clades in the former genogroups as follows: Genogroup E (clades 1 and 3) and Genogroup M (clades 4, 5 and VI). The multivariate analysis of the genogroups revealed a significant association between encephalitis with clades 1 and 3 and with age >14 years old ((P=0.035 and P=0.021, respectively); Table 4.b). Additionally, significantly fewer cases of encephalitis were found in Madrid compared with all other regions studied (P=0.001; Table 4). No associations were found between VZV clades and other variables such as gender and year of detection. No significant trend of the VZV clades over all study period was found.

5. Discussion

In the present study, we performed a novel molecular epidemiological study of cases of VZV infection in patients in Spain with different pathologies, including neurological disease, varicella and varicella zoster, among others. Several studies have demonstrated a regional dominance of specific clades established by many factors such as introduction of strains through immigration to naïve populations. Geographic distribution of clades is supposed to be faded over time because of increased rates of immigration and travel, or by extensive vaccination with live-attenuated clade 2 strains. Eventually, most VZV strains will become mosaic genomes, which would make it challenging to define VZV clades in the future. Our results confirm that clade 1 and 3 are the most predominant clades in Spain, as well as in the European population [15], representing 80.3% of the total VZV cases analyzed. In our study, clade VI was found in 9.9% of patient. In the mentioned European study, Spain contributed with 31 patients with varicella from Catalonia, 23% of which were clade VI [15]. Therefore, it may be interesting to investigate if clade VI distribution responds to a regional pattern of recent immigration and introduction of clades from other geographical areas, since varicella pathology represents recent VZV infection. Significant proportions of clade 1 and 3 cases were obtained from cases of encephalitis and herpes zoster that represent earlier infection and, reinforce the idea that clades 1 and 3 emerged first as predominant clades in Europe. The fact that most cases of clades 4, 5 and VI were found in the area of Madrid supports the hypothesis of the influence of immigration.

We were unable to assign the clade to six clinical samples, despite the fact that the VZV DNA fragments containing the relevant SNPs were fully sequenced. Recent studies have demonstrated that at least some of the VZV clades emerged through recombination [4]. Compared with other human herpesvirus, the VZV genome has an estimated mutation rate ten times lower than its counterpart herpes simplex virus (HSV) and 40 times lower than cytomegalovirus (CMV) [12]. Interestingly, we found that these six cases of VZV had the same SNPs combination in ORF 21, 22 and 50 than the CII/CSF/2909/2011 strain described by Norberg et al [16]. Therefore, our analysis suggests that these

samples were in fact putative interclade 1 and 3 recombinants. As clade 1 and 3 were found as the most common circulating VZV in our study, it is not surprising that these putative recombinants, but no others, were detected. The characteristics of the patients and pathologies associated with the six putative interclade recombinants found suggest that these strains already circulate in the general population. These results do not preclude that other recombinants were present, since the method used in this study only analyzes fragments from three ORFs.

Moreover, other sequencing methods such as the next generation sequencing should be used to detect mixed VZV populations in the same sample, since direct sanger sequencing is not sensitive enough to detect minor (<20%) viral sub-populations. One study performed in a cohort of adults from Africa and Asia that migrated to UK showed a 30% reactivated clade 1 and 3 strains despite a history of varicella supposed to be caused by other clades in the country of origin [18]. Furthermore, cases of varicella caused by co-infection with two different clades have been previously reported, and indicates the potential for recombination, particularly in regions where co-circulation of mixed clades has been identified [19]. In this sense, it is postulated that the introduction of the live-attenuated Oka varicella vaccine could lead to the recombination of wild-type viruses and hence the need for monitoring and characterizing the emerging recombinant VZV strains which could be more virulent. The 4 cases of clade 2 found in this study were in fact from patients who received one dose of VZV vaccine and, we confirmed that the detected strains were vOKA vaccine strains and unfortunately, one of them developed severe neurological disease. However, clade 2 strains have not been found in non-vaccinated patients suggesting that vaccine strains do not circulate among the population.

A limitation of this study is that may not be epidemiologically representative of all cases of VZV disease in Spain since we only analyzed those cases that were sent to the CNM, and thus neurological diseases were overrepresented. It is also possible that more recombinants could be detected if the complete genome was studied. Thus, deep sequencing and phylogenetic studies are needed to detect

other recombinant strains and to assess whether or not all six recombinants have a monophyletic origin since these cases were geographically sparse.

In conclusion, this is the first study that characterizes VZV clade distribution in Spain. Similar to the European population, clade 1 and 3 are the most predominant clades in Spain. The results from this study also indicate that wild-type recombinant VZV circulates in Spain. Although several cases of herpes zoster and one of encephalitis and retinitis are directly related to vaccination, clade 2 vOKA strains might potentially circulate in the population. Thus, molecular epidemiological studies for the surveillance of circulating varicella-zoster viruses are highly recommended.

Transparency declaration

Conflicts of Interest: The authors declare no conflicts of interest.

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Author contribution: David Tarragó: Conceptualization, Methodology, Data curation, Supervision, Formal analysis, Writing–original draft, Writing–review & editing, Funding acquisition and Supervision; Irene González: Data curation and Methodology; Alejandro Molina: Statistical analysis and Writing–review & editing; Pilar Pérez: Writing–review & editing; Juan E. Echevarría: Writing–review & editing; Lante He: Data curation.

All the authors participated in the paper discussion and approved the final version of the manuscript.

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Table 1. Primers used for nested PCR in ORF 21, 22 and 50.

ORF	Primers	Sequence 5'-3'	*Position	Amplicon (bp)
21	vzv21f	TAATGAATTGAGGCGCGGTTTA	33497-33518	502
	vzv21r	CACGTGTAGCTCCAAAAACCTAGG	33976-33999	
21	Nested21f	TGTAAATGCCCCAACCTCAT	33539-33559	406
	Nested21r	ATACAAACGAAACGCCCAGT	33925-33945	
22	ORF22R1f	GGGTTTTGTATGAGCGTTGG	37837-37856	446
	ORF22R1r	CCCCCGAGGTCCGTAATATC	38264-38283	
22	Nested22f	CGTGATATTATAGCATGTCTGGAG	37860-37883	403
	Nested22r	ATATCTCGGTAGTTAGGTATTCCATT	38238-38263	
50	vzv50f	CGCACCCAAAGTGAACATCAT	87736-87756	514
	vzv50r	TCTCGGATGTCAAATATGTTACGA	88227-88250	
50	Nested50f	CGCCAATTAAGCGTATCCAT	87776-87795	446
	Nested50r	GAGAGGCCTGCATCGTTAAA	88202-88222	

*Dumas strain GenBank accession number X04370.

Table 2. Baseline characteristics of the 294 patients included in the study.

Variable	
Gender male, n (%)	148 (59%)
Age, median (IQR)	55 (30-74)
Diagnosis, n (%)	
Colitis	1 (0.3%)
Retinitis	3 (1.0%)
Esophagitis	5 (1.7%)
Encephalitis	153 (52%)
Encephalitis and retinitis	1 (0.3%)
Encephalitis and Varicella	2 (0.7%)
Encephalitis and Herpes Zoster	12 (4.1%)
Pneumonia	3 (1.0%)
Ramsay-Hunt Syndrome	2 (0.7%)
Ramsay-Hunt Syndrome and Encephalitis	2 (0.7%)
Varicella	23 (7.8%)
Herpes Zoster	87 (29.6%)
Region, n(%)	
Madrid	155 (52.7%)
Other:	113 (38.4%)
Castilla-León	35
Galicia	16
Castilla-La Mancha	16
Andalucia	14
València	10
Navarra	9
Extremadura	5
Murcia	4
La Rioja	2
Iles Balears	1
Catalunya	1
Unknown	26
Suspected from VZV vaccine, n	4

Note: Interquartile Range (IQR)

Table 3. Clade and genogroup distribution of the VZV cases diagnosed.

Variable	**Total N=294**
Clade / Genogroup	n (%)
1 / E	132 (44.9%)
1 or 3 / E	56 (19%)
2 / J	4 (1.4%)
3 / E	42 (14.3%)
4 / M	3 (1%)
5 / M	19 (6.5%)
5 or VI / M	3 (1.0%)
rec 1-3 / E	6 (2.0%)
VI / M	29 (9.9%)
VII / M	0 (0%)

Table 4. Statistical analysis: a. Bivariate analysis of the association of the clades with a specific pathology and geographical distribution (Chi Square test). b. Multivariate analysis of the association of the clades with a specific pathology, geographical distribution and age.

Variable	**N (%)**		**Total**	***P***
Clades	**Pathology**			
	Encephalitis	*Other pathologies*		
1 and 3	133 (56.4%)	103 (43.6%)	236	0.01
4, 5 and VI	20 (37%)	34 (63%)	54	
	Region			
	Madrid	Other regions		
1 and 3	119 (77.3%)	97 (88.2%)	216	
4, 5 and VI	35 (22.7%)	13 (11.8%)	48	0.023

Variable	**OR (CI 95%)**	***P***
	Encephalitis	
Clades 1 and 3	2.177 (1.058-4.479)	0.035
Region (Madrid)	0.379 (0.215-0.667)	0.001
Year of detection	0.975 (0.910-1.044)	0.460
Age (>14 years old)	3.605 (1.213-10.709)	0.021