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Short communication

Complementary assays for monitoring susceptibility of varicella-zoster virus resistance to antivirals

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The emergence of varicella-zoster virus (VZV) resistance to current antivirals as acyclovir (ACV) constitutes a hindrance to antiviral treatment effectiveness of VZV infections, especially in immunocompromised patients. The molecular mechanisms of VZV resistance reported so far rely on the presence of mutations within thymidine kinase (TK, ORF36) and DNA polymerase (ORF28) viral genes. The aim of this work was to develop reliable and complementary diagnostic methods to detect VZV antiviral resistance: (i) a genotypic assay based on TK and DNA polymerase genes sequencing, (ii) a plaque reduction assay to determine antiviral 50% effective concentrations, and (iii) a functional assay to evaluate *in vitro* phosphorylation activity of recombinant TKs. As a whole, this study included the analysis of 21 VZV clinical isolates and 62 biological samples from patients experiencing VZV infection. Genetic analysis revealed 3 and 9 new amino acid changes that have not been previously described within the highly conserved TK and DNA polymerase, respectively. Then, VZV isolates bearing newly identified mutations considered as natural polymorphisms were characterized as susceptible to ACV using plaque-reduction assay in MeWo cells. In parallel, the impact of TK changes on ACV phosphorylation activity was examined using a nonradioactive *in vitro* enzymatic assay.

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Varicella-zoster virus (VZV) is a common herpesvirus that is usually known to be responsible for benign varicella and for localized recurrent zoster lesions. VZV infections are associated with significant morbidity and mortality among immunocompromised individuals, especially in HIV-infected patients or in hematopoietic stem cell transplant recipients, because of either disseminated infections or chronic reactivations (Morfin et al., 1999). Acyclovir (ACV) constitutes the first-line therapy for management of VZV infections. ACV, after activation by primophosphorylation by the viral thymidine kinase (TK, encoded by open reading frame 36 [ORF36]), targets the viral DNA polymerase (ORF28) and disrupts the viral genome replication by a chain termination mechanism. Foscarnet (FOS) constitutes an alternative drug that is effective without the phosphorylation by viral TK and that directly inhibits the viral DNA polymerase (Andrei and Snoeck, 2013; Piret and Boivin, 2014). According to these mechanisms of action, viral mutations conferring resistance to anti-VZV drugs have been mapped both in ORF36 and ORF28. However ACV-resistance of VZV strains is mostly mediated by mutations within TK (Piret and Boivin, 2014). To date, few studies have described extensively the natural polymorphism of VZV TK and DNA polymerase and have identified mutations conferring VZV resistance to antivirals (Andrei et al., 2012; Brunnemann et al., 2015; Fillet et al., 1998; Morfin et al., 1999; Sauerbrei et al., 2011; Talarico et al., 1993; Visse et al., 1998). However, the precise interpretation of genotypic resistance assays, based on the identification of specific mutations in TK and DNA polymerase, requires the clear distinction between natural polymorphisms and mutations conferring antiviral resistance. Classically, the undeniable assessment of a presumed resistance mutation is performed by testing recombinant viral

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mutants, generated by marker transfer experiments or bacterial artificial chromosome (BAC) machinery, for drug susceptibility using a standardized phenotypic assay (Brunnemann et al., 2015). For VZV, a trustworthy alternative strategy for the evaluation of the contribution of VZV TK mutations in antiviral resistance is to assess the *in vitro* enzymatic activity of mutant TKs. To date, few methods to study *in vitro* functional activity of VZV TK have been reported: radioactive assays (Ng et al., 2001; Suzutani et al., 2000) and a bacterial colony reduction assay based on restoration of TK-deficient bacteria sensitivity producing functional VZV TK (Sahli et al., 2000). Regarding the herpes simplex virus (HSV), a virus also belonging to the Alphaherpesvirinae subfamily, many methods, either radioactive or non-radioactive ones, have been described for the evaluation of TK phosphorylation activity: radioactive assays based on the use of ^{3}H-labelled thymidine as a substrate or non-radioactive assays indirectly measuring HSV TK activity (Burrel et al., 2012; Frobert et al., 2007, 2005; Malartre et al., 2012; Sauerbrei et al., 2013; Suzutani et al., 2000). The aim of this work was to develop reliable and complementary systems for detection and confirmation of VZV antiviral resistance *viz.*: (i) a genotypic assay based on TK and DNA polymerase gene sequencing, (ii) a plaque reduction assay (PRA) to determine antiviral 50% effective concentrations (EC_{50}), and (iii) a functional assay to evaluate *in vitro* phosphorylation activity of recombinant TKs.

VZV laboratory strain Oka, 21 VZV clinical isolates, and 62 clinical samples were included in the present study. Samples were recovered from distinct patients (median age, 52 years; range, 1–89 and comprising 47 men and 36 women) hospitalized at La Pitié Salpêtrière–Charles Foix University Hospital and experiencing VZV infection, mainly zoster lesions. None of the patients received prior long-term treatment with anti-VZV therapy, at most occasional ACV therapy for treating HSV infections. VZV isolates were obtained from clinical samples by propagation in human fibroblasts (MRC-5) (Harper, 2000).

For genotypic assay, viral DNA was extracted from clinical samples and viral stocks (clinical isolates and laboratory strain Oka) with the MagNA Pure Compact Instrument (Roche Diagnostics, Meylan, France), according to the manufacturer's instructions, and stored at −20 °C for further analysis. TK (ORF36) and DNA polymerase (ORF28) full-length gene amplification (first-round and second-round PCR annealing primers temperature: 52 °C and 59 °C for ORF36 and ORF28, respectively) and sequencing were performed using primers listed in Table 1, with the proofreading enzyme Expand High Fidelity (Roche Diagnostics), according to previously described procedures (Burrel et al., 2010). In order to minimize possible PCR artefacts, all sequences were performed twice on both DNA strands. Nucleotide and amino acid sequences were compared with those of VZV reference strain Dumas (GenBank accession number X04370) (Davison and Scott, 1986). All sequences determined in this study have been deposited in the GenBank database under accession numbers KU529484 through KU529566 and KU529567 through KU529649 for ORF28 and ORF36, respectively.

A plaque reduction assay (PRA) was developed in MeWo (melanoma cell line) cell culture for the measurement of the ACV EC_{50} (Merck, Lyon, France) and FOS (AstraZeneca, Rueil-Malmaison, France), following the previously reported guidelines (Harper, 2000). Briefly, MeWo cells were grown to confluence in 24-well culture plates and then infected with VZV isolates. After 1 h incubation, media containing different concentrations of antiviral compounds were added. Serial dilutions of ACV and FOS were tested, ranging from 5 to 100 μM and 20–200 μM, respectively. The cell cultures were incubated for 5 days, then fixed with 10% formaldehyde, and finally stained with 1% crystal violet in 70% ethanol. The number of viral plaques was counted and EC_{50}s were calculated by the Kärber method. Two distinct ACV-resistant VZV mutants were selected in MRC-5 cells treated with increasing concentrations of ACV by serial passages of the laboratory strain Oka (VZV-R1 mutant) and a clinical isolate (VZV-R2 mutant). Both drug-susceptible laboratory strain Oka and ACV-resistant viruses selected *in vitro* were used as controls to validate this PRA, prior to testing VZV clinical isolates. The resistance thresholds were set at EC_{50} values of 30 μM for ACV and 160 μM for FOS as previously reported (Saint-Léger et al., 2001).

In parallel, a previously published method for the evaluation of *in vitro* phosphorylation activity of HSV recombinant TKs was adapted to VZV recombinant TKs (Burrel et al.,

Table 1
Primers used for amplification and sequence analysis of VZV full-length ORF36 TK and ORF28 DNA polymerase.

Gene	Function	Name	Sequence (5′ → 3′)
ORF36	First-round PCR (outer primers)	VZV_TK-F1	F: ACAGCCCTTTGAACATCCAC
		VZV_TK-R1	R: CGCTGTTACTTGCGCTACAA
	Second-round PCR (inner primers)	VZV_TK-F2	F: GATAACGGCGACAGGAGTTT
		VZV_TK-R2	R: AAGGCGGGATTAAAGGATGT
	Sequence reaction	VZV_TK-A	R: TAGTTGAGGCGATTG GGTGT
		VZV_TK-B	F: CACCAAACCGGATCTTACTCA
		VZV_TK-C	R: CGCGTGCCAGTTGTTAGTT
		VZV_TK-D	F: CGGTTAATCTGCCGTTTGTT
		+ VZV_TK-F2/R2	
ORF28	First-round PCR (outer primers)	VZV_POL-F1	F: GGGAAATCTGTTCACCTCCA
		VZV_POL-R1	R: CCAAAACCCAGTCTGTCGAT
	Second-round PCR (inner primers)	VZV_POL-F2	F: GCGTGTCGGGGGTATAAAAT
		VZV_POL-R2	R: TATGCGGAAACCACAACAAA
	Sequence reaction	VZV_POL-A	R: AGACAGGGTCAAATCCCAAA
		VZV_ POL-B	F: GGATCTCCACGTAGCAAAGC
		VZV_ POL-C	R: CCCAAAAAGCGTACGAGGTA
		VZV_ POL-D	F: AGGACAAAACAGAGCCGTTG
		VZV_ POL-E	R: TATATCGATCCGGTGGTCGT
		VZV_ POL-F	F: CGTTTTCCTCCAACTGTAAAGG
		VZV_ POL-G	R: TCAACGGTCTCATATCTCTGGA
		VZV_ POL-H	F: TCCCGGAATGTCTTTGTAGG
		VZV_ POL-I	R: AGCGACGTTGAAATTGACTG
		VZV_ POL-J	F: GATGTGTTGCATCGGGAAAC
		VZV_ POL-K	R: GACCGCTTGGTACCGTTATT
		VZV_ POL-L	F: ACTCGCTACCGGAACGTATG
		+ VZV_ POL-F2/R2	

F: forward; POL: DNA polymerase; R: reverse; TK: thymidine kinase.

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