

Genotyping of Varicella-Zoster Virus and the Discrimination of Oka Vaccine Strains by TaqMan Real-Time PCR

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Single nucleotide polymorphisms (SNPs) in five genes have been used to identify four major subtypes of wild-type varicella-zoster virus (VZV) A, B, C, and J. Additional SNPs, located in the IE62 major transactivating gene can be used to differentiate the Oka vaccine strain (vOka) from wild-type VZV. Primer-probe sets for the detection of the five polymorphic loci were designed by Applied Biosystems for the ABI 7900HT platform. Probes for each allele were labeled with VIC or 6-carboxyfluorescein fluorogenic markers. Each primer-probe set was validated to establish assay sensitivity and specificity using VZV DNA of predetermined copy number and genotype. Further evaluation was carried out using DNA samples from the vesicle fluid or skin swab of the rash of adult patients with herpes zoster or rashes due to vOka. Assay sensitivity ranged from 10 and 10⁸ copies/ml of VZV DNA (equivalent to 2 to 20 copies per reaction). Statistical analyses showed that for each genotype, a set of two probes clearly differentiated the nucleotide present (allele) at that locus ($P < 0.0001$). It was possible to determine the genotype of wild-type VZV using one of four SNP assays and also to differentiate wild type from vOka using a single SNP assay. The assay can be used for diagnostic and epidemiological studies of VZV, including the differentiation of vOka from wild-type strains, investigation of breakthrough infections, and varicella outbreaks following immunization.

Varicella-zoster virus (VZV) is the etiological agent of chickenpox in childhood and shingles (herpes zoster) following the reactivation of latent virus dormant in the dorsal root ganglia (2). VZV is genetically stable, and one major serotype has been described previously (2). Using single nucleotide polymorphisms (SNPs) located in five open reading frames (ORFs) of VZV, we have identified four major subtypes of wild-type VZV: A (African/Asian), B, C (European), and J (Japanese) (1). Similar results have been described by other groups of investigators, although nomenclature remains to be agreed (5, 13, 18). A live attenuated VZV vaccine, the vOka strain, is derived from a Japanese wild-type genotype J virus. Since 1995 vOka has been licensed for toddler immunization in the United States (4). The vaccine is effective in healthy and immunocompromised individuals, with 95% protection against severe infection and 75% against all infection (11). Complications are rare, the commonest being rashes which occur in 5% of children and 10% of adults (6, 19).

Establishing the vaccine origin of rashes occurring postvaccination is important for the purposes of surveillance (15). Initial methods exploited the geographical SNP variations between the Japanese vaccine strain and wild-type strains found in the United States (10). However, these methods did not

distinguish between the vaccine and some wild-type Japanese strains (16).

An SmaI and an NaeI site in the ORF 62 gene are present in all preparations of the vaccine and in all vaccine virus rashes and are absent from all wild-type strains, whatever their geographical origin (7, 12, 14). In order to maximize detection and typing of low-copy-number vaccine virus in vaccine rashes, we report the development of a sensitive and discriminatory real-time PCR that will facilitate the epidemiological surveillance of vaccine rashes.

MATERIALS AND METHODS

VZV strains and DNA extraction. VZV DNA was extracted (QIAamp DNA MiniKit; QIAGEN, Crawley, United Kingdom) from eight clinical isolates, 79 vesicle fluid samples of patients with shingles, vesicular swabs of the rashes of 14 patients with suspected zoster due to vaccine breakthrough, and commercially available VZV vaccine (MERCK and GSK). The extracted DNA was genotyped as A, B, C, or J using sequence analysis of four regions of the VZV genome, ORFs 1, 21, 50, and 54 (1). The identity of the vaccine strains of VZV was confirmed as vOka by PCR and digestion across the SmaI restriction endonuclease site in ORF 62 (14).

TaqMan SNP assays. For each of the SNPs in Table 1, primer-probe sets

TABLE 1. VZV SNP for TaqMan allelic discrimination assay

Geographic location or source	Genotype	Residue of indicated SNP in ORF:				
		1 SNP 685	21 SNP 33725	37 SNP 66288	60 SNP 101464	62 SNP 106262
Europe	C	G	T	G	C	T
Europe	B	G	C	A	C	T
Africa/Asia	A	G	C	G	A	T
Japan (pOka)	J (J1)	A	C	G	C	T
Japan (European)	J (J2)	A	C	G	C	T
Vaccine (vOka)	J	A	C	G	C	C

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TABLE 2. TaqMan primers and probes evaluated for VZV genotyping

ORF	SNP	Primer (5'-3') ^a	Probe ^b	Identity ^c
1	685	CGTCGCCATCTGGAGTACTAC-F GCGCAACTGAAAATGCAAATGG-R	VIC-ACCCAGTACGTTGCAT FAM-ACCCAGTACATTGCAT	Non-J GT J
21	33725	AGGAACATGGGAGAATGTAAATGC-F TACGTCTCTGACCGTTGTCGTT-R	VIC-ACGTTTTTACATGATAATG FAM-ACATGACAATGTTAAAAT	GT C Non-C
37	66288	CCTACTGGTTTCGATGAAGAACTCA-F CTTCGTGTGTTGTAGGGTTAACCTT-R	VIC-AAAACGTGTTTTCTATCATT FAM-AAAACGTGTTTTTATCATT	Non-B GT B
60	101464	TCTTAACCACGATTCCCGATAGC-F GTATATGAGGCGTGGGACTATGC-R	VIC-ACCCCCGGTATCAA FAM-CACCCCCTGTATCAA	Non-A GT A
62	106262	CGTACACGTGATACTGAGACAAAGC-F GCCGGTTGCTGGTGTG-R	VIC-ATCCCTGGGCCACC FAM-ATCCCCGGGCCAC	Wild vOka

^a F, forward primer; R, reverse primer.

^b Allelic changes in the probe are shown in boldface.

^c GT, genotype.

(Table 2) were made using the Applied Biosystems design service (California). Two fluorogenic minor groove binder probes were used for each locus using the dyes 6-carboxyfluorescein (FAM; excitation, 494 nm) and VIC (excitation, 538 nm) which are easily differentiated in the Applied Biosystems Prism 7900HT PCR system. Real-time PCR was performed using 7.5 µl of TaqMan 2× universal master mix (Applied Biosystems, CA), 0.19 µl of primer-probe (470 nM and 100 µM, respectively), 5.31 µl of RNase- and DNase-free water (Sigma), and 2 µl of sample DNA, in a total volume of 15 µl per single tube reaction. Two wells of a 384 plate (Applied Biosystems, CA) were used for each sample or SNP. DNase-free water used as nontemplate control and DNA of known VZV genotype (appropriate to the SNP of interest) used as a positive control were included in each assay run. Assay conditions were 2 min at 50°, 10 min at 95°C, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Initially, the SNP assay was set up using SDS, version 2.1, software (Applied Biosystems, CA) as an absolute quantification assay, but after assay completion the plate was read using the allelic discrimination settings. Postassay analysis was performed using the SDS software.

SNP assay evaluation. DNA from clinical isolates of known genotype and vaccine strains were tested to assess the ability of each SNP assay to differentiate one allele from another, as well as background and cross talk signals. SNP assays that produced good discrimination were further evaluated using the zoster patient samples, representative of the range of quality and quantity of VZV DNA expected in clinical specimens.

Assay sensitivity. DNA from VZV isolates genotyped as A, B, C, or J by sequencing were quantified using a validated in-house TaqMan viral load assay

(8), and serial dilutions from 10 to 10⁸ copies/ml were used to assess the sensitivity of each SNP assay.

Statistical methods. An unpaired *t* test (GraphPad QuickCalcs; Prism, California) was used to compare the mean and standard deviation of the reactivity for each probe at each locus.

RESULTS

The results of an assessment of the sensitivity of the five probes for each particular genotype at each locus using serially diluted samples of known copy number are shown in Fig. 1. Assay reactivity was directly proportional to copy number (*R*² of >0.95) for all probes. All but one probe was capable of detecting between 2 to 20 copies/reaction. One probe (33725FAM non-genotype C) was relatively insensitive and failed to detect DNA in samples with fewer than 2,000 copies/reaction.

Each SNP assay showed excellent discrimination between genotypes using clinical isolates and vaccine (Fig. 2). The adjusted mean fluorescence (absolute value minus background template control) and standard deviation for each probe at

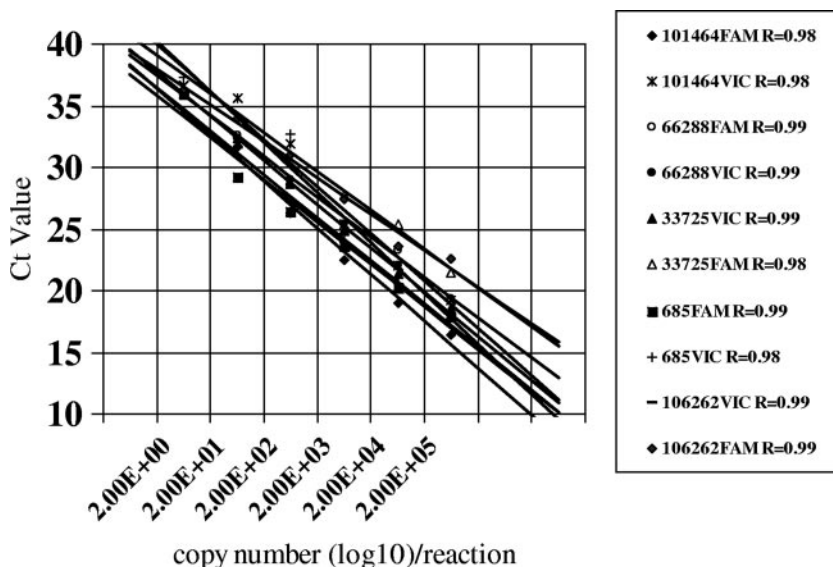


FIG. 1. Efficiency of each TaqMan SNP assay. C₇, cycle threshold.

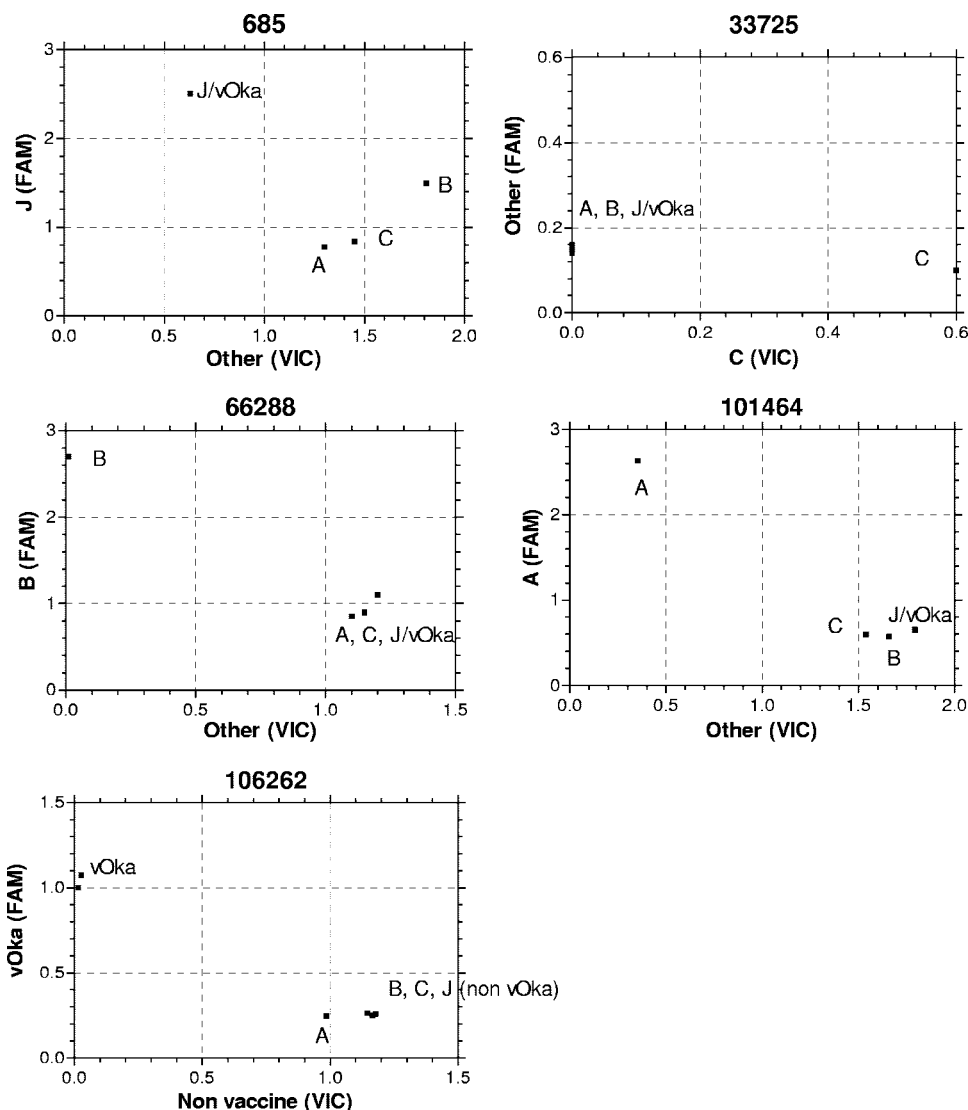


FIG. 2. Allelic discrimination for each genotype at each locus. Results are based on adjusted mean fluorescence of paired wells (absolute value minus background template control).

each locus was calculated for all the zoster and vaccine strains tested (Table 3). Statistical comparison of these values (unpaired *t* test) showed highly significant discrimination ($P < 0.0001$) between probes at each locus. No false-

positive results occurred, although for locus 33725 discrimination of genotype C and non-genotype C viral DNA was based solely on the level of reactivity of the VIC (genotype C specific) probe unless the sample contained high copy numbers of non-

TABLE 3. Analysis of the TaqMan genotype discrimination results of DNA samples derived from vesicle fluid taken from zoster patients

Genotype or source of vesicle fluid sample	Adjusted mean fluorescence (\pm SD) of the indicated probe and genotype for: ^a									
	SNP 685		SNP 33725		SNP 66288		SNP 101464		SNP 106262	
	VIC with non-GT J	FAM with GT J	VIC with GT C	FAM with non-GT C	VIC with non-GT B	FAM with GT B	VIC with non-GT A	FAM with GT A	VIC with WT	FAM with Vac
GT A ($n = 7$)	1.05 (0.29)	0.33 (0.14)	0.01 (0.01)	0.48 (0.10)	0.96 (0.17)	0.25 (0.09)	0.32 (0.17)	2.27 (0.39)	1.00(0.18)	0.10 (0.09)
GT B ($n = 18$)	0.78 (0.45)	0.24 (0.17)	0.00 (0.00)	0.34 (0.12)	0.03 (0.03)	2.13 (0.43)	1.49 (0.08)	0.36 (0.02)	0.90 (0.16)	0.20 (0.12)
GT C ($n = 37$)	0.98 (0.30)	0.28 (0.10)	0.50 (0.22)	0.14 (0.11)	0.91 (0.19)	0.22 (0.06)	1.65 (0.08)	0.39 (0.04)	1.10 (0.25)	0.20 (0.09)
GT J ($n = 17$)	0.07 (0.11)	2.01 (0.64)	0.01 (0.12)	0.17 (0.08)	0.86 (0.17)	0.32 (0.09)	1.68 (0.35)	0.41 (0.13)	1.20 (0.23)	0.20 (0.15)
vOka ($n = 14$)	0.08 (0.05)	2.00 (0.57)	0.01 (0.05)	0.17 (0.05)	0.80 (0.24)	0.32 (0.11)	1.58 (0.24)	0.32 (0.18)	0.10 (0.10)	1.60 (0.35)

^a Values are the mean fluorescence of the probe minus the background control. In all cases, a comparison (unpaired *t* test) of mean (\pm standard deviation) fluorescence values minus the background of each probe at each locus gave a P value of <0.0001 . GT, genotype; WT, wild type; Vac, vaccine.

genotype C DNA owing to the lower sensitivity of the FAM probe.

DISCUSSION

Real-time PCR assays are characterized by a wide dynamic range of quantification and offer a high degree of technical sensitivity and precision (9). No post-PCR steps are required, which avoids the possibility of cross-contamination, and these plate-based assays are easily adaptable for rapid, high-throughput screening. The use of fluorogenic probes for the detection of PCR product during the extension phase of the PCR provides empirical data yielding quantitative results that can be manipulated by statistical procedures to allow accurate quality assurance and control. We used TaqMan real-time allelic discrimination to genotype VZVs using specific primer and probe sets designed around informative polymorphic loci. The resulting assays are sensitive, detecting between 2 and 20 copies per reaction with good genotype specificity. The SNP 22725 assay was able to discriminate genotype C virus but only when used in conjunction with at least one other SNP assay owing to the lack of sensitivity of the non-genotype C (FAM) probe.

The sensitivity of the SNP assays compares with a previously published TaqMan-based assay method for which the limit of detection was 100 copies per reaction for the 107252 SNP of VZV (Oka versus wild type) (3). Five SNPs (SNP 101464 for genotype A, SNP 66288 for genotype B, SNP 33725 for genotype C, SNP 685 for genotype J, and SNP 106262) and the SmaI restriction endonuclease site in ORF 62 of VZV can be used to distinguish vaccine and wild-type VZV. The sensitivity and discrimination of the assay allow genotyping even when copy number is low, as in the case of vaccine virus rashes.

Two groups have described melting curve assays based on real-time PCR using a LightCycler (Roche Applied Science) for the differentiation of vaccine from wild type using SNP 69649 in ORF 38 (17) and SNP 106262 in ORF 62 (12). The geographical SNPs used in our assay provide additional genotype information that will be especially important for monitoring the origin of varicella outbreaks in countries that have good vaccine coverage and low levels of endogenous varicella. In addition, the method provides proof that recombination of vaccine and wild type is not occurring as vaccine is introduced.

In summary, we have described a highly discriminatory and sensitive method for the detection and genotyping of virus from post-Oka vaccine rashes. The method is simple and reproducible and will be useful for postvaccination surveillance.

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REFERENCES

1. Barrett-Muir, W., R. A. Nichols, and J. Breuer. 2002. Phylogenetic analysis of varicella-zoster virus: evidence of intercontinental spread of genotypes and recombination. *J. Virol.* **76**:1971–1979.
2. Breuer, J. 2004. Varicella zoster virus, p53–83. In A. J. Zuckerman, J. E. Banatvala, J. R. Pattison, P. D. Griffiths, and B. D. Schoub (ed.), *Principles and practice of clinical virology*, 5th edition. John Wiley, Chichester, United Kingdom.
3. Campsall, P. A., N. H. Au, J. S. Prendiville, D. P. Speert, R. Tan, and E. E. Thomas. 2004. Detection and genotyping of varicella-zoster virus by TaqMan allelic discrimination real-time PCR. *J. Clin. Microbiol.* **42**:1409–1413.
4. Centers for Disease Control and Prevention. 1996. Prevention of varicella: recommendations of the Advisory Committee on Immunization Practices (ACIP). *Morb. Mortal. Wkly. Rep.* **45**:1–25.
5. Faga, B., W. Maury, D. A. Bruckner, and C. Grosse. 2001. Identification and mapping of single nucleotide polymorphisms in the varicella-zoster virus genome. *Virology* **280**:1–6.
6. Gershon, A. A. 1995. Varicella-zoster virus: Prospects for control. *Adv. Ped. Infect. Dis.* **10**:93–123.
7. Gomi, Y., T. Magana, M. Takahashi, and K. Yamanishi. 2000. Oka varicella vaccine is distinguishable from its parental virus in DNA sequence of open reading frame 62 and its transactivation activity. *J. Med. Virol.* **61**:497–503.
8. Hawrami, K., and J. Breuer. 1999. Development of a fluorogenic polymerase chain reaction assay (TaqMan) for the detection and quantitation of VZV. *J. Virol. Methods* **79**:33–40.
9. Klein, D. 2002. Quantification using real-time PCR technology: applications and limitations. *Trends Mol. Med.* **8**:257–260.
10. LaRussa, P., O. Lungu, I. Hardy, A. Gershon, S. P. Steinberg, and S. Silverstein. 1992. Restriction fragment length polymorphism of polymerase chain reaction products from vaccine and wild-type varicella-zoster virus isolates. *J. Virol.* **66**:1016–1020.
11. Lee, B. R., S. L. Feaver, C. A. Miller, C. W. Hedberg, and K. R. Ehresmann. 1996. An elementary school outbreak of varicella attributed to vaccine failure: policy implications. *J. Infect. Dis.* **190**:477–483.
12. Loparev, V. N., T. Agra, P. R. Krause, M. Takeaway, and D. S. Schmidt. 2000. Improved identification and differentiation of varicella-zoster virus (VZV) wild-type strains and an attenuated varicella vaccine strain using a VZV open reading frame 62-based PCR. *J. Clin. Microbiol.* **38**:3156–3160.
13. Loparev, V. N., A. Gonzalez, M. Deleon-Carnes, G. Tipples, H. Fickenscher, E. G. Torfason, and D. S. Schmid. 2004. Global identification of three major genotypes of varicella-zoster virus: longitudinal clustering and strategies for genotyping. *J. Virol.* **78**:8349–8358.
14. Quinlivan, M. L., A. A. Gershon, S. P. Steinberg, and J. Breuer. 2005. An evaluation of single nucleotide polymorphisms used to differentiate vaccine and wild type strains of varicella-zoster virus. *J. Med. Virol.* **75**:174–180.
15. Sharrar, R. G., P. LaRussa, S. A. Galea, S. P. Steinberg, A. R. Sweet, R. M. Keatley, M. E. Wells, W. P. Stephenson, and A. A. Gershon. 2000. The postmarketing safety profile of varicella vaccine. *Vaccine* **19**:916–923.
16. Takada, M., T. Suzutani, I. Yoshida, M. Matoba, and M. Azuma. 1995. Identification of varicella-zoster virus strains by PCR analysis of three repeat elements and a PstI-site-less region. *J. Clin. Microbiol.* **33**:658–660.
17. Tipples, G. A., D. Safronetz, and M. Gray. 2003. Real-time PCR assay for the detection of varicella-zoster virus DNA and differentiation of vaccine, wild-type and control strains. *J. Virol. Methods.* **113**:113–116.
18. Wagenaar, T., V. T. K. Chow, C. Buranathai, P. Thawatsupha, and C. Grose. 2003. The out of Africa model of varicella-zoster virus evolution: single nucleotide polymorphisms and private alleles distinguish Asian clades from European/North American clades. *Vaccine* **21**:1072–1081.
19. Weibel, R. E., B. J. Neff, B. J. Kuter, H. A. Guess, C. A. Rothenberger, A. J. Fitzgerald, K. A. Connor, A. A. McLean, M. R. Hilleman, and E. B. Buynak. 1984. Live attenuated varicella virus vaccine: efficacy trial in healthy children. *N. Engl. J. Med.* **310**:1409–1411.