Novel genetic variation identified at fixed loci in ORF62 of the Oka varicella vaccine and in a case of vaccine-associated herpes zoster

Running title: Novel genetic variation in the Oka varicella vaccine

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Abstract

The live attenuated Oka varicella vaccine (vOka), derived from clade 2 wild type (wt) virus, pOka, is used for routine childhood immunization in several countries including the United States (US), causing dramatic declines in varicella incidence. vOka can cause varicella, establish latency and reactivate to cause herpes zoster (HZ). Three loci in varicella-zoster virus (VZV) open reading frame (ORF) 62 (106262, 107252, 108111) are used to distinguish vOka from wt VZV. A 4th position (105705) is also fixed for the vOka allele in nearly all vaccine batches. These 4 positions and two vOka mutations (106710 & 107599) reportedly absent from Varivax were analyzed on Varivax-derived ORF62 TOPO TA clones. The wt allele was detected at positions 105705 and 107252 on 3% and 2% of clones, respectively, but was absent at positions 106262 and 108111. Position 106710 was fixed for the wt allele whereas the vOka allele was present on 18.4% of clones at position 107599. We also evaluated the 4 vOka markers in an isolate obtained from a case of vaccine HZ. The isolate carried the vOka allele at positions 105705, 106262 and 108111. However, at position 107252 the wt allele was present. Thus, all of the ORF62 vOka markers previously regarded as fixed occur as the wt allele in a small percentage of vOka strains. Characterization of all four vOka markers in ORF62 and the Clade 2 subtype marker in ORF38 is now necessary to confirm vOka adverse events.

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VZV is the first human herpesvirus for which a vaccine has been licensed. In 1995, the US became the first country to implement routine varicella vaccination for healthy children aged 12 to 18 months (12), resulting in a dramatic decline in varicella morbidity and mortality (9, 33). More recently, a higher dose formulation of the same vaccine seed virus was licensed and recommended for the prevention of HZ in persons ≥60 years of age (4).

Differentiation of vOka from wt virus has become important for at least two reasons. Testing can be used to assess vOka effectiveness by identifying cases of breakthrough varicella caused by wt virus. Breakthrough disease occurred in 3-25% of vOka recipients in outbreak settings (5, 22, 50) and is several-fold more likely to occur among vOka recipients with low VZV IgG levels 6 weeks post-vaccination (<5 gpELISA U/mL) (22, 33)). Strain discrimination testing is also used to document adverse events associated with vOka. Serious adverse events due to vOka are rare, with only 8 laboratory-confirmed reports of meningitis or encephalitis (6, 7, 12, 17, 26, 27, 32, 37) and 7 cases of secondary transmission (13, 17, 18, 24). One of the most common complications post-immunization is a varicella-like rash that occurs, within 1 to 6 weeks post-immunization (6, 17, 43). The incidence of rash is approximately 5% in healthy children (14, 49). vOka can also establish latency and reactivate to cause HZ. While the incidence of wt HZ after vaccination has declined (8, 19, 25, 50), the incidence of HZ caused by vOka is less well defined as most recipients of the vaccine are children, in whom HZ (vOka or wt) is uncommon. In addition, very few HZ cases occurring post-vaccination are identified as attributable to vOka versus wt by laboratory testing.

vOka is a live attenuated virus produced by serial passage of, pOka, in tissue culture (46). Three preparations of vOka are in commercial production, vOka/Biken (Biken Institute, Japan),
Varivax (Merck & Co.) and Varilrix (GlaxoSmithKline (GSK)). The complete DNA sequence of vOka/Biken genome revealed base substitutions at 42 loci compared with pOka, over a third of which clustered in the major viral gene transactivator protein encoded by ORF62 (16). Most of the vOka-specific loci were determined to be mixtures of the wt and vOka nucleotides, revealing that the vOka is a heterogeneous population of viral strains. This has since also been confirmed for the Varivax and Varilrix preparations, which are derived from the same seed stock (48). Genetic variation has been reported between all 3 vaccine preparations and among different lots from the same manufacturer (21, 42, 47, 48). Differences in the production of these vaccine preparations probably accounts for some of this variation. The original vOka/Biken was produced through passage of pOka 11 times in human embryo fibroblast cells at 34°C, 12 times in guinea pig embryo fibroblast cells at 37°C and a further three times in human WI-38 and MRC-5 cells (46). Varivax was produced by a further seven passages of vOka/Biken-infected cells in MRC-5 cells (44), whereas Varilrix was produced through a series of five limiting dilution clonings using cell-free virus resulting in a further 6 passages in W1-38 cells and 8 passages in MRC-5 cells (10).

Five confirmed and 2 provisional wt VZV clades have been described, (1- 5, VI & VII) (3). The clades are differentiated by evaluating a panel of single nucleotide polymorphisms (SNP) in ORFs 1, 21, 22, 50 and 54 (1, 23, 29). However, these SNPs do not differentiate vOka and similar strains from other clade 2 viruses (23). A Pst1 restriction site in ORF 38 across SNP 69349 that is present in clades 1, 3, 4, 5, VI and VII, and in 70% of clade 2 strains, but absent in vOka and 30% of clade 2 wt viruses (20, 29, 45), is required for that purpose. This Pst1 site, together with a Bgl1 restriction site across SNP 95241 in ORF54 were initially used to distinguish vOka from other wt strains in countries such as the US, where no vOka-like (clade 2
Recently, however, clade 2 PstI-wt isolates have been identified in the US and in Australia, rendering that approach problematic (29, 37).

Limits in sensitivity of conventional sequencing methods for detecting low level variant strains (typically <10%) have left unanswered the possibility that observed differences in the SNP profiles of various vaccine preparations may in fact simply reflect a failure to detect variant strains that are present in very low numbers. Whole genome sequencing of the 3 vaccine preparations and early analysis of vOka-associated SNPs was performed using direct Sanger sequencing (16, 48). This method has a detection limit of approximately 10% (21), thus an allele present in a mixture but on <10% of genomes would have appeared fixed. Recent studies have used more sensitive techniques including pyrosequencing, with sensitivity limits ranging on average from 2% to 10% (21, 47) and analysis of vaccine-derived clones (47). The latter method can theoretically detect an allele present on a single genome within a mixture of viruses.

Currently, three loci in ORF62, 106262, 107252 and 108111, are used to distinguish vOka from wt virus 1) because they were reported as fixed for the novel vOka allele in all 3 preparations (16, 21, 30, 48), and 2) because they have been detected in every isolate from cases of lab-documented vOka varicella or HZ. However, a recent study by Thiele et al, (47) reported that 106262 and 108111 were mixed in Varivax, and 107252 was fixed for the novel vOka allele. A fourth position (105705) is reported to be fixed in all but one batch of Varivax (21). Two loci in ORF62 of Varivax, 106710 and 107599 are reported to lack the vOka allele present in Varilrix and/or vOka/Biken (40, 47).

The aim of our study was to analyze full-length ORF62 clones derived from Varivax to further investigate the presence of alleles reported to be absent in this vaccine preparation and, if
detected, to determine whether any of them co-occur in some strains. Related to this we also
describe a case of HZ caused by a unique vOka variant.
Methods

Case report

In March 2011, approximately 23 months after receiving 1 dose of vOka (Varivax, Merck & Co.) (vaccine lot number not indicated), an otherwise healthy 3-year-old female child was hospitalized in New York City, NY. She presented with a rash consisting of 50-249 (exact number not specified) skin lesions on her left arm, affecting dermatomes C5, C6 and T1. The patient’s guardian reported no previous history of varicella or HZ. The patient had been on acyclovir for 2 days prior to specimen collection. A swab was taken from a vesicle 2 days after HZ onset. On-site testing identified presence of VZV and absence of Herpes Simplex Virus DNA by PCR. The patient was found to be VZV IgM negative and IgG positive.

DNA extraction

For the HZ case, total nucleic acid was extracted from 200µl of vesicular fluid using the MagNA Pure LC automated NA purification system (Roche) according to the manufacturers’ instructions and was eluted in 100µl of elution buffer. For evaluation of the ORF62 SNPs on varivax-derived clones, DNA was extracted from a batch of Varivax (lot.LL46F, released in 2008) using a QIAamp DNA Mini Kit (Qiagen LTD., UK) according to the manufacturer’s instructions. Prior to extraction, the lyophilized vOka preparation was re-suspended in the 500µl of the provided hydration buffer. DNA was extracted from 2x200µl and 1x100µl (mixed with 100µl of PBS) aliquots. The 3x200µl aliquots of eluted DNA were merged to form a 600µl DNA pool which was used for subsequent experiments.
Detection of VZV and discrimination between wt and vOka was performed at position 106262 using a Förster resonance energy transfer (FRET)–based realtime PCR assay as previously described (28) and at position 107252 using an in-house FRET assay. All FRET PCRs were performed using the Light Cycler (Roche). A Taqman based real-time allelic discrimination assay, performed using the MX3000p platform (Stratagene) was used to analyze a third SNP (108111) in ORF62 reported to distinguish between vOka and wt strains (40, 48). Position 105705 was characterized by sequencing, which was also used to confirm results of the real-time PCR assays. Primers were designed to sequence SNPs 105705 (5’-caagcgtgtctctgtcgtctg-3’), 106262 (5’-catagtgcctcctcctcct-3’), 106710 (5’-atgatcagaagcctcacatcctccg-3’), 107252 & 107599 (5’-ggtgtctccctaatcttgtcg-3’) and 108111 (5’-tgcgtctgtagttctacctccc-3’). Sequencing reactions were performed using the Big Dye Terminator cycle sequencing kit version 1.1 and analyzed on an ABI prism 3100 Genetic Analyzer (Applied Biosystems) in accordance with the manufacturer’s instructions. SNPs in ORFs 1, 21, 22, 38, 50, and 54 that differentiate the 7 wt clades of VZV was performed as follows: The BglI and PstI sites in ORFs 54 and 38, respectively were characterized using an in-house FRET–based realtime PCR assay. SNPs in ORFs 1, 21, 22 and 50 were analyzed by PCR and sequencing as previously described (1, 29). PCR amplification of the entire ORF 62 (4087bp) was performed using a forward (5’-CCCGCACAGACAGACAGACACT-3’) and reverse (5’-CTGCGAGAGCGTTTGGAAAACT-3’) primer set. PCR master mix included 12.5μl Extensor Hi-Fidelity PCR Master Mix including buffer I (AB-0794, Thermo Scientific), 4μl of forward and reverse primer (each at 6pmol/μl) and 4.5μl of template DNA. PCR reactions were performed in a GeneAmp 9700 PCR system (Applied Biosystems, USA) initiated by 2 minutes at 95°C followed by 10 cycles (94°C, 10
seconds; 55°C, 30 seconds; 68°C 4 minutes) and 20 cycles (94°C, 10 seconds; 55°C, 30 seconds; 68°C 4 minutes, increased by 10 seconds per cycle) of amplification, and ended with a step at 68°C for 7 minutes. The PCR product was visualized by electrophoresis on a 1% gel run at 85 volts for 1.5 hours.

Cloning of Varivax ORF62

Gel purification and cloning of the Varivax-derived ORF62 PCR product was performed using a TOPO XLPCR Cloning Kit with One Shot® TOP10 Chemically competent™ Escherichia coli (KA4750-10, Invitrogen) and suicide gene selection according to the manufacturers’ instructions. Over three hundred colonies were picked and colony PCR using M13 vector primers was performed to amplify a 4335bp fragment (ORF62 insert plus M13 primer and flanking vector regions 132bp from reverse primer site to ORF62 insert and 118bp from forward priming site to ORF62 insert) to confirm the presence of the ORF62 insert. Positions 105705, 106262, 106710, 107599 and 108111 were characterized on each clone by sequencing as described above. Position 107252 was characterized by RFLP analysis using Nae1 restriction endonuclease as described below. ORF62 from pOka was also cloned as described above except only 2 colonies were screened by PCR.

Restriction fragment length polymorphism analysis

The NEBcutter tool (New England Biolabs, [www.neb.com](http://www.neb.com)) was used to verify previously described restriction endonuclease cleavage sites that could be used determine the presence of the wt allele at positions 106262 and 107252 in the ORF62 clones. NEBcutter also generates a
virtual gel image to illustrate how the Restriction fragment length polymorphisms (RFLPs) should look depending on which allele is present.

The vOka alleles at position 106262 and 107252 have been reported previously to create Sma1 and Nae1 restriction endonuclease sites, respectively (15, 28) creating unique vOka and wt RFLPs. In our ORF62 clone, the presence of an additional 8 Sma1 sites produced RFLPs that could not be interpreted by gel electrophoresis. Three additional Nae1 sites were identified in ORF62 at nt 105511 – 105516, nt 105768 – 105773 and nt 108780 – 108785. We substituted the vOka and wt alleles at all 16 vOka loci in ORF 62 and found these additional sites were not affected, nor were any new sites created, by any of the vOka mutations. Using Nae1, RFLPs were produced that clearly differentiated between the presence of the vOka allele and the wt allele (figure 1). Nae1 digestion in the presence of the wt allele resulted in a total of 4 fragments due to the absence of the restriction site at 107252. The sizes of the minor fragments varied slightly depending on the orientation of the ORF62 fragment inserted into the vector (3012bp, 535-552bp or 514-531bp and 257bp). Presence of the vOka allele at 107252 caused cleavage of the Nae1 site resulting in 5 fragments, (1530bp, 1482bp, 535-552bp or 531-514bp & 257bp).

Restriction digest of the PCR product was performed in a 10µl reaction volume consisting of 2 µl of PCR product, 5 U of Nae1 restriction endonuclease (New England Biolabs, USA), 1µl of 10x NEBuffer 4 and 6.5µl Molecular grade water. Reactions were incubated at 37°C for 3 hours and fragments were separated by gel electrophoresis on 1% agarose at 80 volts for 3 hours. The 3 additional Nae1 sites served as an internal control for the enzymatic reaction. Position 107252 was also sequenced in all clones to confirm the RFLP results.
Statistical Calculations

We estimated the probability of occurrence of the wt allele as the number of variant (i.e. wt) alleles that we found divided by the total number of sequences that we analyzed. We assumed a binomial distribution to obtain a 95% confidence interval for the probability, using the calculator at the website http://statpages.org/confint.html#Binomial.

Reference strains

All nucleotide positions referred to are those in strain Dumas (GenBank accession no. X04370; RefSeq NC_001348)
Results

Analysis of HZ case virus

The SNP profile for the VZV isolate obtained from the HZ case is shown in figure 2. DNA was analyzed at positions 105705, 106262, 107252 and 108111 in ORF 62 in order to determine if the virus was vOka-derived or wt. At positions 105705, 106262 and 108111 the virus was found to have the vOka allele confirming it as vOka-derived. However, at position 107252 the virus carried the wt allele. Analysis of SNPs in ORFs 1, 21, 22, 38, 50 and 54 collectively identified the virus as a clade 2 strain with the ORF 38/ORF 54 SNP profile of the vOka.

Analysis of SNPs in Varivax-derived ORF62 clones

The frequency of the wt allele at four loci in ORF62 (105705, 106262, 107252 & 108111) was determined in 304 ORF62 clones derived from a batch of Varivax. At positions 106262 and 108111, no clones with the wt allele were detected. At positions 105705 and 107252, the wt allele was found on 3% (9/304) and 2% (6/304) of clones, respectively. Among the 6 clones with the wt allele at 107252, 3 also carried the wt allele at 105705. The clones were also analyzed at positions 106710 and 107599, both of which are reported to lack the novel vOka allele present in Varilrix and/or vOka/Biken. At position 107599, the vOka allele was found on 18.4% (56/304) of clones but only the wt allele was detected at position 106710.
Effective monitoring for vOka adverse events requires genomic markers that reliably distinguish vOka from wt viruses. The VZV genome is highly conserved, however, and even the most distantly related wt clades have only about 0.1% sequence variation at the DNA level (34, 36).

Complete sequencing of vOka and pOka revealed only 42 SNP that differ between the two viruses (16). vOka has been shown to contain a mixture of strains, and only 4 of 42 vOka-associated SNP were reported as fixed for the vOka allele in all vOka strains (16, 21, 30, 48).

The remaining SNPs occur as mixtures of vOka and wt allele in the vaccine preparations, and the SNP profiles vary both between manufacturers and batch to batch from the same source (21, 42, 47, 48).

The vaccine is known to be attenuated for replication in skin (35), but the genetic basis for attenuation remains elusive. It may involve multiple mutations both within ORF62 and several other genes (reviewed in 39). The presence of the wt alleles is also likely to contribute to the immunogenicity and pathogenicity of the vaccine.

The approach to discriminating vOka from wt strains has evolved with improved understanding of genomic variation in the vOka, and with apparent shifts in the biogeography of wt VZV clades (16, 17, 21, 34, 40, 47, 48). At first, vOka-derived viruses were distinguished using two SNPs located in ORF38 and ORF54 that distinguished the wt clade 2 variant of vOka, (clade 2 Pst1’) from all other wt strains (23). This was an unacceptable approach in Asia, where strains of this subclade were in common circulation, but was initially useful in the US since none of these clades appeared to be present. After pOka and vOka had been completely sequenced, SNPs that could distinguish vOka from all wt strains were identified (16). Based on conventional DNA sequencing methods four SNPs appeared to be fixed in vOka, all of which...
were located in ORF62 (positions 105705, 106262, 107252 and 108111) (16, 30). This finding was supported by the observed SNP profiles of viruses isolated from laboratory-confirmed cases of vOka varicella and HZ, although four isolates were detected with the wt SNP at position 105705 (41). Assays targeting any of the SNPs at 106262, 107252 or 108111 were therefore regarded as the most reliable approach for identifying vOka, particularly if a combination of methods targeting more than one SNP was used. Complicating matters, however, was the observation of a wt clade 5 strain containing the vOka allele at 107252 isolated from varicella cases that had been transmitted from an 86-year-old HZ patient in a long-term care facility (31). Since the index case patient would likely have experienced her primary infection in childhood, this finding suggested that wt strains bearing one of the vOka markers from a clade known to be circulating in North America were present in the early 20th century and are likely to still be circulating today. As such, it has now become necessary to characterize both wt genotyping markers (1, 29) and vOka-specific markers to maximize the reliability of vOka:wt discrimination.

Recognizing the inherent limit in sensitivity of DNA sequencing methods for the detection of mixed alleles, we sequenced a large number of TA clones derived from one lot of vOka to determine whether a small fraction of strains in vOka carried the wt allele at any of the fixed vOka loci. In our study, 2% of vOka clones carried the wt allele at 107252 in ORF62, and 3% of clones carried the wt allele at 105705. None of the 304 ORF62 clones evaluated in this study carried the wt alleles at either 106262 or 108111. A recent study of shorter clones (47), each containing one of the fixed vOka SNPs, found precisely the opposite: 6% of clones had the wt marker at 106262 and 108111, and none of the clones had the wt allele at either 105705 or 107252. While it is unclear whether the discrepant findings reflect lot-to-lot variation,
differences between the experimental approach, the number and type of clones evaluated, or
other factors, it is now apparent that vOka contains small numbers of strains that carry at least
one wt allele at any of the four vaccine markers previously regarded as fixed. Moreover, we
found that 3 of the 6 clones that carried the wt allele at 107252 also carried the wt marker at
105705. On the basis of the findings from our study and that by Thiele and colleges (47),
reliance on a single “fixed” vOka marker would result in a 1-in-17 to 1-in-50 chance of
mischaracterizing a vOka adverse event as a wt infection. A testing algorithm that evaluates all
4 of these SNPs would reduce the risk of mistaken identification by multiple orders of
magnitude. In addition, since one of the vOka markers (107252) has been observed in a North
American clade 5 virus, additional testing to evaluate the Pst1 marker at position 69349 in
ORF38 will also be needed to determine whether an isolate has the SNP profile of vOka-like
clade 2 strains.

We initially screened position 107252 using RFLPs generated by Nae1 restriction
endonuclease. We demonstrated that this approach is a robust, rapid and cost effective screening
tool for characterization of this locus through RFLP patterns generated from the entire OFR62
fragment. The consistency of the additional Nae1 sites in all vOka genomes makes this method
applicable to the analysis of all batches of vOka.

Our observation of a case of vOka HZ illustrates the need for a more complex approach
to discriminating vOka from wt virus. If a method targeting only the 107252 locus had been
employed, this virus would have been identified as wt. Evaluation of SNPs at multiple loci in
ORFs 1, 21, 22, 38, 50, 54, and 62 unambiguously identified the strain as a clade 2 virus of the
vOka/pOka sub-type, (clade 2 Pst1-), with 3 of the 4 vOka markers that were previously
considered fixed.
The Varivax vaccine preparation has also been reported to contain two fixed wt alleles in ORF62 (positions 106710 and 107599) that are present as a mixture of wt and vOka alleles in vOka/Biken and/or Varilrix (16, 40, 48). Since the clones used in our study included the entire ORF62 reading frame, we were also able to evaluate the frequency of these markers in Varivax and to detect any linkage between one or more vOka markers. We detected the vOka allele at 107599 on 18% of clones, which is previously reported to be fixed for the wt in all batches (30, 41, 47, 48). This percentage is well within the detection limits of all currently employed methodologies, and may therefore be indicative of inter-batch variation. None of the 304 clones carried the vOka marker at 106710, consistent with previous observations using DNA prepared directly from the vOka (30, 48). The apparent loss of this vaccine marker may reflect differences in the manufacturing processes used for the three companies that produce this vaccine. The Varivax and Varilrix vaccines underwent additional passages in human cells, which may have introduced selective pressure against the vaccine marker at position 106710.

Another concern for diagnostic approaches to discriminating between vOka and wt strains is the possibility of recombination between vOka and wt virus in co-infected persons. Recombination between wt VZV strains has been reported in vitro and in vivo (1, 2, 11) and phylogenetic analyses of the published complete genomic sequences for VZV have led to the conclusion that recombination has played an important role in the establishment of currently circulating VZV clades (34, 36, 38). Our characterization of the HZ case virus was limited to the analysis of widely scattered SNPs across a large portion of the genome. While the data is not sufficient to make definitive conclusions about recombination, the SNP profile of this virus provided no suggestion that recombination had occurred between vOka and a wt virus.
It is now clear that all 42 of the vOka-associated SNP are present as a mixture of vaccine and wt alleles, albeit at widely variable proportions. The basis for vOka attenuation is therefore likely to be more difficult to ascertain than might have been hoped. A number of the genetic differences between vOka and pOka therefore seem likely to contribute collectively to the reduced pathogenicity of the vaccine. We are currently working to isolate a series of viable clones from the vOka preparation, each with different vOka-associated SNP profiles, for characterization using in vitro and in vivo pathogenicity assays.

In view of our observations, it now seems necessary to evaluate all four of the high-frequency vOka markers in ORF62 (105705, 106262, 107252 and 108111) in addition to the clade 2 subtype marker in ORF38 in order to confidently verify vOka adverse events. Additional genotypic analysis may prove necessary in the future, particularly if the occurrence of vOka:wt recombination is documented.

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Conflict of interest statement
The authors report no conflicts of interest.
References


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