Differentiation of Wild-Type Varicella-Zoster Strains from India and the Oka Vaccine Strain Using a VZV Open Reading Frame – 62 Based PCR-RFLP Technique

Karishma S. Kaushik, Kunal K. Lahiri, Ketoki Kapila, Satish Kumar, Rajiv M. Gupta and Santosh Karade
Department of Microbiology, Armed Forces Medical College; Pune, India

Since the introduction of varicella vaccination in India, surveillance of circulating VZV strains has gained significance. Differentiating wild-type VZV strains from the Oka vaccine strain can be achieved only by molecular genotyping methods. The development of PCR methods for VZV strain differentiation has been hampered by the fact that the VZV genome is highly conserved. We used VZV ORF 62 PCR-RFLP analysis to identify and differentiate wild-type VZV strains in India from the Oka vaccine strain. Digestion of VZV ORF 62 amplicons with SmaI, enabled accurate strain differentiation; the Oka strain was positive for three SmaI sites, compared to two SmaI sites in the wild-type VZV strains that we tested.

Key-Words: Varicella-zoster virus, ORF 62, Oka strain.

Varicella-zoster virus (VZV) is the etiological agent of primary varicella (chicken pox) in childhood; it establishes a latent infection that may be reactivated to cause herpes zoster (shingles). VZV infections are usually benign, but serious and occasionally fatal complications do occur [1]. A live attenuated vaccine against varicella using the Japanese Oka strain is currently licensed for use in India [2]. This vaccine provides protective immunity in more than 90% of healthy adults [3]. Some vaccinees may be incompletely protected against varicella, and when exposed to circulating wild-type VZV strains they can develop "breakthrough" varicella [4]. In addition, several rash-associated clinical syndromes have been described in vaccinees. Vaccinated individuals may experience localized or disseminated rashes within a few weeks following vaccination [5]. In rare cases, transmission of the vaccine strain to secondary contacts has also been described [6]. In addition, the vaccine strain may reactivate, causing a zoster-like illness similar to that caused by reactivation of the wild-type virus [7]. Thus, distinguishing the Oka vaccine strain from circulating wild-type VZV strains is important to monitor potential VZV vaccine-related complications. This would allow us to determine which of these rash syndromes and other side effects are attributable to vaccine strains and which are attributable to the wild-type virus strains.

The development of PCR methods for VZV strain differentiation has been hampered by the fact that the VZV genome is highly conserved. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of PstI and BglI sites in VZV open reading frames (ORFs) 38 and 54, respectively, have been used to differentiate the Oka vaccine strain and wild-type strains [8,9]. More extensive genotyping methods, using amplification analysis of polymorphic repeat regions R2 and R5, have also been analyzed to determine whether they distinguish the vaccine strain from closely related Japanese wild type strains [10]. Argaw et al. identified a single nucleotide polymorphism (SNP) in ORF 62 of the Oka vaccine strain that was absent in the parental isolate from which it was derived [11]. This SNP was caused by a substitution of C for T at position 106262, which established an additional SmaI restriction site in the Oka vaccine strain. This data has provided the basis for developing a new PCR-RFLP technique for differentiation of wild-type VZV strains from the vaccine strain [12]. Thus, Oka-like and non-Oka like wild type strains can be discriminated from the Oka vaccine strain by amplification and digestion of DNA fragments of ORF 62. This method could be universally applied for differentiation between the Oka vaccine and wild-type VZV strains in circulation.

Surveillance of circulating wild-type VZV strains has become important since the introduction of varicella vaccination in India. Differentiation of these strains from the Oka vaccine strain is necessary and can be achieved only by molecular typing methods. We evaluated the utility of the ORF-62-based PCR-RFLP assay to analyze the genotype of circulating wild-type VZV strains in India and differentiate them from the vaccine strain.

Material and Methods

Specimens

Twenty-five patients with VZV infections who attended the Department of Dermatology of our hospital were included in the study. They comprised 22 cases of varicella and three cases of herpes zoster. Vesicle fluid was collected from these patients at the time of presentation. Briefly, each vesicle was gently unroofed using a 22G hypodermic needle. Specimens were transported to the laboratory in Hank’s viral transport medium (VTM) and stored at −20°C. The Oka vaccine strain was obtained from the commercial vaccine preparation Varivax® (Genbiotech, India; Batch no: 200704041 Date of Expiry: 20/10/08) and processed in parallel with the clinical strains.

ORF 62 PCR Assay

Viral DNA was extracted from the clinical specimens using the QIAamp® DNA mini kit (Qiagen, Hilden, Germany),
according to the manufacturer’s instructions. PCR amplification of ORF 62 was performed on all the clinical specimens and the vaccine strain using the PKVL6U-PKVL1L (genome position 106036-106284) set of previously-published primers [12]. The PCR assay was completed in a volume of 100 µL master mix containing 10X PCR buffer (50 mM KCl, 15 mM Tris-HCl), 2.5 mM MgCl₂, 200µM concentration (each) of dATP, dGTP, dCTP and dTTP, and 2.5 units of Taq DNA polymerase (Invitrogen, UK). Each reaction mixture included 0.1 µM of each oligonucleotide primer. For amplification, 200-300 ng of extracted DNA was used as a template. Cycling conditions included an initial denaturation step of 94°C for 5 min, followed by 35 cycles of amplification (94°C for 1 min, 72°C for 1 min) and a final extension step at 72°C for 3 min (Perkin Elmer Cetus). The PKVL6U-PKVL1L primer set offers the advantage of eliminating an annealing step, as at 72°C both primer annealing and polymerase enzyme reaction take place. For detection, 15 µL of PCR product was loaded onto a 10% polyacrylamide gel in Tris-borate-EDTA (TBE) buffer. The gel was run at 150V for 2 hrs; it was then stained with ethidium bromide (0.5 µg/mL in TBE buffer for 30 min).

Restriction Digestion Reaction
Restriction digestion of PCR products was performed in a 25 µL reaction that was comprised of 20 µL PCR product, 2.5 µL (10 units/µL) of restriction enzyme Smal (Sigma, Bangalore, India) and 2.5 µL of accompanying RE buffer. The reactions were incubated at 30°C overnight. The restriction digests were separated by a 10% polyacrylamide gel electrophoresis run at 150V for 2.5 hrs. Gels were stained with ethidium bromide (0.5 µg/mL in TBE buffer for 30 min). The 100-bp DNA ladders (Sigma, India) were used as molecular weight markers.

DNA Sequencing
The nucleotide sequences of PCR products of clinical specimens and the vaccine strain were sequenced with an ABI Prism dye terminator cycle sequencing kit (Applied Biosystems, UK), according to manufacturer’s instructions. The sequences were subjected to BLAST analysis and compared with reference sequences in the GenBank database.

Results
PCR amplification of VZV ORF 62 was achieved in all of the 25 specimens of vesicle fluid that were processed. The primer pair PKVL6U-PKVL1L generated a 268-bp amplicon in both the vaccine Oka strain and clinical VZV strains (Figure 1). Digestion products resulting from Smal RFLP analysis of the amplified 268-bp PCR amplicon produced 112, 79, 41 and 36 bp for the Oka vaccine strain and 153, 79 and 36 bp for all the clinical VZV strains (Figure 2). Thus, fragments obtained from Smal digestion of the ORF 62 of the Oka vaccine strain could clearly be differentiated from fragments obtained from clinical VZV strains. Analysis of nucleotide sequences of the vaccine and wild-type strains revealed a substitution of C for T at position 106262, resulting in a substitution of glycine for arginine. This change leads to the gain of an additional Smal restriction endonuclease cleavage site (CCC-GGG) in the Oka vaccine strain compared to the wild-type VZV strains. Thus, the Oka vaccine strain was positive for the additional Smal restriction site in ORF 62 (Smal⁺), while all the circulating wild-type VZV strains in India were negative (Smal⁻). Nucleotide sequences of the 25 clinical VZV strains and the vaccine strain sequenced in our study were submitted to GenBank (EU264076-EU264101). We found that the wild-type VZV strains in circulation in India can efficiently be differentiated from the Oka vaccine strain with the ORF 62 PCR-RFLP technique.

Discussion
With the introduction of varicella vaccination in India, surveillance of VZV strains occurring in cases of varicella or herpes zoster is needed. Several genetic markers that can detect and differentiate wild-type VZV strains from the vaccine Oka strain have been evaluated previously, such as ORF 6 (AluI), ORF 38 (PstI), ORF 54 (BglII), ORF 62 (Smal, VZV ORF 62 based genotyping NaeI, BssHII) [8-10]. Genetic polymorphisms in ORFs 38 (PstI) and 54 (BglII) have been used widely in vaccine and epidemiological studies [8,13,14].

Wild-type VZV strains circulating in India are of the PstI-BglII genotype, as opposed to the Oka vaccine strain, which has the PstI-BglII genotype [15,16]. However, the limitation of this technique is that it fails to distinguish certain Japanese wild-type strains and isolates from other Asian regions that were misidentified as the Oka vaccine strain [17]. Thus, the ORF 38-54 PCR-RFLP technique is likely to fail in detecting such strains if they are already circulating or subsequently enter the circulating pool of wild-type VZV strains in India. The necessary differentiation appears to be possible by analysis of polymorphisms in ORF 62 of the VZV genome. Argaw et al. identified a SNP in ORF 62 of the Oka vaccine strain that was absent in the parental isolate from which it was derived [11]. This SNP introduced a new Smal restriction site into the Oka vaccine strain and provided the basis for developing a new PCR-RFLP technique for differentiation of wild-type VZV strains from the vaccine strain.

We successfully differentiated circulating wild-type VZV strains in India from the vaccine Oka strain with the ORF-62-based PCR-RFLP method. The vaccine strain was positive for an additional Smal restriction site in ORF 62 (Smal⁺) as opposed to all the circulating wild-type VZV strains analyzed, which were negative (Smal⁻). The ORF 62-based method can be used to characterize wild-type VZV strains presently in circulation in India. In addition, the method will also serve to identify and differentiate Oka-like wild-type strains that may possibly enter circulation and are likely to be missed by less discriminatory methods. An additional advantage of this technique, strain discrimination, can be achieved using a single DNA amplification and a single restriction enzyme digestion, requiring half the cost, time and labor in comparison to the ORF 38-ORF-54 method [12].

ORF 62 is the major VZV regulatory protein, and therefore polymorphisms within this gene have been considered to be a
part of the viral mechanism of attenuation. Wild-type VZV strains display a unique homogeneity at position 106262 of ORF 62, which as demonstrated has a different sequence in the vaccine strain. This could be due to selective pressure against mutations of this nucleotide among the wild-type strains [11]. The largest number of polymorphisms was found in the ORF 62 region of the wild-type strains when compared with the VZV-Dumas ORF 62 region [18]. Thus, substitutions that have accumulated in the ORF 62 region are most likely to be important for differences in the replication and relative attenuation of the Oka vaccine strain as compared to the wild-type VZV strains.

The ORF 62 PCR-RFLP method can be successfully used to genotype and differentiate wild-type VZV strains in India from the Oka vaccine strain. Analysis of additional clinical specimens from different parts of the country will help to strengthen the validity of this approach, particularly in identifying Oka-like wild-type strains that may be in circulation. This method should be a useful tool for definitive differentiation of wild-type VZV strains in India and provide new insight into the molecular epidemiology of VZV infections in our country.

References