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Short communication

# Identification of vaccine-derived polioviruses using dual-stage real-time RT-PCR

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Vaccine-derived polioviruses (VDPVs) are associated with polio outbreaks and prolonged infections in individuals with primary immunodeficiencies. VDPV-specific PCR assays for each of the three Sabin oral poliovirus vaccine (OPV) strains were developed, targeting sequences within the VP1 capsid region that are selected for during replication of OPV in the human intestine. Over 2400 Sabin-related isolates and identified 755 VDPVs were screened. Sensitivity of all assays was 100%, while specificity was 100% for serotypes 1 and 3, and 76% for serotype 2. The assays permit rapid, sensitive identification of OPV-related viruses and flag programmatically important isolates for further characterization by genomic sequencing.

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The Global Polio Eradication Initiative (GPEI) is nearing its goal of interrupting all wild poliovirus (WPV) transmission (World Health Organization, 2013). By 2012, only three countries (Pakistan, Afghanistan, and Nigeria) had never interrupted WPV transmission. Apart from residual localized WPV circulation, all other poliovirus infections worldwide are associated with administration of the live, attenuated Sabin oral poliovirus vaccine (OPV) (Sutter et al., 2013). Most OPV infections are of short duration and confer protective immunity without complication. In rare instances (~1 case per 900,000 first doses administered) phenotypic reversion of the OPV strains may lead to vaccine-associated paralytic poliomyelitis (VAPP) in susceptible OPV recipients and close contacts (Sutter et al., 2013). Vaccine-related viruses excreted by patients with VAPP show only limited sequence divergence from the parental OPV strains. However, more highly divergent vaccine-derived polioviruses (VDPVs), indicative of prolonged replication or circulation, can arise under certain conditions (Kew et al., 2005; Centers for Disease Control and Prevention, 2012). Circulating VDPVs (cVDPVs) are associated with outbreaks in settings where the rates of OPV coverage are low (Kew et al., 2005; Centers for Disease Control and Prevention, 2012). cVDPV outbreaks of all three serotypes have been described, but >80% of isolates have been type 2 cVDPVs (cVDPV2s) (Centers for Disease Control and Prevention, 2012). New cVDPV outbreaks continue to emerge as OPV coverage declines in key high-risk countries and population immunity gaps widen, particularly to type 2 (Centers for Disease Control and Prevention, 2012).

Immunodeficiency-associated VDPVs (iVDPVs) have been isolated from individuals with primary immunodeficiencies (defects in antibody production) who have had prolonged infections, some exceeding 10 years, after exposure to OPV (DeVries et al., 2011; MacLennan et al., 2004). Although fewer than 65 persons have been found since 1961 to excrete iVDPVs, they are at risk of severe paralytic disease, and may serve as a potential source of poliovirus transmission in WPV-free settings (MacLennan et al., 2004; DeVries et al., 2011). Most of the known iVDPV infections have been reported from upper- and middle-income countries where patients have better access to supportive health care. The incidence of new iVDPV infections will likely decline as more countries shift from OPV to inactivated poliovirus vaccine (IPV) (World Health Organization, 2010; Vidor and Plotkin, 2013). Ambiguous VDPVs (aVDPVs) are VDPV isolates from the environment (e.g., sewage) or from persons with no known immunodeficiency and no known link to a VDPV from other than a close contact (Centers for Disease Control and Prevention, 2012). Ambiguous VDPVs continue to be detected in the environment in some settings (possibly signaling the presence of unidentified iVDPV excretors) and in patients with acute flaccid paralysis (AFP) (in some settings signaling early cVDPV emergence) (Centers for Disease Control and Prevention, 2012). The continued

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emergence of VDPVs highlights the need for sensitive surveillance, including improved virologic methods, to assure early detection.

VDPVs are defined as having >1% nucleotide (nt) sequence divergence (i.e., ≥10 nt substitutions) from their parental Sabin strains in the ~900-nt region encoding the major capsid protein, VP1(Kew et al., 2005; Centers for Disease Control and Prevention, 2012). This definition follows from the rapid rate of poliovirus nucleotide sequence evolution of ~1% year (Yang et al., 2005; Jorba et al., 2008; DeVries et al., 2011), and is consistent with at least one year of OPV virus replication (or circulation), considerably longer than the normal period of poliovirus excretion of 4–6 weeks (Alexander et al., 1997; Kew et al., 2005). The demarcation for VDPV2s has been lowered to >0.6% nt sequence divergence in VP1 (i.e., ≥6 nt substitutions) in view of the properties of cVDPV2 isolates from the outbreaks in Nigeria and the Democratic Republic of Congo (Centers for Disease Control and Prevention, 2012; Burns et al., 2013).

Following the 2000–2001 cVDPV1 outbreak in Hispaniola (Kew et al., 2002), the Global Polio Laboratory Network (GPLN) applied two different methods to screen for VDPVs in specimens from AFP patients: (1) one molecular method, usually diagnostic RT-PCR (Yang et al., 1991; Kilpatrick et al., 1996, 1998, 2009), probe hybridization (De et al., 1995), or PCR-restriction fragment length polymorphism analysis (Balanant et al., 1991) to identify polioviruses by their genetic properties and (2) one antigenic method (usually an enzyme-linked immunosorbent assay [ELISA] using specific cross-adsorbed antisera) (van der Avoort et al., 1995) to detect antigenic differences from the OPV strains. Because most VDPVs are antigenic variants (Kew et al., 2005), vaccine-related isolates having "non-Sabin-like", "double-reactive", or "nonreactive" antigenic properties were flagged as candidate VDPVs and further characterized by sequencing of the VP1 capsid region. This screening procedure substantially reduced the need for VP1 sequencing to identify VDPVs and led to the recognition of the cVDPV1 outbreak in the Philippines (Shimizu et al., 2004). However, early (2005–2006) VDPV2 isolates from Nigeria retained their "vaccine-like" antigenic properties in the ELISA, such that the routine screening algorithm did not initially signal any VDPV emergence (Burns et al., 2013). An outbreak was first suspected in 2006 by the frequent isolation from AFP patients of vaccine-related poliovirus type 2, with temporal and geographic clustering in the northern states where OPV coverage rates were known to be low (Wassilak et al., 2011; Burns et al., 2013). The inability of the combined molecular and antigenic assays to identify early outbreak isolates as VDPVs underscored the need for a more sensitive diagnostic method.

Our first approach to developing an RT-PCR method to screen for cVDPVs (Kilpatrick et al., 2004) took advantage of the observation that the large majority of cVDPVs are vaccine/nonvaccine recombinants having some or all of their noncapsid sequences derived from species C human enteroviruses (Kew et al., 2002; Rousset et al., 2003; Shimizu et al., 2004; Kew et al., 2005; Centers for Disease Control and Prevention, 2012), whereas iVDPVs were usually nonrecombinant or vaccine/vaccine recombinants (Kew et al., 2005; Centers for Disease Control and Prevention, 2012). Interpretation of the RT-PCR results, however, was complicated by the occurrence of crossovers in the 3D region at the primer target sequences among some minimally divergent vaccine/vaccine recombinants, and the occurrence of a small number of nonrecombinant cVDPVs (Liang et al., 2006; Yan et al., 2010). Therefore an alternative RT-PCR method targeting capsid region "hot spots" that typically revert in cVDPVs was developed. This new screening method is to be used to identify possible VDPVs once Sabin viruses have been identified using the standard intratypic differentiation methods (Kilpatrick et al., 2009).

The primary targets for each serotype were identified by sequence analysis as those codons frequently changed in VDPVs, compared with the reference Sabin virus sequences (Martín et al., 2000; Kew et al., 2002; Shimizu et al., 2004; Yakovenko et al., 2006; Jegouic et al., 2009; Burns et al., 2013) (Fig. 1). The probes target sites encoding VP1 amino acids 97–103 for Sabin 1 (primary target: aa 99), amino acids 142–147 (primary target: aa 143) for Sabin 2, and amino acids 285–290 (primary target: aa 290) for Sabin 3 target (Fig. 1). All of the targets encode amino acids in surface determinants (Minor, 1990) that are subject to strong selection during replication in the human intestine (Yakovenko et al., 2006). Flanking PCR primers (10 pmol each) were used in conjunction with FAM-labeled TaqMan® probes (Sabin 1, 5 pmol; Sabin 2 and Sabin 3, 20 pmol) in RT-PCR mixes described previously (Kilpatrick et al., 2009). Two stages of amplification were used following the initial cDNA synthesis step, with the first stage (five cycles of 95 °C for 24 s, 44 °C for 30 s, and 60 °C for 24 s) performed at lower annealing and extension temperatures than the second stage (40 cycles of 95 °C for 15 s, 50 °C for 30 s, and 65 °C for 24 s; Fig. 2S). This dual-stage rRT-PCR method increases the

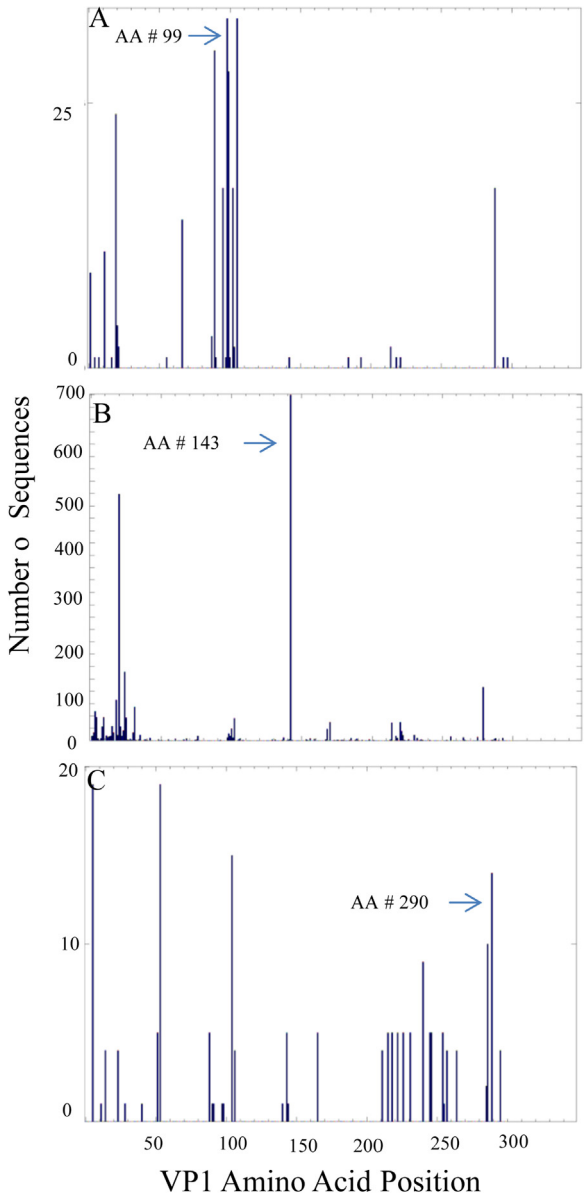


**Fig. 1.** Frequency of changes at each VP1 amino acid position for 34 VDPV1s (A), 702 VDPV2s (B), and 19 VDPV3s (C). The TaqMan probes targeted the identified amino acid as well as amino acids either upstream or downstream to the primary target, depending on the serotype.