Limited and Localized Outbreak of Newly Emergent Type 2 Vaccine-Derived Poliovirus in Sichuan, China

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From August 2011 to February 2012, an outbreak caused by type 2 circulating vaccine-derived poliovirus (cVDPV) occurred in Aba County, Sichuan, China. During the outbreak, four type 2 VDPVs (≥0.6% nucleotide divergence in the VP1 region relative to the Sabin 2 strain) were isolated from 3 patients with acute flaccid paralysis (AFP) and one close contact. In addition, a type 2 pre-VDPV (0.3% to 0.5% divergence from Sabin 2) that was genetically related to these type 2 VDPVs was isolated from another AFP patient. These 4 patients were all unimmunized children 0.7 to 1.1 years old. Nucleotide sequencing revealed that the 4 VDPV isolates differed from Sabin 2 by 0.7% to 1.2% in nucleotides in the VP1 region and shared 5 nucleotide substitutions with the pre-VDPV. All 5 isolates were closely related, and all were S2/S3/S2/S3 recombinants sharing common recombination crossover sites. Although the two major determinants of attenuation and temperature sensitivity phenotype of Sabin 2 (A481 in the 5′ untranslated region and Ile143 in the VP1 protein) had reverted in all 5 isolates, one VDPV (strain CHN16017) still retained the temperature sensitivity phenotype. Phylogenetic analysis of the third coding position of the complete VP1 coding region suggested that the cVDPVs circulated locally for about 7 months following the initiating oral poliovirus vaccine (OPV) dose. Our findings reinforce the point that cVDPVs can emerge and spread in isolated communities with immunity gaps and highlight the emergence risks of type 2 cVDPVs accompanying the trivalent OPV used. To solve this issue, it is recommended that type 2 OPV be removed from the trivalent OPV or that inactivated polio vaccine (IPV) be used instead.

The use of attenuated oral poliovirus vaccine (OPV) for more than 6 decades has reduced the worldwide incidence of polio by >99%; type 2 wild poliovirus (WPV) was eliminated globally in 1999 (1), no type 3 WPV has been reported since 2012, and the transmission of type 1 WPV is now endemic only in Nigeria, Pakistan, and Afghanistan. But with the successful application of OPV immunization, the emergence of vaccine-derived polioviruses (VDPVs) has become a major public health concern, because VDPVs resemble WPVs biologically, can cause paralytic disease in humans, and have the capacity for sustained person-to-person circulation (2, 3). VDPVs were defined as having 1% to 15% nucleotide differences from their parental Sabin strains in the VP1 capsid region of polioviruses at the 17th Informal Consultation on the Global Polio Laboratory Network held in 2011. This arbitrary demarcation represents ~1 year of poliovirus circulation after the initiating OPV dose (4), but it does not imply that poliovirus isolates having <1% nucleotide divergence would lack the capacity to cause paralytic illness in humans or sustain transmission in poorly immunized populations.

For polio eradication programs, the derivatives of the OPV-related polioviruses have been classified into 4 categories (2, 3): (i) vaccine-related polioviruses, which exhibit the closest sequence relationships to the ancestral Sabin OPV strains and include most of the polioviruses isolated from acute flaccid paralysis (AFP) surveillance systems in polio-free countries; (ii) circulating vaccine-derived polioviruses (cVDPVs), which are associated with sustained person-to-person transmission and have caused paralytic cases in which related but nonidentical viruses have been isolated (3); (iii) immunodeficiency vaccine-derived polioviruses (iVDPVs), which are known to be excreted for a prolonged period from the same immunodeficient patient (5, 6); and (iv) ambiguous VDPVs (aVDPVs), which include other VDPVs that cannot be classified into the above 2 VDPV categories. These are viruses that either have been isolated from a single patient without immunodeficiency or are environmental isolates with an unidentified source (2, 7).

The risk for emergence of cVDPVs may be the highest for poliovirus type 2 (8, 9) due to the following. (i) Compared with types 1 and 3, the type 2 OPV strain appears to spread most readily to unimmunized people, as shown by the much higher seroprevalence of poliovirus type 2 found among unvaccinated individuals (10, 11). (ii) Because paralytic attack rates for type 2 poliovirus infections are low, circulation of type 2 VDPVs is the most difficult to detect by AFP surveillance (3). (iii) Type 2 WPV has been eliminated worldwide, suggesting a decrease in the natural immunity of the population to type 2 polioviruses (2). Meanwhile, data from several countries indicated that type 2 vaccine-related viruses from different immunologically normal individuals clustered by serotype, time, or place and followed a common evolutionary pathway (sharing some mutations in the VP1 region) suggestive of type 2 vaccine-related virus circulation occurrence, yet their se-
sequence divergence from the Sabin strain does not exceed 1% (9, 12). Consequently, for avoiding inadequacies of the current cVDPV definition used in the Global Polio Eradication Initiative, which may result in underestimates of the scope of some outbreaks, the World Health Organization (WHO) redefines type 2 VDPV as type 2 polioviruses which differ from Sabin 2 strain by >0.6% in the VP1 region, and all the polioviruses isolated after January 2010 which meet this new definition will be classified as type 2 VDPVs.

In this report, we describe the basic genomic and phenotypic characteristics of type 2 cVDPVs in Aba County, Sichuan, China, during August 2011 to February 2012. This was the first type 2 cVDPV outbreak under the new definition in China. This outbreak was localized and limited in its circulation extent and time.

MATERIALS AND METHODS

Ethics statement. This study did not involve human participants or human experimentation; the only human materials used were stool samples collected from AFP patients and healthy children at the instigation of the Ministry of Health, People’s Republic of China, for public health purposes, and written informed consent for the use of their clinical samples was obtained from the parents of the children involved in this study. This study was approved by the second session of the Ethics Review Committee of the National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention.

Poliovirus isolate classification. In this study, poliovirus isolates are classified as follows: VDPV, Sabin 2-related PV with ≥6 VP1 nucleotide substitutions (≥0.6% divergent); pre-VDPV, Sabin 2-related PV with 3 to 5 VP1 nucleotide substitutions (0.3 to 0.5% divergent), clustered with VDPV geographically and temporally and following the same evolutionary pathway as VDPV. All VDPV and pre-VDPV isolates were included in the description of the type 2 cVDPV outbreak.

Virus isolation and characterization. Polioviruses were isolated by culture in RD cells (human rhabdomyosarcoma cells) and L20B cells (mouse L cells expressing the human CD155 poliovirus receptor [PVR]) and identified by microneutralization with type-specific antisera. Poliovirus isolates were subjected to intratypic differentiation directly using VP1 region sequencing. The complete genome sequences of five type 2 poliovirus isolates were sequenced as described previously (13).

Phylogenetic analysis. VP1 sequence alignments were performed with MEGA software, and phylogenetic trees were constructed by the neighbor-joining method with Kimura’s two-parameter method (version 5.0; Sudhir Kumar, Arizona State University, Tempe, AZ, USA) (14). The reliability of the tree was estimated with 1,000 bootstrap replicates.

Assay for temperature sensitivity. Temperature sensitivities of five Sichuan type 2 cVDPV strains were assayed on monolayers of RD cells in 24-well plates as described previously (15). Briefly, the 24-well plates were inoculated with 50 μl of undiluted virus stocks (P2/Sabin and 5 Sichuan strains). Two different incubators were used; the temperature of one incubator was adjusted to 36°C (optimal temperature for virus propagation), while the other incubator was adjusted to 39.5°C (supraoptimal temperature for virus propagation). After absorption at 36°C or at 39.5°C for 1 h, the unabsorbed viruses were removed, 100 μl of maintenance medium was added to each well, and the plates were continually incubated at set temperatures separately. After 8 h and 24 h postinfection, the plates were harvested, and the 50% cell culture infectious dose (CCID50) was calculated by the endpoint dilution method on RD cell monolayers in 96-well plates at 36°C. Virus isolates showing more than 2-logarithm reductions in titers at different temperatures were considered to be temperature sensitive.

Estimation of the date of the initiating OPV dose. The sequence relationships in the 3rd coding position (closely approximating synonymous substitutions) of the P1 coding region (VP1 to VP4 regions) of all five type 2 isolates were summarized in a phylogenetic tree constructed by Bayesian Markov chain Monte Carlo (MCMC) analysis using the BEAST program (version 1.6.1) (16). The tree was rooted to the Sabin 2 strain, and the time of the initiating OPV dose and divergence of different VDPV branches was estimated from the rate of 3rd-coding-position substitution in the P1 coding region inferred by the MCMC method. The phylogenetic tree was displayed and edited using Figtree software.

Nucleotide sequence accession numbers. The complete genome sequences of the five type 2 Sichuan cVDPV strains described in this study have been deposited in the GenBank database under the accession numbers KJ419273 to KJ419277.

RESULTS

Epidemiologic and clinical background. Circulation of indigenous wild poliovirus (WPV) ceased in China in 1994 (17); along with the other countries in the Western Pacific region, China was certified as free of indigenous WPV in 2000 (18). However, the polio-free status was interrupted by the importation of type 1 WPV from Pakistan in August 2011; the outbreak was stopped 1.5 months later (19).

In that same year, four type 2 cVDPVs occurred in Aba County of Sichuan, China (20). The first patient developed paralysis on 20 August 2011, and a type 2 pre-VDPV was isolated. During October 2011 to February 2012, 3 other AFP patients also yielded type 2 VDPV isolates. In addition, a type 2 VDPV was isolated from a 4-year-old child (Table 1). All 4 case patients were unimmunized children 0.7 to 1.1 years old, and all cases were diagnosed as polio by an expert panel.

Aba County, located in the mountainous area of north Sichuan

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (yr/sex)</th>
<th>Source</th>
<th>No. of doses of OPV</th>
<th>Date of specimen collection</th>
<th>Date of onset</th>
<th>Diagnosis</th>
<th>No. (%) of VP1 nucleotide substitutions</th>
<th>Virus type</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHN15261</td>
<td>0.7/male</td>
<td>AFP</td>
<td>0</td>
<td>20 Sep 2011</td>
<td>20 Aug 2011</td>
<td>Laboratory-confirmed polio case</td>
<td>5 (0.6)</td>
<td>Pre-VDPV</td>
</tr>
<tr>
<td>CHN15284</td>
<td>0.9/female</td>
<td>AFP</td>
<td>0</td>
<td>18 Oct 2011</td>
<td>15 Oct 2011</td>
<td>Laboratory-confirmed polio case</td>
<td>6 (0.7)</td>
<td>VDPV</td>
</tr>
<tr>
<td>CHN16003</td>
<td>1.1/female</td>
<td>AFP</td>
<td>0</td>
<td>19 Jan 2012</td>
<td>7 Jan 2012</td>
<td>Laboratory-confirmed polio case</td>
<td>8 (0.9)</td>
<td>VDPV</td>
</tr>
<tr>
<td>CHN16017</td>
<td>0.8/male</td>
<td>AFP</td>
<td>0</td>
<td>23 Feb 2012</td>
<td>6 Feb 2012</td>
<td>Laboratory-confirmed polio case</td>
<td>11 (1.2)</td>
<td>VDPV</td>
</tr>
<tr>
<td>CHN16019c</td>
<td>4.0/male</td>
<td>Contact of case CHN16003 Unknown</td>
<td>0</td>
<td>8 Feb 2012</td>
<td>8 Feb 2012</td>
<td>Non-AFP</td>
<td>10 (1.1)</td>
<td>VDPV</td>
</tr>
</tbody>
</table>
province, has a large area but a sparse population (83,426 square kilometers with a total population of 0.813 million), which limited the delivery of quality routine immunization services. The annual reported routine immunization coverage of trivalent OPV is more than 95% since 2008 in Aba County. Moreover, an immunization coverage survey of 210 children less than 5 years old in the county after the cVDPV outbreak found that 91% had received three or more doses of OPV. Therefore, immunity gaps may be an important reason for the type 2 cVDPV outbreak (20).

Nucleotide sequence and phylogenetic analysis. Isolate CHN15261 is identified as pre-VDPV because it differs from Sabin 2 at 5 nucleotides in the VP1 region. The isolates CHN15284, CHN16003, CHN16017, and CHN16019c were classified as VDPVs because they differed from Sabin 2 by 0.7% to 1.2% (Table 1). All five isolates shared 5 nucleotide substitutions and were closely related, indicating that they were involved in the same transmission link.

Two nucleotide substitutions identified as key determinants of the attenuated phenotype of the Sabin 2 strain (21) (an A-to-G reversion at nucleotide [nt] 481 in the 5’ untranslated region and a U-to-C reversion at nt 2909 in the VP1 coding region, which caused an Ile143Thr substitution in VP1) had reverted in all five Sichuan type 2 cVDPV strains.

In order to elucidate the diversity and evolution of the Sichuan type 2 cVDPVs, the VP1 sequences of the following viruses were analyzed: 5 Sichuan type 2 cVDPV strains in this study, sporadic type 2 VDPV identified in China earlier (GenBank accession numbers AY948201 and HM107832–HM107835) (7, 22), Egypt type 2 cVDPVs (GenBank accession numbers AF448782 and AF448783) (23), Madagascar type 2 cVDPVs (GenBank accession numbers AM084223 and AM084225) (24), and Nigeria type 2 cVDPVs (GenBank accession numbers JX274980, JX274985, JX275162, and JX275380) (9).

The phylogenetic tree based on the entire VP1 coding region revealed that all five Sichuan type 2 cVDPV strains were much more closely related to each other than to other cVDPVs, could be grouped into a single cluster with pathways of divergence different from Sabin 2 strain, and showed limited divergence from Sabin 2, unlike other cVDPVs in the tree (Fig. 1).

Genomic recombination structure of isolates. Sequencing of the full-length genome of all five Sichuan type 2 cVDPV strains revealed that they were all S2/S3/S2/S3 recombinants sharing common recombination crossover sites, suggesting that these isolates were derived from a common recombinant ancestor strain. The first crossover site mapped to the middle of the 2C region (S2/S3, between nt 4504 and 4508), the second crossover site mapped to the 3’ end of the 2C region (S3/S2, between nt 4771 and 4772), and the third crossover site mapped to the 3’ end of the 3A region (S2/S3, between nt 5257 and 5270) (Fig. 2; also, see Fig. S1 in the supplemental material).

Temperature sensitivity properties of the cVDPV isolates. All five Sichuan type 2 cVDPV strains were compared to Sabin 2 with regard to replication capacity at a supraoptimal temperature (39.5°C) and showed different temperature sensitivity properties.
Sabin 2 was temperature sensitive as expected, with a titer reduction of more than 2 logarithms at 36°C versus 39.5°C, and four isolates showed a markedly lesser effect (titers reduced less than 2 logarithms at 36°C versus 39.5°C). This indicates that the replica-
tion efficiency of these isolates remains the same even at elevated
temperatures and suggests that they had partially lost the temper-
ature-sensitive phenotype (Fig. 3). The exception was VDPV
strain CHN16017, which displayed a growth pattern similar to
that of Sabin 2. This suggests that it had resumed the temperature
sensitivity for growth at 39.5°C in RD cells (Fig. 3).

**Properties in neutralizing antigenic sites.** The amino acid se-
quencies within or near the predicted neutralizing antigenic (NAg)
sites (25, 26) were aligned, including the five Sichuan type 2
cVDPVs, MEF-1, and representative strains of type 2 cVDPVs
from outbreaks in Egypt, Madagascar, and Nigeria. All five Sich-
uan isolates showed the same amino acids in the NAg sites as Sabin
2 (see Fig. S2 in the supplemental material).

**Estimated time of the initiating OPV dose.** A Bayesian
MCMC phylogenetic tree was constructed from the sequences at
the 3rd coding position of the complete P1 region (2,637 nt) of five
Sichuan type 2 cVDPV strains. The 3rd coding position sequences
were compared because they closely approximate synonymous
sites, thereby largely screening out selected changes. The complete
P1 tree diverged from a common ancestral infection estimated to
have occurred around 14 September 2011 (95% highest posterior
density [HPD], 11 August 2011 to 19 September 2011) (Fig. 4). The
rate of fixation of the 3rd-coding-position substitutions into
the P1 region was estimated from the data set to be $5.10^{6}$
3rd-coding-position substitution/site/year, similar to previous es-
timates for the poliovirus capsid region (27). Under the assump-
tion of a strict molecular clock, we estimated that the initiating
OPV dose was given on 8 July 2011 (Fig. 4). The HPD interval for
the date of the initiating OPV dose, calculated from the variance in
the evolution rate, was from 11 February 2011 to 5 August 2011.

MCMC phylogenetic trees based on the complete open reading
frame (ORF) (nucleotides 748 to 7368) and the recombinant por-
tions of the genome (type 3 from nucleotides 4504 to 4771, type 2
from nucleotides 4772 to 5257, and type 3 from nucleotides 5258
to 7368) of these viruses were also constructed. We found that the
topology (the nodes and the branches) of the tree based on the
complete ORF was quite similar to that of the tree based on the
P1 capsid sequences, but the uncertainty (95% HPD) of the node
dates of the initial OPV dose became wider in the complete ORF
tree (data not shown). The topologies of the trees based on each
recombinant interval were similar to that of the tree based on the
P1 capsid sequences, except for the tree based on the first donor
sequence (type 3 from nucleotide 4504 to 4771, which was too
short and quite similar among these virus) (data not shown), sug-

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**FIG 3** Temperature sensitivity test curves of 5 Sichuan type 2 cVDPV isolates.
suggesting that the recombination occurred soon after the initiating OPV dose, probably in the original trivalent-OPV recipient.

DISCUSSION

The VDPVs (both cVDPVs and iVDPVs) that have been identified recently worldwide pose a major challenge for the polio eradication program, especially in “polio-free” countries (3, 5), because their biological properties are indistinguishable from those of the wild polioviruses, including the capacity to cause paralytic disease in human beings, loss of the temperature sensitivity phenotype, and capability of person-to-person transmission. Several studies have been conducted to identify the determinants of attenuation for vaccine-associated strains, but the mechanism underlying the higher transmissibility of polioviruses remains unclear (8, 28).

From August 2011 to February 2012, under the new definition of type 2 VDPV, the first cVDPV outbreak occurred in China. One type 2 pre-VDPV and four type 2 VDPVs were isolated from the cases. These isolates were all newly emergent VDPVs and differed from Sabin 2 by 0.6% to 1.2% nucleotide differences in the VP1 region, and the phylogenetic analysis implied that the cVDPVs circulated for only less than 1 year following the initiating OPV dose. Hence, localization and limitation in circulation extent and time are the characteristics of the cVDPV outbreak in Sichuan which differed from those of earlier reported outbreaks in Egypt (23), Madagascar (24, 29), Democratic Republic of Congo (30), and Nigeria (9, 31), in which more cases were involved and the sequence divergences were larger.

The possibility of antigenic divergence increases along with the replication of OPV strains in human guts (32), and nearly all cVDPVs reported globally have been antigenic variants of OPV strains (9, 23, 33), so WHO adopted a fast intratypic differentiation method (real-time reverse transcription-PCR) to distinguish vaccine-related isolates from VDPV (34). Of the five Sichuan cVDPV isolates, four had partially lost the temperature-sensitive phenotype, but one of the VDPV isolates (strain CHN16017) retained the phenotype of temperature sensitivity, even though it was the most genetically divergent isolate from Sabin 2 and was associated with one of laboratory-confirmed polio cases, showing that key phenotypic properties may fluctuate during the early phase of cVDPV emergence. We assume that the existence of other unknown temperature sensitivity-related determinants could have contributed to this observation, although two known key determinants of the attenuated phenotype of strain CHN16017 reverted to those of the wild type as well as the other 4 isolates.

All five Sichuan type 2 cVDPV strains were vaccine/vaccine (S2/S3/S2/S3) recombinants sharing common recombination crossover sites. This observation addresses a key controversial point in the understanding of cVDPV emergence. Apart from cVDPVs detected in China, which exhibited no recombination of Guizhou cVDPVs (35), and the vaccine/vaccine recombination of Sichuan cVDPVs reported here, all cVDPVs previously described underwent recombination with species C enteroviruses (cVDPVs identified in Egypt, Hispaniola, and the Philippines) (23, 33, 35, 36), prompting the suggestion that recombination with species C enteroviruses is essential to cVDPV emergence and spread. The findings in this report cast some doubt on that assumption and indicate that cVDPVs detected soon after emergence may have properties more typical of vaccine-related strains and that the more extensive evolution (including greater opportunities for recombination) observed elsewhere may be a result of more extensive and prolonged cVDPV transmission. Further virological studies, such as reverse genetic studies, are needed to understand...
the role of nucleotide mutation and genetic recombination in poliovirus evolution and cVDPV emergence.

Although annual province-wide national immunization days (NIDs) have been conducted in Sichuan since 1996 and the annual reported OPV coverage is as high as 95% since 2008, the fact that none of the 4 patients in this cVDPV outbreak was immunized indicates that OPV coverage in some areas is declining and that gaps in population immunity existed which may have established local conditions favoring cVDPV emergence. However, overall population immunity appears to have been sufficiently high to restrict cVDPV transmission to a small extent temporally and geographically. In additional, maintenance of highly sensitive AFP case and virological surveillance permits early detection and a rapid response to interrupt VDPV circulation and restrict transmission.

As a part of the national plan to achieve and maintain a “polio-free” status, China has continued to support an active case-based surveillance system (AFP surveillance) and large-scale annual NIDs. Province-wide immunization in Sichuan that targets all children ages 0 to 3 years has been conducted every December and January since 1996. The cVDPV outbreak in Sichuan of China has important implications in the global initiative to eradicate polio: cVDPVs can emerge and spread in isolated communities with immunity gaps. The detection of the newly emergent cVDPVs in Sichuan highlights the significant role of sensitive field and laboratory surveillance and the risks of type 2 cVDPV emergence accompanying the use of trivalent OPV, and type 2 OPV should be restricted cVDPV transmission to a small extent temporally and geographically.

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