Original Article

Real-time Polymerase Chain Reaction Analysis of Sewage Samples to Determine Oral Polio Vaccine Circulation Duration and Mutation After Mexican National Immunization Weeks

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Presented in part: Infectious Diseases Society of America 49th Annual Meeting, Boston, Massachusetts, October 21, 2011. Abstract 623.

Received December 9, 2011; accepted March 27, 2012; electronically published June 29, 2012.

Background. Oral polio vaccine (OPV) can mutate and cause outbreaks of paralytic poliomyelitis with prolonged replication. After poliovirus eradication, global use of inactivated polio vaccine (IPV) may be needed until all OPV stops circulating. Mexico, where children receive routine IPV but where OPV is given only during biannual national immunization weeks (NIWs), provides a natural setting to study duration of OPV circulation in a community primarily vaccinated with IPV.

Methods. One-liter sewage samples from four separate arroyos (creeks) near Orizaba, Mexico, were collected monthly for 12 months. Concentrated sewage underwent RNA extraction, reverse transcription, and real-time polymerase chain reaction (PCR) to detect OPV serotypes 1, 2, and 3 and their variants containing the serotype-specific point mutation in the 5′ untranslated region associated with neurovirulence.

Results. OPV was detected 3, 4, 5, and 7 months after the May 2010 NIW, but was not detected at 6 or 8 months. A second and third NIW occurred in February 2011 and May 2011, and OPV was detected in the sewage monthly after both of these NIW through July 2011 when collection stopped. The OPV detected was primarily serotype 2 and predominantly contained the point mutations in the 5′ untranslated region associated with increased neurovirulence.

Conclusions. OPV was detected in sewage as late as 7 months after an NIW in a Mexican community primarily vaccinated with IPV, but was not detected at 8 months, suggesting that OPV circulation may have ceased. These data suggest that in communities with high vaccination rates, 1 or 2 years of IPV administration after OPV cessation could be sufficient to prevent outbreaks of paralytic poliomyelitis from vaccine-derived strains.

Key words. Polio; OPV; Mexico; Sewage; PCR

Since 1988, when the World Health Organization proposed a plan to eradicate poliomyelitis, the number of reported annual cases dropped from 350 000 to 650 in 2011 [1, 2]. The last case of naturally acquired wild poliovirus type 2 was reported in 1999, and only 3 countries remain endemic with uninterrupted transmission

[2]. The primary approach for global eradication has been administration of oral polio vaccine (OPV), an inexpensive live attenuated vaccine that is easy to administer and promotes herd immunity as it is shed in the stools of vaccinated children and can spread to community contacts. However, OPV circulation may actually threaten the eradication of poliomyelitis.

OPV can cause vaccine-associated paralytic poliomyelitis (VAPP), in vaccinees or their close contacts, at a rate of about 1 case per 500 000 primary vaccinees [3]. As OPV replicates in the gut, it rapidly acquires serotype-specific point mutations in the stem-loop V of the 5' untranslated region (OPV-1, 480 G to A; OPV-2, 481 A to G; OPV-3, 472 U to C), that revert back to the wild-type sequence at those positions. These neurorevertant mutations, though often encountered in healthy vaccinees, are associated with VAPP. Of more concern are vaccine-derived polioviruses (VDPV), which are 1%–15% divergent from parent OPV strains (0.6%– 15% for serotype 2) and develop with prolonged replication of typically more than one year [4, 5]. In normal circumstances, children vaccinated with OPV and their infected close contacts shed virus for several weeks. However, in communities with incomplete vaccine coverage, OPV and OPV-derived strains can spread person-to-person and circulate for years, allowing the virus to mutate and form circulating VDPV. Eighteen independent circulating VDPV outbreaks have been identified [6, 7], with an attack rate and disease severity similar to wild poliovirus infections [8]. There is no evidence that OPV or inactivated polio vaccine (IPV) fail to induce protective immunity to VDPV.

After wild poliovirus eradication, one strategy to phase out polio vaccination while preventing VDPV outbreaks is global administration of only IPV to maintain high population immunity until all global circulation of OPV and OPV-derived strains ceases. IPV is more expensive than OPV and may provide inferior intestinal immunity; children vaccinated with IPV have been found to have increased OPV shedding after an OPV challenge than children primarily vaccinated with OPV (37% IPV-vaccinated children versus 5% of OPV-vaccinated children have continued shedding after 3 weeks), but less OPV shedding than previously unvaccinated children (of whom 81% were still shedding at 3 weeks) [9]. However, IPV is effective at preventing poliomyelitis, and cannot mutate into neurovirulent forms. The duration of global IPV administration that would be necessary to prevent persistent circulation of OPV and OPV-derived strains is unknown; these data are necessary to determine the cost and feasibility of this strategy.

Mexico, which switched to a primary IPV-only regimen in 2007 (with children receiving IPV as part of a pentavalent vaccine at 2, 4, 6, and 18 months) but administers OPV during semiannual national immunization weeks (NIW), is an ideal setting to study the duration of OPV circulation in a population routinely vaccinated with IPV. Sewage, estimated to contain detectable poliovirus if as few as one in 10 000 people in the community are excreting OPV [10], has been shown to be the most sensitive reservoir for demonstrating OPV circulation in a community [11, 12]. We describe the results from a study in Veracruz, Mexico, in which monthly sewage samples were collected after an NIW and were analyzed by real-time polymerase chain reaction (PCR) to detect OPV and OPV containing the revertant serotype-specific point mutation in the 5' untranslated region associated with increased neurovirulence.

MATERIALS AND METHODS

Study Area and Design

The study area covered urban and rural municipalities (Orizaba, Rio Blanco, Rafael Delgado, and Tlilapan) in Veracruz, Mexico with a total population of 183 855 inhabitants. Of these, 14 573 (7.9%) were 5 years old or younger [13]. Of the 5-year-old children in the area, 88.64% received at least 3 doses of IPV (personal communication, Dr Guadalupe Canales, Orizaba public health epidemiologist). In our August 2010 survey of 72 children, divided evenly among the four municipalities, ranging in age from 3 months to 30 months (median age 15 months), 74% had received all of the IPV doses recommended for their age, 21% had received some (but not all) of the IPV doses recommended for their age, and 5.6% had received no IPV doses. Fifty percent of these 72 children had received a dose of OPV in the May/June 2010 NIW. Eleven percent of these children lived in houses with dirt floors, while 89% lived in homes with cement floors. Nineteen of 57 respondents (33%) did not have flush toilets in their household. On later household visits to 70 of the 72 children, 61% (43/70) lived in overcrowded conditions as defined by CONAPO, the Mexican National Population Council (http://www.conapo.gob.mx/publicaciones/indices/pdfs/001.pdf).

This was a longitudinal study collecting monthly 1-liter sewage samples from 4 creeks (arroyos; Escamela, La Carbonera, Totolitos, and Xalatl) that drained the

study area, after NIWs in which OPV was given to children 5 years old and younger who had already received 2 routine IPV doses. NIWs occurred May 29, 2010 to June 4, 2010, February 15, 2011 to February 19, 2011, and May 23, 2011 to May 27, 2011, but per our survey of local residents, OPV was still being given for about a week after the February 2011 and May 2011 NIWs. Monthly sewage collection occurred from late August 2010 until late July 2011. Escamela and Totolitos drain the urban municipality of Orizaba, La Carbonera drains the urban municipality of Rio Blanco, and Xalatl drains the rural municipalities of Rafael Delgado and Tlilapan. This study received approval from the Stanford Institutional Review Board, the Comisión de Ética and the Comisión de Bioseguridad of the Mexican National Institute of Public Health, and from the Public Health Center of Orizaba, Veracruz.

Sample Processing

Within several hours of collection, sewage samples were brought to the Orizaba laboratory, separated into 50 mL aliquots, and concentrated with a vacuum filtration method using Millipore HAWG 0.45 µm filter membranes (Millipore Inc, Billerica, MA) as described previously [14, 15]. The filtration apparatus was cleaned between aliquots from separate liters of sewage. The funnel, base, and tweezers were washed with detergent and a stiff-bristled brush, rinsed with distilled water, soaked in a 10% bleach solution for 3 minutes, rinsed with distilled water, sprayed with RNAseZAP (Ambion, Austin, TX), rinsed with distilled water, and allowed to completely air dry. If the aliquots could not be concentrated that day, they were frozen at –20°C and concentrated in the next few days.

After concentration, the filter membrane and concentrate were transferred to a cryovial containing 500 µL of guanidine isothiocyanate (GITC) buffer (GITC buffer contained 5 M guanidine thiocyanate, 100 mM EDTA, and 0.5% *N*-lauroyl sarcosine). The cryovials were stored at –80°C until shipment on dry ice to the Maldonado laboratory at Stanford.

Homogenization and RNA Extraction

Homogenization of the concentrated sewage and filter membrane using a QIAshredder spin column (Qiagen, Valencia, CA) and RNA extraction using a RNeasy minikit (Qiagen, Valencia, CA) were performed as described previously with the following exceptions [14]. Three hundred fifty microliters of RLT buffer (Qiagen) containing ß-mercaptoethanol was added to the thawed cryovials of concentrated sewage and vortexed before transferring to the spin columns. Homogenized sewage from 2 adjacent 50 mL sewage aliquots were combined in the ethanol step of the RNA extraction so that the final purified RNA originated from 100 mL of sewage.

Reverse Transcription and Real-time PCR

RNA extracted from the first and last 2 50 mL aliquots for each liter of sewage were analyzed. Reverse transcription of the RNA into cDNA and real-time PCR analysis with 6 assays to detect both the revertant (containing the serotype-specific point mutation in the 5' untranslated region associated with increased neurovirulence) and nonrevertant OPV serotypes 1, 2, and 3 were performed as described previously with the following exceptions [14]. A Biorad CFX384 Real-Time System was used (Bio-Rad Laboratories, Hercules, CA), and a single threshold, set at 400 RFU, was used for detecting fluorescence.

Calculating Lower Limits of Detection

The stocks of positive controls were identical to those described previously [14]. Using a cutoff of $C_T < 35$, the lower limits of detection of the sewage assay were calculated by spiking serial dilutions of cell culture of control nonrevertant and revertant OPV serotypes 1, 2, and 3 into 100 mL of sewage and performing the complete cycle of sewage concentration, RNA extraction, reverse transcription, and real-time PCR.

Interpreting Results

Our real-time PCR assay can detect cycle thresholds (C_T) as high as 40, but we have used a C_T cutoff of <35 to identify samples that contain detectable OPV because a C_T >35 can indicate either very low concentrations of virus or false positives. However, although false positives representing crossreaction of very high concentrations of a different OPV serotypes can have $C_T > 35$ and < 36.5, we saw no false positives among 44 negative controls in the $35 < C_T < 36.5$ range. Consequently, we were confident that samples with C_T in this range indicated low levels of OPV (there were no high concentrations of any serotype that might have crossreacted), and so these samples were included as "borderline positive" in the table. As our revertant proportion equation is not accurate with $C_T > 35$, we could not include a revertant proportion for these samples, but we indicated whether the lower C_T value came from the revertant or nonrevertant assay.

RESULTS

The lower limits of detection of our sewage assay were 1.6, 1.8, and 2.3 log $CCID_{50}$ virus in 100 mL of

sewage for nonrevertant OPV-1, 2, and 3, respectively, and 2.7, 2.1, and 2.5 log $CCID_{50}$ virus in 100 mL of sewage for revertant OPV-1, 2, and 3, respectively. Only intact virus (cell culture) could be detected; naked RNA spiked into the sewage was not detected at any concentration. As we reported previously, there was an inverse linear relationship in the sewage assay between the amount of virus in log $CCID_{50}$ and the C_T value [14], such that lower C_T values indicate a higher concentration of virus.

We detected OPV in sewage 3, 4, 5, and 7 months after the May 2010 NIW, but not at 6 or 8 months (Table 1). OPV was again detected 0.5, 1.5, 2.5, and 3.5 months after the February 2011 NIW and 1 and 2 months after the May 2011 NIW. Thirty of 33 OPV strains detected were serotype 2. OPV serotypes predominantly contained the revertant serotype-specific point mutation at 3, 4, and 5 months, were predominantly nonrevertant at 7 months, and mixed revertant and nonrevertant in the months after the second and third NIWs. In comparing Xalatl (the arroyo draining the two rural municipalities) to the other three arroyos that drained urban municipalities, Xalatl had similar or slightly increased sewage samples with OPV in the 3.5 months directly after an NIW, but no samples with OPV at 4 months after an NIW and beyond. Of note, preliminary data from analysis of stool samples of members of the communities from which the sewage was obtained indicate that the OPV detected was a result of circulation between different members of the community rather than prolonged shedding by an individual member of the community, as different individuals shed OPV during different months and rarely shed OPV longer than 1 month in a row (data not shown). The concentrations of OPV detected in the sewage were too low to sequence in this analysis.

In the $36.5 < C_T < 40$ range, there was a 7% false positive rate among 44 negative controls. Hence, it would be impossible to determine for individual samples whether a C_T in this range was a false positive or indicated very low levels of OPV. Consequently, samples with C_T values in this range were not noted as containing detectable OPV. However, a fourfold increase in percentage of samples with a C_T in this range for all three serotypes was found in the 2 months following the February 2011 NIW as compared to the sewage collected 3–8 months after the first NIW, suggesting that these might represent extremely low concentrations of OPV-derived viruses in the sewage.

DISCUSSION

OPV was detected in sewage up to 7 months after an NIW in a Mexican community primarily vaccinated with IPV, but not at 8 months, indicating that OPV circulation may have ceased by this point. Although the OPV detected 3, 4, and 5 months after the NIW contained predominantly revertant OPV as expected, the OPV detected at 7 months contained predominantly nonrevertant OPV. OPV accumulates revertant mutations with replication over time [3], suggesting that the nonrevertant OPV detected 7 months after the NIW may not have originated from the NIW but may have been imported into the community. If so, circulation of OPV strains from the NIW may have actually ceased by 6 months.

Unfortunately, we have been unable to sequence our isolates to date, which would have been useful to either assess genetic linkage or to evaluate whether there was formation of VDPV. As noted in the Results section, the concentration of the virus we detected was quite low and only just detectable by our real-time PCR assay that amplifies a short section in the 5' untranslated region (varying between 55 and 132 bp long, depending on the serotype). We are still attempting to amplify and sequence the much larger VP1 region (~900 bp long) for both our samples and control virus diluted to similarly low concentrations in order to address the issue of prevalence of VDPV in this community.

The duration of circulation we report is longer than reported in studies in New Zealand and Cuba. In the New Zealand study, stool and sewage were monitored for the presence of OPV after migrating to a national IPV regimen, and OPV was detected in stools up to 4 weeks and in sewage up to 12 weeks after the change [11]. In Cuba, where polio vaccination is based exclusively on semiannual OPV NIWs, OPV was detected in stool samples until between 7 and 15 weeks and in sewage until between 15 and 19 weeks after an NIW [12]. The longer duration of circulation we report could be due to differences in climate and hygiene in Mexico compared to New Zealand, decreased intestinal immunity generated by the IPV primary regimen in Mexico compared to the OPV regimen in Cuba, or because both the Cuba and the New Zealand study used the less-sensitive cell-culture method, rather than real-time PCR, to detect OPV [16]. However, it should be noted that if the OPV seen in the 7-month sewage samples in our study were imported after the NIW as argued above, then the 5-month duration of

Table 1. Sewage Sample Analysis for the Presence of Oral Polio Vaccine Serotypes by Real-Time Polymerase Chain Reaction After 3 National Immunization Weeks

Months Since First (and Second; and Third) NIW:		3	4	5	6	7	8	9 (0.5)	10 (1.5)	11 (2.5)	12 (3.5)	13 (4.5; 1)	14 (5.5; 2)
Serotype	Location	Ct (RP)	Ct (RP)	Ct (RP)	Ct (RP)	Ct (RP)	Ct (RP)	Ct (RP)	Ct (RP)	Ct (RP)	Ct (RP)	Ct (RP)	Ct (RP)
1	Escamela	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	Escamela	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	La Carbonera	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	La Carbonera	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	Totolitos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	Totolitos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**30.57 (0.08)**
	Xalatl	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	Xalatl	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
2	Escamela	Neg	**32.89 (0.99)**	**36.29 (R)**	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	Escamela	Neg	**34.32 (0.98)**	Neg	Neg	**36.04 (NR)**	Neg	Neg	**35.73 (NR)**	Neg	Neg	Neg	Neg
	La Carbonera	Neg	Neg	**34.90 (0.97)**	Neg	Neg	Neg	**34.73 (0.31)**	Neg	Neg	Neg	Neg	Neg
	La Carbonera	Neg	Neg	**36.19 (R)**	Neg	**35.50 (NR)**	Neg	**35.87 (NR)**	**34.63 (0.03)**	Neg	Neg	Neg	Neg
	Totolitos	**33.38 (0.99)**	**33.56 (0.83)**	**35.22 (R)**	Neg	Neg	Neg	**36.01 (R)**	Neg	Neg	Neg	**34.69 (0.97)**	**34.04 (0.98)**
	Totolitos	**32.78 (0.99)**	**35.35 (R)**	Neg	Neg	**34.70 (0.04)**	Neg	**35.86 (R)**	Neg	Neg	Neg	**33.69 (0.99)**	**35.06 (R)**
	Xalatl	**33.01 (0.99)**	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**34.41 (0.02)**	**33.09 (0.02)**	**32.61 (0.01)**	**35.37 (NR)**
	Xalatl	**35.65 (R)**	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**34.29 (0.02)**	Neg
3	Escamela	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	Escamela	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	La Carbonera	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	La Carbonera	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	Totolitos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	Totolitos	Neg	Neg	Neg	Neg	Neg	Neg	**36.19 (R)**	Neg	Neg	Neg	Neg	Neg
	Xalatl	**35.64 (NR)**	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	Xalatl	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Positive and borderline positive results are in bold type. A revertant proportion (RP) close to 1 indicates the oral polio vaccine (OPV) is mostly revertant, and an RP close to 0 indicates the OPV is mostly nonrevertant. RP is inaccurate at cycle threshold (Ct) > 35; for 35 < Ct < 36.5, R or NR indicate whether the lower Ct value was for the revertant or nonrevertant assay, respectively.

Abbreviations: Ct, cycle threshold; NIW, national immunization week; OPV, oral polio vaccine; RP, revertant proportion; Neg, negative for detectable OPV.

[a]NIW took place at months 0, 8.5, and 12.

OPV circulation seen in our study would be very similar to the results from the Cuba study.

OPV serotype 2 was the predominant serotype isolated. Our assay is more sensitive for revertant OPV-2 than for revertant OPV-1 and 3, which likely contributed to this finding. However, the predominance of OPV-2 might also occur because serotyope 2 is better able to replicate and establish intestinal infection than serotypes 1 and 3 [17]. The composition of trivalent OPV was changed from a 10:1:3 ratio of serotypes 1, 2, and 3, respectively to a 10:1:6 ratio in the enhanced trivalent OPV in the late 1980s because serotype 2 replication in the gut was shown to interfere with serotype 3 replication and subsequent seroconversion [17]. The ability of OPV-2 to replicate easily and hence provide high seroconversion rates likely contributed to the eradication of wild poliovirus serotype 2 in 1999. However, this also may have contributed to the finding that the vast majority of circulating VDPV have been serotype 2 [7].

In conclusion, OPV, primarily serotype 2, was detected in sewage as late as 7 months after an NIW in a Mexican community primarily vaccinated with IPV, but not at 8 months, suggesting that OPV circulation may have ceased. The OPV detected predominantly contained the revertant serotype-specific point mutation in the 5' untranslated region. These data suggest that in communities with high vaccination rates, 1 or 2 years of IPV administration after OPV cessation could be sufficient to prevent outbreaks of paralytic poliomyelitis from vaccine-derived strains.

Acknowledgments

We thank the population, participants, and health care workers of the Orizaba Health Jurisdiction, Mexico, for their generous support and cooperation.

Financial support. This work was supported by a grant from the World Health Organization (Protocol ID RPC378, title: "Post-eradication polio vaccination strategies: effect of routine IPV immunization on OPV, VAPP, and VDPV shedding in Mexico after NIDs," PI: Yvonne Maldonado), by an NIH Career Development Award (Protocol ID 1K23AI093678-01, title: "Oral Polio Vaccine Circulation in Mexico," PI: Stephanie Troy), and by the Instituto Nacional de Salud Pública in Mexico.

Potential conflicts of interest. Y. M. has worked on the vaccine advisory board for Merck and Novartis. All other authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. (CDC) CfDCaP. Progress toward interruption of wild poliovirus transmission—worldwide, January 2010–March 2011. MMWR Morb Mortal Wkly Rep **2011;** 60:582–6.
2. Global Polio Eradication Initiative: Polio This Week. http://www.polioeradication.org/Dataandmonitoring/Poliothisweek.aspx. Accessed March 5, **2012**.
3. Minor P, Macadam A, Stone D, Almond J. Genetic basis of attenuation of the Sabin oral poliovirus vaccines. Biologicals **1993;** 21:357–63.
4. Sutter R, Kew O, Cochi S. Poliovirus vaccine-live. In: Paul O, Orenstein W, Plotkin S, eds. Vaccines. 5th ed. Philadelphia: Saunders/Elsevier, **2008.**:631–85.
5. (CDC) CfDCaP. Update on vaccine-derived polioviruses—worldwide, July 2009–March 2011. MMWR Morb Mortal Wkly Rep 2011; 60:846–50.
6. Kew OM, Sutter RW, de Gourville EM, Dowdle WR, Pallansch MA. Vaccine-derived polioviruses the endgame strategy for global polio eradication. Annu Rev Microbiol **2005;** 59:587–635.
7. GPEI. Circulating vaccine-derived poliovirus (cVDPV) 2000–2011. http://www.polioeradication.org/Dataandmonitoring/Poliothisweek/Circulatingvaccinederivedpoliovirus.aspx. Accessed November 9, 2011.
8. Jenkins H, Aylward R, Gasasira A, et al. Implications of a circulating vaccine-derived poliovirus in Nigeria. N Engl J Med **2010;** 362:2360–9.
9. Laassri M, Lottenbach K, Belshe R, et al. Effect of different vaccination schedules on excretion of oral poliovirus vaccine strains. J Infect Dis **2005;** 192: 2092–8.
10. Hovi T, Stenvik M, Partanen H, Kangas A. Poliovirus surveillance by examining sewage specimens: quantitative recovery of virus after introduction into sewerage at remote upstream location. Epidemiol Infect **2001;** 127: 101–6.
11. Huang Q, Greening G, Baker M, et al. Persistence of oral polio vaccine virus after its removal from the immunisation schedule in New Zealand. Lancet **2005;** 366:394–6.
12. Más Lago P, Gary HJ, Pérez L, et al. Poliovirus detection in wastewater and stools following an immunization campaign in Havana, Cuba. Int J Epidemiol **2003;** 32:772–7.
13. INEGI (Instituto Nacional de Estadística G, e Informática). XIII Censo General de Población y Vivienda **2010**. Mexico, 2011.
14. Troy SB, Ferreyra-Reyes L, Huang C, et al. Use of a novel real-time PCR assay to detect oral polio vaccine shedding and reversion in stool and sewage samples after a Mexican national immunization day. J Clin Microbiol **2011;** 49:1777–83.

15. Walters S, Yamahara K, Boehm A. Persistence of nucleic acid markers of health-relevant organisms in seawater microcosms: implications for their use in assessing risk in recreational waters. Water Res **2009;** 43: 4929–39.
16. Corless C, Guiver M, Borrow R, et al. Development, evaluation of a 'real-time' RT-PCR for the detection of enterovirus and parechovirus RNA in CSF and throat swab samples. J Med Virol **2002;** 67:555–62.
17. Maldonado Y, Peña-Cruz V, de la Luz Sanchez M, et al. Host and viral factors affecting the decreased immunogenicity of Sabin type 3 vaccine after administration of trivalent oral polio vaccine to rural Mayan children. J Infect Dis **1997;** 175:545–53.