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Diversity of picornaviruses in rural Bolivia

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Abstract

The family *Picornaviridae* is a large and diverse group of viruses that infect humans and animals. Picornaviruses are among the most common infections of humans and cause a wide spectrum of acute human disease. This study began as an investigation of acute flaccid paralysis (AFP) in a small area of eastern Bolivia, where surveillance had identified a persistently high AFP rate in children. Stools were collected and diagnostic studies ruled out poliovirus. We tested stool specimens from 51 AFP cases and 34 healthy household or community contacts collected during 2002–2003 using real-time and semi-nested RT-PCR assays for enterovirus, parechovirus, cardiovirus, kobuvirus, salivirus, and cosavirus. Anecdotal reports suggested a temporal association with neurologic disease in domestic pigs, so six porcine stools were also collected and tested with the same set of assays, with the addition of an assay for porcine teschovirus. A total of 126 picornaviruses were detected in 73 of 85 human individuals, consisting of 53 different picornavirus types encompassing five genera (all except *Kobuvirus*). All six porcine stools contained porcine and/or human picornaviruses. No single virus, or combination of viruses, specifically correlated with AFP; however, the study revealed a surprising complexity of enteric picornaviruses in a single community.

Introduction

Picornaviruses are among the most common viruses infecting humans. Enteroviruses (EV), including EV species A to D, and rhinovirus species A to C, are the most thoroughly characterized human picornaviruses and cause a wide spectrum of acute clinical disease,

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including undifferentiated febrile illness, respiratory illness, aseptic meningitis, and acute flaccid paralysis (AFP). Human parechoviruses (HPeV) have a similar disease spectrum as the EVs, but routine clinical testing is less common despite increasing evidence of a significant disease burden in infants (Harvala *et al.*, 2010). Zoonotic infection with encephalomyocarditis virus (EMCV) has been reported (Oberste *et al.*, 2009) but testing for this virus is rarely performed in human clinical virology laboratories. No routine testing is available for newer human picornaviruses, which include Saffold viruses (SAFV) (*Cardiovirus*), cosaviruses (CoSV) (also published as "dekavirus"; *Cosavirus*), and "salivirus" (SALV) (also published as "klassevirus"; *Salivirus*) (Adams *et al.*; Knowles *et al.*, 2012).

AFP is a manifestation of many different disease processes with different mechanisms, and multiple causes, both infectious and non-infectious, including paralytic poliomyelitis, certain arboviruses, Guillain-Barré syndrome (GBS), transverse myelitis, certain toxins, and trauma. Surveillance for AFP is recommended by the World Health Organization (WHO) as the standard approach for poliovirus surveillance for the purposes of Global Polio Eradication (Anonymous, 2006). A wide variety of non-polio enteroviruses and other picornaviruses have been identified in stool specimens of AFP cases (Bingjun *et al.*, 2008; Kapoor *et al.*, 2001; Kapoor *et al.*, 2008; Oberste *et al.*, 2006; Saeed *et al.*, 2007; Santos *et al.*, 2002; Shoja *et al.*, 2007). Detection of high-prevalence agents from a non-sterile site is not sufficient to conclusively establish an etiologic link of picornavirus infection with disease; however, given the known ability of many picornaviruses to cause central nervous system (CNS) disease, it is likely that these viruses contribute to at least a fraction of AFP cases. From 1996 to 2006, AFP surveillance activities in Bolivia identified persistently high AFP rates in Hernando Siles province, Chuquisaca Department, a relatively poor, largely agricultural area in southern Bolivia. These unusually high AFP rates—40 to 140 per 100,000 children under age 15, compared to 1.2 to 2.3 per 100,000 for Bolivia as a whole—have raised considerable local public concern. In all cases, wild poliovirus has been ruled out by laboratory testing through the routine AFP surveillance program (Anonymous, 2006); environmental contamination of water and effects of agricultural chemicals were also ruled out. There were anecdotal reports of concurrent outbreaks of paralytic illness in local pigs, though no direct evidence linking human and porcine diseases was available.

The plausibility of a picornavirus etiology and the availability of archived fecal specimens presented an opportunity to assess the role of non-polio picornaviruses in pediatric AFP and the diversity of enteric picornaviruses in children in rural Bolivia.

Results

A total of 49 AFP cases (32 from Chuquisaca, and 17 from Santa Cruz) and 32 contacts (17 from Chuquisaca and 15 from Santa Cruz) were included in the clinical analyses. Of the 49 AFP cases, 26 were reported in 2002 and 23 in 2003. Detailed clinical and epidemiologic information was available for 45 cases. The median age of cases was 5 years (range, 1–21 years) with >90% occurring among children aged ≤8 years; 19 (42%) were male. There was significant geographic difference in seasonality, as all cases from Chuquisaca had onset during February-May, while cases from Santa Cruz were distributed throughout the year,

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with February to May accounting for 58.8% (10 cases) ($p < 0.001$). Most (33 or 75.0%) of the 44 cases for whom available clinical details were sufficient for classification presented with GBS-like paralysis, one case (2.3%) had enterovirus/polio-like paralysis and 10 cases (22.7%) had a mixed clinical picture (Table 1). Clinical presentations of AFP cases in both departments were comparable.

All of the human stools collected were analyzed for picornaviruses (N = 85 total; 51 AFP and 34 contacts). At least one picornavirus was detected in specimens of 73 (85.9%) study subjects, including 37 apparent coinfections with >1 picornavirus, with evidence of up to six different viruses (five different picornaviral genera) in a single patient (Table 2). There were no significant differences between virus detection rates by case/contact status or between departments, suggesting that picornavirus infection was not associated with AFP in these cases. Nevertheless, the study presented an opportunity to examine the diversity of enteric picornaviruses in this population.

Picornaviruses detected in human stools included members of the *Enterovirus*, *Parechovirus*, *Cardiovirus*, *Salivirus*, and *Cosavirus* genera (Tables 2 and 3). EVs were detected in 44 specimens, representing 19 different types from three different EV species (Table 3; Figure 1). Members of EV-A, including coxsackievirus (CV) A5, CVA14, EV76, and EV90, were identified in nine instances (Figure 1E). Nine specimens yielded EV-B, including echovirus (E) 1, E24, E29, E30, E33, and EV106 (Figure 1C). Poliovirus vaccine strains, commonly found in pediatric fecal samples in countries that use live-attenuated, oral polio vaccine, were detected in two samples, one PV2 and one PV3. Other EV-C viruses were also detected, including CVA1, CVA11, CVA17, CVA20, CVA24, EV96, and EV113 (22 detections) (Figure 1B). In two cases, VP1 sequences appeared to be mixtures of EVs and the individual sequences could not be resolved. Two additional specimens were 5'NTR real-time positive with high Ct values near the limit of detection, and negative with the confirmatory/typing EV VP1 assay.

Sixteen human specimens yielded seven types of human parechoviruses (HPeV), including HPeV1, HPeV2, HPeV3, HPeV4, HPeV7, HPeV9, and HPeV12 (Tables 2 and 3; Figure 2A). HPeV4 was the most common HPeV (four human specimens), followed by HPeV7 and HPeV9. The Bolivia HPeV1 VP1 sequences were most closely related to the HPeV1 prototype strain Harris/USA-1956 (84% nucleotide identity [%NT ID] and 95% amino-acid identity [% AA ID]), unlike most recent HPeV1s, which have split into a separate HPeV1 clade (Figure 2A). The two HPeV2 strains from Bolivia are most closely related to the HPeV2 prototype strain Williamson/USA-1956 (86% NT ID and 96% AA ID). Notably, the Bolivia HPeV4 VP1 carboxyl-termini lacked the RGD motif found in all other HPeV4s in GenBank (data not shown), and had instead a variant consensus carboxyl-terminal end (17 amino-acids). We were unable to grow these variant HPeV4s in cell culture, unlike the prototype HPeV4 that grows readily in Vero and other cell types. The Bolivia HPeV4s shared six identical amino acids at the putative VP1-2A cleavage site (NPFEDE / S) and significant amino acid similarity in the remainder of the carboxyl-tail (data not shown) with HPeV1 and HPeV5 variants from the Netherlands and with HPeV15 from Bangladesh (Oberste *et al.*, 2013). The single HPeV3 found in Bolivia also lacked the RGD motif. We

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did not attempt to grow this virus in culture. HPeV1s and HPeV2s found in Bolivia all contained the RGD motif.

Cardiovirus was detected in 21 human specimens (Table 2), including 16 detections of members of the species *Theilovirus*, and five detections of members of the *Encephalomyocarditis virus* (EMCV) species. Partial VP1 sequencing was successful for 11 of 16 theilovirus-positive specimens and all were identified as SAFV (Table 3; Figure 2B). SAFV1 was the most common type identified, with five positive stools, followed by SAFV2, SAFV9, and SAFV4 (Figure 2B). Phylogenetically, SAFV1s split into two Bolivia clades, sharing 87.9 to 89.1% NT ID between them (99.1 to 100 % AA ID). Both clades had from 77.3 to 79.2 % NT ID (94.4 to 95.8 % AA ID) with the 1981 SAFV1 prototype strain from the United States (NC009448). The two SAFV2 strains clustered with SAFV2 from Brazil. One SAFV sequence appeared to be a mixture and four other specimens were SAFV 5'NTR real-time positive, but negative in the SAFV VP1 assay. All EMCV-positive specimens had very high threshold cycle (Ct) values (from 40 to 45) near the limit of detection (Ct of 45), indicating very low genome copy number, and could not be independently confirmed with the EMCV VP1 sequencing/typing assay.

Human cosaviruses (CoSV) were detected in 44 human stool specimens (Ct values from 30 to 44.7), 35 of which yielded clean partial VP1 sequences (Tables 2 and 3, Figure 3A–E). Five CoSV sequences appeared to be mixtures, which could not be resolved and four specimens with high CoSV real-time Ct values were not amplified by the CoSV VP1 sequencing assay. Three CoSVs were typed as CoSV A3 (77 to 85.2% NT ID and 93.2 to 100% AA ID), three as CoSV A8 (76–94.5% NT ID and 88–97.6% AA ID), two as CoSV A17 (78.8–90% NT ID and 93.4–98.2% AA ID), one as CoSV A7 (77.9% NT ID and 95% AA ID) and two as CoSV D4 (80–99.5% NT ID and 88.5–90.7% AA ID). CoSV species A was the most common CoSV detected (15 viruses; eleven types), followed by CoSV E (13 viruses; nine types), CoSV D (six viruses; three types), and CoSV B (one virus). Analysis of the partial VP1 sequences of the Bolivia CoSVs suggests that 22 of them can be classified as 15 new types (nine CoSV E, three CoSV A, two CoSV D and one CoSV B). All of their partial VP1 sequences have <75% NT ID and <88% AA ID to all previously characterized CoSV types (Holtz *et al.*, 2008; Kapoor *et al.*, 2008).

A single specimen was positive by 5'NTR real-time RT-PCR targeting human kobuvirus and salivirus but not canine, murine, porcine or bovine kobuviruses (in silico analyses only) (Tables 2 and S1), with the partial VP1 sequence having 79.8% NT ID (88% AA ID) to the prototype SALV A1 (GQ507022) and 92.4% NT ID (96% AA ID) to a candidate SALV A2 (JQ898343) from Thailand (2009), sequenced directly from sewage (Ng *et al.*, 2012). Based on the partial VP1 sequence analyses, all three of these viruses are SALV A1. None of the human specimens tested positive for teschovirus.

Picornaviruses were detected in all six pig specimens (Table 2). Partial VP1 sequences from two of these were porcine enteroviruses (PEV) (Figure 1D), one of which may represent a new PEV type, having only 73% NT ID (76% AA ID) to the closest PEV in GenBank (GQ502355), while the other PEV shared 78% NT ID (86% AA ID) with PEV-10 (GQ502355). Parechovirus was detected in three pig specimens; two were identified as

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HPeV4 (Figure 2A), but the third (Ct = 38.6) produced no VP1 amplicon, so the type could not be determined. One cardiovirus of TMEV species was detected (Ct = 38), but VP1 sequence could not be obtained. CoSV with a partial VP1 sequence that was a close match to a Bolivia CoSV sequence from a human specimen (98.9% NT ID and 97.6% AA ID) was detected in one pig stool (Figure 3B). Teschovirus was detected and identified in three of six pigs with no further characterization. No human kobuviruses or saliviruses were detected in the pigs. Four picornaviruses were detected in a single pig, including teschovirus, PEV (Ct = 25), CoSV (Ct = 42.5), and HPeV4 (Ct = 35.4).

The four human and one porcine HPeV4 with complete VP1 sequences were 97 to 100% NT ID (97.4 to 100% AA ID) to one another and 73.0 to 73.9% NT ID (84.1 to 85.8% AA ID) to the HPeV4 prototype (AM235750). With <75% NT ID and <88% AA ID to the HPeV4 prototype, the Bolivia HPeV4s could possibly be classified as a new type (Nix *et al.*, 2010); however, most of the difference between the prototype and the Bolivia HPeV4s was due to variation in 17 amino acids (51 nucleotides) at the carboxyl-terminal end. When these 17 amino acids were omitted from the analysis, the Bolivia HPeV4s were 89.3 to 91.1% AA ID (75.9 to 76.8% NT ID) to the HPeV4 prototype strain.

Discussion

Our study demonstrates that picornavirus diversity in human and animal feces is clearly much more complex than previously known. Nevertheless, our findings suggest that picornaviruses are not a major contributor to the very high AFP rates observed in the Hernando Siles province of Bolivia. The basis for this conclusion includes the predominance of GBS-like presentation usually not consistent with picornavirus etiology, tremendous diversity of identified picornaviruses without any predominant agents, substantial overlap of results between cases and contacts, or between clinical subtypes, and lack of significant differences between the departments with high and low incidence. The involvement of picornaviruses in individual cases cannot be ruled in or out based on the available data.

Molecular methods detect a wider range of picornaviruses than cell culture alone (Begier *et al.*, 2008) and several of the picornaviruses found in this study have not been cultured to date (CoSV, SALV, and new HPeV types), thereby confirming the superior performance of molecular approaches for surveillance, diagnostics, and genotyping of picornaviruses. Several other studies, using both classical and molecular methods, have detected EV, HPeV, SAFV, and CoSV in fecal samples from AFP cases (Bingjun *et al.*, 2008; Junttila *et al.*, 2007; Kapoor *et al.*, 2001; Oberste *et al.*, 2006; Saeed *et al.*, 2007). Most previous studies, including our own, used specific PCR assays to detect members one or two genera (usually only *Enterovirus*), due to specificity, low cost, and potential for high throughput, though some investigators are starting to use metagenomics methods to expand the range of viruses that can be detected (Victoria *et al.*, 2009), with limitations of higher per-sample cost and lower overall throughput. Our approach was a compromise between specific PCR and metagenomics, allowing the possibility of detecting seven different genera within the family *Picornaviridae*. In areas where conditions are favorable for fecal-oral transmission, one should expect to find a wide variety of enteric picornaviruses. As metagenomic approaches

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become more cost effective, a wider range of potential pathogens can be detected in large studies, facilitating better assessment of disease association.

A diverse range of EVs was identified in this study (Figure 1), including 19 different types, among them a newly identified type, EV-C113, which along with EV-A90 were the most common viruses identified in the study, and another recently discovered type, EV-B106. Of the four EV species EV-C was the most common. No EV-D viruses were identified.

Our study also demonstrated substantial diversity of HPeVs in Bolivia (Figure 2A). One of the seven different types identified had a distinct genetic characteristic, specifically, the absence of the classical RGD integrin-binding motif, shown to be the receptor ligand for HPeV1 and possibly the receptor ligand for HPeV2, 4, 5, and 6 as well. This is distinct from all other previously described HPeV4, but similar to HPeV3 and HPeVs 7 through 16, which lack the RGD motif in all VP1 sequences published to date (Benschop *et al.*, 2008). The absence of the RGD motif in these viruses suggests that these viruses may interact with integrins by a different mechanism or use a different receptor altogether. The carboxyl-terminal differences between the HPeV4 prototype and the Bolivia viruses may be the result of intertypic recombination and another HPeV. Natural intertypic recombination at the 3' end of the P1 region has been reported for polioviruses (Blomqvist *et al.*, 2003; Blomqvist *et al.*, 2010).

Very few human clinical virology laboratories are capable of testing for cardiovirus. With the exception of the recent reports of SAFV (TMEV species) in human feces and respiratory specimens (Abed & Boivin, 2008; Drexler *et al.*, 2011; Drexler *et al.*, 2008; Jones *et al.*, 2007; Nielsen *et al.*; Zoll *et al.*, 2009), almost all cardiovirus testing is for animal specimens in the context of EMCV epizootics (Maurice *et al.*, 2005). Recent studies have reported EMCV infection in two human cases from Peru (Oberste *et al.*, 2009) and 21% EMCV seroprevalence among persons sampled in the same area of Peru (Czechowicz *et al.*, 2011). Both cardiovirus species were identified in Bolivia human samples. All EMCV-positive specimens in our study had very high Ct values approaching the assay's limit of detection (Ct of 45 or approximately 10 EMCV genomic copies), suggesting a very low viral load and no type data was acquired from these specimens. The low levels of EMCV detected may be indicative of environmental contamination with rodent materials, resulting in "pass-through" virus and not indicative of virus replication in the human intestinal tract. SAFV1, the most common cardiovirus genotype identified (Figure 2B), was the overall third most common virus found among study subjects. SAFV2, detected in North and South America, Europe, and Asia, has been the most commonly identified genotype to date (Abed & Boivin, 2008; Blinkova *et al.*, 2009; Chiu *et al.*, 2008; Drexler *et al.*, 2008; Itagaki *et al.*, 2010; Zoll *et al.*, 2009). SAFV2 has been associated with respiratory disease (Abed & Boivin, 2008; Chiu *et al.*, 2008), and with invasive disease, including two fatalities (Drexler *et al.*, 2011; Nielsen *et al.*). All SAFV types have been associated with gastroenteritis, often in coinfections with other known viral pathogens. Additional studies are needed to determine the prevalence of SAFV and zoonotic EMCV infection in humans, define the associations with human disease, and to estimate the human disease burden due to cardioviruses.

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CoSV, found in a large proportion of study subjects, were equal to EV in prevalence. Quantitative real-time RT-PCR showed high CoSV viral load in stool, ranging from approximately 4×10^4 to 4×10^9 per gram stool (data not shown), indicating probable CoSV replication in the intestinal tract. CoSV diversity, based on partial VP1 sequences, was the broadest of all picornavirus genera identified, encompassing 21 types in CoSV-A, -B, -D, and -E (Table 3; Figure 3). Picornavirus typing using partial VP1 sequences is generally reliable; however, because the ICTV Picornavirus Study Group requires at least complete VP1 sequence for consideration of new types, the new CoSV types from Bolivia must be considered tentative until complete VP1 sequences have been acquired. These studies are in progress.

A single salivirus, SALV A1 was identified in the Bolivia samples. SALV A1 has not been isolated in culture, and represents the only definitive type identified so far worldwide. SALV seroprevalence in the United States, investigated using an IgG ELISA, was 8.6% in a pediatric cohort and 21.8% in an adult cohort (Greninger *et al.*, 2009), suggesting infection is relatively common.

The inclusion of pig specimens was related to anecdotal reports of frequent paralytic illness in pigs in communities with high human AFP rates. Three pig illnesses which are associated with encephalitis and can present with paralysis have been repeatedly documented in the area: pseudorabies (Aujeszky's disease), caused by porcine herpesvirus 1; classical swine fever, caused by a flavivirus; and Teschen-Talfan disease, caused by teschoviruses (Anonymous, 2011; 2012a; b; World Organization for Animal Health). None of these viruses infects humans but, because people in these communities often have very high exposure to pigs due to their economic importance, potential zoonotic transmission of other porcine viruses (e.g. EMCV) must be considered. Our study did not identify porcine viruses in any of the human specimens. However, we detected carriage of human picornaviruses, including HPeV, CoSV, and SAFV, in pigs. The HPeV4 and CoSV found in pig specimens were clearly the same respective viruses found in the human specimens (Figure 2A–B, and 3B) but the possibility of human picornavirus replication in the pigs can only be systematically addressed through animal studies. Though the study is limited by the amount and quality of clinical and epidemiologic data available for diseased pigs and the small number of specimens, the data suggest zoonotic porcine picornaviruses are an unlikely cause of the disease in humans

The limitations of this study include lack of true controls as only contacts of AFP cases were tested, which are likely to share exposures, and consequently viral flora, with cases. In addition, stool is not the optimal specimen for picornavirus disease association studies due to the high proportion of asymptomatic infections and because disease generally affects other organ systems. The zoonotic transmission aspect of the study was limited by the lack of clinical and epidemiologic data for the pigs and the small number of specimens.

The cause of very high AFP rates in Hernando Siles province of Chuquisaca Department in Bolivia remains unclear, but most likely is not due to picornavirus infections. A potential non-infectious cause worth additional exploration could be the toxins from *Karwinskia* plants common in Central and South America. The *K. humboldtiana* fruits contain toxins

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known to cause GBS-like illness, and their consumption has been associated with relatively high rates of AFP in some Central American countries (Martinez *et al.*, 1998; Ocampo-Roosens *et al.*, 2007). A representative of *Karwinskia* genus, *K. oblongifolia* grows in this area of Bolivia (Serrano & Terán, 2000) and its fruit is commonly consumed by children.

Materials and Methods

Study subjects

AFP cases and their contacts from Hernando Siles province reported to the national AFP surveillance system during 2002–2003 were included in the study. Hernando Siles is a poor, difficult to access, agricultural region with mountainous terrain, a subtropical climate, and a population of approximately 36,000 (~0.4% of the national population, 34.6% aged <15 years; 2001 census, http://apps.ine.gob.bo/censo/entrance.jsp). Most people live in isolated communities established in river canyons and in the towns of Huacareta and Monteagudo, the provincial capital. Available specimens from AFP cases and their contacts from Santa Cruz Department in eastern Bolivia, which does not have a high AFP rate, were tested for comparison.

Clinical and epidemiologic data for the AFP cases were obtained from national AFP surveillance records during a 2007 site visit. Details for the contacts of cases were not available. The available clinical data were limited because of the lack of specialist neurology examinations for all but two cases and the absence of imaging or nerve conduction studies to confirm the diagnosis and further characterize the subtype of AFP. Additionally, no cerebrospinal fluid was taken for laboratory examination or virus diagnostic studies. Clinical subtypes of AFP were classified as GBS-like, enterovirus/poliovirus-like, and “mixed.” Cases with ascending, symmetric, more distal than proximal paralysis which progressed over several days and often had sensory involvement were considered as having GBS-like-paralysis. Cases with descending, asymmetric, more proximal than distal paralysis were considered as having EV/polio-like paralysis. Cases with features of both categories were classified as having a “mixed” subtype. Chi-square and Fisher’s test were used for statistical comparisons, as appropriate.

Specimen collection

Stool specimens included in the study were collected from AFP cases and their household or community contacts by the Ministry of Health, aided by PAHO-Bolivia, following World Health Organization guidelines for AFP case investigation (Anonymous, 2006). Additionally, six fecal specimens were collected from domestic pigs in the affected communities in Hernando Siles.

Picornavirus molecular detection and identification

RNA was extracted from stool suspensions, as previously described (Nix *et al.*, 2006), and tested for enterovirus, parechovirus, cardiovirus, kobuvirus, salivirus, and cosavirus by genus- or genus/species-specific Taqman® real-time RT-PCR assays targeting the 5’ non-translated region (5’NTR) (Kilpatrick *et al.*, 2009; Nix *et al.*, 2008; Oberste *et al.*, 2009) (Table S1). Teschovirus testing was performed by using a 5’-NTR nested RT-PCR (Zell *et*

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al., 2000), with the addition of PCR amplicon sequencing to confirm the identity of the correct-sized PCR product.

In vitro transcribed single-stranded RNA controls were produced from PCR products as described previously (Nix *et al.*, 2008). RNA control transcripts were used to generate standard curves for estimating genome copy numbers in the stool specimens. RT-PCR primers and the viral RNAs used to make DNA templates for the *in vitro* RNA transcription reactions are shown in Table S2.

Real-time PCR-positive specimens were confirmed by nested or semi-nested RT-PCR targeting a portion of the genome encoding the VP1 capsid protein (S3–S6), followed by amplicon sequencing for EV (Nix *et al.*, 2006), HPeV (Nix *et al.*, 2010), cardiovirus (Oberste *et al.*, 2009), kobuvirus, salivirus, and cosavirus. Virus type was determined by comparison of the VP1 amplicon nucleotide sequences with VP1 sequences of the reference strains for each genus by script-driven sequential pairwise comparison using the program Gap (Wisconsin Sequence Analysis Package, version 11.0, Accelrys, Inc., San Diego, CA). Deduced VP1 amino acid sequences were compared by a similar method. VP1 nucleic acid sequences were aligned using the Pileup program (Wisconsin Package) and phylogenetic relationships were inferred by the neighbor-joining method implemented in MEGA, version 4.0 (Kumar *et al.*, 2001), using the Kimura 2-parameter method (Jones *et al.*, 1992; Kumar *et al.*, 2001). Support for specific tree topologies was estimated by bootstrap analysis with 1000 pseudo-replicate data sets. Nucleotide and amino acid identity matrices were calculated using MegAlign (DNAStar, version 8, Madison, WI).

The nucleotide sequences described here have been deposited in the GenBank sequence database, accession no. JX219484-JX219590. Four nucleotide sequences used to identify virus types were rejected by GenBank due to their short lengths (<200 base pairs) (Table S9).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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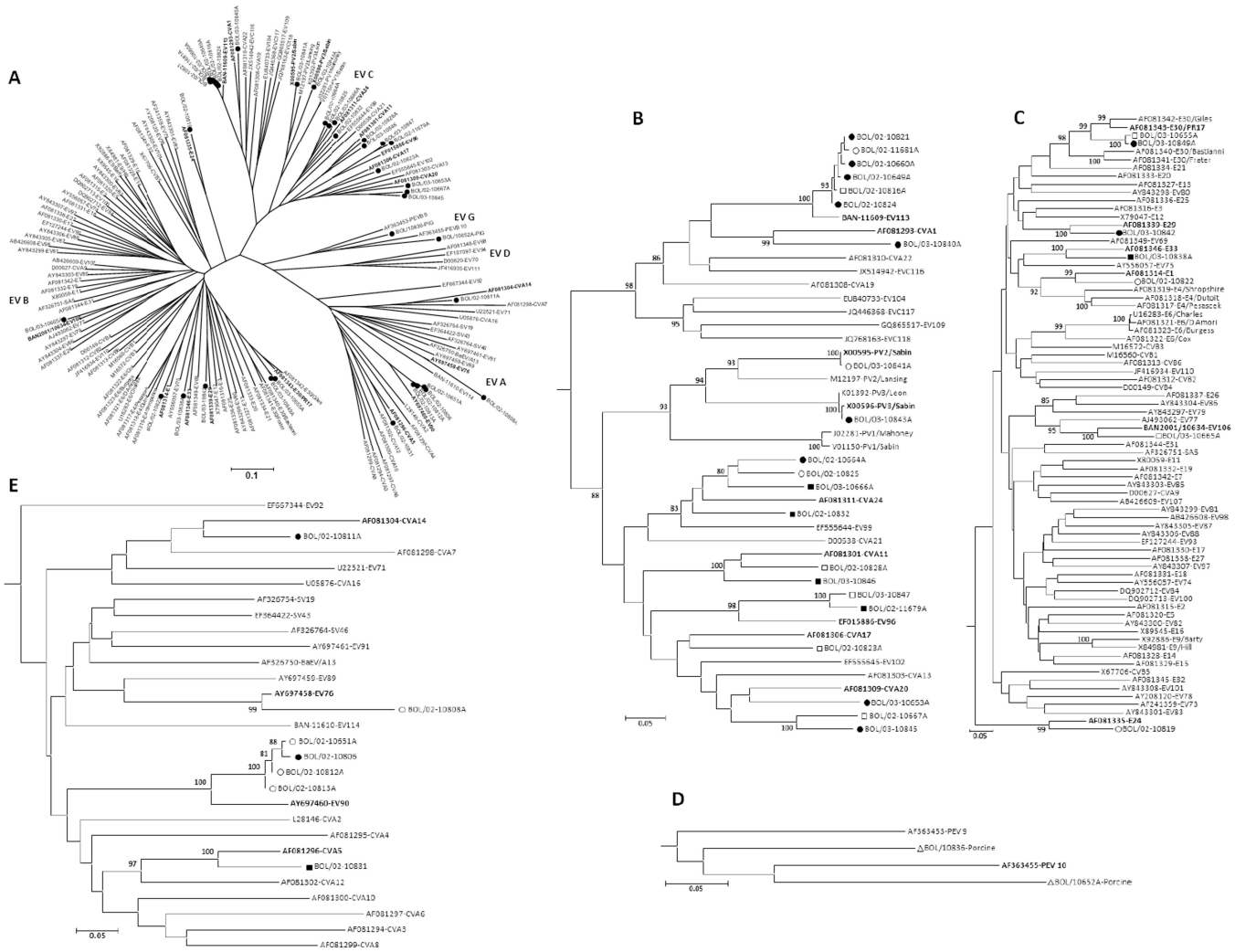

Figure 1.

Phylogenetic relationships among human enteroviruses detected in Hernando Siles province. The VP1 nucleic acid tree was constructed by the neighbor-joining method implemented in MEGA, version 4, using the Kimura 2-parameter substitution model (Jones *et al.*, 1992) and 1000 bootstrap pseudoreplicates. E, echovirus; EV, enterovirus; CV, coxsackievirus. A. The radial tree shows the distribution of EV species (EV-A, -B, -C, -D, and porcine EV-G) typed from Bolivia (filled circles). B-E. Subtrees of EV-C, EV-B, EV-G, and EV-A respectively. Chuquisaca: solid circle = AFP case, hollow circle = contact, hollow triangle = porcine; Santa Cruz: solid square = AFP case, hollow square = contact. Prototype viruses detected in Bolivia are in bold. Only full length, double-stranded partial VP1 sequences were included for phylogenetic analyses.

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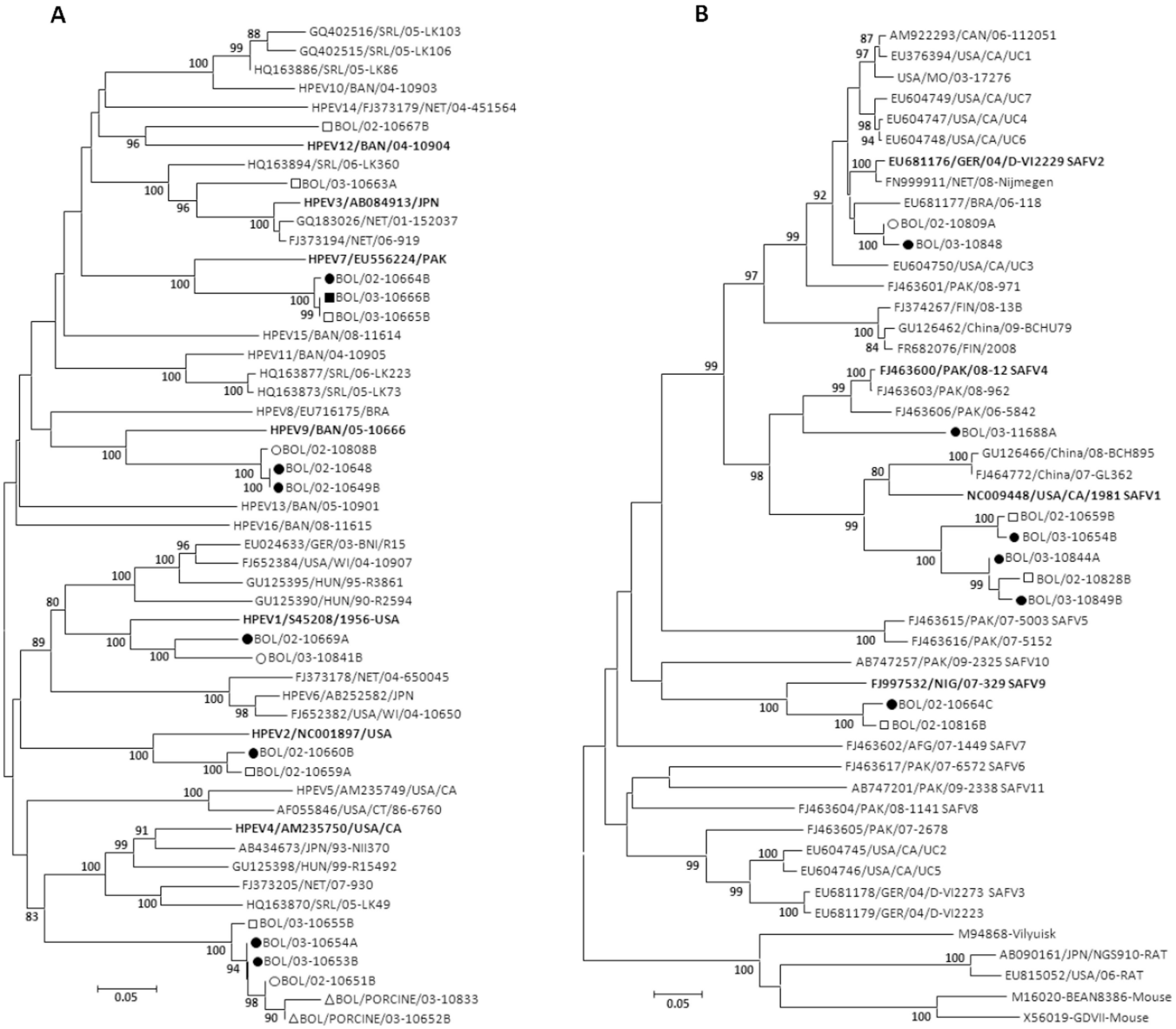

Figure 2.

A. Phylogenetic relationships among human parechoviruses detected in Hernando Siles province. B. Phylogenetic relationships among human cardioviruses detected in Hernando Siles province. The VP1 nucleic acid trees were constructed by the neighbor-joining method implemented in MEGA, version 4, using the Kimura 2-parameter substitution model (Jones *et al.*, 1992) and 1000 bootstrap pseudoreplicates. Chuquisaca: solid circle = AFP case, hollow circle = contact, hollow triangle = porcine; Santa Cruz: solid square = AFP case, hollow square = contact. Prototype viruses detected in Bolivia are in bold. Only full length, double-stranded partial VP1 sequences were included for phylogenetic analyses.

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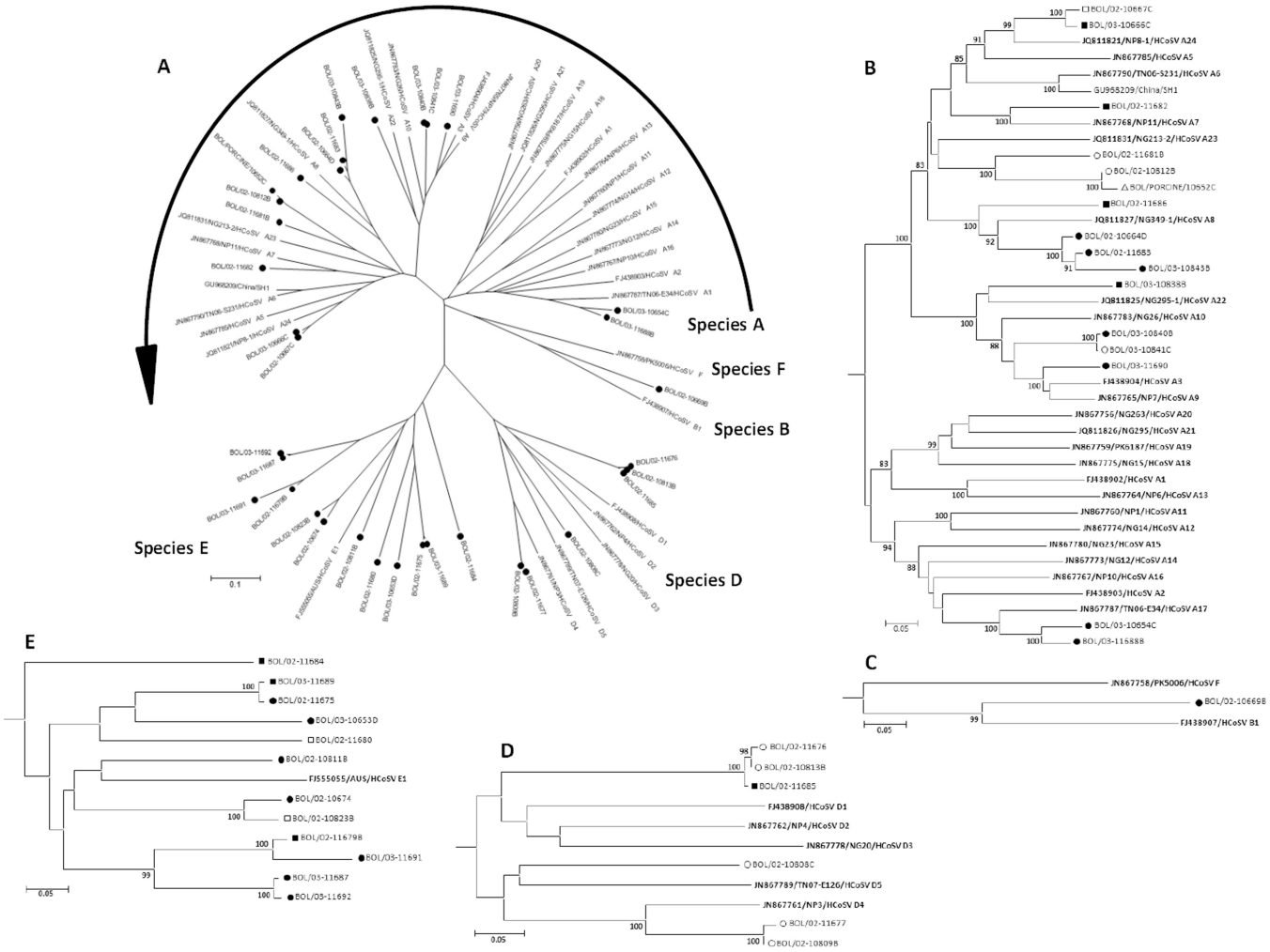

Figure 3.

Phylogenetic relationships among human cosaviruses (CoSV) detected in Hernando Siles province. The VP1 nucleic acid tree was constructed by the neighbor-joining method implemented in MEGA, version 4, using the Kimura 2-parameter substitution model (Jones *et al.*, 1992) and 1000 bootstrap pseudoreplicates. A. The radial tree shows the distribution of CoSV species (CoSV-A, -B, -D, and -G) typed from Bolivia (filled circles). B-E. Subtrees of CoSV-A, -B, -D, and -E respectively. Chuquisaca: solid circle = AFP case, hollow circle = contact, hollow triangle = porcine; Santa Cruz: solid square = AFP case, hollow square = contact. All proposed prototype CoSVs are shown in bold. Only full length, double-stranded partial VP1 sequences were included for phylogenetic analyses.

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Table 1

Clinical characteristics of AFP cases from Chuquisaca and Santa Cruz departments, Bolivia, 2002–2003.

Characteristics	N (%)
Duration of progression, mean (range)	6 (1–15) days
Ascending paralysis	33/43 (76.7%)
Descending paralysis	2/43 (4.7%)
Simultaneous paralysis in all 4 limbs	8/43 (18.6%)
Symmetric paralysis	36/43 (83.7%)
Paresthesias	5/24 (20.8%)
Sensory deficits	19/30 (63.3%)
Muscle pain	36/40 (90.0%)
Febrile/”infectious” prodrome	24/43 (55.8%)
Fever at paralysis onset	20/43 (46.7%)
Residual paralysis/weakness (>70 days after onset)	28/43 (65.1%)
Death	2/43 (4.7%)

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Table 2

Picornavirus detection rates in stool specimens of AFP cases and their contacts, Chuquisaca and Santa Cruz departments, Bolivia, 2002–2003.

Categories	Total		Chuquisaca		Santa Cruz	
	No.	%	No.	%	No.	%
Total subjects	85	100.0	51	100.0	34	100.0
Any picornavirus (+)	73	85.9	47	92.2	26	76.5
Enterovirus (+)	44	51.8	30	58.8	14	41.2
Parechovirus (+)	16	18.8	10	19.6	6	17.6
Cardiovirus (+)						
SAFV	16	18.8	9	17.6	7	20.6
EMCV	5	5.9	3	5.9	2	5.9
Cosavirus (+)	44	51.8	30	58.8	14	41.2
Kobuvirus (+)	0	0	0	0	0	0
Salivirus (+)	1	1.2	1	2.0	0	0
>1 picornavirus (+)	37	43.5	21	41.2	16	47.0
AFP cases	51	100.0	32	100.0	19	100.0
Any picornavirus (+)	43	84.3	28	87.5	15	79.0
Enterovirus (+)	22	43.1	15	46.9	7	36.8
Parechovirus (+)	8	15.7	7	21.9	1	5.3
Cardiovirus (+)						
SAFV	11	21.6	8	25.0	3	15.8
EMCV	4	7.8	3	9.4	1	5.3
Cosavirus (+)	27	52.9	17	53.1	10	52.6
Kobuvirus (+)	0	0	0	0	0	0
Salivirus (+)	1	2.0	1	3.1	0	0
>1 picornavirus (+)	20	39.2	14	43.8	6	31.6
Contacts of AFP cases	34	100.0	19	100.0	15	100.0
Any picornavirus (+)	30	88.2	19	100.0	11	73.3
Enterovirus (+)	22	64.7	15	78.9	7	46.7
Parechovirus (+)	8	23.5	3	15.8	5	33.3

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Categories	Total		Chuquisaca		Santa Cruz	
	No.	%	No.	%	No.	%
Cardiovirus (+)						
SAFV	5	14.7	1	5.3	4	26.7
EMCV	1	2.9	0	0	1	6.7
Cosavirus (+)	17	50.0	13	68.4	4	26.7
Kobuvirus (+)	0	0	0	0	0	0
Salivirus (+)	0	0	0	0	0	0
>1 picornavirus (+)	17	50.0	10	52.6	7	46.7

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Table 3

Picornaviruses detected and sequenced (VP1 gene region) from human stool specimens[a].

Genus/Species	Type	No. Sequenced	Chuquisaca	santa Cruz	AFP	Contact
Enterovirus/EV-A	CVA5	1	0	1	1	0
Enterovirus/EV-A	CVA14	1	1	0	1	0
Enterovirus/EV-A	EV-A76	1	1	0	0	1
Enterovirus/EV-A	EV-A90	6	6	0	1	5
Enterovirus/EV-B	E1	1	1	0	0	1
Enterovirus/EV-B	E24	3	3	0	1	2
Enterovirus/EV-B	E29	1	1	0	1	0
Enterovirus/EV-B	E30	2	1	1	1	1
Enterovirus/EV-B	E33	1	0	1	1	0
Enterovirus/EV-B	EV-B106	1	0	1	0	1
Enterovirus/EV-C	PV2 [b]	1	1	0	0	1
Enterovirus/EV-C	PV3 [b]	1	1	0	1	0
Enterovirus/EV-C	CVA1	1	1	0	1	0
Enterovirus/EV-C	CVA11	3	1	2	2	1
Enterovirus/EV-C	CVA17	1	0	1	0	1
Enterovirus/EV-C	CVA20	3	2	1	2	1
Enterovirus/EV-C	CVA24	4	2	2	3	1
Enterovirus/EV-C	EV-C96	2	0	2	1	1
Enterovirus/EV-C	EV-C113	6	5	1	4	2
Parechovirus/HPeV	HPeV1	2	2	0	1	1
Parechovirus/HPeV	HPeV2	2	1	1	1	1
Parechovirus/HPeV	HPeV3	1	0	1	0	1
Parechovirus/HPeV	HPeV4	4	3	1	2	2
Parechovirus/HPeV	HPeV7	3	1	2	2	1
Parechovirus/HPeV	HPeV9	3	3	0	2	1
Parechovirus/HPeV	HPeV12	1	0	1	0	1
Cardiovirus/TMEV	SAFV 1	5	3	2	3	2

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Genus/Species	Type	No. Sequenced	Chuquisaca	santa Cruz	AFP	Contact
Cardiovirus/TMEV	SAFV 2	2	2	0	1	1
Cardiovirus/TMEV	SAFV 4	2	1	1	2	0
Cardiovirus/TMEV	SAFV 9	2	1	1	1	1
Salivirus	SALV A1	1	1	0	1	0
Cosavirus	CoSV A3	3	3	0	2	1
Cosavirus	CoSV A7	1	1	0	1	0
Cosavirus	CoSV A8	3	3	0	3	0
Cosavirus	CoSV A17	2	2	0	2	0
Cosavirus	CoSV A24	2	0	2	1	1
Cosavirus	CoSV D4	2	2	0	0	2
Cosavirus	New (15 genotypes)	22	14	8	14	8

[a] Only viruses detected and successfully sequenced (VP1 gene region) from human specimens are listed here.

[b] All polioviruses were identified as Sabin-like by sequencing.