The molecular epidemiology of type 1 poliovirus in Central African Republic

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An increase in the incidence of acute flaccid paralysis cases associated with wild-type 1 poliovirus occurred in children in the city of Bangui, Central African Republic (CAR), in 1993 and 1994. Genetic relationships of 33 isolates were analysed by restriction fragment length polymorphism and by sequencing the VP1/2A junction region (150 nucleotides) of the viral genome. Two distinct genotypes, A and B, were co-circulating in 1993, while in 1994 only a third genotype, C, was observed. Comparison of the sequences found, with those of the sequences from isolates from neighbouring and other endemic countries revealed that genotype A isolates were related to strains from Egypt (90.7% identity), genotype B isolates to strains from Kenya (96.7% identity), Sudan and Egypt, and genotype C isolates to strains from various countries in western and southern Africa (89.0% identity). Genotypic diversity and genetic linkage with strains from neighbouring countries indicate intense poliovirus circulation and transmission that does not respect national borders. Therefore, eradication of poliomyelitis from CAR can only be achieved by a coordinated multinational strategy that stops poliovirus circulation in the whole of Africa.

Introduction

In 1988, the World Health Organization (WHO) set the goal of eradication of the disease poliomyelitis and its cause, the wild polioviruses, by the year 2000. Although much progress has been made towards this goal, in many parts of the developing world, especially in Africa, poliomyelitis is still endemic, and paralytic poliomyelitis continues to be an important disease affecting many children. In 1995, 3751 cases of poliomyelitis were reported (WHO, 1996). Key elements of the eradication program are the improvement of vaccination coverage, and surveillance of wild poliovirus circulation by virological laboratory analysis. Investigations to trace the origin of the detected strains are important in epidemiological surveillance. Nucleotide sequence comparison of amplified cDNA of the VP1/2A region in the viral RNA is an excellent method for such studies (Kew et al., 1990). The poliovirus genome has been reported to evolve rapidly and with a constant rate on passage through humans (Nottay et al., 1981). Genotypes of polioviruses have been defined as groups of strains that show more than 85% sequence identity in the VP1/2A region (Rico-Hesse et al., 1987). Transmission pathways can be inferred from the pattern of nucleotide variation among strains, and outbreaks can be traced using genomic analysis (Oostvogel et al., 1994; van Niekerk et al., 1994).

In Central African Republic (CAR), the expanded programme on immunization (EPI) was introduced in 1988, but reported coverage with oral polio vaccine is less than 50% (Ministry of Health, personal communication). We have reported an increase in incidence of acute flaccid paralysis (AFP). At the same time a change in the type of wild poliovirus circulating in the city of Bangui was observed (Gouandjika et al., 1995). In 1991 and 1992, wild-type 3 poliovirus was isolated from 12 of 370 stool samples (in 4/5 patients with AFP, and in 8/365 healthy children), while in 1993 and 1994 (37 AFP cases in total) only wild-type 1 poliovirus was found. Wild-type 1 poliovirus was isolated from 59 (17.2%) of the 343 stool samples: in 22 (59.1%) of the 37 patients with AFP (two strains were isolated from the same patient), and in 36
(11.7%) of 306 healthy contacts (children) of the patients. In this report, we present the molecular characterization of wild-
type 1 poliovirus strains isolated during this period, the
transmission patterns and the origin of the various genotypes
circulating in CAR.

Methods

■ Virus strains. Polioviruses were isolated in the WHO Regional
Reference Center for central Africa (Institut Pasteur, Bangui, CAR) from
stool specimens of patients with AFP, and from apparently healthy
contacts, in 1993 and 1994 in the city of Bangui. Viruses were isolated on
HEp-2c cells and typed as type 1 polioviruses using a standard microplate
neutralizing assay (WHO, 1992). The intratypic differentiation was
carried out using the neutralizing index test with specific neutralizing
monoclonal antibodies previously described (Crimi et al., 1983). A total of
59 wild-type 1 polioviruses were isolated. No Sabin-like poliovirus
was isolated (Table 1).

■ Restriction fragment length polymorphism (RFLP) assay.
For the screening, 33 strains (18 from AFP patients and 15 from healthy
contacts) were analysed using the RFLP assay of a reversed-transcribed
genomic fragment amplified by PCR.

The supernatant of HEp-2c cells infected with poliovirus (106
TCID50/ml) and clarified by centrifugation (10 min, 1600 g) was used for
analysis. We used two sets of primers purchased from the Organic
Chemistry Unit (Institut Pasteur, Paris). For RFLP in the VP1 capsid
region (RFLP1) including the antigenic site 1 coding segment, we used
downstream primer UC1 at nt 2861–2881 and upstream primer UG1 at
nt 2402–2421, delimiting a 480 nt segment (2402–2881), according to
the method described previously (Balanant et al., 1991). For RFLP in the
virus polymerase-encoding region (RFLP3d), the downstream primer
(UC8) has the sequence 5' GATGTCTCTCTCTCTCTCCCC at nt
6355–6376, and the upstream primer (UG7) has the sequence 5'
TGTAGGCTCTAGCTTCCC at nt 6355–6376. Reverse transcription (RT), PCR amplification
and restriction mapping, using three enzymes (Haelll, Hpyll and Ddel) for
RFLP1 and three enzymes (Haelll, Rsal and Xba1) for RFLP3d, were
carried out as described previously (Balant et al., 1991). Restriction
profiles obtained with different restriction enzymes were compared with
profiles of the homotypic poliovirus Sabin-1 vaccine reference strain
(LS-c,2ab) and of the wild-type 1 poliovirus Mahoney reference strain.

■ Determination of the nucleotide sequence

Primers. In order to generate templates for sequencing, a 290 nt frag-
ment located in the VP1/2A junction, encompassing a 150 nt fragment
(3296–3445) used for genotype analysis, was amplified using
poliovirus-specific primers. Primer 2A at nt 3235–3254, previously
described by Rico-Hesse et al. (1987) was used as the reverse primer.
Forward primer PVP_CR2 (position 3508–3527, 5' AAGAGGTCTCTCT-
ATTCCACAT) was chosen from a conserved region of the poliovirus
VP1 region after analysis of published Sabin and wild-type sequences
(Chezi, 1996).

Virus RNA extraction for PCR. Nucleic acid was extracted from 160 µl
of infected tissue culture fluid. Then, 40 µl of 5 x lysis buffer (250 mM
Tris–HCl pH 8.3, 350 mM KCl, 25 mM MgCl2, 2.5% NP40) was added
to each tube and tubes were incubated on ice for 15 min. Nucleic acid
was extracted once with phenol, once with phenol–chloroform (1:1)
and once with chloroform–isoamyl alcohol (24:1). The aqueous superna-
tants were used as templates for PCR reactions and subsequently
stored at −70°C.

<table>
<thead>
<tr>
<th>Table 1. Intratypic differentiation of type 1 polioviruses by neutralization index with strain-specific antibodies</th>
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<tbody>
<tr>
<td>Poliovirus strain</td>
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<tr>
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</tr>
<tr>
<td>P1 Mahoney</td>
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<tr>
<td>Sabin LS-c,2ab</td>
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<tr>
<td>1CAR93</td>
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<tr>
<td>17CAR93</td>
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<tr>
<td>2CAR93</td>
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<tr>
<td>175CAR94</td>
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<tr>
<td>177CAR94</td>
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</tbody>
</table>

* NI, The difference between the log virus titre in the absence and the
presence of a constant concentration of antibody.
† l0, Anti-LSc,2ab monoclonal antibodies recognizing the VS epitope;
l0, Anti-Mahoney monoclonal antibodies recognizing the Vwa epitope
(mean values of two individual experiments).

Reverse transcription and PCR (RT–PCR). Reverse transcription and PCR
were performed in a single step in a 100 µl reaction volume. An RT–
PCR master mix containing 10 mM Tris–HCl pH 8.3, 1.5 mM MgCl2,
50 mM KCl, 20 pmol of each primer, 200 µl of each deoxyribonucleo-
sidetriphosphate, 10 units RNase inhibitor (Boehringer Mannheim), 5 units
AMV reverse transcriptase (Boehringer Mannheim) and 2.5 units Taq
polymerase (Boehringer Mannheim) for each reaction was prepared and
aliquoted in 95 µl volumes into thin-walled 0.5 ml reaction tubes. Re-
action mixtures were overlaid with 3 drops of light mineral oil (Sigma)
and kept on ice. RNA templates were heat-denatured (80°C, 2 min),
and snap-cooled on ice for 5 min. Denatured RNA template (5 µl) was
Fig. 1. (a) RFLP1 profiles (analysis in the VP1 capsid region) of wild-type 1 polioviruses isolated in CAR. cDNA fragments (480 nt) were separately digested with *Hae*III, *Hpa*II or *Dde*I. See also Table 2. (b) RFLP3d profiles (analysis in the virus polymerase encoding region) of wild-type 1 polioviruses isolated in CAR. cDNA fragments (291 nt) were separately digested with *Rsa*I, *Hae*III or *Xba*I. See also Table 2.

added to each reaction tube. RT–PCR amplification was performed using the following programme on a Biometra Trioblock thermal cycler: one cycle of reverse transcription (42 °C, 45 min), one cycle of denaturation (95 °C, 3 min), 30 cycles of denaturation (95 °C, 30 s) – annealing (56 °C, 45 s) – elongation (72 °C, 1 min), followed by one cycle of elongation (72 °C, 7 min).

For analysis of the amplicons, 10 µl of amplified products was electrophoresed at 10 V/cm through 2.5% agarose gels in 0.5 x Tris-borate–EDTA buffer. Gels contained 0.4 µg/ml ethidium bromide and were visualized under shortwave UV light.

**Sequencing of amplified products.** Amplified products were purified from agarose gels using the Mermaid kit (Bio 101). Briefly, 40 µl of amplified products was electrophoresed on a 1.5% Biogel (Bio 101) in Mermaid buffer, and the target band excised from the gel. DNA was purified from the agarose slice according to the manufacturer's instructions, and eluted in a final volume of 20 µl distilled water.

DNA (5 µl) was used for sequencing. Dideoxy sequencing of purified PCR products was performed using the Sequenase PCR-product sequencing kit (US Biochemical) with [32P]dATP. Amplified products were sequenced using primer 2A, and sequences confirmed by repeat sequencing with primer PVPCR2. Sequencing products were resolved on 8% acrylamide gels containing 7 M urea and visualized by autoradiography (βmax film, Amersham). Sequence data were read manually and entered into DNASIS (Hitachi) analysis software. The sequences of the PCR products were compared to those of reference type 1 poliovirus strains to determine genotype.

**Data analysis.** A dendogram of sequence relatedness between isolates from CAR and other African countries was constructed by the
method of least squares using the KITSCH programme from the PHYLIP phylogenetic interference package (Felsenstein, 1993). In the dendrogram, 11 strains were included (24115/KEN93, 24094/SUD93, 18643/PAK91, 08659/IND91, 24122/EGY93, 08659/IND91, 21197/PAK91, 11236/EGY91, 11257/EGY91, 08425/ISRR88 and 11300/EGY91) that have previously been sequenced by a non-radioactive protocol (Mulders et al., 1995).

Results

Classification by RFLP

Thirty-three wild-type 1 poliovirus strains isolated during 1993 and 1994 were analysed by RFLP assay. The characteristic RFLP1 and RFLP3d profiles generated after restriction digestion were compared. The RFLP profiles revealed that all the CAR isolates were of non-Sabin origin. Within type 1 poliovirus strains isolated during this period we detected three clusters according to RFLP3d profile groups (Fig. 1).

The poliovirus strains isolated during this period we detected three clusters according to RFLP3d profile groups (Fig. 1). The characteristic RFLP1 profile showed two DNA fragments (365 and 105 nt) after HaeIII digestion; the 2a cluster (three strains) with two DNA fragments (118, 95 and 78 nt). Strains belonging to group 2 were isolated during the same period (March–April 1993). It was noted that three strains belonging to group 2 (20CAR93, 24CAR93 and 25CAR93) were isolated from healthy contacts of an AFP patient with a group 1 strain (4CAR93).

Group 3. The RFLP1 profile was characterized by two DNA fragments (279 and 201 nt) after HpaII digestion. It was subdivided in four clusters: cluster 3a (eight strains isolated from November 1993 to August 1994) showed two DNA fragments (310 and 170 nt) after HaeIII digestion; cluster 3b (one strain) different from 3a by cleavage after Rsal digestion by RFLP3d assay; cluster 3c (four strains isolated between February and March 1994) with an RFLP1 profile showing three fragments (310, 95 and 75 nt) after HaeIII digestion; and cluster 3d (two strains isolated in April 1994) different from 3c by cleavage after HaeIII digestion by RFLP3d assay (150, 121 and 20 nt). It was noted that the 76CAR94 strain was isolated from a healthy contact of an AFP patient with 4eCAR94 strain belonging to cluster 3c.

Sequencing

In order to compare the nucleotide sequences (150 nt) encoding the VP1/2A junction region, 22 type 1 poliovirus CAR strains screened by RFLP were selected. Restriction enzyme profiles, clinical and epidemiological data were used to select the strains (Table 3). Nucleotide sequences were compared with the corresponding sequences of 24 other

Table 2. RFLP profiles of wild-type 1 polioviruses isolated in CAR (1993–1994)

<table>
<thead>
<tr>
<th>Strains</th>
<th>RFLP1</th>
<th>RFLP3d</th>
<th>RFLP Profile</th>
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<tr>
<td></td>
<td>HaeIII</td>
<td>HpaII</td>
<td>Ddel</td>
</tr>
<tr>
<td>P1 Mahoney</td>
<td>252/228</td>
<td>279/201</td>
<td>480</td>
</tr>
<tr>
<td>Sabin LS-c.2ab</td>
<td>228/141/111</td>
<td>279/201</td>
<td>360/120</td>
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<td>1CAR93, 3CAR93, 4CAR93, 5CAR93, 6CAR93</td>
<td>365/105</td>
<td>480</td>
<td>480</td>
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<td>480</td>
<td>480</td>
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<td>13CAR93, 15CAR93, 20CAR93</td>
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<td>310/170</td>
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<td>279/201</td>
<td>480</td>
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<td>45CAR94</td>
<td>310/170</td>
<td>279/201</td>
<td>480</td>
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<tr>
<td>2CAR94, 6CAR94, 46CAR94, 50CAR94</td>
<td>310/95/75</td>
<td>279/201</td>
<td>480</td>
</tr>
<tr>
<td>76CAR94, 169CAR94</td>
<td>310/95/75</td>
<td>279/201</td>
<td>480</td>
</tr>
</tbody>
</table>
Type 1 poliovirus genotypes

strains (Africa, Middle-East and Indian subcontinent), and with the Sabin-1 vaccine strain LS-c.2ab (Fig. 2). We generated a dendrogram of sequence relationship between the isolates. There appeared to be three distinct branches in addition to that of the vaccine strain (Fig. 3). Each branch represented one genotype (> 15 % sequence difference). Within each genotype, a high percentage of genetic identity was found, thus indicating a direct transmission link. Type 1 poliovirus isolates were distributed in suburbs of Bangui and there was no apparent geographical correlation between genotype clusters (Fig. 4).

Genotype A comprised five strains isolated in March and April 1993; two strains (20CAR93 and 24CAR93) diverged by 2.6% from the others. These two strains were isolated from healthy contacts of an AFP patient with genotype B strain. The strains belonging to group 2 by RFLP were classified in this genotype. These strains are closely related to the genotype described by Mulders et al. (1995) including older strains from Egypt (11236EGY91) and the Middle-East (8425ISR88). CAR isolates show a high degree of identity with the isolate 11236EGY91 (90.7%).

Genotype B comprised six strains also isolated in March and April 1993. The maximum sequence divergence among the strains was 1.3% (6CAR93), and three strains were identical. Isolates present in group 1 by RFLP were classified into genotype B. These strains clearly belong to the genotype 4 described by Mulders et al. (1995). CAR isolates showed a high degree of identity with the isolates 24115KEN93 (96.7%) and 24094SUD93 (94.7%).

Genotype C included 11 strains isolated later (between November 1993 and August 1994). During this period, sequence variation of the genotype C strains was limited to 2 nucleotides (1.3%) and six strains had an identical sequence, indicating the clonal spread of one variant. Isolates belonging to group 3 by RFLP were classified into this genotype C. CAR isolates from genotype C grouped in a sub-Saharan African genotype which currently includes isolates from Namibia,
Discussion

Between 1992 and 1993 a change in the wild poliovirus circulation in the city of Bangui, CAR (type 3 to type 1) was observed. During the 1993–1994 period, AFP case registration and type 1 poliovirus isolation increased in Bangui and its adjacent regions.

To go, Angola and Zaire. These 11 CAR strains showed the highest degree of identity (89%) with 1994 isolates from Central Angola (5384AN94) and showed between 83% and 88% identity with current Namibian isolates (Chezzi, 1996). They showed 87% identity with the 757ZAI95 strain isolated during the 1995 Zaire outbreak (adjacent country).
suburbs. This epidemic activity occurred in a population with insufficient vaccine coverage (< 50%). The concomitant circulation of two distinct genotypes of type 1 poliovirus in 1993 and their replacement by a third genotype in 1994 could be identified from RFLP assay and sequence analysis of the VP1/2A junction region. In each of the three genotypes observed in CAR, strains have a high degree of identity (> 98%), indicating an epidemiological link between cases (Rico-Hesse et al., 1987). A similar situation occurred in southern Saudi Arabia in 1989 (Kew et al., 1993) and in the Caucasus region (Lipskaya et al., 1995), where type 1 poliovirus outbreaks were associated with several genotypes. Recently, Li et al. (1995) reported genetic heterogeneity of poliovirus in China with a dominant genotype responsible for each outbreak. This genetic heterogeneity has been described in endemic regions by previous investigators (Kew et al., 1993; Mulders et al., 1995).

Polioviruses may cluster geographically and different genotypes may be isolated in adjacent communities. Our data collected in a limited area around Bangui showed no correlation between genotypes and geographical origin of patients and contacts. The high genotype diversity between type 1 poliovirus strains isolated from the same geographical area (city of Bangui) during a short period could be explained by the presence of different reservoirs that maintain transmission of variant genotypes over several years.

In 1993, two genetically different wild-type 1 poliovirus strains were co-circulating in the same reservoirs, as docu-
mented by the isolation of genotype A virus from three contacts of a patient with genotype B isolate. Two of these contacts, however, came from another, neighbouring suburb. This indicates a high diffusion of poliovirus, and the presence of at least two independent but overlapping community reservoirs. Similar observations were reported from Pakistan, where four genotypes of type 1 poliovirus were found within the city of Karachi (Mulders et al., 1995). Multi-chain transmission and circulation of different genotypes between adjacent countries are usually observed (Kew et al., 1995). The high genotype diversity between type 1 poliovirus isolated in Bangui, and their genetic linkage with other strains circulating in neighbouring countries suggests that transmission of genotypes introduced from different origins occurred in CAR. For genotype B, the CAR strains have a high degree of identity with strains isolated during the same period from Kenya, Egypt and Sudan, and with older strains from Asian countries. The closest match was observed with the 24115KEN93 isolate (97.4%) which was found to be identical to 24087SUD93 (Mulders et al., 1995). A high percentage identity was also observed with 24094SUD93, indicating that Sudan strains could represent the ancestors of genotype B strains isolated in Bangui. Importation of strains from Sudan to the north of Kenya and to CAR is probably the result of movements of large populations due to political unrest. As for genotype A, genetic sequencing showed that strains isolated in Bangui are related to older Egyptian strains (11236EGY91) and to Middle-Eastern strains. The sequence divergence (9%) is more important between these strains isolated within a 2 year period, indicating a continuous silent transmission of polioviruses. Within regional foci of endemicity, each genotype may persist for many years.

Since November 1993, a third genotype (genotype C) has been isolated. Genotype C appeared to be more closely related to strains circulating in southern Africa, but sequence divergence was observed between CAR isolates and polioviruses circulating within Zaire or within more distant areas (Angola). This indicates that separate evolution of the same genotype via multiple chains of transmission may occur when vaccination coverage is not sufficient to interrupt transmission.

This genotypic heterogeneity may exist prior to epidemics, but during epidemic outbreaks one specific genotype is usually predominant. When vaccination coverage is not sufficient, and the population of susceptibles is large enough to maintain poliovirus circulation for an extended period of time, a genotype may displace another within the course of a single epidemic. In CAR since 1993, incomplete immunization of the population was favourable for the circulation of the genotype C.

This report has shown the presence of several reservoirs maintaining transmission. In the central African area the circulation of three genotypes at endemic level is extending into neighbouring countries. RFLP has documented the continuous occurrence of mutations in various parts of the viral genome (VP1 and 3dPol regions), and also in strains that by sequencing of only the VP1 region were indistinguishable. These molecular analyses help to define the extension of poliovirus genotypes, to study the pathways of poliovirus transmission, and to develop strategies for eradication. In an enclaved country such as CAR, the efforts to eradicate poliomyelitis and its cause, the wild poliovirus, cannot be successful without a multinational strategy of eradication. This strategy includes the national immunization days synchronized among neighbouring countries that are scheduled in many African countries concurrently in 1996.

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