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Sequence Note

Epidemic Expansion of HIV Type 1 Subtype C and Recombinant Genotypes in Tanzania

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HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) subtypes are distributed unevenly across African nations.[1] In East and Central African countries such as Uganda, Rwanda, Kenya, and Tanzania, the HIV-1 epidemic has involved two HIV-1 subtypes, A and D. In contrast, HIV-1 subtype C has dominated the rapidly expanding epidemic in Malawi and South Africa.[1–3] The relative roles played by virological, behavioral, and host determinants in the epidemic expansion of any particular HIV-1 subtype are unclear. Characterization of the transmissibility and pathogenic potential of distinct HIV-1 genetic subtypes is currently under investigation in many regions of the world. Careful surveillance of genetic subtypes prevalent in a given population is one particularly important approach to better understand the biological properties of different HIV-1 subtypes.

The presence of HIV-1 subtypes A and D in asymptomatic carriers and AIDS patients from several geographical locales in Tanzania has been previously described.[4–7] An analysis of samples collected in 1988 showed that 10 of 15 (67%) envelope V3 sequences from Tanzanian samples were found to belong to subtype D. The remaining five samples (33%) belonged to HIV-1 subtype A.[4] In Dar es Salaam, *vpu* and *env* sequences from 8 of 10 AIDS patients (80%) clustered with subtype D viruses and the remaining 2 (20%) with subtype A.[5] A study in northern Tanzania reported that the *env*-encoded gp41 regions from 12 samples also clustered with HIV-1 subtypes A and D.[6] In another report from northern Tanzania, four of eight (50%) envelope sequences sampled encompassing the C2V3 region belonged to subtype A and the other half to subtype D.[7] Envelope sequences from HIV-1-infected individuals of Tanzanian origin, but living in Sweden, showed that three of four samples were HIV-1 subtype C and the remaining sample was HIV-1 subtype A.[8] Even though this study suggested that HIV-1 subtype C was now present in Tanzania, it could not establish definitively that these infections were acquired in Tanzania, nor were the cases documented as thoroughly as the samples described in this sequence note. In the present study, we describe HIV-1 subtypes A, D, C, and recombinants (C/A, C/D, A/D) in infants from Dar es Salaam, Tanzania.

Whole blood was collected from 387 infants, ranging from 6 to 62 weeks of age, attending the Muhimbili Medical Center neonatal clinic in Dar es Salaam. The infants were participating in a randomized trial to determine whether vitamin supplements could reduce the rate of mother-to-child transmission of HIV-1. Samples analyzed corresponded to the latest blood sample available from each infant. Peripheral blood mononuclear cells (PBMCs) were separated by centrifugation on Ficoll density gradients in Dar es Salaam and frozen cells were transferred to the laboratory in Boston. PBMCs were thawed, washed, and counted. HIV infection in the infants was determined by polymerase chain reaction (PCR) using the Amplicor HIV-1 detection kit (Roche Diagnostic Systems, Nutley, NJ) with modifications that increased primer sensitivity to non-B HIV-1 genotypes.[9] All samples that gave a PCR signal according to the manufacturer standards were then used for envelope PCR amplification and sequence analysis. Two HIV-1-negative samples were included with each cell lysate preparation. Twenty microliters of crude cell extract was used for heminested PCR amplification with primers located in the envelope region of HIV-1. The first PCR amplification was done with primer vpu157 (nucleotides 5754–5774 according to HxB2 numbering[10]) and env1556 (nucleotides 7361–7328). Four microliters of the first PCR amplification was subjected to heminested PCR with primers env768 (nucleotides 6535–6559) and env1556. The amplification cycles for both reactions lasted for 3 min at 94°C, followed by 39 cycles of 1 min at 94°C, 1 min at 52°C, 3 min at 72°C, and a final extension cycle for 10 min at 72°C. Two separate reactions of an HIV-1-negative cell extract and a reaction without DNA were

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included with every PCR reaction. Amplified products were confirmed by Southern blot hybridization using internal ^{32}P-labeled probes. Amplicons were gel purified and cloned into a TopoVector (Invitrogen, San Diego, CA). Four *env* clones from each sample were sequenced in both directions by automated cycle sequencing with dye terminators using an ABI 373 system (Applied Biosystems, Foster City, CA). Six hundred and fifty nucleotides of each individual clone were subjected to phylogenetic analysis and recombination analysis through comparisons with the C2–C4 regions of previously classified HIV-1 subtypes A, B, C, D, E, F, G, and H[10] (Table 1).

Eighty-six of 387 infants tested (22.2%) were found to be HIV-1 positive by the Amplicor test and confirmed by in-house PCR. Envelope sequences were obtained from 71 infants. Genotype classification by phylogenetic trees revealed that all 71 samples clustered inside or next to control sequences belonging to HIV-1 subtype A, D, or C. Samples that clustered next to a specific subtype group did not subcluster within that group were further analyzed using the recombination identification program (RIP) (Table 1).[10] This analysis revealed the presence of recombinant genotypes between A, D, and C parent genomes. Consequently, all samples in this sequence note were subjected to subtype classification by both phylogenetic trees and RIP analysis. In addition, analyses of previously described sequences from Tanzania[4–7] using similar algorithms were conducted to confirm the presence of HIV-1 subtypes A, D, and C as well as to rule out the presence of recombinant genomes.

We found that phylogenetic analysis alone was not able to detect recombinant genomes since they were located as outliers of HIV-1 subtype A, D, or C clusters. Identification of recombinant genomes was possible only when RIP analysis was performed on these samples (Table 1). Twenty-five samples (35.2%) were classified as subtype A by phylogenetic and RIP analysis. The range of age for these infants was 6 to 62 weeks (mean of 17 weeks). Twelve samples (16.9%) were classified as HIV-1 subtype D with an age range of 10 to 50 weeks (mean of 21 weeks). Twenty-three samples (32.4%) with ages ranging from 6 to 51 weeks (mean of 18 weeks) were classified as HIV-1 subtype C by the same analysis. Although this study analyzed a fragment of one locus of the HIV-1 genome (650 base pairs), recombinant genotypes accounted for 11 samples (15.5%) in this study. The ages of these infants ranged from 6 to 26 weeks (mean of 13 weeks). Nine of these recombinant envelope fragments (82%) contained HIV-1 subtype C sequences (two C/A and seven C/D recombinant) and the other two were of the A/D genotype variety. Similar results were obtained independent of the clone used for the analysis. Without exception, clones belonging to one sample always clustered together and away from any other clone or control sequence used in the analysis. None of the sequences from the infants sam-

TABLE 1. HIV-1 SUBTYPES BASED ON PHYLOGENETIC AND RECOMBINANT IDENTIFICATION PROGRAM ANALYSIS OF SEQUENCES FROM TANZANIAN SAMPLES

Report[a]	*Year of sampling*[b]	*Location*[c]	*HIV-1 loci*[d]	*HIV-1 subtypes by RIP analysis*[e]			
				A	*D*	*C*	*Rec.*
4	1988	Turiani, Iringa, Das es Salaam, Kilimanjaro	102–111 bp *env* V3	5 (33.3%)	9 (60.0%)	1[f] (6.7%)	0 (0.0%)
5	1988	Dar es Salaam	231 bp *env* V3	2 (20.0%)	8 (80.0%)	0 (0.0%)	0 (0.0%)
6	1995[g]	Moshi	50 aa *env* gp41	4 (33.3%)	8 (66.7%)	0 (0.0%)	0 (0.0%)
7	1987/1994	Mara region	336 bp *env* C2–V3	4 (50.0%)	3 (37.5%)	0 (0.0%)	1 (12.5%)
Present study	1996/1997	Dar es Salaam	650 bp *env* C2–C4	25 (35.2%)	12 (16.9%)	23 (32.4%)	11 (15.5%)

[a]Numbers correspond to the reference number.
[b]Year of sampling according to report.
[c]Geographical location where the samples were obtained.
[d]HIV-1 loci used in the study. bp, Base pairs; aa, amino acids.
[e]Frequency of HIV-1 subtypes among samples subjected to RIP analysis. Percentages are shown in parentheses. Detection of intersubtype recombinants was performed using the recombinant identification program (RIP) from the Los Alamos HIV sequence database.[10] RIP parameters included a 50-base window, 90% threshold for statistical significance, and stripped gaps between sequences. Each sample was compared with a master alignment (comprising consensus sequences from HIV-1 subtypes A, B, C, D, E, F, G, and H) obtained from the Los Alamos HIV sequence database. In addition, each sample was compared with consensus sequences from two putative subtype parent sequences (A–D, A–C, or C–D, respectively) and an outgroup sequence (SIVcpzGAB or HIV-1 MN).
[f]This sample, TZ10, clustered with HIV-1 subtype C controls by phylogenetic and RIP analysis.
[g]J. Shao, personal communication, 1997.
Nucleotide sequences were deposited in GenBank under accession numbers AF038051–AF038121.

ples clustered together with any sequence present in our sequence database and none of the clones from samples with recombinant genomes showed evidence of nonrecombinant sequences.

The results described here allow us to conclude that subtypes A, C, D, and their recombinants are currently present in the general population of Dar es Salaam, Tanzania. Since our findings of HIV-1 subtype C contrasted with the relative absence of subtype C in previous studies, we performed similar phylogenetic and RIP analyses to confirm the absence of subtype C and recombinants in earlier samples from Tanzania (Table 1). We were able to identify 1 HIV-1 subtype C and 1 HIV-1 C/D recombinant in 42 previously reported sequences. Sample TZ10 was not identified as subtype C since only controls of HIV-1 subtypes A, B, and D were used in their analysis.[4] The other sample, TZ1585, was classified as HIV-1 subtype D by phylogenetic analysis,[7] but the RIP analysis showed that this sample corresponded to a C/D recombinant.

The fact that subtype C genotypes, including recombinants containing a portion of subtype C, were found in about 45% of the infected newborns, as opposed to only 2 of the 45 samples (4.4%) described earlier, supports the hypothesis that different HIV-1 genotypes might be spreading at different rates in this area. A 1994 report of *vpu* and *env* sequences from 10 AIDS patients in the same geographical location as the present study classified 8 samples as HIV-1 subtype D and 2 samples as HIV-1 subtype A.[5] Phylogenetic and RIP analyses of these 10 samples did not show evidence of HIV-1 subtype C or HIV-1 recombinant genomes.

The rate of perinatal transmission from HIV-1-positive mothers infected with distinct subtypes or recombinant viruses is not known at the present time. However, it is likely that the virus subtype detected in the infants indicates the presence of at least that subtype in the mother. We were able to detect recombinant sequences in infants as young as 6 weeks, which suggests that these recombinant viruses were probably transmitted from their mothers. If we assume that all HIV-1 subtypes are equally transmitted from mother to infants, HIV-1 subtype C appears to have been introduced after HIV-1 subtypes A and D were already established in Dar es Salaam and has quickly become one of the major subtypes present in that city. This assumption is supported by the elevated numbers of infants infected by HIV-1 subtype C and the apparent decrease in the proportion of those infected by subtype D. The rate of HIV-1 subtype D in all four previous studies range from 37.5 to 80.0% of all samples analyzed,[4–7] as opposed to 16.9% in the current study. Conversely, if we assume that subtype C accounts for a minority of infections in pregnant women and the general population of Dar es Salaam, our results would suggest that HIV-1 subtype C (alone or its recombinants) showed higher rates of transmission from mother to infants than HIV-1 subtype A, D, or A/D recombinants. This assumption is supported by the fact that HIV-1 subtype C and its recombinants (C/A and C/D) account for 45% (23 individuals infected with HIV-1 subtype C and 9 with subtype C recombinants) of infant infections in a geographical locale where only HIV-1 subtypes A and D once predominated.[4,5] We cannot rule out that the differences between our results and those of four previous reports on the subtypes prevalent in Tanzania may be due to geographic heterogeneity of the population sampled, or to an artifact of sensitivity related to the different primers used for PCR amplification. However, the overwhelming number of infections by HIV-1 subtype C makes this possibility unlikely. Based on 650 bp of *env*, we cannot rule out that the number of recombinant genomes is higher than the 15.5% reported. It has been described that about 10% of the HIV-1 isolates sequenced over large regions including several genes are intersubtype recombinants.[11,12] It is plausible that additional samples would be classified as recombinant if other HIV-1 genes were analyzed. Since our analysis was done directly from the infant peripheral blood mononuclear cells (PBMCs) to avoid tissue culture artifacts, these findings suggest that recombinant viruses are efficiently transmitted from infected mothers to infants.

To determine accurately the prevalence of HIV-1 subtype C and its recombinants in Tanzania and their ability to be transmitted from mother to infant, further studies are needed of the HIV-1 subtypes present in transmitting and nontransmitting mothers. These estimates can be subsequently compared with the HIV-1 subtypes present in infected newborns.

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