

## Development and Evaluation of a DNA Enzyme Immunoassay Method for *env* Genotyping of Subtypes A through G of Human Immunodeficiency Virus Type 1 Group M, with Discrimination of the Circulating Recombinant Forms CRF01\_AE and CRF02\_AG

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The tools currently available for genetic subtyping of human immunodeficiency virus type 1 are laborious or can be used only for the analysis of a limited number of samples and/or subtypes. We developed and evaluated a molecular biology-based method using subtype-specific oligonucleotide probes for *env* genotyping of subtypes A through G, CRF01\_AE, and CRF02\_AG. DNA enzyme immunoassay (DEIA) genotyping is based on nested PCR amplification of the 5' end of the *env* gene (proviral DNA), followed by subtype-specific hybridization and immunoenzymatic detection on microplates. DEIA genotyping was validated with a large number of samples ( $n = 128$ ) collected in Europe (France;  $n = 47$ ), West-Central Africa (Cameroon;  $n = 36$ ), and West Africa (Senegal;  $n = 45$ ). Three different formats, depending on the distribution of subtypes in the three countries, were developed. The results were compared with those obtained by sequencing of the V3-V5 region and phylogenetic analysis or an *env* heteroduplex mobility assay. Additional sequencing and phylogenetic analyses of the DEIA region (the first codon of the *env* coding sequence to the middle of conserved region C1 of gp120) were performed to investigate the reasons for discrepancies. Intense and highly specific reactions between the oligonucleotide probes and the corresponding samples were observed. Overall, correct identification was achieved for 107 of 128 samples (83.6%). One sample was not amplified, 10 (8%) were nontypeable (NT), and 10 (8%) were misidentified. Six of the 10 discordant samples were further investigated by phylogenetic analysis, which indicated that these samples corresponded to recombinants involving the *env* 5' end and the V3 and V5 regions of the two parental clades. Sequencing of NT samples showed numerous differences between sample and probe sequences, resulting in a lack of hybridization, and revealed the limitations of the selected probes in terms of specificity and sensitivity. We demonstrated the feasibility of DEIA genotyping: six subtypes plus the two most prevalent circulating recombinant forms were discriminated by using the 5' end of the *env* gene. This method can be adapted to the local situation by including only probes that correspond to the prevalent strains.

Phylogenetic analyses have classified human immunodeficiency virus (HIV) type 1 (HIV-1) strains into three groups: M (for major), which is responsible for the HIV-1 pandemic; O (for outlier); and N (for non-M/non-O). O and N strains are mainly isolated from Cameroonian patients (9, 39, 54). Group M isolates are diverse and have been subdivided into genetic subtypes approximately equidistant from each another (29; HIV sequence Database [<http://hiv-web.lanl.gov>]). The increasing complexity of the sequences of HIV-1 group M isolates, the occurrence of recombinant forms responsible for major epidemics, and the existence of divergent clusters within subtypes have made it necessary to reevaluate the nomenclature. A revised nomenclature system was recently proposed. That system defines 9 "pure" subtypes (subtypes A to D, F to H, J, and K) with no identified recombination and 11 circulat-

ing recombinant forms (CRFs), in which members of a lineage that have identical mosaic structures and that play an important role in the pandemic are grouped (49; HIV Sequence Database). Subtypes E and A1bNg in the initial classification are, in fact, two major CRFs now designated CRF01\_AE and CRF02\_AG, respectively (49). The notion of subsubtypes has also been defined to refer to distinct lineages very closely related to a particular subtype lineage but genetically distant enough to be regarded as a new subtype. Subsubtypes A1 and A2 have been recognized (21), as have subsubtypes F1 and F2, found in Brazil and Europe and in Cameroon, respectively (59). Due to their previously described close relationship, subtypes B and D could be considered subsubtypes B1 and B2, respectively; we refer to them here as subtypes B and D, respectively, to remain consistent with the designations in published studies (49).

This extraordinary degree of diversity complicates diagnosis, disease monitoring, and treatment (1–3, 5, 16–18, 33, 34, 40, 53). It may also affect biological properties such as infectious-

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TABLE 1. Characteristics of subtype-specific probes

Probe	Subtype(s) identified	Oligonucleotide sequence <sup>a</sup>	T <sub>m</sub> (°C)
SAG1	CRF02_AG	5'-ATA TTT TGG ATA ATG ATA ATT TGT AAT GC-3'	53.7
SA2	CRF02_AG, A, CRF01_AE	5'-AAA GAY GCA GAG ACC ACC C-3'	55.2
SAE1	CRF01_AE	5'-AGA CAC AGA TGA ATT GGC CAA-3'	53.9
SB2	B	5'-GAM GGR GAY CAG GAA GAA TT-3'	51.2
SBD3	B, BD, D	5'-ATG CTC CTT GGG ATR TTR ATG-3'	52
SD1.1	D	5'-AGA GTG AGG GRG ATA GAG AGG AAT T-3'	59.5
SC1	C	5'-ATC TTA GGC TTT TGG ATG ITA ATG-3'	54
SF6	F	5'-GGG AAA TGG GGC CTT TTA TT-3'	53.2
SG1	G	5'-CAT ATA GTA CTG AAA GCC ATA ATG T-3'	54.6

<sup>a</sup> Degenerate bases (in boldface) are defined as follows: Y, C + T; R, = A + G; M, A + C; I, inosine.

ness, transmissibility, and immunogenicity, making continuous molecular biology-based epidemiology studies necessary. The diversity of HIV has also been shown to be an important epidemiological marker. Large-scale studies are required to follow the dynamics of the pandemic and are probably also required for vaccine design. HIV-1 strains can be subtyped (i.e., subtypes and CRFs can be identified) directly by genomic analysis (genotyping) or indirectly by analysis of the antibody response of the host by using synthetic peptides corresponding to the V3 loop of gp120 (V3 serotyping) (7, 10, 42, 51). Serological assays have been shown to be useful tools in some geographical areas, but they discriminate only a limited number of antigenically different subtypes (4, 6, 11, 12, 20, 22, 30, 52, 55, 63). Genome analysis based on DNA sequencing followed by phylogenetic analysis is the most informative method for subtyping and is considered the “gold standard.” Although this is becoming easier and is now partly automated, it remains

time-consuming and expensive and requires specific equipment, rendering it inappropriate for large-scale studies. The *env* and, recently, the *gag* heteroduplex mobility assays (HMAs) have been developed as alternative methods (15, 23); the HMA technique is nonetheless laborious and allows the analysis of only a limited number of samples, and interpretation may be difficult. Other genetic methods can be used in large-scale studies but discriminate fewer subtypes. These methods include subtype A-specific PCR, restriction fragment length polymorphism analysis, combinatorial melting assay, and probe hybridization (19, 26, 28, 35, 38, 41, 43, 47, 48, 56, 57, 60). Probe hybridization has been demonstrated to be effective for rapid screening of the prevalent subtypes in Uganda (subtypes A and D) and Thailand (subtype B and former subtype E) (47, 56). The aim of this study was to develop and to evaluate the feasibility of a similar methodology adapted to the identification of the six major subtypes prevalent world-

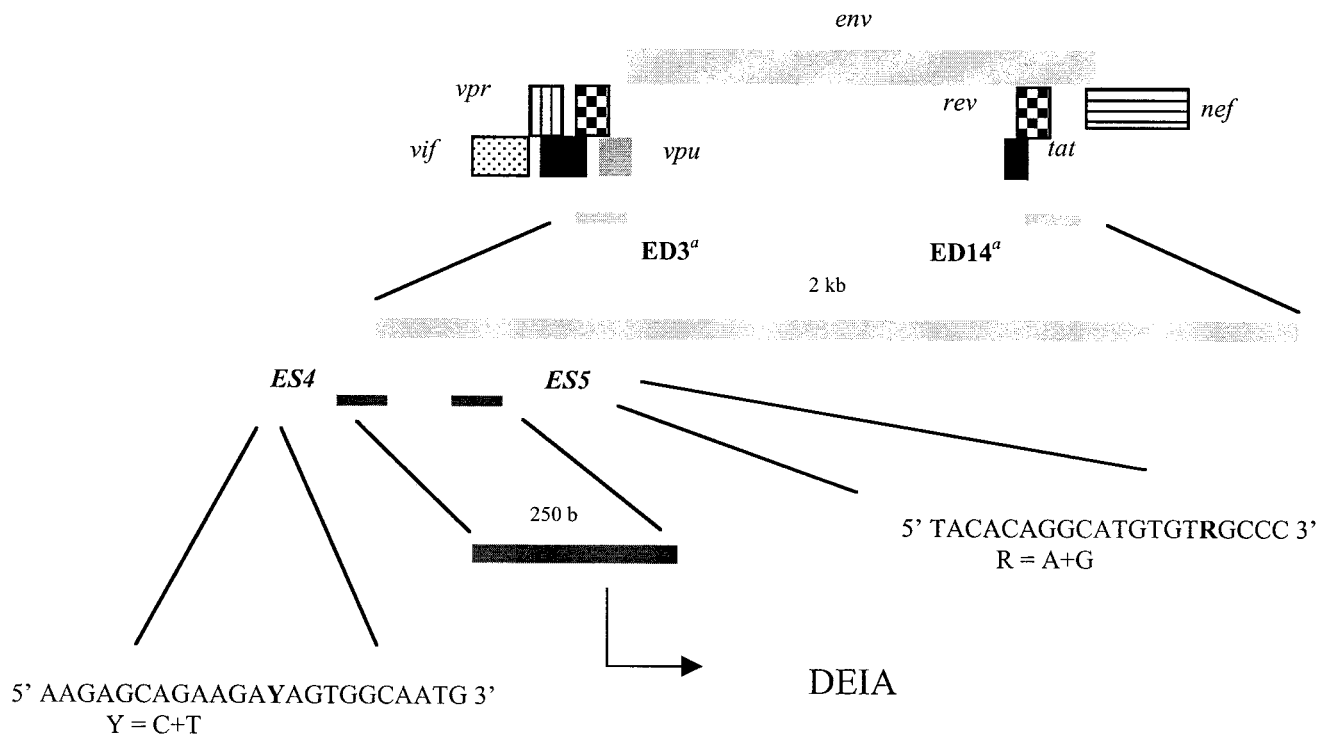


FIG. 1. Principle of genotyping of HIV-1 by DEIA. An *env* fragment of 250 bp is amplified by nested PCR and is then used for subtype-specific detection and identification by DEIA. <sup>a</sup>, primers ED3 and ED14 corresponded to those used in the *env* HMA (15).

wide (subtypes A through G), which also allows the discrimination of CRF01\_AE and CRF02\_AG. We wanted to include CRF discrimination in our objectives because CRF02\_AG is now recognized as responsible for the subtype A epidemic in West and West-Central Africa, and no specific tool except *gag* HMA has been available to track this epidemic (23).

We adapted the DNA enzyme immunoassay (DEIA) technique previously described as relevant for hepatitis C virus (HCV) genotyping (25, 31, 61). This technique is based on the hybridization of amplified proviral DNA with subtype-specific probes used to coat microtiter plate wells, followed by detection with a mouse monoclonal anti-double-stranded DNA antibody. We validated DEIA genotyping using a large number of samples collected in Europe (France), West-Central Africa (Cameroon), and West Africa (Senegal).

#### MATERIALS AND METHODS

**Sample collection.** A total of 131 HIV-1 samples whose envelopes (V3-V5) were genetically characterized were analyzed. Fifty samples were obtained from in- or outpatients, mainly African, attending the Bichat Hospital in northern Paris (European samples). The genetic subtypes of these samples were identified by HMA ( $n = 47$ ) or sequencing ( $n = 3$ ). Among these 50 samples, the genetic subtype distribution was as follows: 15 were subtype A-like, 10 were subtype B, 6 were subtype C, 5 were subtype D, 4 were subtype F, 4 were subtype G, 3 were subtype H, and 3 were CRF01\_AE. Discrimination between subtype A and CRF02\_AG in the envelope was not possible since the genetic subtypes were identified by HMA.

An additional 81 samples (African samples) were obtained from patients attending the Military Hospital in Yaounde, Cameroon ( $n = 36$ ), and patients attending one of the three major hospitals in Dakar, Senegal ( $n = 45$ ). For all these samples the genetic subtype was identified by sequence analysis; and the subtype distribution was as follows: 19 were subtype A, 3 were subtype B, 3 were subtype C, 9 were subtype D, 7 were subtype F, 12 were subtype G, and 28 were CRF02\_AG.

**Peripheral blood mononuclear cell isolation, DNA extraction, and reference subtyping.** Peripheral blood mononuclear cells were separated on a Ficoll gradient. They were washed twice in phosphate-buffered saline (PBS), pelleted, and stored at  $-80^{\circ}\text{C}$ . DNA was extracted as described previously (32).

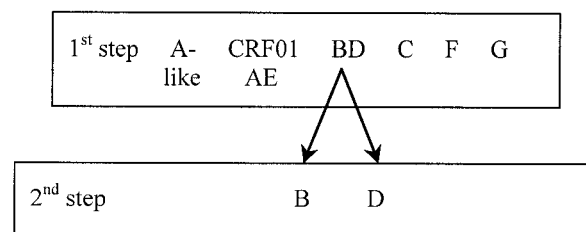
The *env* subtypes of 47 European samples were determined by *env* HMA as follows: 1  $\mu\text{g}$  of DNA was used for PCR amplification with primers ED3 and ED12. The second round of amplification was performed with 2  $\mu\text{l}$  of the first-round product and primers ES7 and ES8, as described by Delwart et al. (15). Subtype reference samples were amplified with second-round primers and 10 ng of plasmids A to H as templates. HMA was performed as described previously (14).

Three European samples and all African samples were genotyped by sequencing of the V3-V5 region of the *env* gene (700 bp, amplified with HMA primers ED5 and ED12 as outer primers and primers ES7 and ES8 as inner primers), followed by phylogenetic analysis, under the conditions described previously (62).

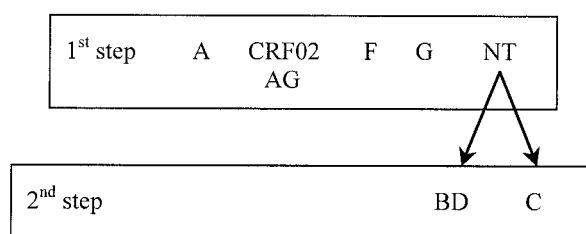
**DEIA genotyping. (i) Choice of subtyping region and selection of identification probes.** The subtype-specific probes were selected by analyzing the entire consensus sequences of the *env* genes of pure subtypes A to D and F to G and the two CRFs, CRFs AE and AG (HIV Sequence Database). The criteria for probe specificity were set on the basis of previous experience with the DEIA genotyping of HCV (25, 31, 61). Probes were 20 to 30 bases long and were designed such that the melting temperatures ( $T_m$ s) of all probes were close, with intersubtype differences of at least 20% (about 4 different bases), and the region corresponding to the probes was conserved for a given subtype. The most suitable region presenting these characteristics comprised the first codon of the *env* coding sequence (*env* cds) to the middle of conserved region C1 of gp120 (DEIA region).

A set of different probes specific for this region was defined. Single subtype-specific probes for subtypes C, F, and G were easily identified (Table 1). Unique probes for subtypes B and D could not be found. The lack of specificity of the subtype D probe, which also detected non-B, non-D subtypes, was associated in part with a high level of sequence similarity between subtypes B and D. Therefore, samples of subtypes B and D (called subtype BD) were first distinguished with a common subtype BD-specific probe (probe SBD3), followed, if a positive

A



B



C

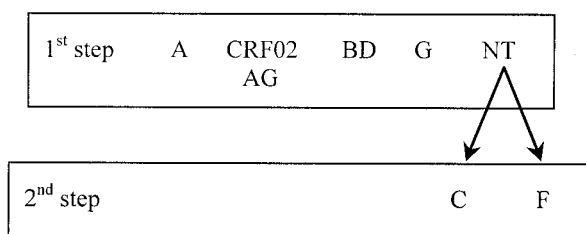


FIG. 2. Schematic diagram of the three DEIA formats developed for subtyping of samples from Europe (France; A), West-Central Africa (Cameroon; B) and West Africa (Senegal; C).

result was obtained, by specific identification with subtype B- and subtype D-specific probes (Table 1).

It was difficult to find a specific probe for subtype A due to the close relationship between this subtype, CRF01\_AE, and CRF02\_AG. The 5' end of the *env* gene of the two CRFs is indeed partly subtype A-like (HIV Sequence Database). A common probe (probe SA2) that identifies subtype A, CRF01\_AE, and CRF02\_AG was therefore identified, with discrimination between subtype A and the two CRFs being achieved with additional CRF-specific probes, designed on the basis of CRF-specific signature sequences (Table 1).

After preliminary assays, nine probes (probes SA2, SAE1, SAG1, SBD3, SB2, SD1.1, SC1, SF6, and SG1) were selected for the detection and identification of samples of subtype A, CRF01\_AE, and CRF02\_AG; samples belonging to either subtype B or D; samples of subtype B; samples of subtype D; and samples of subtypes C, F, and G (Table 1). They were synthesized, 5' biotinylated, and purified by high-pressure liquid chromatography (Eurogentec, Seraing, Bel-

TABLE 2. DEIA genotyping interpretation chart

Probe	Reactivity to the following subtype <sup>a</sup> :							
	A	CRF01_AE	CRF02_AG <sup>b</sup>	B	D	C	F	G
SA1	●	●	●	●				○
SAE1		⊕						○
SAG1			●	●				
SBD3				●	●			
SB2				⊕				
SD1.1					⊕			
SC1						●		
SF6							●	
SG1	○							●

<sup>a</sup> Symbols: ●, dominant reactivity; ⊕, cross-reactivity always present; ○, cross-reactivity sometimes present.

<sup>b</sup> Three different patterns of reactivity were observed.

gium). All probes were 20- to 29-base oligonucleotide probes. The probe sequences and  $T_m$ s are presented in Table 1. Nine oligonucleotides with sequences complementary to those of the nine probes were used as positive controls for hybridization.

(ii) **Subtype-nonspecific PCR.** A nested PCR for the region that spans the 5' end of the *env* gene (DEIA region) of all genetic subtypes A through H and the two CRFs (CRFs AE and AG) was performed to amplify a 250-bp fragment encompassing the sequences of the identification probes (Fig. 1) (HIV Sequence Database). The primer sets used were primers ED3 and ED14 as outer primers (as described for HMA) and primers ES4 and ES5 as inner primers (designed for DEIA), (see the sequences in Fig. 1). For the first round, 10  $\mu$ l of DNA template was added to a 90- $\mu$ l reaction mixture consisting of 10 mM Tris-HCl (pH 9), 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl<sub>2</sub>, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 20 pmol of each primer, and 3.5 U of Expand high-fidelity *Taq* polymerase (Roche Diagnostics, Mannheim, Germany). Ten microliters of product from the first round was used for the second round in 100  $\mu$ l of the same mixture used for the first-round PCR and 2.5 U of *Taq* polymerase (Promega, Madison, Wis). The first-round PCR was performed as previously described by Delwart et al. (14); a first denaturation step for 2 min at 94°C was followed by 3 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min and 32 cycles of 94°C for 15 s, 55°C for 45 s, and 72°C for 1 min, with a final extension of 5 min at 72°C. The conditions for the second round were a denaturation step of 2 min at 94°C, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, with a final extension of 7 min at 72°C.

(iii) **DEIA procedure.** The DEIA was based on the hybridization of amplified single-stranded DNA (ssDNA) with oligonucleotide subtype-specific probes attached to the surfaces of microtiter plate wells by a streptavidin-biotin bond. Hybrids of the probe and the ssDNA were detected with a mouse monoclonal anti-DNA antibody. Antibody binding was detected by means of a colorimetric immunoenzymatic reaction.

The DEIA was performed with a GEN-ETI-K PS0001 DEIA kit, according to the manufacturer's instructions (DiaSorin, Saluggia, Italy). Three parameters had to be defined: the concentration of the identification probe used to coat the microplates, the concentration of the complementary oligonucleotides, and the hybridization temperature. After calibration, the coating concentrations of the identification probes were defined according to the probes used. The concentrations were 0.25 ng/ $\mu$ l for probes SA2, SAE1, SBD3, SD1.1, SC1, and SG1; 0.75 ng/ $\mu$ l for probe SB2; and 0.05 ng/ $\mu$ l for probes SAG1 and SF6. The optimal concentration of the complementary oligonucleotides was defined as 2.5 ng/ $\mu$ l, and the hybridization temperature was 45°C.

Three formats of the assay, one for each sample collection (i.e., a Europe, a Cameroon, and a Senegal format) were evaluated (Fig. 2). The number and type of probes were adapted to the predominant circulating subtypes.

(a) **Europe format.** The Europe format used subtype-specific probes SA2, SAE1, SBD3, SB2, SD1.1, SC1, SF6, and SG1 to identify subtype A-like samples (subtype A and CRF02\_AG samples), CRF01\_AE samples, and subtype B, D, C, F, and G samples. A first step discriminated six subtypes (subtype A-like, CRF01\_AE, and subtypes BD, C, F, and G), as follows (Fig. 2A). Wells of a streptavidin-coated microplate were sensitized with 100  $\mu$ l of probes appropriately diluted in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]); one probe per strip for strips 1 to 6 and 7 to 12) by incubation for 20 h at 4°C. The wells were washed five times with PBS containing Tween 20 (PBS-TW; DiaSorin). Before the assay, the products of the nested PCR were denatured for 15 min at 97°C. They were cooled on ice for at least 10 min before DEIA analysis.

Hybridization buffer (100  $\mu$ l, containing Denhardt's solution, SSC [0.15 M NaCl plus 0.015 M sodium citrate], Tris-HCl, and EDTA; DiaSorin) was added to each well. We then added 10  $\mu$ l of a negative control (NC) consisting of Tris-HCl, MgCl<sub>2</sub>, and bovine serum albumin (DiaSorin) to wells 1 to 6 of the first line. Ten microliters of each of the six complementary oligonucleotides was added as a positive control to wells 1 to 6 of the second line. Ten microliters of the ssDNA sample (one sample per six wells) was added to each well of the next lines and to each well of all lines on strips 7 to 12. A cardboard lid was placed over the plate to prevent evaporation, and the microplate was incubated for 1 h at 45°C ( $\pm$ 1°C). The wells were then washed five times with PBS-TW. One hundred microliters of an anti-double-stranded DNA antibody solution (1:50 dilution in PBS and fetal calf serum; DiaSorin) was added, and the plates were incubated for 30 min at 30°C ( $\pm$ 1°C) after they were covered with a cardboard lid. The wells were then washed five times with PBS-TW. One hundred microliters of the enzyme tracer solution (protein A conjugated to horseradish peroxidase [1:50 dilution in PBS-fetal calf serum]; DiaSorin) was added, and the plates were incubated for 30 min at room temperature after they were covered with a cardboard lid. The wells were washed five times with PBS-TW, and the reaction was detected by incubation with 100  $\mu$ l of a 1:1 chromogen-substrate solution of tetramethylbenzidine and hydrogen peroxide for 30 min at room temperature in the dark. Color development was stopped by adding 1 N H<sub>2</sub>SO<sub>4</sub> (200  $\mu$ l), and the  $A_{450}$  and  $A_{630}$  were read. After preliminary experiments, the cutoff value was defined as the absorbance for the NC + 0.400.

A second step was performed for the BD-specific probe-reactive samples to distinguish between subtypes B and D. The same conditions used for the first step was applied, except that the cut-off value for the well coated with probe D1.1 was defined as the absorbance for the NC + 0.150.

In this Europe format, we were able to carry out an initial screening of 14 samples per microplate.

(b) **Cameroon format.** The Cameroon format involved two steps: the first used probes SA2, SAG1, SF6, and SG1, corresponding to the predominant genotypes in this country. The nontypeable (NT) samples (those with absorbance values below the cutoff) were then analyzed with probes SBD3 and SC1 (Fig. 2B). The same experimental conditions described above for the Europe format were applied for the Cameroon format. In the Cameroon format, we were able to carry out an initial screening of 22 samples per microplate.

(c) **Senegal format.** The Senegal format was performed as described above, except that the subtype BD-specific probe replaced the subtype F-specific probe for the first step; the NT samples were further analyzed with subtype C- and F-specific probes (Fig. 2C). In this format, we were able to carry out an initial screening of 22 samples per microplate.

(iv) **Probe reactivity.** The intersubtype relationships discussed above precluded direct simple subtyping with single reactivity to the specific probe. However, a reproducible profile of probe reactivity including cross-hybridization made it possible to define a chart representing a specific pattern of hybridization for each subtype or CRF (Table 2). A genotype was then assigned according to this chart, with any reactivity profile different from those in this chart being classified as indeterminate.

**DNA sequencing of the *env* cds-C2 region.** DNA sequencing and phylogenetic analysis of the *env* 5' end of NT, indeterminate (similar absorbances with two probes), and discordant samples were performed to assign a subtype to the DEIA region. An 820-bp fragment was amplified from proviral DNA by nested PCR with primers ED3 and ED14 as the outer primers and primers ES4 and PSA2 (5'-CCATTTAACAGCAGTTGAGTTG-3') as the inner primers. The

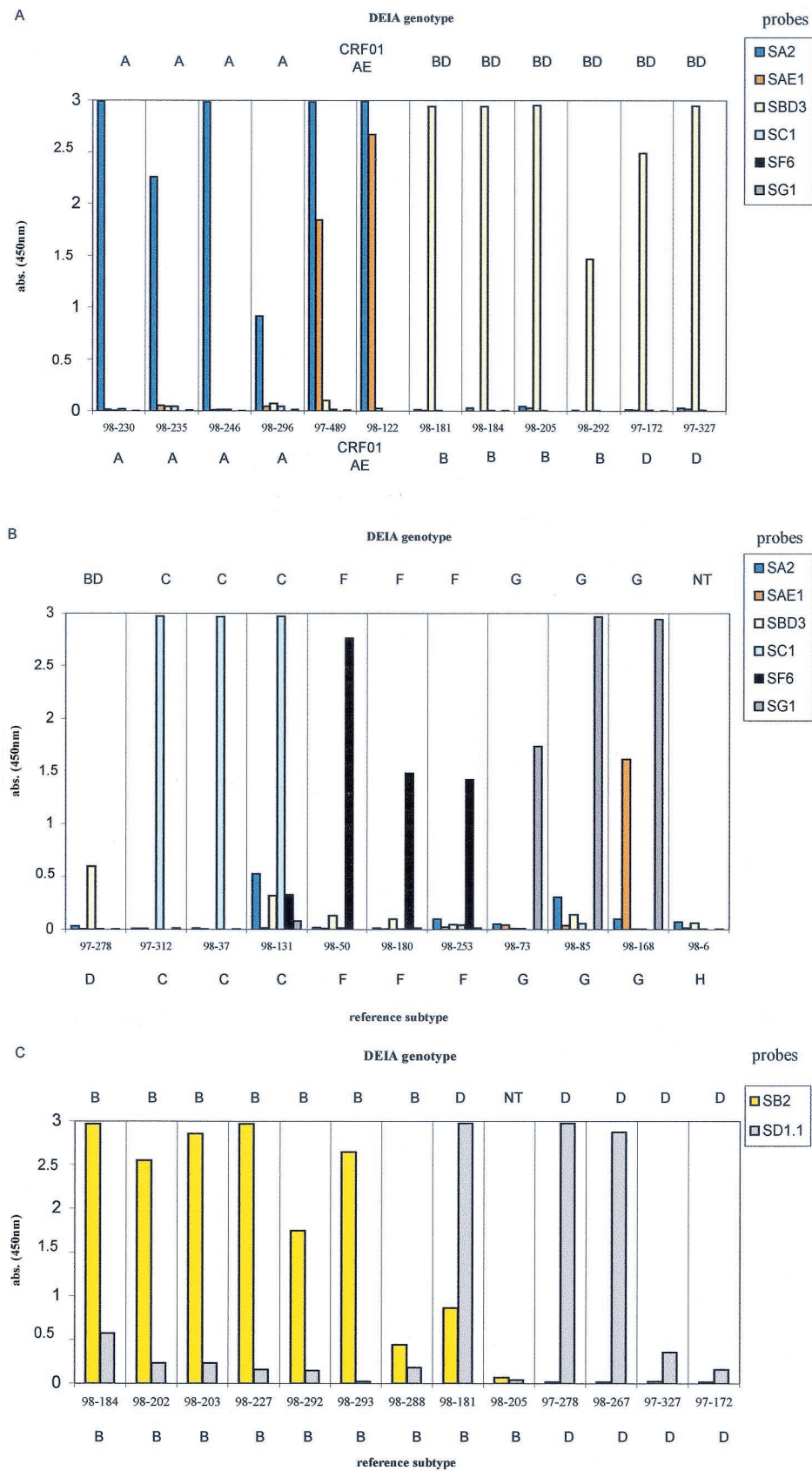


FIG. 3. Representative profiles of probe reactivities with European samples. The genotype determined by DEIA is given at the top, and the reference subtype is given at the bottom. (A and B) The first screening discriminated subtype A-like, CRF01\_AE, and subtype BD, C, F, and G samples. (C) Specific identification of subtypes B and D from the samples reactive with subtype BD-specific probes was performed in a second step with individual B- and D-specific probes. abs., absorbance.

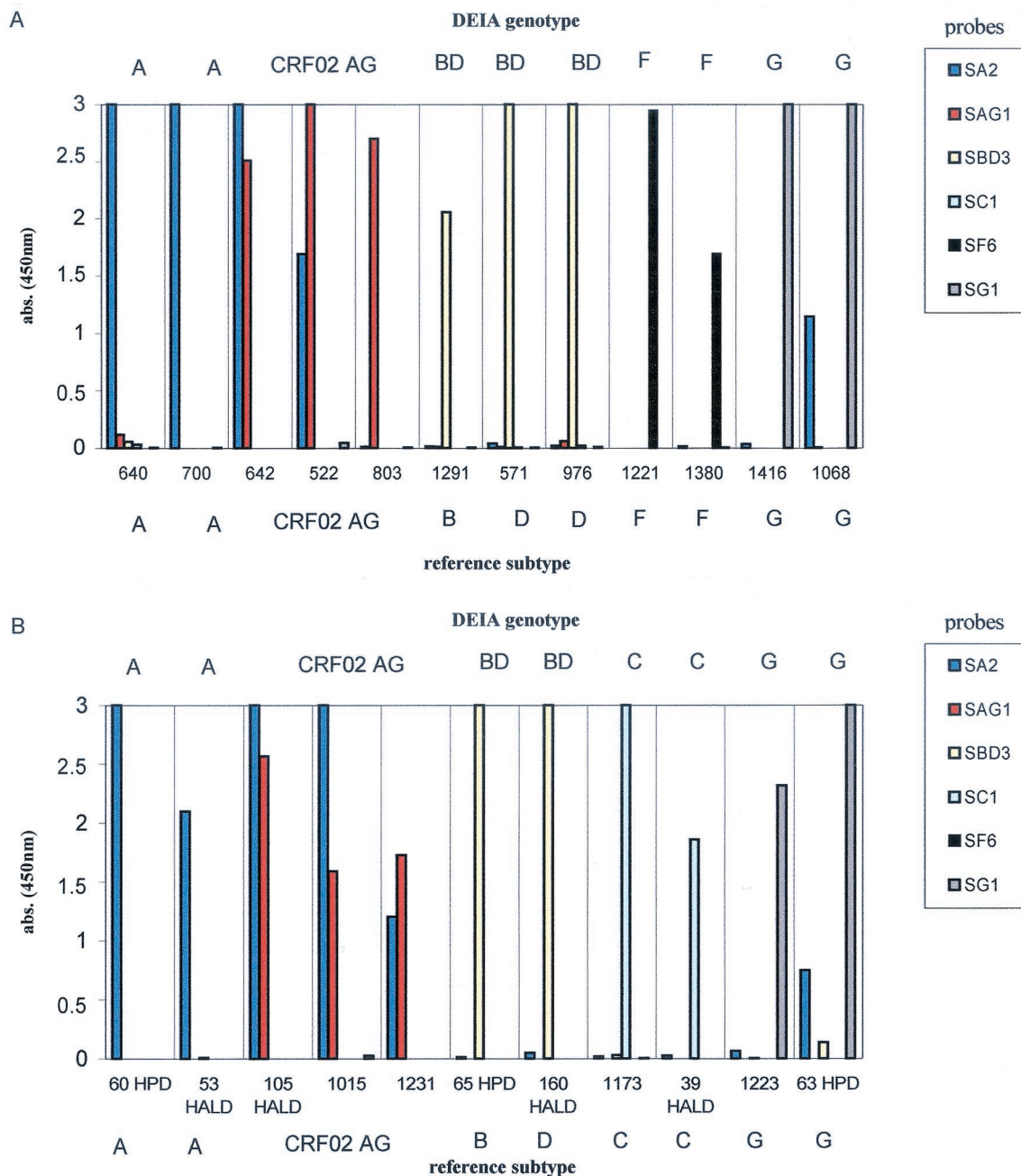


FIG. 4. Representative profiles of probe reactivity with Cameroonian samples (A) and Senegalese samples (B). The DEIA genotype is given at the top, and the reference subtype is given at the bottom. abs., absorbance.

first round was performed with the Expand Long Template PCR system (Roche Diagnostics), according to the manufacturer's instructions. The second round was performed with *Taq* DNA polymerase (Promega), as follows: 1 to 5 µl from the first-round amplification was used for the second round with the inner primers, the reaction mixture consisting of 50 mM KCl, 10 mM Tris-HCl (pH 9), 0.1% Triton X-100, 1.8 mM MgCl<sub>2</sub>, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 2.5 U of *Taq* polymerase, and 20 pmol of each primer. The PCR conditions were 92°C for 5 min, followed by 38 cycles at 92°C for 20 s, 50°C for 30 s, and 72°C for 2 min, with a final extension step at 72°C for 10 min. The amplified fragments were purified with the QIAquick gel extraction kit (QIAGEN, Courtabouef, France) and were directly sequenced with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase FS (Perkin-Elmer, Roissy, France) on an Applied Biosystems 373A automatic DNA sequencer (Stretch model).

**Phylogenetic analysis.** Nucleotide sequences were aligned with the CLUSTAL W program with minor manual adjustments to take into account the protein sequences (58). Regions that could not be aligned unambiguously due to length or sequence variability were omitted from the analysis. Phylogenetic trees were produced by the neighbor-joining method, and the reliability of the branching orders was assessed by the bootstrap approach with the CLUSTAL W program. Genetic distances were calculated by Kimura's two-parameter method (27).

**RESULTS**

**Probe reactivity.** The reactivity profiles of representative European and African samples are presented in Fig. 3 and 4. Subtype BD-, C-, and F-specific probes produced intense and

TABLE 3. Correlation between *env* HMA or V3-V5 sequencing and DEIA genotyping of the European collection

Genotype by <i>env</i> HMA or sequencing	No. of samples with the following <i>env</i> genotype by DEIA <sup>a</sup> :									Total ( <i>n</i> = 50)	
	A-like <sup>b</sup>	CRF01_AE	BD	B	D	C	F	G	NT		
									PCR positive		PCR negative
A-like <sup>a</sup>	<b>13</b>							1	1	15	
CRF01_AE		<b>3</b>								3	
B			<b>9<sup>c</sup></b>	<b>7<sup>d</sup></b>	<b>1<sup>d</sup></b>				1	10	
D			<b>4<sup>c</sup></b>		<b>4<sup>d</sup></b>				1	5	
C						<b>4</b>			1	6	
F							<b>4</b>			4	
G								<b>4</b>		4	
H									2	3	

<sup>a</sup> Boldface numbers indicate correct identification.

<sup>b</sup> Genotype A-like samples corresponded to both subtype A and CRF02\_AG.

<sup>c</sup> Discrimination of subtype B or D samples from the other subtypes with a common BD-specific probe.

<sup>d</sup> Identification of the subtype BD probe-reactive samples with B- or D-specific probes.

highly specific signals with samples of the subtypes corresponding (Fig. 3A, 3B, and 4). Among the subtype BD-reactive samples from the European collection, the additional identification of subtype B samples was accurate, with high absorbance values, whereas specific identification of subtype D samples was characterized by lower absorbance values (two of four samples had absorbances of <0.4; Fig. 3C). This result was probably due to a higher variability of subtype D sequences and led to a lowering of the cutoff value (absorbance for the NC + 0.150).

We analyzed the results for the European and African samples for subtype A, CRF01\_AE, and CRF02\_AG, separately. According to the interpretation chart, in the absence of the CRF02\_AG-specific probe, 11 of the 13 European samples were correctly identified. Subtype A-like samples (subtypes A and CRF02\_AG samples) reacted specifically with the subtype A-specific probe, giving intense signals (Fig. 3A). The three CRF01\_AE samples were discriminated on the basis of their strong reactivities with the CRF01\_AE-specific probe (Fig. 3A). In the African sample collections, 11 of the 13 correctly identified subtype A samples reacted with the subtype A-specific probe, giving an intense signal without cross-hybridization (Fig. 4). For the 26 correctly identified CRF02\_AG samples, two major patterns of reactivity were observed (Table 2; Fig. 4): dominant reactivity with the subtype A-specific probe and cross-hybridization to the CRF02\_AG-specific probe (21 samples) and dominant reactivity with the CRF02\_AG-specific probe and cross-hybridization to the subtype A-specific

probe (4 samples). For one sample, only reactivity to the CRF02\_AG-specific probe was observed.

Subtype G samples reacted dominantly with the SG1 probe, but various degrees of cross-reactivity to SAE1 and/or SA2 probes were observed (Table 2; Fig. 3B and 4).

**Comparison between reference subtyping and DEIA genotyping.** The results of DEIA genotyping were compared to those obtained by *env* HMA genotyping or *env* sequencing for the 131 samples.

**(i) Subtyping of the European collection.** The Europe format included eight probes for the identification of *env* subtype A-like samples, CRF01\_AE, and the B, C, D, F, and G genotypes (Table 3). We did not distinguish CRF02\_AG because it was not distinguished by *env* HMA. Due to the predominance of subtype B in Europe, specific identification of subtypes B and D with the set of BD-, B-, and D-specific probes was required.

Of the three subtype H samples, one was not amplified and two were nonreactive, providing information about the specificities of the probes. Six of the 47 samples were identified as NT (1 was not amplified and five gave weak signals below the cutoff). Thirty-nine samples (83.0%) were correctly identified. All CRF01\_AE, subtype F, and subtype G samples were correctly identified; 13 of 15 (86.6%) subtype A-like samples reacted specifically with the subtype A-specific probe and one was genotyped as subtype G by DEIA. Thirteen of the 15 subtype B and D samples (86.6%) were discriminated with the common BD-specific probe. The subsequent use of probes

TABLE 4. Correlation between *env* V3-V5 genotyping and DEIA genotyping for the African collections

Genotype by <i>env</i> sequencing	No. of samples with the following <i>env</i> genotype by DEIA <sup>a</sup>								Total ( <i>n</i> = 81)
	A	CRF02_AG	BD	C	F	G	I <sup>b</sup>	NT <sup>c</sup>	
A	<b>13</b>	4				2			19
CRF02_AG	1	<b>26</b>						1	28
B or D			<b>11</b>					1	12
C				<b>2</b>		1			3
F					<b>6</b>			1	7
G						<b>10</b>	1	1	12

<sup>a</sup> Boldface indicates correct identification.

<sup>b</sup> I, indeterminate, i.e., similar reactivity with subtype A- and subtype G-specific probes.

<sup>c</sup> The NT samples were amplified by nested PCR, but the absorbance value was below the cutoff.

TABLE 5. Molecular biology-based analysis of NT samples, misidentified samples, and samples with indeterminate results<sup>a</sup>

Sample collection	Identification	Subtype by:			% Difference <sup>b</sup>	Note		
		<i>env</i> HMA or V3-V5 sequencing and phylogenetic analysis	<i>env</i> DEIA	<i>env</i> cds-C2 sequencing and phylogenetic analysis				
Europe	<b>98 289</b>	A <sup>c</sup>	<b>G</b>	<b>G</b>	15	Reactive with CRF02_AG-specific probe, if present		
	98 238	A <sup>c</sup>	NT	CRF02_AG				
	98 181	B <sup>c</sup>	BD or D	B			10	Lower specificity of subtype D-specific probe
	98 205	B <sup>c</sup>	BD or NT	B			5	Lower sensitivity of subtype B-specific probe
	98 210	C <sup>c</sup>	NT	C	33			
Cameroon	<b>CMMP 1432</b>	A	<b>G</b>	<b>G</b>	24			
	CMMP 1367	D	NT	D				
Senegal	<b>SN 191HPD</b>	A	<b>CRF02_AG</b>	<b>CRF02_AG</b>	28	Lower specificity of CRF02_AG-specific probe		
	<b>SN 65HALD</b>	A	<b>CRF02_AG</b>	<b>CRF02_AG</b>				
	<b>SN 19HPD</b>	A	<b>CRF02_AG</b>	<b>CRF02_AG</b>				
	SN 103HPD	A	CRF02_AG	A				
	<b>SN 1048SC</b>	A	<b>G</b>	<b>G</b>				
	SN 46HALD	CRF02_AG	A	CRF02_AG			Four bases + two insertions different from the CRF02_AG-specific probe	
	SN 1128	CRF02_AG	NT	CRF06_cpx			Reacted dominantly with subtype C-specific probe, if present	
SN 165HPD	C	G	C	Similar reactivities to subtype A- and subtype G-specific probes				
	SN 99HALD	G	I	G				

<sup>a</sup> The reference subtype, the DEIA genotype, and the *env* 5' end subtype of 16 analyzed samples are reported. Samples for which identical subtypes were found by DEIA genotyping and DEIA region sequencing are indicated in boldface. Further explanations of discrepancies are reported in the last column.

<sup>b</sup> Difference between sample sequence and probe sequence specific for subtype identification.

<sup>c</sup> Reference subtype determined by *env* HMA.

specific for subtypes B and D correctly identified seven of nine subtype B samples and four of four subtype D samples (Table 3).

(ii) **Subtyping of African collections.** For clarity, the results obtained with the Cameroon and Senegal formats are presented in Table 4.

(a) **Cameroon format.** Thirty-two of 36 samples (88.9%) were correctly identified by use of the Cameroon format. A first screening of the four predominant subtypes (subtype A, CRF02\_AG, and subtypes F and G) with the corresponding probes discriminated 27 samples, with 1 being misidentified as subtype G instead of subtype A. One subtype F, one subtype G, and the seven subtype B or D samples were NT; they were further analyzed in a format containing subtype BD- and C-specific probes. Six of seven subtype B or D samples were correctly identified; the other three samples remained NT.

(b) **Senegal format.** Due to the nonnegligible prevalence of subtypes B and D in Senegal, the subtype BD-specific probe instead of the subtype F-specific probe was included in the first step. Thirty-six of 45 samples (80%) were correctly identified. Seven samples were misidentified with the CRF02\_AG-specific probe (four samples of genotype A), the subtype G-specific probe (two samples of genotypes A and C, respectively), and the subtype A-specific probe (one sample of genotype CRF02\_AG); and three samples (one CRF02\_AG sample and two subtype C samples) were NT. The NT samples were further discriminated by an assay with subtype C- and subtype F-specific probes; the two subtype C samples were correctly identified, and the CRF02\_AG sample remained NT. One subtype G sample was indeterminate, displaying similar reactivities with subtype A- and subtype G-specific probes.

**Molecular basis of DEIA genotyping results.** DNA sequencing followed by phylogenetic analysis of the *env* cds to the C2 region of gp120 was performed to assign a subtype to the DEIA region and to account for discrepancies. This was possible for 16 of the 21 samples which were NT ( $n = 6$ ) or misidentified ( $n = 10$ ) by the DEIA (Table 5; Fig. 5). The subtype deduced from phylogenetic analysis of the *env* cds to the C2 region correlated with the DEIA genotype for 6 of the 10 misidentified samples (Table 5). This revealed the occurrence of recombination involving the 5' end of the *env* gene and the V3 and V5 regions of the two parental subtypes (Fig. 5 and 6). For one sample (sample SN 165HPD), misidentification was due to the absence of the subtype C-specific probe in the format; this sample reacted dominantly to the subtype C-specific probe when that probe was present and cross-hybridized with the subtype G-specific probe (data not shown).

It was possible to sequence the DNA of the DEIA region for 6 of the 11 NT or indeterminate samples. Three samples (samples 98 210, CMMP 1367, and SN 1128) showed too many differences between sample and complementary probes sequences (more than 20%), probably resulting in a lack of hybridization (Table 5). One of these samples (sample SN 1128) presented a particularly complex *env* pattern (subtypes A, G, J, and K) involving the V3-V5 region from CRF02\_AG associated with the 5' end of *env* from the recently described CRF06\_cpx (Fig. 5 and 6) (36). For one European sample (sample 98 238) identified as subtype A-like by *env* HMA, phylogenetic analysis of the 5' end of *env* revealed that the DEIA region was related to CRF02\_AG (Fig. 5). This sample was initially NT by the European format, but subsequent analysis with the CRF02\_AG-specific probe showed that the sam-





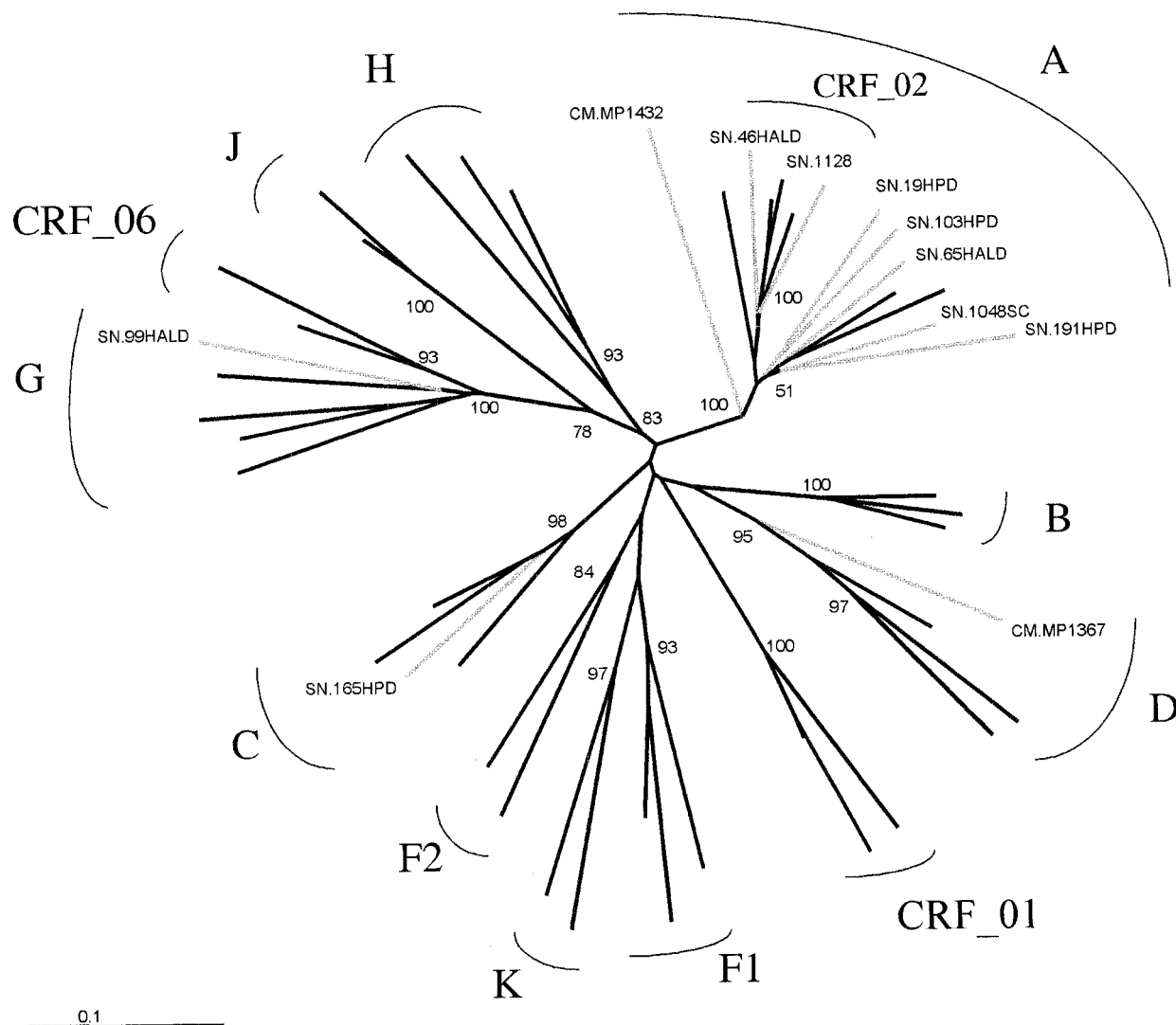


FIG. 6. Phylogenetic tree based on 670 unambiguously aligned nucleotides from the V3-V5 *env* region of the 11 discordant samples from the Senegalese and Cameroonian collections (Table 5), for which the reference subtype was determined by *env* sequencing and phylogenetic analysis.

of recombinations involving the 5' end of *env* and the V3 and V5 regions (seven samples) or the presence of too many differences between the sample and the probe sequences, resulting in a lack of hybridization (two samples). For two other samples, the samples were misidentified or NT due to the absence of specific probes in the format.

The data show that DEIA genotyping is a potentially useful tool suitable for rapid and simple *env* genotyping. Previous studies demonstrated that probe hybridization is an effective technique for molecular biology-based epidemiological analyses (47, 56). These studies were based on the distinction of only two prevalent subtypes, whereas our results indicate that identification of the six major subtypes and discrimination of the two most prevalent CRFs are possible. The adaptation of the assay to give three different formats, corresponding to the subtype distributions in three countries, showed that DEIA genotyping can be modulated as required. This is of value for monitoring the dynamics of the epidemic and the spread of the various subtypes. For instance, HIV is becoming more diverse

in Western countries such as France and Sweden, where the prevalences of non-subtype B isolates recently reached 15 and 30%, respectively (6, 55). Different algorithms can be defined for the prescreening of a limited number of predominant subtypes within a large panel of specimens according to the local context or for the screening of a larger number of subtypes within a selected collection. Samples NT or indeterminate by DEIA can be further analyzed by sequencing and phylogenetic analysis.

This methodology has several advantages. It can be used for the direct analysis of the genome, which is more precise than serotyping and which takes advantages of the simplicity of the enzyme-linked immunosorbent assay (ELISA) technique. Microplate detection is less labor-intensive than HMA or sequencing and requires simple ELISA equipment, and the results are based on the simple identification of reactive and nonreactive samples. This would facilitate the screening of numerous samples in large population-based epidemiology studies, especially in developing countries, where sequencing

and HMA are difficult to perform and V3 serotyping is limited to a few antigenically different V3 subtypes. In countries where subtypes A, C, F, and G are prevalent, their serologic discrimination is inefficient due to cross-reactivity between subtype-specific peptides (24, 46, 50). Our results suggest that DEIA genotyping could be a valuable tool for distinguishing these subtypes. The identification of specific nucleotide signature sequences made it possible to discriminate associated subtypes such as subtype A, CRF01\_AE, and CRF02\_AG. The simple and efficient identification of CRF02\_AG is particularly important because this form accounts for between 60 and 84% of the subtype A samples identified from West and West-Central Africa (37). CRF02\_AG seems to have already spread widely and accounts for a large proportion of the subtype A-like variants found worldwide. The recent development of a *gag* HMA makes discrimination of such variants possible (23); however, it involves additional *gag* analysis of subtype A-like samples identified by *env* HMA. The discrimination of CRF02\_AG by DEIA with the *env* gene may be of value because it is faster and simpler.

As for any subtyping method, DEIA has limitations. DEIA genotyping is based on 20- to 30-base probes and analyzes only a single short region of the *env* gene. Nonetheless, our results showed that the selected region may provide the same information provided by the longer fragment used in the *env* HMA technique. The misidentification of 6 of 10 samples was accounted for by recombination events that were detectable only by analysis of the entire *env* gene. Another limitation is the genetic variability of HIV-1, which increases with the duration of the epidemic; thus, subtype-specific probes should be regularly reevaluated and adapted. The DEIA region was found to be less variable than other parts of the *env* gene, particularly the V3 region, when the probes were designed. This reduced variability might reduce this limitation.

The occurrence of recombination is a major obstacle to HIV-1 subtyping; it is linked to the prevalence, dynamics, and duration of the epidemic in the region concerned. The description in particular of numerous *gag* and *env* recombinants makes subtyping less informative if it is based on only one gene. The development of a rapid and simple method for the identification of *gag* and *env* recombinants in one step is necessary. We are therefore developing a *gag* DEIA genotyping system. However, these data showed that discordant results could be due to intragene recombinations. In countries where the epidemic is relatively old and dynamic, with a high level of diversity and at least 30% recombinant forms, such as in the Democratic Republic of Congo (62), DEIA genotyping may be unsuitable. In such situations, the sequencing of entire genes remains the only suitable technique for correct identification.

In conclusion, this study demonstrated the feasibility and relevance of an HIV-1 DEIA genotyping approach. The primers, probes, and formats developed in this work can be used as they are. However, the high rate of evolution of HIV-1 and its worldwide distribution require the definition of probes adapted to a regional context rather than the unrealistic permanent use of definitively identified universal subtype-specific probes.

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