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Sequence Note

Study of HIV Type 1 *gag/env* Variability in The Gambia, Using a Multiplex DNA Polymerase Chain Reaction

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ABSTRACT

A multiplex DNA PCR assay was developed for the simultaneous first-round amplification of HIV-1 *gag* and *env* fragments for the heteroduplex mobility assay (HMA). This assay was compared with the conventional amplification assay, using DNA extracted from PBMC samples from 30 HIV-1-seropositive individuals from The Gambia, who were enrolled between 1992 and 1997. From 27 of 30 (90%) samples both *gag* and *env* HMA fragments were amplified simultaneously. In one sample only the *gag* HMA fragment could be amplified by multiplex DNA PCR, and in two samples amplification was negative for both *gag* and *env* HMA in multiplex as well as the mono-DNA PCR. Of the 28 Gambian isolates subtyped by *gag/env* HMA or by sequencing and phylogenetic analysis, the majority (19 of 28; 68%) were intersubtype recombinant. Fifteen of 28 (53%) samples were circulating recombinant form (CRF) CRF02.AG variants. Two isolates clustering with the previously documented Gambian isolate GM4 (previously described as an *env* GC recombinant) are classified as *gag* A/*env* J recombinants.

THE GAMBIA in West Africa has a rising HIV-1 seroprevalence of 0.5% and a stable HIV-2 seroprevalence of 1.1%. The aim of this study was to determine the prevalent HIV-1 group M subtypes, circulating recombinant forms (CRFs), and intersubtype recombinant forms (IRFs) in The Gambia by *env* and *gag* heteroduplex mobility assay (HMA), and to evaluate a multiplex DNA polymerase chain reaction (PCR) assay for this simultaneous amplification of HIV-1 *gag* and *env* fragments as first-round amplification products for the HMA.

Thirty HIV-1-seropositive Gambian patients ($CD4^+$ T cell count, <200 $CD4^+$ cells/μl of blood) attending the Medical Research Council (MRC) hospital clinic between 1992 and 1997 and who had no history of antiretroviral therapy were recruited in The Gambia. The serostatus of the patients was determined by a combined enzyme-linked immunosorbent assay (ELISA) (ICE HIV-1.0.2; Murex Biotech Limited, Dartford, UK) and when positive by type-specific HIV-1 and/or HIV-2 assays confirmed by PEPTI-LAV1-2 (Sanofi Diagnostic Pasteur, Marnes la Coquette, France). This study was approved by the MRC Scientific Coordinating Committee (SCC), and ethical approval was obtained from The Gambia government/MRC Ethical Committee.

DNA was extracted from cryopreserved peripheral blood mononuclear cells ($\geq 2 \times 10^6$ peripheral blood mononuclear cells [PBMCs]) by a salting-out method (Puregene DNA extraction kit; Gentra Systems, Minneapolis, MN). Cell lysis solution was initially added to samples at the MRC laboratories. Samples were then transported at room temperature to the Institute of Tropical Medicine (ITM, Antwerp, Belgium), where DNA extraction was continued. DNA was successfully extracted from all samples as monitored by agarose gel electrophoresis, ethidium bromide staining, and visualization under UV radiation. Five of 100 μl of extracted DNA was used as template for the multiplex DNA PCR assay, whereby the *gag*

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and *env* fragments were coamplified using previously described *gag* (H_1G777 + H_1P202)[1] and *env* (ED5 + ED12)[2] primers at a concentration of 0.2 μM each. The multiplex DNA PCR mix contained 1× PCR buffer, 1.5 mM $MgCl_2$, 2.5 U of *Taq* DNA polymerase (PE Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands), and 0.2 mM dNTP mix (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) in a total volume of 50 μl. Amplification was conducted at 94°C for 2 min followed by 35 cycles of 94°C for 15 sec, 55°C for 30 sec, 72°C for 60 sec, and a final extension of 7 min at 72°C. The PCRs were carried out in a PTC-200 thermal cycler (Biozym, Roosendaal, The Netherlands). Nested PCR, using 1 μl of the first-round multiplex DNA PCR product as template, was conducted in separate reaction tubes to amplify the 0.46-kb *gag* or 0.7-kb *env* HMA fragment, as described previously.[1,2]

Both *gag* and *env* HMA amplification products were obtained in the multiplex DNA PCR from 27 of 30 (90%) Gambian samples. In one sample (GMB 24), only the *gag* HMA fragment could be amplified by the multiplex DNA PCR; the yield of *env* HMA fragment obtained in a mono-PCR was insufficient for HMA purposes. In two samples (GMB 1 and 10) amplification was negative for both *gag* and *env* by multiplex DNA PCR and by mono-PCR, whereas in both samples amplification of the smaller *env* fragment encoding C2V3 was successful.

Both *gag* HMA[1] and *env* HMA[2] were performed as described previously. Of 28 Gambian isolates, 16 could be classified by *gag* HMA as CRF02.AG, 7 as subtype A, 2 as subtype B, 2 as subtype C, and 1 as subtype D (Table 1).

The V3–V5 envelope sequences (ES7/ES8) amplified from 27 Gambian isolates were analyzed by *env* HMA: 21 of the samples were A# (A#, subtype A and CRF02.AG could not be differentiated by *env* HMA), 2 were subtype B, 2 were subtype C, and 1 was subtype G (Table 1). One isolate, GMB 22, which showed an overall intersubtype migration pattern, could not be assigned a definitive subtype by *env* HMA. The PCR products of four samples (GMB 1, 10, 22, and 24) were analyzed by sequencing followed by phylogenetic analysis of the C2V3 *env* region to determine the subtype in case no (GMB 1 and 10) or insufficient (GMB 24) HMA DNA PCR product was obtained, or if the HMA result was inconclusive (GMB 22). The newly documented sequences were aligned with 20 *env* C2V3 sequences representing HIV-1 group M subtypes A, B, C, D, F, G, H, J, CRF02.AG, and CRF01.AE. In addition, the Gambian isolate GM4, described as a complex *env* GC intersubtype recombinant by Bobkov *et al.*,[3] was included in the alignment. Sequences were aligned with CLUSTAL W[4] and the resulting alignments were edited using the dedicated comparative sequence editor (DCSE).[5] Regions that could not be aligned unambiguously due to substantial length or sequence variation were omitted from the analysis. For distance calculations (Jukes and Cantor), tree construction (neighbor joining), and bootstrap analysis the software package TREECON[6] was used. A bootstrap value greater than 70% based on 2000 bootstrap trees was considered supported. A C2V3 *env* phylogenetic tree based on the aligned sequences was constructed.

Isolates GMB 1 and GMB 10 were, respectively, classified as subtypes C and D (Table 1), and isolates GMB 22 and 24 clustered with GM4, the *env* intersubtype recombinant reported by Bobkov *et al.*[3] This relationship was supported by a 98% bootstrap value. Whereas the GM4 isolate was previously classified as an *env* GC intersubtype recombinant, availability of subtype J reference sequences now identified GM4 as well as GMB 22 and 24 as clustering with subtype J isolates (result not shown). To verify the intersubtype recombinant structure of GM4 and GMB 22 and 24, larger *env* and *gag* fragments were also analyzed: the *env* 1.2-kb ED5–ED12 fragment encoding V1 to V5 of gp120 of GMB 22 and 24[2] and the 1.3-kb *gag* fragment covering AA1p17–AA8p1 (according to HIV-1 ELI; accession number P04592) of GMB 22 were amplified. Amplification was performed with primer HG00 (5′-cc*ggatcc*-*c*gggactagcggaggctagaag-3′) and H1P202 in the first round,[1] and with HGHMA1 (5′-atgggtgcgagagcgtcagtatt-3′) and HGHMA 1317[1] in the second round. Cycling conditions were

TABLE 1. *gag* AND *env* SUBTYPE OF GAMBIAN ISOLATES[a]

	Subtype		
	gag	env	
Sample	*HMA*	*HMA*	*C2V3*
GMB 1	PCRneg	PCRneg	C
GMB 2	CRF02.AG	A#	
GMB 3	CRF02.AG	A#	
GMB 4	CRF02.AG	A#	
GMB 5	CRF02.AG	A#	
GMB 6	B	B	
GMB 7	CRF02.AG	A#	
GMB 8	CRF02.AG	A#	
GMB 9	C	C	
GMB 10	PCRneg	PCRneg	D
GMB 11	A	A#	
GMB 12	CRF02.AG	A#	
GMB 13	CRF02.AG	G	
GMB 14	CRF02.AG	A#	
GMB 15	A	A	
GMB 16	CRF02.AG	A#	
GMB 17	CRF02.AG	A#	
GMB 18	A	A	
GMB 19	CRF02.AG	A#	
GMB 20	A	A#	
GMB 21	CRF02.AG	A#	
GMB 22	A	ND	J
GMB 23	CRF02.AG	A#	
GMB 24	A	U	J
GMB 25	CRF02.AG	A#	
GMB 26	C	C	
GMB 27	D	A#	
GMB 28	A	A#	
GMB 29	B	B	
GMB 30	CRF02.AG	A#	

Definitions: A#, Subtype A could not be differentiated from CRF02.AG by *env* HMA; U, Unclassified; ND, not done.

[a] Subtypes for *env* were based on the 700-bp (ES7/ES8) HMA fragments covering V3–V5 or C2V3 *env* sequencing and/or phylogenetic analysis, and for *gag* were based on the 460-bp (H_1G1584-G17) HMA fragment covering AA132p24–AA40p7 (according to HIV-1 ELI; accession number K03454).

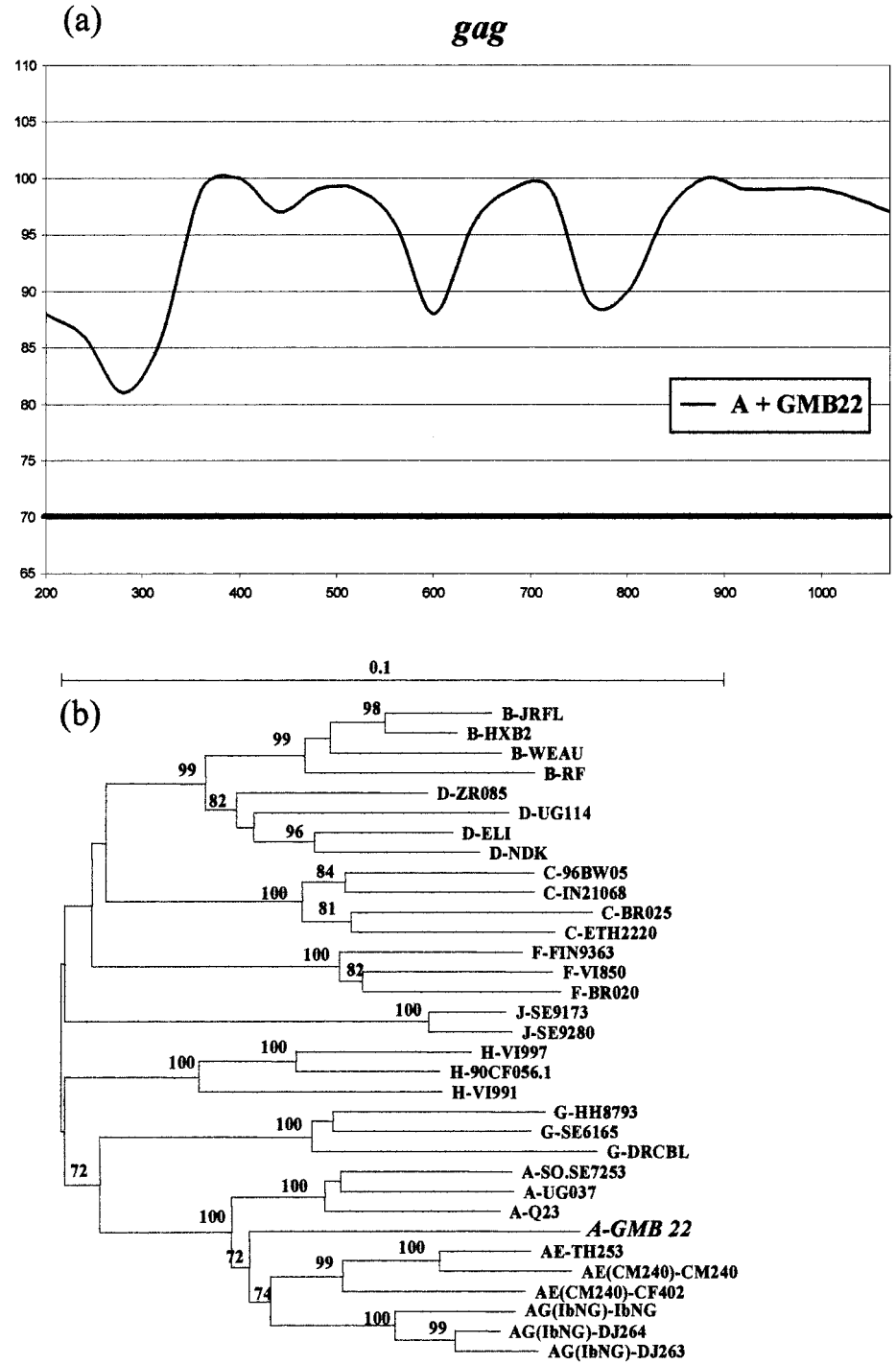
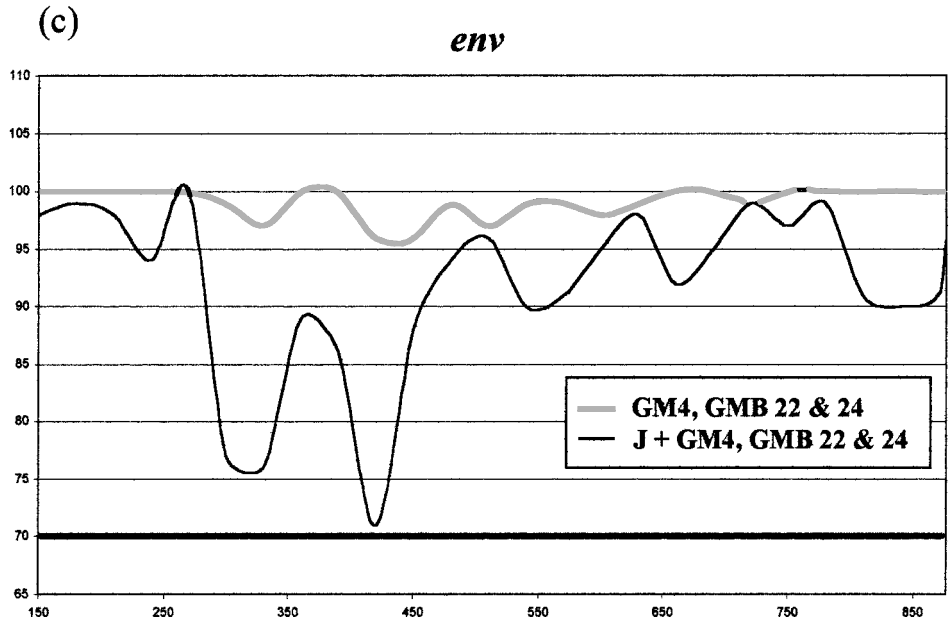


FIG. 1. (**a**) Bootscanning of the bootstrap value of the cluster containing both subtype A references U455, SE7253, Q23, UG037, and the GMB 22 sequence for the *gag* region. Alignment positions and bootstrap values (%) are depicted on the *x* and *y* axes, respectively. (**b**) Phylogenetic tree of the 1319-bp *gag* fragment covering AA1p17–AA8p1 (according to HIV-1 ELI; accession number P04592) of Gambian isolate GMB 22 aligned with previously reported *gag* sequences representing HIV-1 subtypes A, B, C, D, F, G, H, J, CRF02.AG, and CRF01.AE. Gambian isolate GMB 22 is indicated in italic. Two thousand bootstrap samples were analyzed. Bootstrap values are given as percentages at the internodes if they exceed the 70% level. The distance between two sequences is obtained by summing the lengths of the connecting horizontal branches, using the scale on top. The tree is rooted arbitrarily between subtypes.



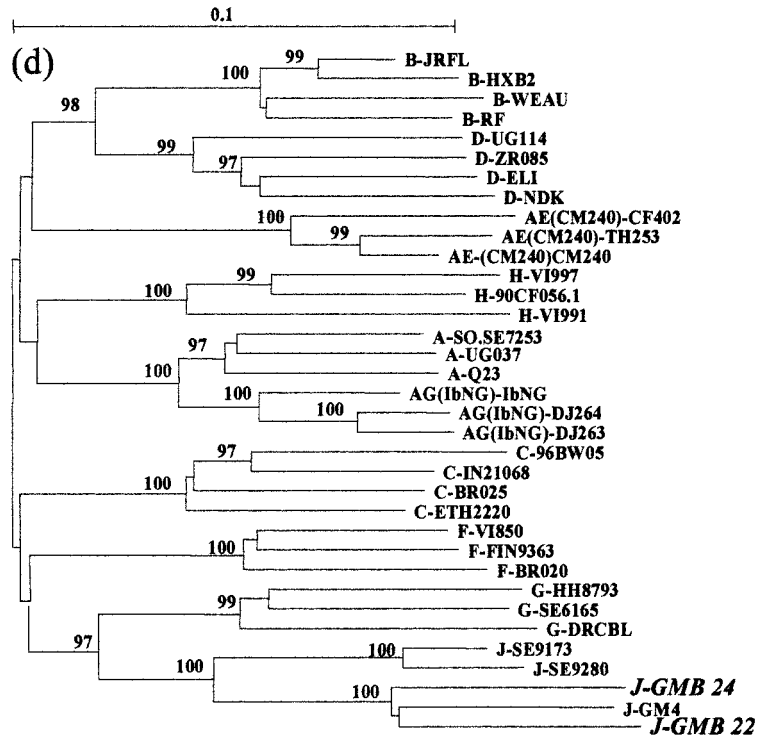


FIG. 1. *Continued.* (**c**) Bootscan plot of the 1.1-kb *env* V2–V5 region of the cluster containing the Gambian sequences GM4, GMB 22 and 24, and the cluster containing both subtype J references SE9280 and SE9173 and the Gambian sequences. Alignment positions and bootstrap values (%) are depicted on the *x* and *y* axes, respectively. (**d**) Phylogenetic tree of Gambian isolates GM4, GMB 22 and 24, based on 1.2-kb V2–V5 *env* sequences aligned with previously reported *env* sequences representing HIV-1 subtypes A, B, C, D, F, G, H, J, CRF02.AG, and CRF01.AE. Newly documented Gambian isolates are indicated in italic. Phylogenetic analysis as described in (**b**). The nucleotide sequence data were deposited in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under the following accession numbers: AJ276694–AJ276698.

as follows: 2 min at 94°C; 35 cycles each of 30, 30, and 120 sec at 94, 50, and 72°C, respectively; and a final extension for 7 min at 72°C in the first round; for the second round the same cycle protocol was used but the extension time was reduced to 90 sec. The PCR product was cloned, sequenced, and analyzed by bootscanning[7] and phylogenetic analysis, using the references stated above. Bootscanning was performed in order to assess the bootstrap support of particular nodes in each region of the alignment. Distances were calculated with the Kimura two-parameter model, with a transition-to-transversion ratio of 2.0, and trees were constructed by the neighbor-joining algorithm. A sliding window of 400 bp (*gag*) or 300 bp (*env*) was used, advanced in steps of 40 and 30 bp, respectively, and 100 bootstrap replicates were analyzed for each window. Bootscanning of the *gag* gene fragment of GMB 22 revealed classification as subtype A, as is apparent from Fig. 1a, which shows the bootstrap support for the node joining GMB 22 with the subtype A references. This was confirmed by phylogenetic analysis of the entire *gag* fragment (Fig. 1b). In the case of GMB 24 only the *gag* HMA fragment could be analyzed. Sequence analysis and phylogenetic analysis indicated that GMB 22 and 24 clustered (data not shown). The bootscan support of the node joining the *env* gene fragments of GM4 and GMB 22, and 24, and the support for the node joining these three isolates with the subtype J references (Fig. 1c), indicated that these three isolates were similar to each other and that they cluster with the subtype J references over the entire sequence range, which was confirmed by phylogenetic analysis (Fig. 1d). On the basis of these findings GM4 and GMB 22, and 24 were classified as *env* subtype J. As such GM4 and GMB 22 and 24 are *gag* A/*env* J recombinants based on the analyzed fragments (Table 1). For GM4 no *gag* sequences were available.

Overall, among the 28 Gambian isolates analyzed by the *gag/env* HMA and/or by phylogenetic analysis, we observed that the majority (19 of 28; 68%) were intersubtype recombinants, or which CRF02.AG (15 of 19; 79%) was most common (Table 1). In view of the fact that it is currently not possible to differentiate subtype A and CRF02.AG by *env* HMA, and since subtyping accounts only for analyzed genome regions, the frequency of recombinants may still be underestimated.

The multiplex DNA PCR assay coupled with the *gag/env* HMA can serve as a tool for the identification of recombinant HIV-1 isolates in countries where more than one HIV-1 subtype is cocirculating. The multiplex DNA PCR assay in combination with the *gag* and *env* HMA makes large-scale HIV-1 subtyping fast, simple, and more economical. Lack of differentiation between subtype A and CRF02.AG by *env* HMA underestimates the true prevalence rate of intersubtype recombinants and highlights the need either to improve experimental conditions for *env* HMA or to investigate other genome regions to allow this differentiation. This study is the first to document a high prevalence of intersubtype recombinants in The Gambia. We have identified two additional "GM4-like" isolates. However, we have no knowledge at this time whether these isolates are epidemiologically linked to the GM4 isolate. These preliminary results might have implications for vaccine design and research in The Gambia, if confirmed with a larger number of samples.

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