AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 14, Number 10, 1998, pp. 877–883
Mary Ann Liebert, Inc.

HIV Type 1 Diversity and the Reliability of the Heteroduplex Mobility Assay

IBTISSAM LOUSSERT-AJAKA,[1] ELISABETH MENU,[2,3] CRISTIAN APETREI,[1,4] MARTINE PEETERS,[5] FLORENCE DAMOND,[1] PHILIPPE MAUCLÈRE,[6] JOSEF EBERLE,[7] CÉCILE BRENGUES,[5] SENTOB SARAGOSTI,[8] FRANÇOISE BARRÉ-SINOUSSI,[2] FRANÇOISE BRUN-VÉZINET,[1] and FRANÇOIS SIMON[1]

ABSTRACT

We investigated HIV-1 diversity by means of heteroduplex mobility assay (HMA) genotyping. We studied 199 samples from patients originating from 26 countries and living in France. The HMA successfully genotyped 182 (91%) of these samples, as follows: 77 (42%) subtype A, 57 (31%) subtype B, 5 (3%) subtype C, 5 (3%) subtype D, 8 (4%) subtype E, 22 (12%) subtype F, 5 (3%) subtype G, and 3 (2%) subtype H. We were not able to genotype 12 samples by means of the HMA. These latter strains were sequenced, and phylogenetic analyses revealed that they were highly divergent subtype A-, D-, or G-related strains. Eight (of 12) subtype D strains were indeterminate by HMA, owing to the broad intrasubtype diversity, suggesting that new reference subtype D plasmids are required, as previously proposed. Thirty-seven strains belonging to the different subtypes were sequenced, and the results showed perfect concordance with the HMA results. Interlaboratory quality controls confirmed the reliability of the HMA for HIV-1 subtyping, despite the extensive viral variability. However, plasmid selection must be continuously revised to cover viral diversification.

INTRODUCTION

At least 10 HIV-1 group M subtypes (or clades) have so far been defined on the basis of relations among *env* gene sequences.[1,2] The potential biological significance of these genetic clusters remains to be established. The study of HIV-1 subtypes is a prerequisite for vaccine trials, in order to establish the most appropriate antigen formulation.[3] Moreover, HIV antibody screening tests may lack sensitivity for HIV-1 group O samples,[4] and in the early seroconversion phase in non-B subtype HIV-1 infection.[5] The study of HIV-1 subtype distribution also allows worldwide viral spread to be monitored: subtype prevalence can vary significantly from country to country and within population groups, and can change with time.[6]

Although sequencing remains the most informative method for determining the HIV-1 subtype, it is costly and lengthy, and requires highly qualified personnel. Alternative, less sophisticated methods of differentiating HIV-1 subtypes include serotyping methods,[7,8] the probe hybridization assay,[9] and the heteroduplex mobility assay (HMA).[10,11] The latter is considered the most effective after sequencing, being highly specific, rapid, and standardizable. Several molecular epidemiology studies have been performed with this technique, and the results show that the HMA is a reliable method for the study of HIV diversity in different populations.[12–14]

The French National Agency for AIDS Research (Agence Nationale de Recherches sur le SIDA, ANRS; Paris, France) has decided to sponsor a prospective study of HIV-1 diversity in France, based on HMA genotyping in different laboratories, followed by sequencing of distant isolates. To prepare for this study, we evaluated the performance of the HMA in clinical practice, by (1) assessing the reliability of this technique for

[1]Laboratoire de Virologie, Hôpital Bichat-Claude Bernard, 75018 Paris, France.
[2]Institut Pasteur, Unité de Biologie des Rétrovirus, 75724 Paris Cedex 15, France.
[3]Laboratoire de Biologie Cellulaire et Moléculaire de la Relation Materno-Foetale, Hôpital Antoine Béaclère, 92140 Clamart, France.
[4]Virus Laboratory, School of Medicine, "Gr. T. Popa" University, R-6600 Iasi, Romania.
[5]Laboratoire des Rétrovirus ORSTOM, 34032 Montpellier, cedex 1, France.
[6]Laboratoire National de Santé Publique et de Référence, Centre Pasteur, Yaoundé, Cameroon.
[7]Max von Pettenkofer Institute, University of Munich, D-80336 Munich, Germany.
[8]Institute Cochin de Génétique Moléculaire, INSERM, Hôpital Cochin, Paris, 75014 Cedex 14, France.

Fonds Documentaire ORSTOM
010014699
Fonds Documentaire ORSTOM
Cote : B*14699 Ex : 1

genotyping HIV-1 strains from patients originating from various geographic regions, (2) comparing the HMA results with those of sequence analysis, and (3) performing an interlaboratory quality control study.

MATERIALS AND METHODS

Samples

Patients were selected according to the ANRS program requirements. Briefly, all new cases of HIV-1 infection diagnosed in the virology laboratory of the Bichat-Claude Bernard Hospital (Paris, France) were included. Samples were serotyped with an HIV-1 subtype-specific enzyme immunoassay (EIA) based on blockade by an excess of peptide in liquid phase.[7,12] All patients infected by non-subtype B strains (mainly originating from African countries), subtype B-infected African patients, and patients with nonserotypable strains confirmed as being HIV-1 seropositive between January 1, 1995 and June 30, 1996 were included, together with 37 subtype B-infected Caucasian patients and 18 subtype F-infected Romanian children and adults,[15] to assess HMA reliability. The study group thus consisted of 199 patients originating from 26 countries on 4 continents, as follows: Europe ($n = 70$), North Africa ($n = 11$), Central Africa ($n = 51$), West Africa ($n = 57$), Asia ($n = 3$), and the Caribbean ($n = 7$).

PBMC isolation and DNA extraction

Peripheral blood mononuclear cells (PBMCs) were separated on a Ficoll gradient. After two washes in phosphate-buffered saline (PBS), cells were pelleted and stored at −80°C. DNA was extracted as previously reported.[16]

Polymerase chain reaction and heteroduplex mobility assay

One microgram of DNA was first subjected to polymerase chain reaction (PCR) amplification using the ED3/ED12 primer pair. When ED3/ED12 amplification failed we used the ED5/ED14 primer pair.[13] The second round of amplification was performed with 5 μl of the first-round product, using the ES7/ES8 primer pair described by Delwart *et al.*[13] Subtype reference plasmids were amplified using second-round primers and 10 ng of plasmids A to H as templates.[13] The heteroduplex mobility assay was performed as previously described.[10,13] HLA-DQα amplification was performed on all samples, as previously described.[17]

Samples with a low yield or negativity in PCR were subjected to XL-PCR (Perkin-Elmer [Norwalk, CT] kit), using primers HPOL4235[18] and LSiGi (5′-TCAAGGCAAGCTTTATTGAGGCTTAAGCAG-3′). LSiGi is located in the long terminal repeat (LTR) (at the 9598–9627 position, corresponding to the HIV-1 HXB2 isolate). One microgram of DNA was subjected to XL-PCR amplification according to the manufacturer instructions. Nested PCR was performed with the ES7/ES8 primer pair as previously described.[13]

Assessment of HMA reliability

Intralaboratory evaluation: Sequencing and phylogenetic analysis. Sequence analysis was performed on all nongenotypable viruses ($n = 12$) and 37 strains identified as belonging to different subtypes in HMA.

PCR products were purified (gel extraction kit; Qiagen, Chatsworth, CA) and used as a template for sequencing with primers ES7 and ES8 in the Prism Ready Reaction Amplitaq FS dye terminator kit (Perkin-Elmer) on an automated DNA sequencer (373A sequencer; Applied Biosystems Foster City, CA). Multiple alignments of DNA and protein sequences were done using the Clustal W program[19] with manual editing. The sequences were gapstripped and a pairwise matrix was generated with the DNADIST program (PHYLIP package, version 3.5c).[20] Tree topology was inferred by neighbor joining with the Kimura two-parameter distance matrix (PHYLIP) and a transition/transversion ratio of 2. Bootstrap analysis was performed with the SEQBOOT (100 resamplings), DNADIST, NEIGHBOR, and CONSENSE programs (PHYLIP package). Maximum likelihood trees were created using the PHYLIP DNAML 3.56 program with a transition-to-transversion ratio of 2.

Recombination events were checked using the Recombinant Identification Program (RIP).[21]

Interlaboratory evaluation: HMA quality control. Two groups of samples formed the quality control panel: 12 HMA-indeterminate samples and 13 samples genotyped by HMA as subtypes A to H. This panel will be subjected to a national HMA quality control study. It was first tested in three laboratories to assess the reliability of the approach. The participants' first-round amplification strategy differed. In the Montpellier ORSTOM laboratory and the Pasteur Institute laboratory, the first amplification step was done using the primer pairs ED5/ED12 and ED3/ED14.[13] The corresponding proviral DNA of all these samples was sequenced in the V3–V5 region. A larger quality control study including all laboratories performing the HMA in France is in progress.

RESULTS

Heteroduplex mobility assay

Viral DNA Amplification. The genotyping strategy is presented in Fig. 1. The PCR was performed using the ED3/ED12 primer pair on all the available samples ($n = 199$) from patients originating from Africa, Asia, and the Caribbean, and on a subset of samples from European patients. In the 17 cases in which nested amplification with ES7/ES8 primers failed, ED5/ED14 primers were used for the first PCR.[10] Using both sets of primers we succeeded in amplifying the viral DNA in 190 of the 199 samples (96%). The remaining nine samples originated from Africans or from Caucasians sexually infected by African subjects. All nine PBMC samples were subjected to XL-PCR, which successfully amplified four of them. Nested PCR was then performed with inner primers ES7/ES8. All samples were positive for HLA-DQα amplification.

HMA genotyping results. The HMA unambiguously genotyped 182 of the 194 samples (93.4%), whereas in 12 cases the HMA results were uninterpretable. These samples were sequenced for further phylogenetic analysis. There was no relation between ED3/ED12 PCR failure and HMA-indeterminate results, as all samples amplified with ED5/ED14, and all but

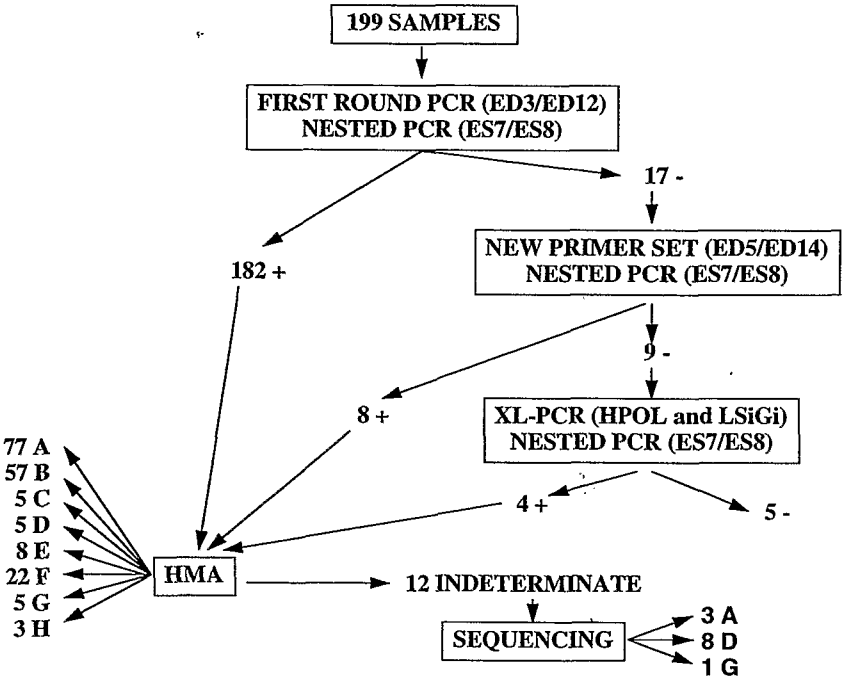


FIG. 1. PCR amplification strategy used for HMA genotyping in the virology laboratory of Bichat-Claude Bernard Hospital.

one (BCB64) of those amplified with XL-PCR, were correctly genotyped.

The HMA genotyped 77 (42%) of the samples as subtype A, 57 (31%) as subtype B, 5 (3%) as subtype C, 5 (3%) as subtype D, 8 (4%) as subtype E, 22 (12%) as subtype F, 5 (3%) as subtype G, and 3 (2%) as subtype H. Table 1 summarizes the HMA results according to the geographic origin of the patients. The patients with HMA-indeterminate results originated from West or Central Africa ($n = 10$), or were Europeans ($n = 2$) infected by African subjects.

HMA reliability

Sequence analysis. The sequences of 49 samples were determined to assess the reliability of HMA genotyping results. This study included the samples that gave an indeterminate HMA result ($n = 12$), and 37 samples genotyped by HMA (8 A, 2 B, 2 C, 1 D, 1 E, 19 F, 2 G, and 2 H).

Sequence analysis confirmed the HMA results for all the genotyped samples. Sequencing and phylogenetic analyses using representative strains of the different subtypes allowed us to determine the HIV-1 subtype of all the samples with indeterminate HMA profiles. The HMA-indeterminate samples clustered with low bootstrap values with subtypes A ($n = 3$), D ($n = 8$), and G ($n = 1$). To investigate the reasons for the indeterminate HMA results, we further analyzed these strains.

Phylogenetic analysis of HMA-indeterminate samples. Phylogenetic trees were constructed using the C2V3 *env* sequences of HMA-indeterminate viruses and a wide range of subtype A (Fig. 2a), subtype D (Fig. 2b), and subtype G (Fig. 2c) strains. Representative group M strains were also included in these trees, together with unclassified, highly divergent *env* sequences from the HIV database.[1] As shown in Fig. 2a, three HMA-indeterminate samples classified as subtype A outliers in the preliminary phylogenetic analysis were highly divergent strains

TABLE 1. HETERODUPLEX MOBILITY ASSAY RESULTS ACCORDING TO THE GEOGRAPHIC ORIGINS OF PATIENTS

	Subtype									
Origin	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*IND*[a]	*Total*
Europe	5	39			2	21	1		2	70
North Africa	3	5	1			1				10
Central Africa	28	1	3	2	3		2	3	6	48
West Africa	39	7	1	3			2		4	56
Asia					3					3
Caribbean	2	5								7
Total:	77	57	5	5	8	22	5	3	12	194

[a]IND, HMA-indeterminate samples.

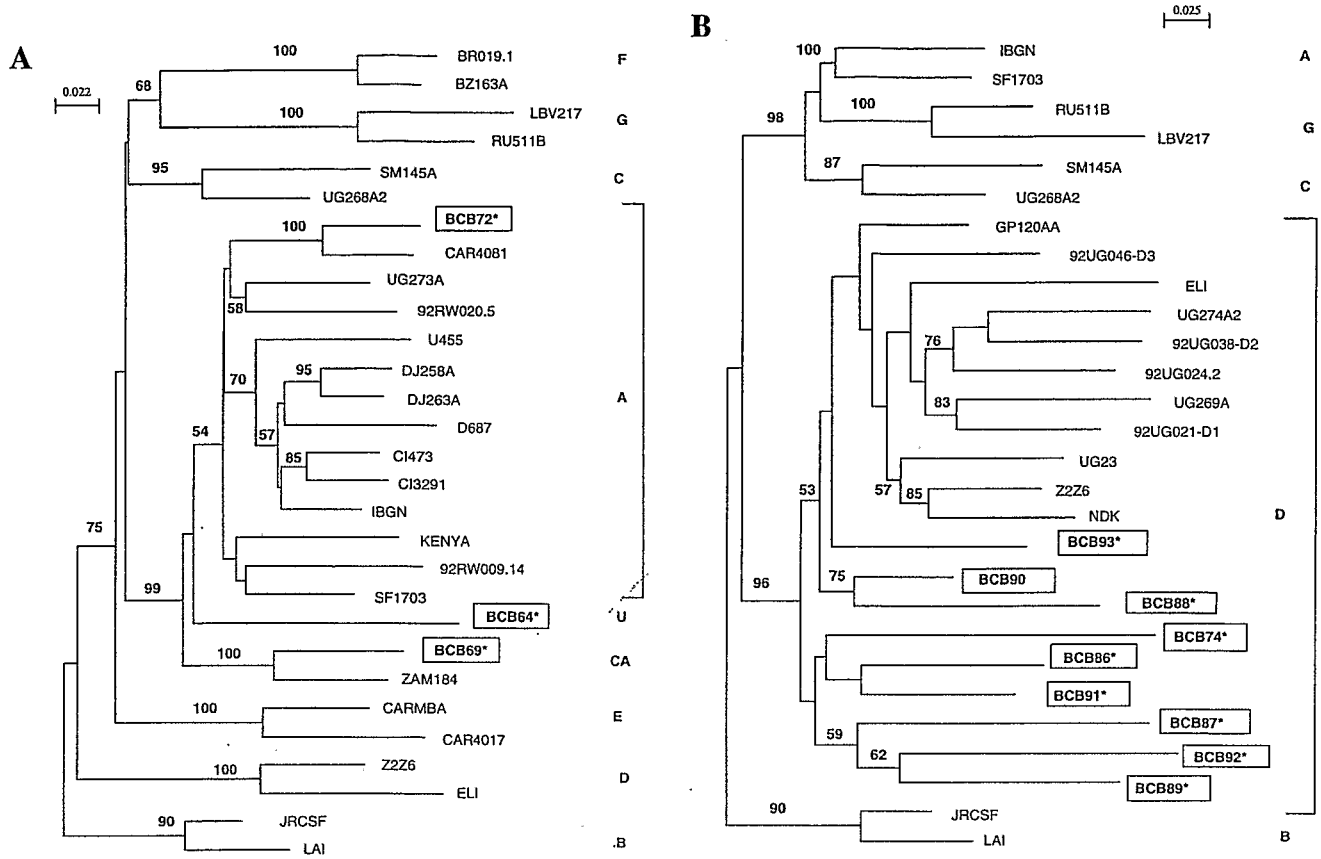


FIG. 2. Phylogenetic trees derived from the comparison of C2V3 *env* nucleic acid sequences of subtype A (a), subtype D (b), and subtype G (c). There were 444, 430, and 449 positions after gapstripping for the subtype A, subtype D, and subtype G trees, respectively. Corresponding sequences from the HIV database[1] for different HIV-1 group M subtypes, and for unclassified strains, were included in the tree construction. Sequences determined in this study are boxed. HMA-indeterminate samples are designated by asterisks (*). Tree topologies were inferred by neighbor joining with the Kimura two-parameter distance matrix (PHYLIP). The numbers at nodes indicate the frequency with which each node occurred in 100 bootstrap replicates, and are restricted to values greater than 50%. Brackets and letters on the right indicate the subtype classification. GenBank accession numbers for the newly reported sequences are Z95438–Z95473.

and clustered with unclassified sequences originating from recombinant strains.[1] The first strain (BCB72) was isolated from a patient from the Ivory Coast and clustered with strain CAR4081 (Fig. 2a), with a high bootstrap value (100%). The genetic distance between BCB72 and subtype A ranged from 14.4 to 28.0%, whereas the distance between BCB72 and CAR4081 was 9.3%. The latter was considered as U (uncertain) before being shown to be an A/G recombinant strain,[1] but it was classified as subtype A on the basis of the envelope region used to construct our tree. The clustering of BCB72 and CAR4081 as subtype A outliers in our preliminary analysis, together with the indeterminate HMA pattern of BCB72, raised the question of whether BCB72 was a recombinant virus. The second strain (BCB69) originated from a Malian patient. The genetic distance between this strain and subtype A ranged from 18.7 to 24.5%. The shortest distance was found with recombinant strain ZAM184 (12.1%), with which it clustered with a high bootstrap value (100%). ZAM184 is an A/C recombinant strain.[1] The *env* sequence of strain ZAM184 used in this analysis had a recombinant structure corresponding to subtypes A (239 bp) and C (246 bp). The third strain (BCB64), which was also isolated from a patient from the Ivory Coast, gave an uncertain classification result. The phylogenetic analysis revealed that this strain did not cluster with any of the subtype A strains from the HIV databases. Similar results were obtained for these three strains by using maximum likelihood analysis (data not shown).

By sequencing we identified 13 subtype D samples in our study group, only 5 of which were correctly genotyped by HMA (Table 1). The status of subtype D reference plasmids (D1, D2, and D3) used in our study was confirmed by sequencing.

Two HMA-indeterminate, highly divergent subtype D strains were isolated from patients originating from Cameroon (BCB88) and Cape Verde (BCB93). Interestingly, the phylogenetic analysis revealed that one of these HMA-indeterminate subtype D strains (BCB88) and an HMA-genotyped isolate

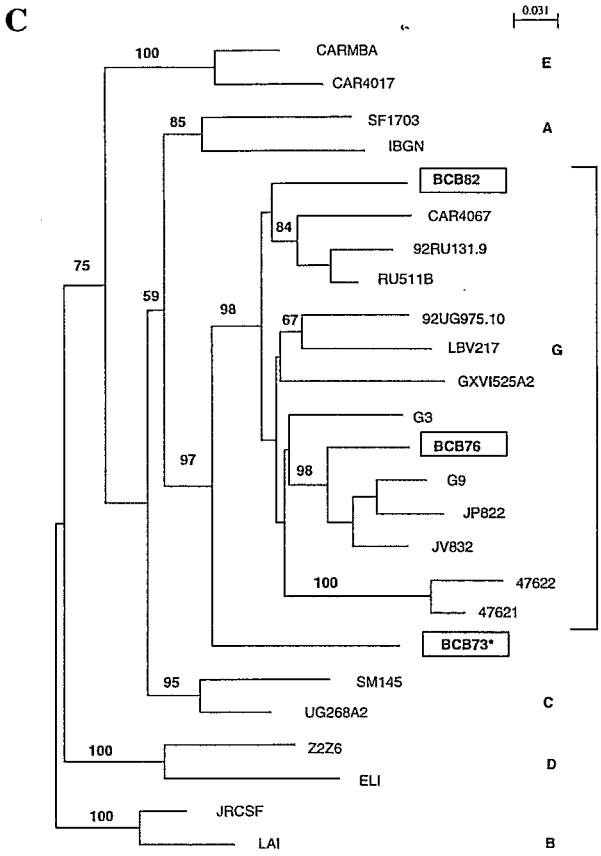


FIG. 2. (continued)

(BCB90) were highly related (bootstrap value of 75). Although the reasons for this segregation of HMA results are not clear, the discrepancy observed between HMA and the phylogenetic analysis for patient BCB88 could be explained by the presence of insertions in the ES7/ES8 regions of this isolate relative to the reference sequences. Figure 3 depicts the amino acid alignments for these three subtype D strains and the D1, D2, and D3 plasmids. As can be observed, HMA-indeterminate strain amino acid sequences are longer than subtype D plasmid sequences and the BCB90 sequence, the insertions in the nucleotide sequences forming gaps and thus generating indeterminate patterns.

The presence in our study group of six new indeterminate subtype D strains (BCB74, BCB86, BCB87, BCB89, BCB91, and BCB92) drastically influenced the efficiency of subtype D genotyping by means of the HMA. A possible explanation for the failure to genotype the subtype D strains is the heterogeneity of our study group. The neighbor-joining tree (Fig. 2b) illustrating the relationship between subtype D strains revealed two groups separated by a high bootstrap value (96) within this subtype. Six HMA-indeterminate samples originating from West Africa grouped together, separately from the subtype D HMA reference plasmid group (Fig. 2b). Although this phylogenetic group was not defined by high bootstrap values, it was also found by using the maximum likelihood method (data not shown). The failure of HMA genotyping in these cases might thus be explained by sequence data. The mean genetic distance was 27% (range, 15–37%) within the divergent D group, 29.5% (range, 20–38%) between this group and the three subtype D reference plasmids, and 20% (range, 17–25%) between the three reference plasmids and other subtype D strains. HMA-indeterminate samples originated from patients from West and Central Africa, whereas the three subtype D reference plasmids are from Uganda and so do not cover all subtype D diversity.

Sequence analysis of the latest HMA-nontypable strain (BCB73) classified this strain as a distant subtype G (Fig. 2c). The genetic distance between BCB73 and subtype G was 28% (range, 21–34%), whereas it was 20% within subtype G (range, 6–25%).

The analysis by RIP of the breaking points in the HMA-indeterminate sequences revealed continuous structures for all but one sequence (BCB69). This later sequence presented a configuration close to that of ZAM184, an A/C recombinant strain, the same recombination junction being shared by the two sequences. Sequencing of the complete genome of distant strains (BCB69, BCB72, BCB64, and BCB73) is underway to determine if they correspond to new subtypes or intrasubtype recombinants.

HMA quality control study

All HMA-indeterminate samples and a panel of HIV-1 isolates belonging to subtypes A to H were subjected to blind HMA testing in three separate laboratories. In the first amplification step the primers used by the virology laboratory of Bichat-Claude Bernard Hospital (ED3/ED12 or ED5/ED14) differed from those used by the other participants, i.e., ED5/ED12 or ED3/ED14, as recommended by Delwart *et al.*[13] However, the

```
UG038    IIVQLNESVTISCARPFYAIRQ--KTPIGQGQVLYTTK-KIGRIGQAHCNITEADWKKTLQQVAKKLRDLLNITAVIFKP
UG021    IIVHLNESVPINCTRPYDKSY---RTPIGVGRASYTTR-IKGDIRQAHCNISGEKWNKTLQQVAVKLRDLLNQTAIIFKP
UG046    IIVQLNESVPINCSRPYENRR---RTPIGLGQAYYTTK-LKGYIRPAHCNISGAEWNKTLQQVAKKLGDLFNQTTIIFQP
BCB88    IIVQLNEAINITCIRPSNNTRKRIRIGIGRGQALYTTDNIIGDIRKAHCNISEAKWNNTLQKIADKLKELYNNKTIIFRP
BCB90    IIVQLNETVEINCTRPNNNRK---SIHLGPGQAFYTS-NIIGDIRQAHCNISGEKWNNTLQRLAIKLRNLLNKTTIIFKP
BCB93    IIVQLSDPVEINCTRPNYKRQ---RTGIGQGQALYTTR-INGDIRKAHCNISRTAWNTTLRQVAEKLGNLANITTIIFKP

UG038    SSGGDPELTTHSFNCGGEFFFCNTSQLFNSTWSN--TSNS-TNN------TIK-PCRIRQIINLWQEVGKAMY      140
UG021    SSGGDPEITTHSFNCGGEFFYCNTSGLFNNVWTSNSTIGANGI-------T---PCRIKQIINMWQGVGKAMY      139
UG046    HSGGDPEITTHSLNCGGEFFYCDASRLFNRTTSS--T-G---VNNS----TIK-PCRIKQIINMWQGVGKAMY      138
BCB88    SSGGDLEITTHSFNCGGEFFYCNTSGLFNRTFIG--TPENSTSNG-----TIHIPCRIKQIINMWQGVGKAMY      146
BCB90    SSGGDPEITTHSVNCGGEFFYCNTSKLFNSTNDNS-T-GSNDSIL-----T---PCRIKQIINMWQGVGKAMY      139
BCB93    SSGGDPEITTHSFNCGGEFFYCNTTQLFNNTWKNG-TLRGPNITGPNKN-TI-IPCRIKQIINMWQGAGKAMY      146
```

FIG. 3. Deduced amino acid alignment for the subtype D plasmids (D1–D3) and three subtype D samples, two of which (BCB88 and BCB93) give HMA-indeterminate patterns.

second step of the amplification strategy was identical in the three laboratories (ES7/ES8 primer pair). All genotyped samples in this quality control exercise were correctly identified by HMA in all three laboratories. All but two HMA-indeterminate samples were not recognized by the three participants. The remaining HMA-indeterminate samples (subtype D, BCB88 and BCB93) yielded discordant results: one laboratory identified both strains as subtype D, the second identified only one (BCB88), as subtype D, and neither sample was identified by the third participant. To investigate the reasons for these discrepancies, serial dilutions of PBMC DNA prior to the nested PCR were tested in order to generate products derived from a less complex mixture or a single variant;[22] the two samples giving discordant results were then genotyped as subtype D.

DISCUSSION

This study confirms that HMA is an effective HIV-1 subtyping method, classifying 91% of 199 clinical HIV-1 isolates.

The strategy we used (Fig. 1) positively amplified all but five samples originating from African patients. In these five cases the failure of XL-PCR amplification could have been due to a low viral load, or to the existence of highly divergent HIV-1 strains in our study group.

In 12 cases the HMA failed to establish the HIV-1 genotype despite positive PCR amplification. The samples that gave indeterminate HMA results were either from African patients or from Caucasians sexually infected by African partners. They mainly corresponded to subtype D or to highly divergent strains with an uncertain classification, as demonstrated by sequence analysis. These data are in agreement with a report on HMA-nontypable samples from patients infected by divergent subtype A and D viruses.[23] Our phylogenetic analysis indicated that these viruses were highly divergent strains; this was the case of the three HMA-nontypable subtype A samples tested. The detection of highly divergent subtype A-related viruses in our study group might be explained by the large number of samples from patients originating from Africa, where subtype A prevails. Despite the wide intrasubtype A variability, HMA remained a reliable subtyping approach, as it succeeded in genotyping 77 subtype A viruses in our study group. Also, despite the puzzling structure of recombinant strains, the HMA oriented our subsequent investigations of three very divergent strains, two of which were related to recombinant strains.

As regards subtype D viruses, the high proportion of HMA-indeterminate results was related to problems with the reference plasmids, which were constructed with *env* sequences of viruses from Uganda, a site selected by the World Health Organization (WHO) for HIV characterization with a view to vaccine trials.[24] On the basis of the sequence analysis, HMA plasmids containing subtype D *env* sequences from Uganda are unlikely to be the most appropriate for genotyping subtype D viruses, which circulate in other parts of Africa,[25,26] as well as in Europe[12,27–29] and the United States.[30] It thus seems useful to add subtype D plasmids originating from West and Central Africa to the HMA kit.

The last HMA-indeterminate sample also diverged from other previously reported subtype G strains, which may explain its no identification by HMA (Fig. 2c). Subtype G reference plasmids were selected when a limited number of subtype G sequences were available.[31] HMA subtype G detection might thus be improved by the use of new reference plasmids.

Intralaboratory HMA quality control showed no discrepancy between the HMA and sequencing results. Interlaboratory analysis proved to be useful for the assessment of HMA reliability in a national survey. The three participants in the interlaboratory quality control correctly genotyped HIV-1 strains belonging to different subtypes. None of the participants was able to identify the highly divergent subtype A strains or the very divergent subtype D strains that formed a separate group within the subtype D cluster. However, different results were observed for two subtype D samples.

Previous reports have suggested that the HMA is an effective method for evaluating the genetic diversity of HIV-1 in different geographic regions.[10–14] However, the technical performance of the HMA may be influenced by the relative prevalence of the different subtypes in the target population. The HMA gives excellent results for viruses from populations in which a particular subtype prevails, such as HIV-1 subtype B in western European countries and the United States,[12,13] and HIV-1 subtype E in Vietnamese drug users.[14] Similar results were obtained in populations in which divergent HIV-1 subtypes have recently created a founder effect, such as subtype E in Thailand[11] and subtype G in Russia.[32] Our study confirms that the HMA gives excellent results for the detection of the prevalent subtype in Caucasian patients originating from western countries (subtype B) and Romania (subtype F). Conversely, extensive viral variations might create problems in Africa, where different HIV subtypes have been circulating longer.

Our study showed that as HIV-1 diversification appears to be a continuous and dynamic phenomenon, the HMA should be adapted periodically by renewing the plasmids, as proposed by Delwart *et al.*[10]

ACKNOWLEDGMENTS

This work was supported by the ANRS (Agence Nationale de Recherches sur le SIDA) (Grant 96009). C.A. is a postdoctoral fellow of the ANRS. We thank Frederic Bibolet (ORSTOM, Montpellier) for advice on phylogenetic analyses.

REFERENCES

1. Myers G, Hahn BH, Mellors JW, *et al.*: *Human Retroviruses and AIDS 1995: A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences.* Los Alamos National Laboratory, Los Alamos, New Mexico, 1995.
2. Kostrikis LG, Bagdades E, Cao Y, Zhang L, Dimitriou D, and Ho DD: Genetic analysis of HIV-1 strains from patients in Cyprus: Identification of a new subtype designated subtype I. J Virol 1995;69:6122–6130.
3. WHO Network for HIV Isolation and Characterization: HIV type 1 variation in World Health Organization-sponsored vaccine evaluation sites: Genetic screening, sequence analysis, and preliminary biological characterization of selected viral strains. AIDS Res Hum Retroviruses 1994;10:1327–1343.
4. Loussert-Ajaka I, Ly TD, Chaix ML, Ingrand D, Saragosti S, Couroucé AM, Brun-Vézinet F, and Simon F: HIV-1/HIV-2 ELISA

seronegativity in HIV-1 subtype O-infected patients. Lancet 1994;343:1393–1394.
5. Apetrei C, Loussert-Ajaka I, Descamps D, Damond F, Saragosti S, Brun-Vézinet F, and Simon F: Lack of screening test sensitivity during HIV-1 nonsubtype B seroconversions. AIDS 1996;10: F57–F60.
6. Kalish ML, Lou C-C, Weniger BG, Limpakarnjanarat K, Young N, Ou C-Y, and Schochetman G: Early HIV type 1 strains in Thailand were not responsible for the current epidemic. AIDS Res Hum Retroviruses 1994;10:1573–1575.
7. Barin F, Lahbabi Y, Buzelay L, Lejeune B, Baillou-Beaufils A, Denis F, Mathiot C, M'Boup S, Vithayashai V, Dietrich U, and Goudeau A: Diversity of antibody binding to V3 peptides representing consensus sequences of HIV-1 genotypes A to E: An approach for HIV-1 serological subtyping. AIDS Res Hum Retroviruses 1996;12:1279–1289.
8. Pau CP, Kai M, Holloman-Candal DL, Luo CC, Kalish ML, Schochetman G, Byers B, George JR, and the WHO Network for HIV isolation and Characterization: Antigenic variation and serotyping of HIV type 1 from the World Health Organization-sponsored HIV vaccine sites. AIDS Res Hum Retroviruses 1994;11:1369–1377.
9. Ou CY, Takebe Y, Weniger BG, Luo CC, Kalish ML, Auwanit W, Yamazaki S, Gayle HD, Young NL, and Schochetman G: Independent introduction of two major HIV-1 genotypes into distinct high-risk populations in Thailand. Lancet 1993;341:1171–1174.
10. Delwart EL, Shpaer EG, Louwagie J, McCutchan FE, Grez M, Rübsamen-Waigmann H, and Mullins JI: Genetic relationship determined by a DNA heteroduplex mobility assay: Analysis of HIV-1 *env* genes. Science 1993;262:1257–1261.
11. Wasi C, Herring B, Raktham S, Vanichseni S, Mastro TD, Young NL, Rübsamen-Waigmann H, von Briesen H, Kalish ML, Luo C-C, Pau C-P, Baldwin A, Mullins JI, Delwart EL, Esparza J, Heyward WL, and Osmanov S: Determination of HIV-1 subtypes in injecting drug users in Bangkok, Thailand, using peptide-binding enzyme immunoassay and heteroduplex mobility assay: Evidence of increasing infection with HIV-1 subtype E. AIDS 1995;9:843–849.
12. Simon F, Loussert-Ajaka I, Damond F, Saragosti S, Barin F, and Brun-Vézinet F: HIV type 1 diversity in northern Paris, France. AIDS Res Hum Retroviruses 1996;12:1427–1433.
13. Delwart EL, Busch MP, Kalish ML, Mosley JW, and Mullins JI: Rapid molecular epidemiology of human immunodeficiency virus transmission. AIDS Res Hum Retroviruses 1995;11:1081–1093.
14. Menu E, Lien TTX, Lafon ME, Lan NTH, Müller-Trutwin MC, Thuy NT, Deslandres A, Chaouat G, Trung DQ, Khiem HB, Fleury HJA, and Barré-Sinoussi F: HIV type 1 Thai subtype E is predominant in South Vietnam. AIDS Res Hum Retroviruses 1996;12:629–633.
15. Apetrei C, Loussert-Ajaka I, Collin G, Letourneur F, Duca M, Saragosti S, Simon F, and Brun-Vézinet F: HIV-1 subtype F sequences in Romanian children and adults. AIDS Res Hum Retroviruses 1997;13:363–365.
16. Loussert-Ajaka I, Chaix ML, Korber B, Letourneur F, Gomas E, Allen E, Ly TD, Brun-Vézinet F, Simon F, and Saragosti S: Variability of HIV type 1 group O strains isolated from Cameroonian patients living in France. J Virol 1995;69:5640–5649.
17. Erlich HA and Bugawan TL: HLA DNA typing. In: *PCR Protocols: A Guide to Methods and Applications* (Innis MA, Gelfand DH, Sninsky JJ, and White TJ, eds.). Academic Press, New York, 1990, pp. 261–271.
18. Fransen K, Zhong P, De Beenhouwer H, Carpels G, Peeters M, Louwagie J, Janssens W, Piot P, and van der Groen G: Design and evaluation of new, highly sensitive and specific primers for polymerase chain reaction of HIV-1 infected primary lymphocytes. Mol Cell Probes 1994;8:317–322.
19. Thompson JD, Higgins DG, and Gibson TJ: CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673–4680.
20. Felsenstein J: *PHYLIP (Phylogeny Interference Package)* version 3.5c. Department of Genetics, University of Washington, Seattle, Washington, 1993.
21. Siepel AC, Halpern AL, Macken C, and Korber BT: A computer program designated to screen rapidly for HIV type 1 intersubtype recombinant sequences. AIDS Res Hum Retroviruses 1995;11: 1413–1416.
22. Edmonson PL and Mullins JI: Efficient amplification of half-genome sized fragments of human immunodeficiency virus from infected tissue samples. Nucleic Acids Res 1992;20:4933.
23. Novitski V, Arnold C, and Clewely JP: Heteroduplex mobility assay for subtyping HIV-1: Improved methodology and comparison with phylogenetic analysis of sequence data. J Virol Methods 1996;59:61–72.
24. Bachmann MH, Delwart EL, Shpaer EG, Lingenfelter P, Singal R, Mullins JI, and the WHO Network for HIV isolation and characterization: Rapid genetic characterization of HIV type 1 strains f rom four World Health Organization-sponsored vaccine evaluation sites using a heteroduplex mobility assay. AIDS Res Hum Retroviruses 1994;11:1345–1353.
25. Potts KE, Kalish ML, Bandea CI, Orloff GM, St. Louis M, Brown C, Malanda N, Kavuka M, Schochetman G, Ou CY, and Heyward WL: Genetic diversity of human immunodeficiency virus type 1 strains in Kinshasa, Zaire. AIDS Res Hum Retroviruses 1993; 9:613–618.
26. Murphy E, Korber B, Georges-Courbot MC, You B, Pinter A, Cook D, Kieny MP, Georges A, Mathiot C, Barré-Sinoussi F, and Girard M: Diversity of V3 region sequences of human immunodeficiency virus type 1 from Central African Republic. AIDS Res Hum Retroviruses 1993;9:997–1006.
27. Arnold C, Barlow KL, Parry JV, and Clewely JP: At least five HIV-1 sequence subtypes (A, B, C, D, A/E) occur in England. AIDS Res Hum Retroviruses 1995;11:427–429.
28. Leitner T, Escanilla D, Marquina S, Wahlberg J, Broström C, Hansson HB, Uhlén M, and Albert J: Biological and molecular characterization of subtype D, G, and A/D recombinant HIV-1 transmissions in Sweden. Virology 1995;209:136–146.
29. Lukasov VV, Cornelissen MTE, Goudsmit J, Papuashvilli MN, Rytik PG, Khaitov RM, Karamov EV, and de Wolf F: Simultaneous introduction of distinct HIV-1 subtypes into different risk groups in Russia, Byelorussia and Lithuania. AIDS 1995;9:435–439.
30. Gao F, Yue L, Hill SC, Robertson DL, Graves AH, Saag MS, Shaw GM, Sharp PM and Hahn, BH: HIV-1 sequence subtype D in the United States. AIDS Res Hum Retroviruses 1994;10:625–627.
31. Delwart EL, Herring B, Rodrigo AG, and Mullins JI: Genetic subtyping of human immunodeficiency virus using a heteroduplex mobility assay. PCR Methods Appl 1995;4:S202–S216.
32. Bobkov A, Cheingsong-Popov R, Garaev M, Rzhaninova A, Kaleebu P, Beddows S, Bachmann MH, Mullins JI, Louwagie J, Janssens W, van der Groen G, McCutchan F, and Weber J: Identification of an *env* G subtype and heterogeneity of HIV-1 strains in the Russian Federation and Belarus. AIDS 1994;8:1649–1655.

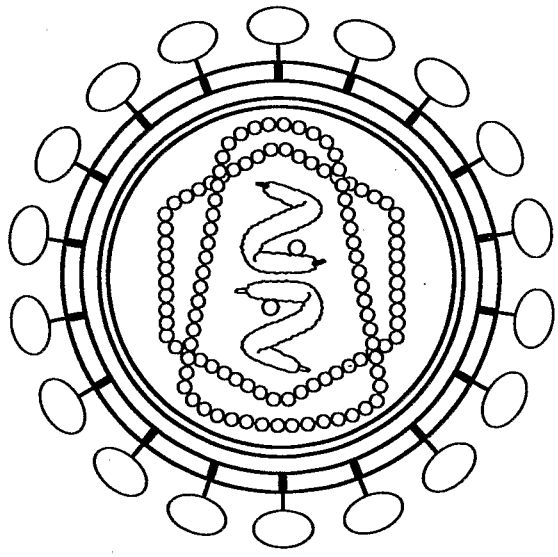
Address reprint requests to:
François Simon
Laboratoire de Virologie
Hôpital Bichat-Claude Bernard
46 rue H. Huchard
75018 Paris, France

Volume 14 Number 10 July 1, 1998

ISSN: 0889-2229

AIDS RESEARCH AND HUMAN RETROVIRUSES

Editor: DANI BOLOGNESI, Ph.D.



[PM 859]

[illegible] JUL 1998