THE GLOBAL HIV-1 EPIDEMIC comprises the circulation of two HIV-1 groups, group M (major) and group O (outlier). HIV-1 group M viruses are widespread; group O viruses have a rather limited spread thus far. Eight HIV-1 group M subtypes have been described. In Europe, subtype B is prevalent but multiple subtypes are reported to cocirculate in Belgium, France, Sweden, Finland, The Netherlands, and Russia. We previously documented the long-standing presence in Belgians of multiple HIV-1 subtypes other than subtype B. Among the HIV-1-infected individuals in Belgium homosexual behavior was highly correlated (96%) with subtype B infection, as was observed in other western countries. Among heterosexuals (31%), a large variety of subtypes was observed, with subtype A (39%) the most prevalent. Our data suggested that HIV was introduced into the Belgian heterosexual population through sexual contact with people from sub-Saharan Africa. Here we report on the C2V3 env genetic and phylogenetic analysis of HIV-1 circulating among 96 individuals of Belgian nationality, seen at the Institute of Tropical Medicine (Antwerp, Belgium), and diagnosed as HIV-1 infected between 1985 and 1994. Sex, age, and CD4 cell count at the time of blood sampling were recorded, as well as the route and origin of infection. We compared the genetic subtype classification obtained by C2V3 env phylogenetic analysis with the previously obtained heteroduplex mobility assay (HMA) classification.

Virus isolation, preparation of DNA template for polymerase chain reaction (PCR) using the Isoquick extraction kit (EurosClone, Seraing, Belgium), PCR amplification, and direct sequencing were performed. In the case of untraceable sequences, the PCR fragment was cloned and sequenced.

A phylogenetic tree was constructed on the basis of 279 positions of a C2V3 env alignment of the Belgian strains documented in this study, and 2 strains representative of each HIV-1 group M subtype, A through H (Fig. 1). Six isolates (VII1234, VII850, VII1197, VI961, VI11358, and VI991), of which VII1234 and VII1358 could not be subtyped by HMA, were previously documented for the HIV-1 env region encompassing V3, V4, and V5, and the start of gp41. VI11358 was classified as an HIV-1 subtype C virus on the basis of phylogenetic analysis of this region, but was unclassified by C2V3 env phylogeny. The patient contributing VII11358 was probably infected through heterosexual contact in Kinshasa (Democratic Republic of Congo). Three other isolates, VII1144, VII1126, and VII1325, could not be classified by HMA or C2V3 env phylogenetic analysis as belonging to any existing subtype. VII1325 was previously documented as unclassified by gag gene phylogeny. VII1126, VII1325, and VII1035 (a sample from the individual infected with VII1144, taken 7 months earlier), were kept for full-length genome analysis to identify their recombinant nature or their classification as new HIV-1 subtypes. The individual infected with VII1126 had heterosexual contacts with prostitutes in the Democratic Republic of Congo. The man infected with VII1325 is a sailor with sexual contacts in Ivory Coast, Democratic Republic of Congo, and Tenerife. The man infected with VII1144 travels frequently to Africa (Democratic Republic of Congo) and Asia. For three individuals multiple HIV-1 subtype introduction was documented. Isolate VII1309 was classified by HMA as subtype D, but has in the phylogenetic tree a position borderline to subtype E or D (Fig. 1). Sequence analysis revealed a background sequence suggestive of a second amplification product. Reanalysis of the obtained HMA (ED5–ED12) PCR product, covering the gp120-encoding fragment, by 5% polyacrylamide gel electrophoresis revealed the presence of two PCR products differing in length. Cloning, sequencing, and phylogenetic analysis of these fragments revealed the presence of variants of subtypes C and D (Fig. 2a). The individual infected with VII1309 had heterosexual contacts in Kigali (Rwanda). Isolate VI563 was previously analyzed by HMA (ES7–ES8) as a subtype A isolate. Direct sequencing and phylogenetic analysis of this PCR fragment, using different annealing temperatures, resulted in either a subtype A or subtype...
FIG. 2. Phylogenetic trees indicating subtype classification of multiple infected individuals; (a) V11309 and V1563; (b) V1822. See the caption to Fig. 1 for details. The number of bootstrap trees out of 100 replications supporting a particular phylogenetic group in more than 70% is placed alongside the node considered.

B variant. Direct sequencing, using magnetic beads to separate both DNA strands prior to sequencing, resulted in a subtype A sequence for the biotinylated positive strand, using primer H1E203 (5' GTAAATTTCTAAATCCCTCCCTCGAG 3') as sequencing primer at an annealing temperature of 50°C, whereas the sequence obtained from the negative strand in the supernatant, using primer H1E110 (5' AATGGCAGTCTAGCATGATCA- GAA 3'), was not readable. Cycle sequencing of the same PCR fragment, using primer H1E203 at 60°C, resulted in a subtype B sequence, whereas the sequence obtained with primer H1E110 remained unreadable. Subsequent cycle sequencing using primer H1E204 (5' GATGGGAGGGCATATATTGCGT 3'), 199 bp downstream of primer H1E203, again resulted in a subtype A sequence (Fig. 2a). Furthermore, the unreadable sequences obtained by primer H1E110 could be resolved as a superposition of both subtype A and B sequences. The differential outcome of subtype classification was due to mismatches located at the H1E203 primer-binding site of the subtype A variants, whereby cycle sequencing at 60°C allowed amplification only of the subtype B variants. For isolate V1822, HMA using ES7–ES8 primers spanning the C2V5 region gave inconclusive results, shifting equally well with representatives of both subtypes A and B. HMA using ED31–ED33 primers spanning the C2C3 region indicated subtype E classification. Cloning of the ES7–ES8 and ED31–ED33 HMA fragments and sequencing of the C2V3 env part suggested triple infection with subtypes A, B, and E (Fig. 2b). The person infected with V1822 was a sailor (a cook), homosexually infected in 1989.

The predicted amino acid sequence of the Env C2V3 region for the documented strains is presented in Fig. 3. A high di-

FIG. 1. Phylogenetic tree based on 279 aligned positions of HIV-1 C2V3 env sequences of Belgian isolates (italic) and sequences representative of HIV-1 group M subtypes A through H and simian immunodeficiency virus SIVcpz-gab. Regions that could not be aligned unambiguously, owing to length or sequence variability, were omitted from the analysis. Starting from the alignment, a distance matrix corrected for multiple mutations per site (Jukes and Cantor) was constructed. Tree topologies were inferred by neighbor joining. Confidence values for individual branches of the resulting tree were determined by a bootstrap analysis in which 1000 bootstrap trees were generated from resampled data. Distance calculation, tree construction, and bootstrap analysis were realized with the software package TREECON. The root of the tree is placed so as to equalize its distance to the outgroup sequence SIVcpz-gab and its average to the HIV-1 sequences. The distance between two sequences is obtained by summing up the lengths of the connecting horizontal branches, using the scale on top. The number of bootstrap trees out of 1000 replications supporting a particular phylogenetic group in more than 50% is placed alongside the node considered. V1832 and V1860 are follow-up samples of V1712 and V1090, respectively. Countries from which the strains are collected are indicated by a code and precede the strain names: BE, Belgium; GH, Ghana; ZR, Zaire (now Democratic Republic of Congo); CM, Cameroon; GA, Gabon; TH, Thailand; FR, France; IN, India; ZA, South Africa; BR, Brazil; CY, Cyprus; US, United States. The nucleotide sequence accession numbers were deposited in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under the following accession numbers: AJ238140–AJ238235.
Fig. 3. Amino acid sequence alignment of Env C2V3 regions of the Belgian strains and subtypes A through H consensus sequences as compared with the overall HIV-1 group M consensus sequence.12 Amino acid identity is represented by dashes; points are introduced for alignment of the sequences; carets show where the N-linked glycosylation sites are found in the consensus. The transmission route for each patient is indicated in parentheses: 1, homosexual contact; 2, heterosexual contact with a person from an endemic region; 3, intravenous drug use; 4, transfusion; 5, hemophilia; 6, partner of hemophiliac patient; 7, child of seropositive mother; 8, other; 9, unknown.

University of octameric sequences at the tip of the V3 loop was observed, suggestive of multiple introductions of HIV-1 in the country. Octameric sequences are documented in this study that were previously undocumented for subtype A: HMGPQVQV (V1560), TMGPQAVI (V1114); subtype B: TMGPQAVI (V1412), TMGPQAVI (V1518), TMGPQAVI (V1546), HMGAVFTV (V1612), TIGPGKAF (V1713), IGPGQAV (V1819), HAVGPGQI (V835), HMGPGQAI (V843), SLGPQAVL (V844), HMGPGQAV (V1714), and a sequence containing a GPGAV duplication, TMGPQAVFVPGF (V485); subtype D: HMGQGIAL (V865), HMGQGIAL (V890), HMGQGIAL (V897); subtype F: HLAGGRAF (V507), HLGPGQGF (V850), RIGPGQFR (V1167); subtype H: HFGPGQFAF (V1997).13

In conclusion, subtype A, B, C, D, E, F, G, and H variants, displaying a great variety of octameric sequences at the tip of the V3 loop, are cocirculating in Belgium. C2V3 env sequencing and phylogenetic analysis largely confirm the HMA subtyping. Five isolates of 96 were reclassified by HMA, of which 4 remained unclassified by C2V3 env phylogenetic analysis. For three additional isolates the genetic subtype classification depended on the applied methodology and suggested dual or triple infection with different subtypes. Altogether, the circulation of all subtypes in Europe, together with the frequency at
which they occur, indicate that nonsubtype B HIV-1 variability needs to be taken into account for future diagnostic assay development, antiviral drug therapy, and vaccine design for Europe as well as for the rest of the world.

ACKNOWLEDGMENTS

This work was supported by grants from the Flanders Institute for Biototechnology-VIB, and the Fonds Wetenschappelijk Onderzoek (Grant G.013497).

REFERENCES


FIG. 3. Continued

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