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V3 Serotyping of HIV-1 Infection: Correlation With Genotyping and Limitations

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Summary: HIV-1 V3 serotyping is a classification of immunodeficiency viruses based on antibody binding to V3 peptides that allows obtaining information on circulating subtypes that could be important for population-based epidemiologic studies. Recently, several laboratories have developed V3 enzyme-immunoassays (EIAs) using V3 peptides of subtypes A to E. In the present study, the utility of including additional peptides of subtypes F to H to the EIA was evaluated on a panel of 203 well-characterized serum samples from patients with diverse geographic origins (22 countries) and known HIV-1 genotype (79 A, 61 B, 21 C, 7 D, 7 E, 21 F, 6 G, 1 H). The results indicate a high predictive value (ppv) for serotypes B (≥0.86), D (1) and E (0.88), and confirm the difficulty of predicting genotype A or C based on serotype A or C. Results also indicate that inclusion of the F peptide in the V3 EIAs may be useful (ppv = 0.61), but introduction of peptides G and H failed to demonstrate significant sensitivity or specificity for these subtypes. Correlation between serotyping and amino-acid sequences of the V3 region from 103 samples allowed the identification of key amino-acids that appear essential for subtype-specific seroreactivity. **Key Words:** V3 serotyping—HIV-1—Genotyping.

HIV-1 variability is a serious obstacle for the development of a broadly effective vaccine, which protects against HIV-1 strains from different geographic locations. Two groups of strains have been characterized in this lineage: the main (M) group and the outlier (O) group. The O group variants are located in a limited area of Africa but recently, a few HIV-1 O isolates have been reported in France, Spain, and the United States (1–3). By phylogenetic analysis of the *env* gene, at least eight subtypes (A to H) of the M group have been identified (4–6). Results of multiple descriptive molecular epidemiology studies in Africa indicate that all HIV-1 subtypes are present in this continent. Subtype A and to a lesser extent subtype D are prevalent in the broad east–west belt across sub-Saharan Africa; the subtype C viruses appear to be highly prevalent along the length of Africa's east coast (7). Other genetic subtypes (B, E, F, G, and H) are diversely distributed over the sub-Saharan African region (8–11). In the Americas, most HIV-1 strains belong to subtype B; reports have described the introduction of subtypes A, D, and E in North America (12–14). Subtypes F, C, and E are found in South America (15–17). In Europe, most HIV-1 isolates belong to subtype B. However, several Western European countries have significant numbers of infections with HIV-1 subtypes other than B, and an increasing prevalence of non-B subtypes has been recently reported (18–21). In

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Eastern Europe, the presence of less predominant subtypes of HIV-1 (e.g., subtypes F, G, H) has been described (16,22,23) but the major and growing epidemics are caused by subtypes B and A (24–25). In Southeast Asia, subtypes B and E are prevalent but increasingly infections caused by subtype C have been reported in China and India (26–28). The complexity of HIV-1 diversity has been compounded by the recognition of intersubtype recombinants (29).

For most molecular epidemiologic studies, HIV-1 subtyping has been conducted using the sequencing of genomic fragments amplified by polymerase chain reaction (PCR) or by the heteroduplex mobility assay (HMA) (30,31). Although these reference methodologies allow direct subtype classification, they are time consuming, expensive, technically difficult, and do not allow rapid analysis of numerous samples. Both methods may be considered insensitive to identify intersubtype recombinants. Moreover, the immunologic relevance of genotypes has not been established (32). Development of rapid and simple laboratory methods may greatly facilitate such molecular epidemiology studies. Serotyping of HIV-1 by antibody (Ab) binding to the V3 region has been proposed as such an alternative approach (33–37). It may have an advantage in identifying subtypes based on antigenic properties and therefore might be more relevant for evaluating antigenic diversity critical to vaccine development. We earlier reported the development of a simple and rapid subtype-specific enzyme immunoassay (SSEIA), which used V3 consensus sequences of subtypes A to E (20,33).

The aim of the present study was to determine whether an SSEIA using V3 consensus sequences from subtypes A to H would yield higher concordance between serotype and genotype on a panel of diverse samples. Our data confirm that V3 serotyping is useful in the detection of a limited number of serotypes (38) and we have been able to identify subtype-specific signature sequences for serologic typing.

MATERIALS AND METHODS

Peptides

Eight V3 peptides were synthesized. These peptides corresponded to the consensus sequences of the five major subtypes of HIV-1 group M (A–E) described by Myers et al. in 1992 (4), and the recently described subtypes F to H (5). These were 30–amino acids long except V3-D, which contained 29 residues. The sequences were as follows:

V3-A NNTRKSVHIGPGQAFYATGDIIGDIRQAHC
V3-B NNTRKSIHIGPGRAFYTTGEIIGDIRQAHC
V3-C NNTRKSIRIGPGQTFYATGDIIGDIRQAHC
V3-D NNTRQRTHIGPGQALYTTGRIIGDIRQAHC
V3-E NNTRTSITIGPGQVFYRTGDIIGDIRQAHC
V3-F NNTRKSIHLGPGQAFYATGDIIGDIRKAHC
V3-G NNTRKSITIGPGQAFYATGDIIGDIRQAHC
V3-H NNTRKSIRIGPGQAFHAIGAIIGDIRQAHC

The peptides were made according to the solid-phase procedure developed by Merrifield on an automated peptide synthesizer (Applied Biosystems 431A, Perkin-Elmer, Applied Biosystem Division, Foster City, CA, U.S.A.), with 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids and hydroxymethylphenoacetic-polystyrene resin (39). After synthesis, the resin support and the side-chain-protecting groups were removed with trifluoracetic acid, using distilled water, phenol, ethanedithiol, and thioanisole as scavengers. After cleavage, the peptides were purified by reverse phase chromatography on C8 columns (Aquapore octyl, 20 μm, 100 × 10 mm, Applied Biosystems). Purity of preparations was confirmed by both the presence of a single sharp peak on high-power liquid chromatography (HPLC) analysis using C8 columns (Aquapore octyl, RP-300, 7 μm, 220 × 4.6 mm, Applied Biosystems) and amino acid analysis. Peptide compositions met expectations.

Immunoassays

We previously described the development of an SSEIA that uses the principle of blocking by an excess of peptide in the liquid phase (33). Two SSEIAs were performed for the present study, the first using five V3 sequences ($SSEIA_{A-E}$) and the second using eight V3 sequences ($SSEIA_{A-H}$).

In the $SSEIA_{A-E}$ assay, wells of polyvinyl microtiter plates (Falcon, Becton Dickinson, Meylan, France) were coated with an equimolar mixture of the five V3 peptides (0.5 μg/ml each in 0.05 *M* bicarbonate buffer, pH 9.6; 100 μl/well) by incubation for 20 hours at 37°C. The wells were washed 3 times with phosphate-buffered saline (PBS) containing 0.5% Tween 20 (PBS-TW) and the unoccupied sites of the wells were saturated with PBS containing 2% new-born calf serum (NBCS) by incubation for 45 minutes at 37°C. Serum samples were diluted 1:100 in 0.01 *M* sodium phosphate buffer, pH 7.4, containing 0.75 M NaCl, 10% NBCS, and 0.05% Tween 20 (PBS-TW-NBCS). Each sample was tested in seven wells in the presence of various blocking solutions. Ten μl of a 100 μg/ml solution (in PBS-TW-NBCS) containing V3 peptide of either A, B, C, D, or E subtype was first added to wells 1 to 5, respectively. Ten microliters of a 100 μg/ml solution of an equimolar mixture of the five peptides (theoretically, 100% blocking) was added to well 6. Ten microliters of PBS-TW-NBCS (theoretically, 0% blocking) was added to well 7. Next, 100 μl of diluted sera was added to each well, incubated for 30 minutes at room temperature and the wells were washed five times with PBS-TW. Peroxidase-conjugated goat F(ab')2 anti-human Ig (TAGO, Burlingame, CA, U.S.A.; 100 μl of a 1:5,000 dilution in PBS-TW-NBCS) was added and incubated for 30 minutes at room temperature. The wells were washed three times with PBS-TW and the reaction was revealed by incubation with hydrogen peroxide-*o*-phenylenediamine (H_2O_2-OPD) for 15 minutes at room temperature. The color development was stopped with 2N H_2SO_4 and the optic density value (OD) was read at 492 nm.

The percentage inhibition of binding induced by each of the five peptides for every serum sample was calculated using the following formula:

$$\frac{\text{OD without blocking (well 7)} - \text{OD in presence of peptide } x}{\text{OD without blocking (well 7)} - \text{OD in presence of the 5 peptides (well 6)}} \times 100$$

The $SSEIA_{A-E}$ thus indicates the immunodominant subtype reactivity as the peptide with the strongest blocking capacity, and also a serologic profile defined by the five values of inhibition (% inhibition by peptide A/% inhibition by peptide B/% inhibition by peptide C/% inhibition by peptide D/% inhibition by peptide E).

The $SSEIA_{A-H}$ assay was used in the same experimental conditions as above, except that wells were coated with an equimolar mixture of the 8 V3 peptides (0.4 μg/ml each in 0.05 *M* bicarbonate buffer, pH 9.6; 100 μl/well). Each serum sample diluted 1:100 was tested in 10 wells in the presence of various blocking solutions. Ten microliters of 100 μg/ml solutions (in PBS-TW-NBCS) of peptides A to H were first added to wells 1 to 8, respectively. Ten microliters of a 100 μg/ml solution of an equimolar mixture of the eight peptides (theoretically, 100% blocking) was added to well 9. Ten microliters of PBS-TW-NBCS (theoretically, 0% blocking) was added to well 10. The following steps were similar to those used for $SSEIA_{A-E}$. The percentage inhibition of binding induced by each of the eight peptides for every serum sample was calculated using the following formula:

$$\frac{\text{OD without blocking (well 10)} - \text{OD in presence of peptide } x}{\text{OD without blocking (well 10)} - \text{OD in presence of the 8 peptides (well 9)}} \times 100$$

The $SSEIA_{A-H}$ indicated the immunodominant subtype reactivity as the peptide with the strongest blocking capacity and also a serologic profile defined by the eight values of inhibition.

Subtypes determined by SSEIA were further designated as serotypes. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated for every SSEIA taking results of genotyping as the standard criterion.

Serum Samples

The study included 203 samples from HIV-1–infected patients (Table 1). Each serum sample came from patients whose infecting strain was genotyped. This included 79 genotype A samples, 61 genotype B samples, 21 genotype C samples, 7 genotype D samples, 7 genotype E samples, 21 genotype F samples, 6 genotype G samples, and 1 genotype H sample, which had been collected in three different laboratories

Bichat Hospital, Paris

Eighty samples were gathered from both inpatients and outpatients. A wide diversity of HIV-1 strains isolated in this hospital in northern Paris has been previously described (20). Geographic origin was known for 36 patients: Algeria (1 patient), Burkina-Faso (2 patients), Cambodia (1 patient), Cameroon (2 patients), the Cape Verde islands (1 patient), Congo (3 patients), Côte d'Ivoire (9 patients), Democratic Republic of Congo (formerly Zaire, 7 patients), France (5 patients), Gambia (3 patients), Ghana (1 patient), and Mali (1 patient). An additional set of 17 samples from Romania was included (40). Genotyping was performed on every sample by HMA as described (20). V3 sequences were obtained from 19 of 80 samples.

ORSTOM, Montpellier

Seventy-two samples from French-born individuals infected overseas or from African patients living in France (41). They were infected in Cambodia (5 patients), Cameroon (1 patient), Chad (2 patients), Côte d'Ivoire (2 patients), Democratic Republic of Congo (2 patients),

TABLE 1. *Geographic origin and genotype of the 203 samples analyzed*

Geographic origin	Genotype							
	A	B	C	D	E	F	G	H
Algeria	—	—	—	—	—	1	—	—
Burkina-Faso	2	—	—	—	—	—	—	—
Cambodia	—	—	—	—	6	—	—	—
Cameroon	1	1	—	1	—	—	—	—
Cape Verde	—	—	—	1	—	—	—	—
Chad	—	2	—	—	—	—	—	—
CAR	1	—	—	1	—	1	—	—
Congo	2	—	—	1	—	—	—	—
Côte d'Ivoire	9	1	—	1	—	—	—	—
Djibouti	5	8	18	—	—	—	—	—
France	1	18	—	—	—	1	—	—
French Guyana	2	2	—	—	—	—	—	—
Gabon	—	1	—	—	—	—	—	—
The Gambia	3	—	—	—	—	—	—	—
Ghana	1	—	—	—	—	—	—	—
Mali	1	—	—	—	—	—	6	—
Mayotte Island	—	1	—	—	—	—	—	—
Romania	—	—	—	—	—	17	—	—
Senegal	25	—	3	—	—	—	—	—
Thailand	—	—	—	—	1	—	—	—
U.S.A.	—	1	—	—	—	—	—	—
Zaire	7	—	—	1	—	—	—	1
Unknown	19	26	—	1	—	1	—	—
Total (%)	79 (39)	61 (30)	21 (10)	7 (3,5)	7 (3,5)	21 (10,5)	6 (3)	1 (0,5)

CAR, Central African Republic; Zaire, currently Democratic Republic of Congo.

Djibouti (33 patients), France (16 patients), Gabon (1 patient), French Guyana (4 patients), the island of Mayotte off the African coast (1 patient), Central African Republic (3 patients), Thailand (1 patient), and the United States (1 patient). An additional set of six samples was from African patients from Mali. Genetic subtypes were determined using HMA; strains were further characterized by sequencing and phylogenetic analysis of the C2-V3 region. V3 sequences were available from 56 of 72 patients (41).

Harvard School of Public Health, Boston

Twenty-eight samples from Senegalese female commercial sex workers (CSW) (42). Genetic subtyping was performed by DNA sequencing of amplified products in the C2-V3 envelope region followed by phylogenetic analysis. DNA was extracted from the peripheral blood mononuclear cells (PBMCs). To amplify approximately 350 base pairs (bp) in the C2-V3 envelope region, a nested PCR was performed using two sets of primers: WT1 5'GCTGGTTTTGCGATTCTAAAGTGTA (6884–6908, positions relative to HXB2) and WT2 5'CAATAGAAAAATTCCCCTCCACAAT (7353–7377), for the first round; and published primers, KK30–KK40 (43) for the second round. The PCR product was purified and directly sequenced using the second round primers (KK30 and KK40). When necessary, the purified product was cloned in pCR2.1 vector (T/A cloning, Invitrogen, San Diego, CA, U.S.A.). Sequences were determined by dye terminator cycle sequencing using Taq polymerase (Perkin-Elmer, Applied Biosystems Division) and an automatic sequencer ABI 373A (Perkin-Elmer, Applied Biosystem Division). In all cases, sequences were determined for both strands of DNA and in many instances clones were sequenced. Multiple alignments were performed with the Clustal package (Clustal W, version 1.6) with minor manual adjustment when necessary (41,44). Phylogenetic analysis was performed by the neighbor-joining method and reliability was estimated from 1000 bootstrap resamplings. Representative sequences of various HIV subtypes A to I were included in the analysis (6). The nucleotide sequences have been submitted to GenBank (accession numbers pending). The Senegalese samples were tested only in the $SSEIA_{A-E}$ due to small volumes available. Subtypes determined by HMA and/or nucleic acid sequencing in this paper are further designated by genotype.

RESULTS

Correlation Between Genotyping and Serotyping

In the study, 175 serum samples were tested in both $SSEIA_{A-E}$ and $SSEIA_{A-H}$. Serum samples from 28 Senegalese CSW were tested only with $SSEIA_{A-E}$. In both assays, the same six samples (3.4%) were nonreactive (Table 2); they were considered nontypable, due to lack of reactivity to V3 peptides. The $SSEIA_{A-E}$ and $SSEIA_{A-H}$ identified correctly 89 (50.9%) and 93 (53.1%) samples, respectively. Most misidentifications observed with the $SSEIA_{A-E}$ were due to genotype A samples, which were identified as serotype C (37 cases), and genotype F samples, which were identified as serotype A (18 cases). The low number of correctly identified samples in the $SSEIA_{A-E}$ resulted from 28 of 175 samples being from patients infected by genotypes F to H whose corresponding antigens were not included in the assay. Surprisingly, the introduction of peptides V3-F to V3-H did not significantly improve the number of correctly identified samples. Clearly, peptides V3-G and V3-H were not well adapted to the assay and objective. Only 2 of 6 genotype G samples were correctly identified (Table 2) and the single genotype H sample reacted to V3-B. Moreover, these two peptides induced the misidentification of 19 samples from patients infected by isolates genotyped A (8 cases), B (8 cases), or C (3 cases). Introduction of peptide V3-F was more relevant. Although this antigen also induced misidentification, mainly in genotype A samples (7 cases), it allowed the identification of 20 of 21 genotype F samples (95.2%; Table 2).

Based on these data, we calculated the sensitivity, specificity, positive predictive value (ppv), and negative predictive value (npv) of both serotyping assays in this

TABLE 2. *Correlation between genotyping and serotyping ($SSEIA_{A-E}$-A-and $SSEIA_{A-H}$-B-) using samples from Bichat Hospital, Paris, and ORSTOM, Montpellier, France*

A)

Genotype	$SSEIA_{A-E}$ A	B	C	D	E	NT	Total (n = 175)
A	**10**	6	37	—	1	—	54
B	2	**50**	5	—	—	4	61
C	—	—	**18**	—	—	—	18
D	2	—	—	**4**	—	1	7
E	—	—	—	—	**7**	—	7
F	18	1	1	—	—	1	21
G	4	—	2	—	—	—	6
H	—	1	—	—	—	—	1

B)

Genotype	$SSEIA_{A-H}$ A	B	C	D	E	F	G	H	NT	Total (n = 175)
A	**3**	4	31	—	1	7	4	4	—	54
B	1	**42**	5	—	—	1	7	1	4	61
C	—	—	**15**	—	—	—	1	2	—	18
D	—	—	—	**4**	—	2	—	—	1	7
E	—	—	—	—	7	—	—	—	—	7
F	—	—	—	—	—	**20**	—	—	1	21
G	—	—	1	—	—	3	**2**	—	—	6
H	—	1	—	—	—	—	—	—	—	1

NT, Non typable
Values over 0.7 are in bold
SSEIA, subtype-specific enzyme immunoassay.

TABLE 3. *Comparison of sensitivity, specificity, predictive values between $SSEIA_{A-E}$ and $SSEIA_{A-H}$*

	$SSEIA_{A-E}$				$SSEIA_{A-H}$			
Subtype	Sensitivity	Specificity	ppv	npv	Sensitivity	Specificity	ppv	npv
A	0.19	**0.79**	0.28	0.68	0.06	**0.99**	**0.75**	**0.70**
B	**0.82**	**0.93**	**0.86**	**0.91**	0.69	**0.96**	**0.89**	**0.85**
C	**1**	**0.71**	0.29	**1**	**0.83**	**0.76**	0.29	**0.98**
D	0.57	**1**	**1**	**0.98**	0.57	**1**	**1**	**0.98**
E	**1**	**0.99**	**0.88**	**1**	**1**	**0.99**	**0.88**	**1**
F	—	—	—	—	**0.95**	**0.92**	0.61	**0.99**
G	—	—	—	—	0.33	**0.93**	0.14	**0.98**
H	—	—	—	—	0	**0.96**	0	**0.99**

Values >0.7 are in **bold.**
SSEIA, subtype-specific enzyme immunoassay; ppv, positive predictive value; npv, negative predictive value.

panel of diverse samples (Table 3). In both assays, a high ppv (>0.85) was observed for serotypes B and E. Serotype D had an excellent ppv (1) due to its specificity but a weak sensitivity (0.57). In the $SSEIA_{A-E}$, serotypes A and C had a low ppv (0.28 and 0.29, respectively) due to the frequent misidentification of genotype A samples that reacted as serotypes C (37 of 54 samples) and misidentification of most genotype F samples, which reacted as serotypes A (18 of 21 samples). This was in part corrected in the $SSEIA_{A-H}$, which showed an increase in the ppv of serotype A to 0.75 due to the sensitivity of serotype F (0.95). However, misidentification of genotype A samples as serotype C was not corrected.

Correlation Between V3 Amino-Acid Sequence and Serotyping

V3 sequences of viruses corresponding to 75 of 175 samples were available. Samples were grouped according to the serotype based on SSEIA results and V3 sequences were aligned to correlate amino acid sequences with serologic reactivity (Fig. 1).

We focused the analysis on the peptide located between position 312 and 324 at the tip of the V3 loop because the N-terminal and C-terminal parts of the loop are highly conserved in all subtypes and this area has been previously shown to contain the V3 epitopes (45–47). An important sequence homology was observed within each serologic group (Fig. 1; Table 4). If one amino-acid difference was allowed, 14 of 16 serotype B samples had the sequence xxIxI/MGPG**R**/KAF, 17 of 21 serotype A or F samples had the sequence xG/Sx**HI**/LGPG**QAF**, 16 of 22 serotype C samples had the sequence KS/Gx**RI**/MGPG**QT**/AF, the 5 serotype E samples had the sequence **TS**IxIGPG**QV**F, and the 2 serotype D samples exhibited the sequence **Q**x**T**xI/MGPG**QAL**. Based on these data, some residues conserved in most of the samples of the same serotype, but absent in the other serotypes, could be considered as essential for the serotype specificity (Table 4). These were indicated in bold previously. An arginine (or a lysine) residue at position 322 was found mainly in B-reactive samples. A leucine residue in place of a phenylalanine at position 324 associated with a glutamine-322, a threonine-314, and a glutamine-312 was characteristic of serotype D. Serotypes A and F reactivity appeared associated with histidine, glutamine, and alanine at positions 315, 322, and 323, respectively, whereas serotype C reactivity was associated with arginine, glutamine, and threonine or alanine at the same positions. Serotype E was characterized by the presence of glutamine and valine at positions 322 and 323, respectively, associated with threonine at position 312. The antigenic specificity associated to these residues at these crucial positions might explain the discrepancies between genotyping and serotyping. For instance, two genotype D samples which were found in serotype A did not harbor the characteristic leucine-324, which was replaced by a tyrosine residue or a phenylalanine residue, nor the glutamine-312, which was replaced by an arginine or a lysine. The genotype A samples that appeared as C serotypes had an arginine-315, whereas the geno-

FIG. 1. V3 sequences of viruses corresponding to 75 samples (Bichat Hospital, Paris, and ORSTOM, Montpellier) are grouped according to serotype based on subtype-specific enzyme immunoassay $(SSEIA)_{A-H}$ results. Subgroups are defined according to the genotype results. Residues are numbered according to the system used by Ratner et al. (56). The sequences of the peptides used in the SSEIAs are in **bold** at the upper part of each group, and sequences of the viruses were compared with this peptide (one lane per sample). *Dots* represent insertions, and *dashes* represent residue identity. In the group of serotypes A and F, sequences were compared with peptide F.

Genotype n = 75	$SSEIA_{A-E}$ n = 75	$SSEIA_{A-H}$ n = 75																												
		Peptide V3-B	**N**	**N**	**T**	**R**	**K**	**S**	**I**	**H**	**I**	**G**	**P**	**G**	**R**	.	.	**A**	**F**	**Y**	**T**	**T**	**G**	**E**	**I**	**I**	**G**	**D**	.	**I**	**R**	**Q**	**A**	**H**	**C**
B	B	B	-	-	-	-	R	G	-	-	-	-	-	-	-	.	.	-	-	-	A	-	-	D	-	-	-	-	.	-	-	-	-	Y	-
B	B	B	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	-	-	-	A	-	-	G	-	T	-	-	.	-	-	-	-	-	-
B	B	B	-	-	-	-	-	-	-	P	-	-	-	-	-	.	.	-	-	-	A	-	-	D	-	-	-	-	.	-	-	-	-	Y	-
B	B	B	-	-	-	-	-	G	-	P	-	-	-	-	-	.	.	-	L	-	A	-	-	-	-	-	-	-	.	-	-	-	-	-	-
B	B	B	-	-	-	-	-	G	-	P	-	-	-	-	-	.	.	-	-	-	A	-	-	D	-	-	-	-	.	-	-	-	-	-	-
B	B	B	-	-	-	-	-	-	-	S	-	-	-	-	-	.	.	-	-	-	A	-	-	D	-	-	-	-	.	-	-	-	-	-	-
B	B	B	-	-	-	-	L	-	-	S	-	-	-	-	-	.	.	-	-	-	A	-	-	A	-	-	-	-	.	-	-	-	-	-	-
B	B	B	-	-	-	-	-	-	-	T	-	-	-	-	-	.	.	-	-	F	A	-	-	D	V	-	-	-	.	-	-	-	-	-	-
B	B	B	-	-	-	-	Q	-	-	T	-	-	-	-	-	.	.	-	-	F	A	-	-	-	-	-	-	N	.	-	-	-	-	Y	-
B	B	B	-	-	-	-	-	-	-	-	M	-	W	-	-	.	.	-	-	-	-	-	-	A	-	-	-	-	.	-	-	-	-	-	-
B	B	B	-	-	-	-	-	R	-	P	M	-	-	-	-	.	.	-	-	-	-	-	-	-	V	-	-	-	.	-	-	R	-	-	-
B	B	B	-	-	-	-	R	-	-	S	-	-	L	-	-	Y	N	R	V	-	-	-	-	M	-	-	-	-	R	L	-	K	-	Y	-
B	B	B	-	-	-	-	-	G	V	-	-	-	-	-	K	.	.	T	W	-	A	-	-	-	-	-	-	-	.	-	-	-	-	-	-
B	B	B	-	-	-	-	-	R	-	T	M	-	-	-	K	.	.	V	Y	-	-	-	-	-	V	-	-	-	.	-	-	R	-	-	-
B	B	B	-	-	-	-	-	-	-	Y	-	-	-	-	K	.	.	-	-	H	-	-	D	R	-	-	-	-	.	-	-	-	-	-	-
H	B	B	-	-	-	-	-	-	-	R	-	-	-	-	Q	.	.	-	-	-	A	-	-	D	-	V	-	N	.	-	-	-	-	-	-
		Peptide V3-C	**N**	**N**	**T**	**R**	**K**	**S**	**I**	**R**	**I**	**G**	**P**	**G**	**Q**	.	.	**T**	**F**	**Y**	**A**	**T**	**G**	**D**	**I**	**I**	**G**	**D**	.	**I**	**R**	**Q**	**A**	**H**	**C**
A	C	C	-	-	-	-	T	-	L	-	-	-	-	-	-	.	.	A	-	-	-	-	-	-	V	-	-	-	.	-	-	K	-	-	-
A	C	C	-	-	-	-	-	-	V	-	-	-	-	-	-	.	.	A	-	-	-	-	-	-	-	-	-	-	.	-	-	-	-	-	-
A	C	C	-	-	-	-	R	-	V	-	-	-	-	-	-	.	.	V	-	-	-	-	-	-	-	-	-	K	.	-	-	-	-	-	-
B	C	C	-	-	-	-	-	G	-	H	M	-	-	-	R	.	.	A	-	H	-	-	-	-	-	V	-	-	.	-	-	-	-	R	-
B	C	C	-	-	-	-	-	G	-	H	-	-	-	-	R	.	.	A	C	-	T	-	-	G	-	V	-	-	.	L	-	K	-	-	-
B	C	C	-	-	-	-	-	G	-	H	M	-	W	-	R	.	.	A	-	-	T	-	-	A	-	-	-	-	.	-	-	-	-	-	-
B	C	C	-	-	-	-	-	G	-	P	M	-	-	-	R	.	.	A	-	-	-	-	-	E	-	-	-	-	.	-	-	K	-	-	-
B	C	C	-	-	-	-	-	R	-	S	-	-	-	-	R	.	.	A	V	-	T	-	-	Q	-	-	-	-	.	-	-	-	-	-	-
C	C	C	-	-	-	-	-	-	V	-	M	-	-	-	R	.	.	-	-	-	-	-	-	-	-	-	-	-	.	-	-	-	-	-	-
C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	A	-	-	-	-	N	G	-	-	-	-	.	-	-	A	-	-	-
C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	-	-	-	-	-	-	-	-	-	-	-	.	-	-	-	-	-	-
C	C	C	-	-	-	-	-	-	M	-	-	-	-	-	-	.	.	-	-	-	-	-	-	A	-	-	-	-	.	-	-	-	-	-	-
C	C	C	-	-	-	-	-	-	M	-	-	-	-	-	-	.	.	-	-	-	-	-	-	-	-	-	-	-	.	-	-	-	-	-	-
C	C	C	-	-	-	-	-	-	M	-	-	-	-	-	-	.	.	-	-	-	-	-	-	-	-	-	-	-	.	-	-	-	-	Y	-
C	C	C	-	-	-	-	-	-	M	-	-	-	-	-	-	.	.	-	-	-	-	-	-	-	-	-	-	-	.	-	-	-	-	-	-
C	C	C	-	-	-	-	R	-	M	-	-	-	-	-	-	.	.	-	-	-	-	-	-	-	-	-	-	-	.	-	-	-	-	-	-
C	C	C	-	-	-	-	-	-	V	-	-	-	-	-	-	.	.	-	-	-	-	-	-	-	-	-	-	-	.	-	-	K	-	Y	-
C	C	C	-	-	-	-	-	-	V	-	-	-	-	-	-	.	.	-	-	-	-	-	-	-	-	-	-	-	.	-	-	-	-	-	-
C	C	C	-	-	-	-	Q	-	-	-	-	-	-	-	-	.	.	-	-	-	-	-	-	-	-	-	-	-	.	-	-	K	-	-	-
C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	-	-	-	-	-	-	-	-	-	-	-	.	-	-	-	-	-	-
C	C	C	-	-	-	-	E	-	-	-	-	-	-	-	-	.	.	-	-	-	-	-	-	-	-	-	-	-	.	-	-	L	-	-	-
G	C	C	-	-	-	-	-	-	-	P	F	-	-	-	-			A	-	-	-	-	-	-	-	-	-	-	.	-	-	-	-	-	-
		Peptide V3-D	**N**	**N**	**T**	**R**	**Q**	**R**	**T**	**H**	**I**	**G**	**P**	**G**	**Q**	.	.	**A**	**L**	**Y**	**T**	**T**	.	**R**	**I**	**I**	**G**	**D**	.	**I**	**R**	**Q**	**A**	**H**	**C**
D	D	D	Y	K	I	-	-	-	-	G	-	-	Q	-	-	.	.	-	-	-	-	-	.	-	-	N	-	-	.	-	-	K	-	-	-
D	D	D	-	-	-	-	-	G	-	-	M	-	-	-	-	.	.	-	-	F	-	N	.	K	V	-	-	N	.	-	-	R	-	-	-
		Peptide V3-E	**N**	**N**	**T**	**R**	**T**	**S**	**I**	**T**	**I**	**G**	**P**	**G**	**Q**	.	.	**V**	**F**	**Y**	**R**	**T**	**G**	**D**	**I**	**I**	**G**	**D**	.	**I**	**R**	**Q**	**A**	**H**	**C**
E	E	E	-	-	-	-	-	-	-	N	-	-	-	-	-	.	.	-	-	-	-	-	-	-	-	-	-	-	.	-	-	K	-	Y	-
E	E	E	-	-	-	-	-	-	-	P	-	-	-	-	-	.	.	-	-	-	-	-	-	-	-	-	-	-	.	-	-	K	-	Y	-
E	E	E	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	-	-	-	-	-	-	E	-	-	-	-	.	V	-	R	-	Y	-
E	E	E	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	-	-	-	-	-	R	-	-	T	-	-	.	-	-	K	-	Y	-
E	E	E	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	-	-	-	-	-	-	-	-	-	-	-	.	M	-	K	-	Y	-
		Peptide V3-A	**N**	**N**	**T**	**R**	**K**	**S**	**V**	**H**	**I**	**G**	**P**	**G**	**Q**	.	.	**A**	**F**	**Y**	**A**	**T**	**G**	**D**	**I**	**I**	**G**	**D**	.	**I**	**R**	**Q**	**A**	**H**	**C**
		Peptide V3-F	**N**	**N**	**T**	**R**	**K**	**S**	**I**	**H**	**L**	**G**	**P**	**G**	**Q**	.	.	**A**	**F**	**Y**	**A**	**T**	**G**	**D**	**I**	**I**	**G**	**D**	.	**I**	**R**	**K**	**A**	**H**	**C**
A	A	A	-	-	-	-	H	G	-	-	I	-	-	-	-	.	.	P	L	-	T	-	.	R	-	-	-	-	.	-	-	Q	-	-	-
A	A	F	-	-	-	-	-	G	-	-	I	-	-	-	-	.	.	-	I	-	-	-	-	-	V	-	-	-	.	-	-	Q	-	-	-
A	A	F	-	-	-	-	Q	G	V	-	I	-	-	-	-	.	.	-	I	-	T	-	.	R	-	-	-	N	.	-	-	Q	-	-	-
A	A	F	-	-	-	-	-	G	-	-	I	-	-	-	-	.	.	-	-	-	-	-	-	-	V	-	-	N	.	-	-	Q	-	-	-
A	A	F	-	-	-	-	R	G	-	-	I	-	-	-	-	.	.	-	-	-	T	-	.	-	-	-	-	-	.	-	-	Q	-	-	-
D	A	F	E	-	K	-	R	R	T	P	I	-	L	-	-	.	.	-	Y	-	T	-	.	K	L	K	-	Y	.	-	-	P	-	-	-
D	A	F	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	-	-	-	T	S	.	N	-	-	-	-	.	-	-	Q	-	-	-
F	A	F	-	-	-	-	-	G	-	-	-	-	-	-	-	.	.	-	-	-	-	-	-	-	-	-	-	-	.	-	-	R	-	-	-
F	A	F	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	-	-	-	T	-	-	-	-	-	-	N	.	-	-	-	-	-	-
F	A	F	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	-	-	-	-	-	-	-	-	-	-	-	.	-	-	-	-	-	-
F	A	F	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	-	-	-	-	-	-	E	-	-	-	-	.	-	-	-	-	Y	-
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F	A	F	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	-	-	-	-	-	-	-	-	-	-	N	.	-	-	-	-	-	-
F	A	F	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	-	-	-	-	-	-	-	-	-	-	N	.	-	-	Q	-	-	-
F	A	F	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	-	-	-	-	-	-	-	-	-	-	-	.	-	-	-	-	-	-
F	A	F	Y	-	-	-	-	R	-	-	I	-	-	-	R	.	.	S	-	-	T	-	S	N	-	Q	-	-	.	-	-	-	-	Y	-
F	A	F	-	-	-	-	-	-	-	Y	V	-	-	-	R	.	.	-	-	Q	T	-	D	K	-	-	-	-	.	-	-	-	-	-	-
F	B	F	-	-	-	-	-	-	-	Y	-	-	-	-	H	.	.	-	-	H	T	-	-	R	-	-	-	-	.	-	-	-	-	-	-
F	C	F	-	-	-	-	-	-	-	-	-	A	-	-	-	.	.	-	-	-	-	-	-	-	-	-	-	-	.	-	-	-	-	-	-
G	A	F	-	-	-	-	-	-	-	-	F	A	-	-	-	.	.	-	-	-	-	-	-	-	-	-	-	-		-	-	Q	-	-	-
G	A	F	-	-	-	-	-	-	-	S	F	A	-	-	-	.	.	T	-	-	-	-	-	G	-	-	-	N		-	-	Q	-	-	-
		Peptide V3-G	**N**	**N**	**T**	**R**	**K**	**S**	**I**	**T**	**I**	**G**	**P**	**G**	**Q**	.	.	**A**	**F**	**Y**	**A**	**T**	**G**	**D**	**I**	**I**	**G**	**D**	.	**I**	**R**	**Q**	**A**	**H**	**C**
A	B	G	-	-	-	-	R	-	F	H	-	-	-	-	-	.	.	-	-	-	-	-	-	A	-	-	-	K	.	-	-	-	-	-	-
A	C	G	-	-	-	-	R	-	-	R	-	-	-	-	-	.	.	-	-	-	-	D	-	-	-	-	-	N	.	-	-	-	-	-	-
A	C	G	-	-	-	-	R	-	-	R	-	-	-	-	-	.	.	S	L	-	-	P	-	E	-	-	-	-	.	-	-	-	-	Y	-
B	A	G	D	-	-	-	R	-	-	P	-	-	-	-	R	.	.	-	-	-	-	-	-	-	-	-	-	N	.	-	-	L	-	-	-
B	B	G	-	-	-	-	-	-	-	P	-	-	-	-	R	.	.	-	-	-	-	-	-	-	-	-	-	-	.	-	-	-	-	Y	-
G	A	G	-	-	-	-	-	-	-	H	-	-	-	-	-	.	.	-	-	-	-	-	-	E	-	-	-	-	.	-	-	-	-	-	-
		Peptide V3-H	**N**	**N**	**T**	**R**	**K**	**S**	**I**	**R**	**I**	**G**	**P**	**G**	**Q**	.	.	**A**	**F**	**H**	**A**	**I**	**G**	**A**	**I**	**I**	**G**	**D**	.	**I**	**R**	**K**	**A**	**H**	**C**
A	C	H	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	-	-	Y	-	T	-	E	-	-	-	-	.	-	-	D	-	-	-
C	C	H	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	T	-	Y	-	T	-	D	-	-	-	-	.	-	-	Q	-	-	-
C	C	H	-	-	-	-	E	-	-	-	-	-	-	-	-	.	.	T	-	Y	-	T	-	D	-	-	-	-	.	-	-	Q	-	-	-

TABLE 4. *Subtype-specific determinants of the different seroreactivity categories*

Residue number[a]	311				315		320						325		
Serotype															Frequency[b]
B	...	R	x	x	I	x	I/M[c]	G	P	G	R/K	A	F	Y	... 14/16 (87.5%)
A/F	...	R	x	G/S	x	**H**[d]	I/L	G	P	G	Q	A	F	Y	... 17/21 (81.0%)
C	...	R	K	S/G	x	**R**	I/M	G	P	G	Q	T/A	F	Y	... 16/22 (72.7%)
E	...	R	**T**	S	I	x	I	G	P	G	Q	V	F	Y	... 5/5 (100%)
D	...	R	Q	x	**T**	x	I/M	G	P	G	Q	A	**L**	Y	... 2/2 (100%)

[a] Residues are numbered according to Ratner et al. (56).
[b] Number of identical sequences/number of samples in this serotype (percent).
[c] Two amino-acids found at this position; first is predominant.
[d] Key amino-acids are in bold.

type A samples that were found A or F serotypes had an histidine residue at this position.

To further analyze the frequent serotype C among genotype A samples we studied the correlation between genotype and serotype in a group of 28 samples from Senegalese CSW. Among them, 5 of 6 genotype A samples that were serotype A had the histidine-315 and 4 of 6 had an alanine-323 (Fig. 2). In contrast, 3 of 3 genotype C samples, which were serotype C had the arginine-315 associated in 2 cases with threonine-323. All 17 genotype A samples that were serotype C had arginine-315, associated in 13 cases with threonine-323. We also observed that most serotype A samples had a glycine-313 whereas serotype C samples had a serine at this position.

Five genotype B samples were serotype C, although they did not harbor the C "specific" peptide described above (Fig. 1). Interestingly, they harbor related sequences, especially showing glycine-313, histidine-315, methionine-316, arginine-322, and alanine-323; four were collected from patients infected in the same geographic area, Djibouti.

DISCUSSION

Description of the genetic diversity of HIV-1, particularly the recent identification of genetic clades or subtypes of the virus, led to the necessity of developing efficient tools to identify subtypes rapidly to study the epidemiologic consequences of this diversity. Nucleotide sequencing of portions of the genome remains the standard criterion for classification, but simpler assays are necessary to get information on large populations and to study changes of subtypes distribution over time. Due to its immunogenicity, its variability, and its biologic importance, the V3 region was proposed as a target for serologic subtyping. Several laboratories have developed different formats of V3 EIAs (33–35,45), which have shown similar results independent of the format (48,49). All these EIAs used V3 peptides of subtypes A to E. The more recent description of subtypes F to H led us to modify this assay by including additional peptides representative of these subtypes. Two assays, $SSEIA_{A-E}$ and $SSEIA_{A-H}$, were evaluated using a panel of well-characterized serum samples from patients with known genotype. The origins of the patients were highly diverse, providing the opportunity to study the correlation between serotyping and genotyping under challenging conditions. We therefore believe that this evaluation was conducted on a difficult sample in term of diversity, reflecting a situation far more diverse than what would actually be encountered in any single place in the world.

The results indicate that introduction of peptides G and H do not allow efficient identification of samples corresponding to genotypes G and H, although numbers were small. Moreover, they frequently induced misidentification of samples from patients infected by A, B, or C strains. We cannot exclude that this was caused by only a limited number of sequences related to these two genotypes being known, and therefore the G and H consensus sequences may not be representative of these circulating subtypes. In contrast, due to high sensitivity, peptide F appeared useful with 20 of 21 genotype F samples correctly identified. It can therefore be recommended that peptide F should be included in V3 EIAs when seroepidemiologic studies of HIV-1 diversity are conducted in areas where this subtype is prevalent, such as Latin America or Romania. However, due to the fact that most of our F samples were collected from a single country (Romania), we cannot exclude that the strong correlation between genotype and serotype observed for subtype F could result from geographic bias.

Our results confirm a high ppv for serotypes B, D, and E (>0.80). Therefore in areas where one or several of these subtypes are highly prevalent, V3 serotyping represents an adapted approach for broad epidemiologic studies. Such studies have been conducted in Thailand (50,51) and more recently in France where an increasing prevalence of non-B serotypes was observed between

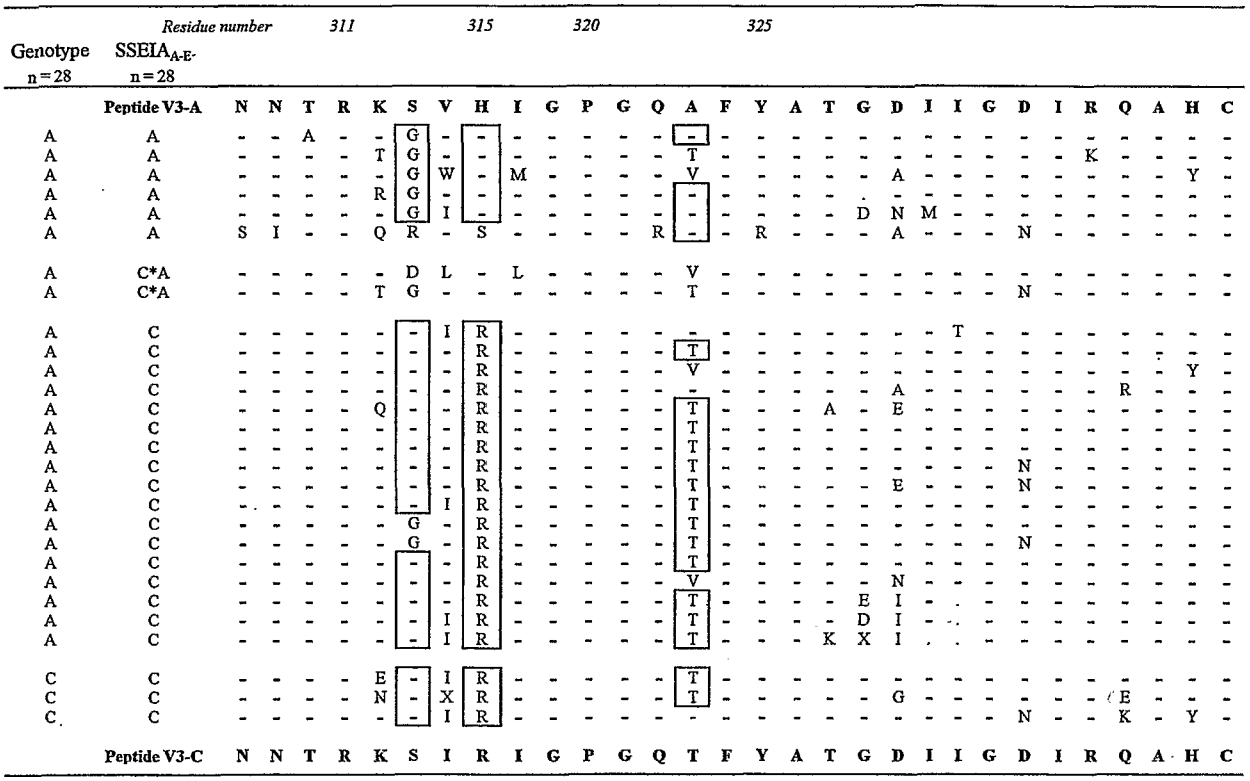

FIG. 2. V3 sequences of viruses corresponding to the 28 Senegalese samples are grouped according to the serotype based on subtype-specific enzyme immunoassay $(SSEIA)_{A-E}$ results. Subgroups are defined by serotypes and genotype results. Residues are numbered according to the method of Ratner et al. (56). Consensus sequence of the peptide V3-A is provided in **bold** (*top*). Sequences of the viruses (one lane per sample) were compared with this peptide. *Dashes* represent residue identity. Positions highly different between serotypes A and C are framed. Sequence of the peptide V3-C is at the bottom. C*A indicates dominant reactivity to peptide C with high cross-reactivity to peptide A.

1985 and 1995 (52). Our results also confirm the difficulty in distinguishing A and C subtypes by V3 serology, frequently reported in previous studies (33–35,48,49). In addition, in areas such as Africa, where G and H subtypes can be expected, our data indicate that patients carrying these viruses would probably react as A, B, or C serotypes. Similarly, samples of genotype F would react generally as an A serotype if the V3 EIA does not include a F-specific peptide. Such a case was previously reported in a Caribbean patient with hemophilia who was infected by an F variant but whose serum sample reacted as an A serotype by $SSEIA_{A-E}$ (53).

Correlation between serotyping and genotyping was further analyzed using amino acid sequences of the V3 region, which were available for 103 samples. Data indicated that V3 serotyping was closely related to the V3-loop amino acid sequence of the analyzed sample. In other words, misidentification by V3 serotyping mainly reflects the difference between genetic analysis and antigenicity, which are not directly linked, as has been shown using a phenetic analysis (38,54) and in a previous study conducted in Tanzania (49). Our study allowed us to identify key amino acids, which appear essential for subtype-specific seroreactivity. They are located on both side of the tip of the V3-loop in the peptide xxxxxGPGxxx (Table 4). This observation is in agreement with the structural analysis done by crystallography and epitope mapping that showed that amino acids on both sides of GPG participate to binding of Fab to the V3 epitope or epitopes (46,47,55). Our results clearly indicate that most of serotype A and F samples harbor the sequence xG/sxHI/LGPGQAF, whereas most serotype C samples have the sequence KS/GxRI/MGPGQT/AF. The presence of histidine-315 or arginine-315 and alanine-323 or threonine-323 appear critical for the serotype specificity. However, if these serotype-specific signature sequences can explain most of the results, the correlation is not perfect. It cannot be excluded that other residues might also influence antigenicity, even indirectly by modifying presentation of the entire loop. In this respect, our data indicates that residues at positions 312 and 313 may be important and worthy of further study.

In conclusion, this study further characterizes the limitations of V3 serotyping and our results indicate that V3 serology can discriminate among a limited number of serotypes. Therefore, in locations where a few serologically distinguishable serotypes are prevalent, large population-based epidemiology studies can be performed with a high degree of confidence. Other approaches, different from both V3 serology and genetic characterization, are necessary to distinguish HIV strains based on biologic properties such as neutralization. Such data would help to define composition of future multivalent vaccines if the ongoing efforts on a subtype B vaccine lead to encouraging results.

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