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Published in:
AIDS

DOI:
10.1097/00002030-199812000-00001

Lack of correlation between V3-loop peptide enzyme immunoassay serologic subtyping and genetic sequencing

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Objective: To compare the performance of V3-loop peptide enzyme immunoassay (PEIA) methodologies from four different laboratories for subtyping HIV-1, and to determine the causes for the lack of correlation between V3-loop PEIA serotyping and subtyping by sequencing.

Materials and methods: Synthetic peptides derived from the amino-acid consensus sequences of the V3-loop of group M strains representing genetic subtypes A–F as well as reference strains were evaluated in PEIA by four different laboratories for their ability to accurately determine the subtype in a panel of 85 sera obtained from persons infected with known HIV-1 subtypes (28 subtype A, 34 subtype B, four subtype C, 10 subtype D, seven subtype F, one each of subtype H and G). Furthermore, the V3 loop of the corresponding virus was compared with the V3 loop of the peptides used in PEIA.

Results: The correlation between HIV-1 subtyping by sequencing and V3-loop PEIA from the different laboratories varied considerably for the different HIV-1 subtypes: subtype A (46–68%), B (38–85%), C (75–100%), D (29–50%), and F (17–57%). A 70% agreement between PEIA and sequencing subtypes was observed for samples with the concordant presence of the same octameric sequences in the V3 loop of the virus and the V3 loop of the peptide used in PEIA; however, only 42% of specimens with different V3-loop octameric viral and peptide sequences yielded concordant results in V3-loop serotyping and genetic subtyping.

Conclusion: Our results indicate that V3-loop PEIA methodologies used in different laboratories correlate poorly with genetic subtyping, and that their accuracy to predict HIV-1 subtypes in sera of Belgian individuals infected with different HIV-1 subtypes (A, B, C, D, F, G and H) vary considerably. The poor correlation between serotyping and genetic subtyping was partly due to the simultaneous occurrence of subtype-specific octameric sequences at the tip of the V3 loop of viruses belonging to different genetic subtypes.

AIDS 1998, **12**:1405–1412

Keywords: V3-loop peptide enzyme immunoassay subtyping, genetic subtypes, peptides

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Sponsorship: This work was supported by Fonds Wetenschappelijk Onderzoek (grants G.3301.96 and G.0134.97), EC project IC18-CT96-0110, IC18 CT97-02476, European AIDS-Virus Serosurveillance Programme (EASP), contract no. BMH4-CT96-1559.1; R.C.P. was supported by the Wellcome Trust, UK.

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Date of receipt: 16 January 1998; revised: 16 April 1998; accepted: 27 April 1998.

Introduction

The development of simple and rapid techniques for large-scale HIV-1 subtype characterization in specific geographic areas is valuable in order to better understand the epidemiology of HIV-1 subtype distribution, which may facilitate the evaluation of potential candidate HIV-1 vaccines. Although nucleic acid sequencing is the technique of choice for characterizing viruses genetically, several less cumbersome and less expensive techniques have been developed, including the heteroduplex mobility assay (HMA) [1], restriction fragment length polymorphism [2], and oligonucleotide probe hybridization [3]. However, these methods require a PCR intermediary step, thus making them less attractive for large-scale application, especially in field settings. More than 32 million persons are thought to be HIV-infected worldwide; however, only a few thousand HIV-1 strains have been characterized genetically by nucleic acid sequencing [4]. The V3-loop peptide enzyme-linked immunosorbent assay (PEIA) serology promises to be an attractive alternative. PEIA has been used previously to determine genetic subtypes in some geographic areas [5,6] and in the World Health Organization HIV vaccine trial sites [6–9]. Subtyping using PEIA in countries such as Thailand where only two subtypes of HIV-1 circulate has been shown to be very effective [6,9]. However, this technique has not been successful in population-based studies in areas where several antigenically related subtypes of HIV-1 cocirculate, due to a high level of cross-reactivity [10,11]. PEIA methodologies for HIV-1 subtyping vary from one laboratory to another; both in the concentration of peptides used for coating, choice and length of peptides, and dilution factors of both specimens and reagents. Studies are lacking on comparative evaluation of the different techniques used by several laboratories on a well-characterized panel of serum or plasma specimens. Such studies may provide clues on which peptides, reagents, or PEIA protocols are suitable for obtaining accurate results in large-scale subtyping in specific populations. In this study, we report on a multicentre evaluation of different PEIA protocols to subtype a panel of plasma samples from Belgian HIV-infected individuals whose infecting virus had been characterized by genetic sequencing, and also to examine the causes of the poor correlation between HIV-1 serotyping by V3-loop PEIA and genetic subtyping.

Materials and methods

Collaborating laboratories

Four laboratories participated in the study, including the Department of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium (lab 1); the Department of Genito-Urinary Medicine and Communicable Diseases, St Mary's Hospital Medical School, London, UK (lab 2); the Division of AIDS, STD and TB Laboratory Research, Centers for Disease Control and Prevention, Atlanta, Georgia, USA (lab 3); and the Laboratoire de Virologie URA CNRS 1334, Université François Rabelais, Tours, France (lab 4).

V3-loop peptides

The amino-acid sequences of the peptides used by the collaborating laboratories are shown in Table 1. The peptides used by labs 1, 2 and 4 corresponded to subtype A–E consensus sequences described by Myers and colleagues in 1992 [12]. The subtype F peptide used by labs 1 and 2 was derived from the consensus sequence for subtype F, reported in the 1994 Los Alamos Database [13]. The peptides differed from 14- or 15-mer in length (labs 1 and 2) to 29- or 30-mer in length (lab 4). The peptides used by lab 3 were according to representative strains of subtypes A–F including ZR.6649 (subtype A), MN (subtype B), IN.757 (subtype C), UG2.121 (subtype D), TH1.8659 (subtype E), and RO.14018 (subtype F). Aspartic acid was added at the amino-terminus of the peptides. Synthesis and purification of these peptides used has been previously described by each participating group [7,8,10,14].

V3-loop peptide protocols

The protocols used by the participating laboratories have been published previously. Labs 1 and 2 used a

Table 1. V3-loop peptides used by the four laboratories.

Laboratory	V3-loop peptides
Subtype A	
Lab 1	KSV**HIGPGQAF**YAT
Lab 2	KSV**HIGPGQAF**YAT
Lab 3	DKSV**HIGPGQAF**YAT
Lab 4	NNTRKSV**HIGPGQAF**YATGDIIGDIRQAHC
Subtype B	
Lab 1	KSI**HIGPGRAF**YTT
Lab 2	KSI**HIGPGRAF**YTT
Lab 3	DKRI**HIGPGRAF**YTT
Lab 4	NNTRKSI**HIGPGRAF**YTTGEIIGDIRQAHC
Subtype C	
Lab 1	KSI**RIGPGQTF**YAT
Lab 2	KSI**RIGPGQTF**YAT
Lab 3	DKSI**RIGPGQTF**YAT
Lab 4	NNTRKSI**RIGPGQTF**YATGDIIGDIRQAHC
Subtype D	
Lab 1	RQRT**HIGPGQAL**YTT
Lab 2	RQRT**HIGPGQAL**YTT
Lab 3	DQST**HIGPGQAL**YTT
Lab 4	NNTRQRT**HIGPGQAL**YTTRIIGDIRQAHC
Subtype E	
Lab 1	DTSI**TIGPGQVF**YRT
Lab 2	DTSI**TIGPGQVF**YRT
Lab 3	DTSI**TIGPGQVF**YRT
Lab 4	NNTRTSI**TIGPGQVF**YRTGDIIGDIRQAHC
Subtype F	
Lab 1	RKSI**HLGPGQAF**YTT
Lab 2	RKSI**HLGPGQAF**YTT
Lab 3	DKSI**HLGPGQAF**YAT
Lab 4	–

two-step procedure [7,10]. An indirect V3 peptide-binding assay was employed initially as a primary screening assay. Subsequently, an antigen-limiting enzyme-linked immunosorbent assay in which V3 peptides are coated at a concentration of 10, 1, 0.1, and 0.01 μg/ml in 20 mmol/l carbonate buffer was used as a secondary assay to discriminate cross-reactivity if the screening assay was equivocal [7].

Lab 3 also used a two-step procedure [8]. All samples were first screened for different antibody populations at a sample dilution of 1 : 400, and samples that were reactive to two or more peptides (i.e., with an optical density difference of 10% or lower) were retested at a dilution of 1 : 1600. If a sample was initially negative to all peptides, it was retested at 1 : 100 dilution.

The protocol for lab 4 included a subtype-specific antibody assay (HIV-1 SSEIA) that was based on the principle of blocking by excess peptide in liquid phase [14].

Patient sera

A total of 85 plasma specimens collected at the outpatient clinic of the Institute of Tropical Medicine, Antwerp, Belgium were used in this study. Samples were selected from patients of Belgian nationality, from whom sociodemographic data and information on the mode of transmission were available [15]. The genetic subtype of the corresponding viruses obtained from the same blood samples as the plasma had been previously determined at the Institute of Tropical Medicine by HMA [15], and confirmed by sequencing and phylogenetic analysis of the *env* region encoding C2–V3 [16]. These samples were part of a larger study, whereby genetic characterization by *env* sequencing was compared with HMA. Samples that were suggestive of dual infection by different HIV-1 subtypes, or *env* genetic recombination, were excluded from this study in order to exclude the high cross-reactivity that may be caused by these events. Aliquots of the serum samples were sent, blinded, on dry ice to all the participating laboratories, and V3-loop PEIA was performed according to the protocols established in individual laboratories. The distribution of HIV-1 subtypes in the 85 samples studied was as follows: 28 subtype A, 34 subtype B, four subtype C, 10 subtype D, none subtype E, seven subtype F, one subtype G, and one subtype H.

Analysis of data

Genetic subtyping by C2–V3 encoding *env* sequencing and phylogenetic analysis, was taken as a 'gold standard'. A subtype was defined based on the highest antibody binding (optical density/cut-off ratio) at the lowest antigen concentration or at the highest serum dilution. A serum sample was considered correctly subtyped by PEIA if antibody reactivity was highest with a given peptide representing the particular subtype with which the patient was infected.

Results

Capacity to accurately identify HIV-1 subtypes

The results of serological subtyping obtained by each laboratory for the sera tested are shown in Table 2. Of the sera that were classified as subtype A by sequencing, 67.8% (19 out of 28), 64.3% (18 out of 28), 52.2% (12 out of 23), and 46.4% (13 out of 28) were correctly identified by labs 1–4, respectively (Table 2). It is noteworthy that a substantial number of subtype A sera [25% (seven out of 28, labs 1 and 2), 30.4% (seven out of 23, lab 3), and 50% (14 out of 28, lab 4)] cross-reacted with the subtype C peptide, and were thus inaccurately classified. Of the 34 subtype B sera, 38.2, 50, 82 and 85.3% were accurately classified by labs 2, 1, 4, and 3, respectively. A higher number (13 out of 34, 38.2%) of subtype B sera were non-reactive by PEIA in labs 1 and 2, compared with two (5.9%) out of 34 for lab 3, and five (14.7%) out of 34 for lab 4. All subtype C sera were correctly classified by labs 2, 3, and 4 (all four); however, lab 1 misclassified one subtype C serum as subtype A. Of the subtype D sera, 50% (five out of 10, labs 1 and 2), 40% (four out of 10, lab 4), and 28.6% (two out of seven, lab 3) were correctly identified by V3-loop PEIA. The subtype F V3-loop peptide was included for labs 1, 2, and 3: lab 1 classified correctly 57.1% (four out of seven) of specimens, and only 28.6% (two out of seven) and 16.6% (one out of six) were scored correctly by labs 2 and 3, respectively. The overall correlation between serological subtyping by PEIA and genetic subtyping for all the sera tested was 56.5, 49.4, 64.4, and 58.3% for labs 1, 2, 3 and 4, respectively. The mean optical density/cut-off values for the sera that were correctly subtyped by PEIA was not significantly different from those of sera that were incorrectly subtyped (data not shown).

Comparison of octameric sequences

We compared the amino-acid sequences used in the V3-loop peptides of PEIA and those obtained by sequencing a 14 amino-acid sequences (positions 10–23) in the V3-loop for each of the patients' viruses. The V3-loop amino-acid sequences were grouped according to subtypes and within a subtype the octamers present at the tip of the V3-loop were subdivided into three groups: octamers identical to the octamers in the peptides used in PEIA; octamers previously reported for a particular subtype; and newly identified octamers hitherto not reported in the Los Alamos Database [17]. We found that only a few specimens had a similar octamer for each subtype as the peptides used in PEIA (highlighted octamers in Table 2 versus those in Table 1): subtype A, five (17.8%) out of 28; subtype B, 11 (32%) out of 34; subtype C, one (25%) out of four; subtype D, four (40%) out of 10; subtype F, one (14%) out of seven (Table 2).

Table 2. Comparison of the genetic and serological subtype results obtained in four different laboratories, with the V3-loop amino-acid sequences of the corresponding HIV-1 isolates from patients from whom the sera were serologically subtyped by peptide enzyme immunoassay.

Sample	HIV-1 isolate identification	Subtype	Amino-acids 10–23	Serological subtype by the four laboratories			
				1	2	3	4
Same octamer as peptide A							
1	VI1031	A	KSI**HIGPGQAF**YAT	A	A	A	A
2	VI1047	A	KSV**HIGPGQAF**YAT	A	A	A	A
3	VI1198	A	KSV**HIGPGQAF**HAT	A	D	A	A
4	VI1199	A	KGI**HIGPGQAF**NAT	A	A	A	C
5	VI1319	A	KSI**HIGPGQAF**YAT	A	A	ND	C
Octamer previously reported for subtype A							
6*	VI529	A	KSV**RIGPGQAF**YAT KSV**RIGPGQTF**YAT	C	C	E	C
7	VI537	A	KSI**RIGPGQAF**YAT	NR	NR	B	NR
8	VI559	A	KGI**HIGPGRAF**YAL	A	A	B	A
9	VI712	A	KGV**HIGPGQTF**YAT	A	A	A	A
10	VI805	A	QSV**RIGPGQAF**YTT	A	A	A	A
11	VI832	A	KGV**HIGPGQTF**YAT	A	A	A	A
12	VI860	A	KSV**RIGPGQTF**YAT	NR	A	C	C
13	VI955	A	KSV**RIGPGQAF**YAT	A	A	A	A
14	VI1007	A	KSF**RIGPGQSF**YAT	C	C	C	C
15	VI1034	A	KSI**RIGPGQVF**YAG	A	A	C	C
16	VI1090	A	KSV**RIGPGQTF**YAT	C	C	C	C
17	VI1122	A	KSI**RIGPGQAF**YAT	A	A	A	A
18	VI1130	A	KSV**RIGPGQAF**YAT	A	A	A	A
19	VI1139	A	RSV**RIGPGQAF**YGT	A	C	A	C
20	VI1243	A	KSI**RIGPGQAF**FAT	A	A	C	A
21	VI1280	A	KGI**HIGPGRAF**YGT	A	A	A	C
22	VI1318	A	RSI**RIGPGQAF**YAT	A	A	ND	A
23	VI1320	A	KSW**RIGPGQTF**YAT	C	C	ND	C
24	VI1325	A	KGV**HIGPGQTF**YAT	A	A	ND	A
25	VI1360	A	QRI**HIGPGRAF**HTT	C	C	ND	C
Octamer not previously reported for subtype A							
26	VI566	A	KGI**HMGPGQVF**YAT	C	C	C	C
27	VI857	A	KSV**HMGPGQAF**YAT	C	NT	C	C
28	VI1120	A	QST**RIGPGQTL**YTT	A	A	B	C
Same octamer as peptide B							
29	VI322	B	KSI**HIGPGRAF**YTT	B	B	B	B
30	VI323	B	KSI**HIGPGRAF**YTT	B	B	B	B
31	VI339	B	KGI**HIGPGRAF**YTT	B	B	B	B
32	VI479	B	KGI**HIGPGRAF**YTT	B	B	B	B
33	VI536	B	RRI**HIGPGRAF**YTT	B	B	B	B
34	VI552	B	KSI**HIGPGRAF**YTT	B	A	B	B
35	VI601	B	KSI**HIGPGRAF**YTT	NR	NR	NR	NR
36	VI688	B	KSI**HIGPGRAF**YAT	NR	NR	E	NR
37	VI774	B	KSI**HIGPGRAF**YAT	B	B	B	B
38	VI811	B	RSV**HIGPGRAF**YTT	C	B	B	B
39	VI826	B	KSI**HIGPGRAF**YTT	B	B	B	B
Octamer reported for subtype B							
40	VI334	B	KRV**TMGPGRVY**YTT	NR	NR	B	B
41	VI452	B	KSI**PIGPGRAF**YAR	NR	D	B	B
42	VI543	B	KSI**PMGPGKAF**YAT	B	D	A	B
43	VI704	B	KRI**SIGPGRAF**RTA	D	D	B	B
44	VI750	B	RGI**SIGPGRAF**YAT	B	B	B	B
45	VI765	B	KSI**NIGPGRAF**YAT	NR	NR	A	NR
46	VI771	B	KGI**HIGPGKAF**YAH	B	B	B	B
47	VI782	B	KSI**SMGPGRAF**RTR	NR	NR	B	B
48	VI810	B	KGI**HMGPGRAF**YAT	NR	NR	B	B
49	VI840	B	KSI**PMGPGKAF**YTT	B	B	B	B
50	VI863	B	KSI**NIGPGRAF**YTT	B	NR	B	B
Octamer not previously reported for subtype B							
51	VI412	B	KRI**TMGPGRVF**YTT	B	B	B	B
52	VI518	B	KSI**PMGPGRAI**FAT	NR	NR	B	A
53	VI546	B	RSI**PIGPGKAI**YAT	B	A	B	B
54	VI609	B	KSI**HIGPGKAI**YTT	NR	NR	B	B
55	VI612	B	GRI**HIGRGRTF**SAT	NR	NT	B	B
56	VI713	B	KSI**TIGPGKAF**YAT	NR	NT	NR	NR
57	VI819	B	KSI**SIGPGRAV**YAT	NR	NR	B	NR
58	VI833	B	KSI**HIGPGSAI**YTT	B	B	B	B
59	VI835	B	KSI**HVAPGGTI**YAT	C	NR	B	B
60	VI843	B	KSI**HMGPGGAI**YAT	A	D	B	B

Table 2. (continued)

Sample	HIV-1 isolate identification	Subtype	Amino-acids 10–23	Serological subtype by the four laboratories			
				1	2	3	4
61	VI844	B	KRI**SLGPGRVL**YTT	B?	NR	B	B
62	VI845	B	PGR**FVGPGRAF**YTT	NR	NR	B	B
Same octamer as peptide C							
63	VI882	C	ESI**RIGPGQTF**YAT	C	C	C	C
Octamer reported for subtype C							
64	VI829	C	ESV**RIGPGQAF**YAT	C	C	C	C
65	VI849	C	KSI**RIGPGQAF**YAT	C	C	C	C
66	VI1044	C	KSI**RIGPGQAF**YAT	A	C	C	C
Same octamer as peptide D							
67	VI656	D	KSI**HIGPGQAL**HTT	A	A	A	A
68	VI693	D	QGT**HIGPGQAL**FTN	D	D	ND	D
69	VI722	D	QST**HIGPGQAL**FTT	D	D	D	D
70	VI1091	D	QGI**HIGPGQAL**YTT	D	D	B	A
Octamer reported for subtype D							
71	VI824	D	EGI**HIGPGRAF**FTT	D	D	D	D
72	VI973	D	RSI**HIGPGRAF**YAT	B	B	ND	B
73	VI1234	D	KGV**HMGPGRVF**YAT	NR	NR	B	B
Octamer not previously reported for subtype D							
74	VI865	D	KSI**HMGPGQAL**YAS	A	A	F	A
75	VI902	D	KGI**HIGPGRSF**YTT	NR	NR	B	A
76	VI979	D	KGI**HIGPGQSL**FTT	D	D	ND	D
Same octamer as peptide F							
77	VI1310	F	KSI**HLGPGQAF**YAT	F	F	B	A
Octamer reported for subtype F							
78	VI850	F	KGI**HLGPGQIF**YAT	F	A	A	A
79	VI961	F	KSI**PLGPGQAF**YTT	F	A	ND	A
80	VI1052	F	KSI**HLGPGRAF**YAT	A	D	B	B
Octamer not previously reported for subtype F							
81	VI507	F	KSI**HLAPGRAF**YAT	A	A	A	A
82	VI1206	F	RSV**RIGPGQAF**YGT	A	A	F	A
83	VI1267	F	KSI**RIGPGQAF**YAT	F	F	A	A
Octamer not previously reported for subtype G							
84	VI1197	G	KSI**GFGPGQAF**YAT	NR	NR	C	ND
Octamer reported for subtype H							
85	VI991	H	KSI**RIGPGQAF**YAT	A	A	A	A

*Two different octamers were documented for the same sample. Incorrect predictions are indicated as follows: NR, non-reactive; NT, not typable (no distinction was made between two or more peptide reactivities); ND, not determined.

Effect of different octameric sequences

Next, we examined the effect of differences in the octameric sequence present at the tip of V3 loop of the viruses and the peptides used in PEIA for accurate serologic subtyping by PEIA. We observed that the capacity of PEIA to accurately classify a given specimen increased in proportion to the percentage homology between the octameric sequence of the V3 loop of the patients' virus and the octamer in the peptide. For instance, the proportion of subtype A specimens correctly identified by PEIA decreased from 84 to 63%, and 10.7% for sera with 100, 87.5 and 75% homology, respectively, between the patients' virus octameric sequence and that of the peptide (data not shown). Indeed, of five (samples 1–5) of the 28 genetic subtype A viruses presenting the same octameric sequence (HIGPGQAF; Table 2) as the subtype A peptide used in PEIA (Table 1), 84% (16 out of 19) PEIA results were correctly identified by the four laboratories. However, six (sera 9, 10, 11, 13, 17 and 18) of the 28 genetic subtype A specimens presenting a different octamer to that used in the subtype A peptide (previously reported for subtype A) were correctly identified as subtype A by PEIA in all four laboratories. Two additional specimens not analysed by lab 3 (samples 22 and 24) were correctly identified by labs 1, 2 and 4. Three isolates (samples 9, 11 and 24) had the HIGPGQTF octameric sequence, and five isolates (samples 10, 13, 17, 18 and 22) had the RIGPGQAF octameric sequence. Of the 23 patients (samples 6–28; Table 2) infected with HIV-1 subtype A virus whose octameric sequence differed from the octamer present in the subtype A peptide, only 52% (46 out of 88) PEIA results were correctly identified as subtype A by all four laboratories. On average, the percentage homology between the octamer amino-acid sequence at the tip of the V3-loop isolates (samples 6–28; Table 2) and the octamer in the subtype A peptide was 82% (data not shown). Of the four subtype A isolates (samples 6, 12, 16 and 23) with a similar octameric sequence at the tip of the V3-loop to the octameric sequence of the subtype C peptide, 80% (12 out of 15) of PEIA results were misclassified as subtype C by all four laboratories.

Of the subtype B viruses with 100% homology between the virus and peptide octamer, 77% (34 out of 44; samples 29–39) of PEIA results were accurate, whereas only 58% (53 out of 92; samples 40–62) were accurate for specimens with a 72% homology in peptide and virus octameric sequences. For subtype C samples, the accuracy of PEIA decreased from 100% (all four) to 92% (11 out of 12) for samples 63–66 (Table 2) for specimens with a 100 and 87% homology, respectively, between the octameric sequence of the viruses and peptides. Similarly, for subtype D specimens, the accuracy of PEIA decreased from 60% (nine out of 15) to 32% (seven out of 22) for samples 67–70 and samples 71–76 (Table 2) for octameric sequence homologies of 100 and 73%, respectively. Finally, for subtype F specimens, the accuracy of PEIA decreased from 50% (two out of four) to 22% (five out of 23) for serum sample 77 and samples 78–83, for which the percentage homology between isolate and peptide F octamers was 100% and at average 81%, respectively.

Frequency of subtype-specific peptide octamers

To further determine whether the discrepancy observed between genetic subtyping and serological subtyping by PEIA was due to the simultaneous occurrence of a similar subtype-specific octameric sequences at the tip of the V3-loop peptide in HIV-1 isolates belonging to different genetic subtypes, we analysed the V3-loop amino-acid sequences documented in this study, as well as the V3-loop sequences of genetically subtyped isolates described in the Los Alamos Database [17]. The subtype A-specific octameric sequence (HIGPGQAF) was mainly present in this subtype (61 out of 236, 26%), followed by 6% (four out of 64) of subtype F, and 0.2% (two out of 1009) of subtype B (Table 3).

Similarly, the subtype B octameric sequence was mainly present in subtype B viruses; even though only 280 (28%) out of 1009 subtype B isolates had this specific octamer. The subtype C octamer was frequent mostly for subtype C (83 out of 123, 67%), 51 (22%) out of 236 subtype A, and three (12%) out of 24 subtype G; the subtype D octamer was frequent mostly in subtype D, even though only 15% (17 out of 116) subtype D isolates contained the peptide D octamer. Finally, the subtype F octameric sequence was mostly frequent in subtype F viruses, however, only 45% (29 out of 64) contained the peptide F octamer.

In a detailed analysis of only the specimens documented in our study, it was striking to observe that the subtype A-specific octameric sequence, RIGPGQAF, was present at the tip of the V3-loop of nine isolates classified as subtype A (samples 6, 7, 10, 13, 17, 18, 19, 20, 22), and also three subtype C viruses (samples 64, 65 and 66), two subtype F viruses (samples 82 and 83), and one subtype H virus (sample 85; Table 2). Of the nine subtype A viruses with the RIGPGQAF octamer at the tip of the V3-loop, the four laboratories correctly subtyped 24 (68.6%) out of 35 results by PEIA. Of the three subtype C-infected individuals, the four laboratories correctly identified 92% (11 out of 12) by PEIA; however, only 37% (three out of eight) of the three subtypes F viruses were correctly identified by the laboratories, the remaining 63% (five out of eight) were classified as subtype A by PEIA, as well as the subtype H specimen (sample 85). Intrigued by this observation, we searched further for the octamer RIGPGQAF distribution among V3-loop sequences reported in the Los Alamos Database [17]. The octamer RIGPGQAF was present in 22.9% (54 out of 263) of subtype A and 24.4% (30 out of 123) of subtype C strains (Table 3).

Discussion

This study was aimed at determining the capacity of different laboratories' PEIA methodology to correctly identify HIV-1 subtypes in a panel of 85 samples whose infecting HIV-1 subtypes (A, B, C, D, F, G and H) had been determined by both HMA and sequencing. Our results indicate that the capacity of PEIA to correctly identify a given genetic subtype in the specimens varies considerably depending on the laboratories. Subtype A isolates were best identified by lab 1 (67.8%); subtype B was best identified by lab 3 (85.3%), followed by lab 4 (82.3%); subtype C specimens were all correctly subtyped by labs 2, 3, 4; subtype D specimens were

Table 3. Frequency of occurrence of identical octameric sequences at the tip of the V3-loop in HIV-1 isolates of different genetic subtypes reported in this study and the Los Alamos Database.

Octamer amino-acid sequences	No. isolates with the peptide octamer in V3 loop									
	A (n = 236)*	B (n = 1009)	C (n = 123)	D (n = 116)	E (n = 124)	F (n = 64)	G (n = 24)	H (n = 2)	U (n = 15)	Total
HIGPGQAF (peptide A)	61	2				4				67
HIGPGRAF (peptide B)	6	280		4		3			2	295
RIGPGQTF (peptide C)	51		83	1	2	1	3		1	172
HIGPGQAL (peptide D)		2		17			1			20
HLGPGQAF (peptide F)	2	1				29				32
RIGPGQAF	54		30		1	2	1	1	1	90

*Total number of V3-loop amino-acid sequences of subtype A isolates as mentioned in the Los Alamos Database [17] plus the V3-loop amino-acid sequences generated during this study.

best identified by labs 1 and 2 (50%); and subtype F strains were best predicted by lab 1 (57.1%).

Several factors may explain the differences in the results of PEIA by different laboratories, including the protocol used, the choice of peptides and coating conditions, the type of assays, and possibly problems associated with peptide conformation. For instance, in labs 1 and 2, the same peptides were used but the protocols differed with respect to substrate and coating conditions, this resulted in an overall slightly better subtype prediction, 60% (lab 1) versus 56% (lab 2). Furthermore, labs 1 and 2, which used the same peptides, were more successful in predicting subtype A samples than labs 3 and 4.

In our study, there was considerably discordance between serological subtyping by PEIA and genetic sequencing, which is consistent with other previous studies [10,11]. Several reasons, on the basis of our results, may account for these discordant observations. First, several HIV-1 genetic subtypes share common or identical octamer amino-acid sequences at the tip of the V3 loop. In a recent study, the main cause of discordant results in HIV serotyping and genetic subtyping was the existence of highly similar V3-loop amino-acid sequences in different genetic subtypes [18]. Similar V3-loop sequences among subtypes A and C have been reported [19]. In addition, a phenetic cluster consisting of V3-loop amino-acid sequences from subtype A and C viruses has been described [20]. Indeed, we showed that octamer RIGPGQAF was common among 15 different viral isolates (nine subtype A, three subtype C, two subtype F, and one subtype H; Table 2). Second, the difference between the octameric sequence of the V3-loop peptide used in PEIA and that of the V3-loop sequences of the patients' viruses is critical for the outcome of serological subtyping. We observed, on average, a high correlation of 70% between serological subtyping and sequencing when concordant octameric sequences were present in the peptides and the V3 loop of the patients' viruses. In contrast, only 42% correlation was observed from specimens with discordant octameric sequences of the peptide and the virus. Finally, antibody cross-reactivity and polyspecificity to certain peptides could contribute to the discordance between serotyping results and genetic subtyping. This phenomenon appears to be common; recently, HIV-1 V3 serotyping has been described as a method to identify HIV-1 serotypes. This is another system to group HIV-1 isolates based on antibody binding to V3 peptides and is different from genetic classification [20,21]; V3 serotypes are closely related to only viral subtypes B, C, and E.

Taken together, our results clearly showed that PEIA cannot be used to predict viral subtypes in geographical regions where multiple subtypes of HIV-1 co-exist. In fact, V3-loop consensus sequences are based on the number of samples analysed and the geographic region(s) where the specimens originate. For instance, the subtype F consensus V3-loop peptide is dominated by strains obtained from the recent epidemic in Romania, and although each subtype F virus from this country has a closely related V3-loop sequence to this consensus peptide, subtype F viruses from other countries are considerably different; indeed, we have reported previously mean genetic nucleotide distances of up to 27% in the C2–V3 region of subtype F viruses from Cameroon compared with the subtype F viruses from Romania [22]. However, V3-loop peptide serology may be useful for the study of HIV-1 in countries where few genetic subtypes and less genetic diversity exist, as has been documented in Thailand [6], and recently in Côte d'Ivoire [23] and South Africa [24].

Therefore, we propose to improve PEIA subtyping by extended collaborative studies in which larger numbers of samples from diverse geographical regions will be genetically characterized by phylogenetic analysis of nucleic acid sequences and used as gold standard in PEIA subtyping. New peptides representative of different genetic subtypes will be synthesized and tested. Statistical methods such as polygram analysis [25] will be used to select amino-acid quadruplets in the immunodominant regions of *env* that are specific for a particular genetic subtype, and can be helpful in the design of new peptides. Peptides will be synthesized containing these amino-acid quadruplets. Subsequently, PEIA reactivity patterns of a single serum against multiple peptides will be analysed by spectral map analysis [25]. The latter will allow us to group sera of patients infected with a similar HIV-1 subtype, based on the homology of reactivity patterns obtained in PEIA using different peptides.

In conclusion, our findings demonstrate that V3-loop PEIA correlates poorly with genetic subtyping in sera of Belgians infected with different HIV-1 subtypes. The poor correlation between serotyping and genetic subtyping was partly due to the simultaneous presence of subtype-specific octameric sequences at the tip of the V3 loop of viruses belonging to different genetic subtypes, and the discordance between the peptides used in PEIA serotyping and that of the patients' virus.

Acknowledgement

The authors thank Dr Chou-Pong Pau (Division of AIDS, STD, and TB Laboratory Research, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA) and Simon Lister (Imperial College School of Medicine at St Mary's, London, UK) for their technical support, and Ciska Maeckelbergh for typing the manuscript.

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