Ann Ist Super Sanità 2011 | Vol. 47, No. 4: 424-428
DOI: 10.4415/ANN_11_04_15

Performance of V3-based HIV-1 sero subtyping in HIV endemic areas

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Summary. HIV-1 serosubtyping based on reactivity to peptides from the V3 region of gp120 is a low-cost and easy to perform procedure often used in geographical areas with high prevalence and incidence of HIV infection. We evaluated the performance of V3-based serotyping on 148 sera from 118 HIV-1-infected individuals living in Uganda, with estimated dates of seroconversion. Of the 148 tested samples, 68 (46.0%) specifically reacted with only one of the V3 peptides included in the test (SP), 64 (43.2%) did not react with any peptide (NR) and 16 (10.8%) reacted with two or more peptides (CR). According to the estimated seroconversion date, the large majority of samples collected early after infection belonged to the NR group. These samples had also a low Avidity Index. In contrast, samples collected later after infection belonged mainly to CR and SP groups and had also a higher avidity index. These results indicate that the performance of V3-based assays depends on maturation of HIV-specific immune response and can be significantly lowered when these tests are carried out on specimens collected from recently infected individuals.

Key words: HIV-1, antigenic variation, classification, diagnosis, serum.

Riassunto *(Performance dei test sierologici basati sulla reattività a peptidi della regione V3 nella sotto tipizzazione di HIV-1 in aree altamente endemiche per l'infezione da HIV)*. La sottotipizzazione di HIV-1 basata sulla reattività dei sieri di pazienti infettati da HIV ai peptidi della regione V3 della gp120 di HIV-1 è una procedura spesso utilizzata per sottotipizzare le varianti virali che circolano in aree geografiche con alta prevalenza ed incidenza di infezione da HIV. Allo scopo di valutare l'efficacia e l'affidabilità di questo approccio diagnostico in individui che risiedono in queste regioni, lo abbiamo applicato su 148 sieri ottenuti da 118 individui ugandesi infettati da HIV-1, con data di sieroconversione nota. Dei 148 sieri saggiati, 68 (46,0%) reagivano specificamente ad un solo peptide e potevano così essere facilmente sottotipizzati (sieri SP), ma 64 (43,2%) non reagivano ad alcun peptide (sieri NR) e 16 (10,8%) reagivano a due o più peptidi (sieri CR) e non potevano essere sottotipizzati. Quando venivano considerate le date di sieroconversione, la grande maggioranza dei campioni raccolti da individui con infezione recente apparteneva al gruppo dei sieri NR che mostravano anche un basso Indice di Avidità (AI) per gli anticorpi anti-HIV. I campioni raccolti, invece, durante le fasi più tardive della malattia appartenevano principalmente ai gruppi CR e SP e mostravano un AI più alto. Questi risultati indicano che l'efficacia e l'affidabilità del metodo di sottotipizzazione delle varianti di HIV-1 basato sulla reattività dei sieri ai peptidi della regione V3, dipendono dalla maturazione della risposta immune specifica per HIV e possono significativamente essere molto basse quando si saggiano sieri da individui con un'infezione recente.

Parole chiave: HIV-1, variazione antigenica, classificazione, diagnosi, siero.

INTRODUCTION

Continuous investigation of HIV-1 subtypes' dynamics in geographical areas with a high incidence of HIV infection and broad virus heterogeneity is important because different HIV-1 subtypes may influence the efficacy of potential vaccines against HIV, the performance of tests to detect HIV infection and the monitoring of HIV-infected patients [1]. A number of techniques, including molecular characterization of PCR-amplified genomic sequences and the HMA (heteroduplex mobility assay), have proved to be highly sensitive in identifying HIV-1 subtypes. However, due to high costs and complexity of these procedures in a poor resource setting, as it is often common in

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highly HIV endemic areas, alternative, cost-effective, easy and rapid to perform serological methods, which are based on antibody cross-recognition of synthetic peptides from the V3 variable region of the HIV-1 envelope glycoprotein gp120, have been proposed and used [2]. Nonetheless, antibody cross-recognition can be influenced by the variability of the V3 sequence due to the high heterogeneity of circulating HIV variants [3]. In addition, it is known that avidity of antibodies against HIV antigens is low early after infection, but increases over time as the infection progresses [4, 5]. Thus, V3-based tests could have a weak performance when sera from recently HIV-infected individuals are tested, a common event in areas highly endemic for HIV infection.

In order to evaluate whether V3-based serological testing can be a reliable approach for serotyping in these areas, we investigated the performance of two V3-based serological assays on samples from HIV infected people living in Uganda, a country highly endemic for HIV infection, where several different HIV-1 subtypes and recombinants circulate [6, 7].

MATERIALS AND METHODS

Participants and serum samples

A total of 148 sera from 118 individuals were collected at the Joint Clinical Research Centre in Kampala, Uganda. Individuals were derived from two independent cohort studies conducted in Uganda (PAVE Study and HC-HIV Study) [8, 9]. These studies were reviewed and approved by local Institutional Review Boards and all participants provided an informed consent. Individuals were 31 male military recruits and 87 women of reproductive age. These individuals seroconverted during the above described studies and seroconversion date was estimated as the midpoint between the dates of the last negative and the first positive HIV test. The interval between the two dates never exceeded 180 days. Specimens were considered HIV-positive when reactive using commercial EIA, confirmed by Western Blotting and/or PCR.

Sera collected within or after 180 days from the estimated seroconversion date were considered as derived from recently or chronically infected individuals, respectively, as previously described [10]. For 30 of the 118 individuals, two serial sera after seroconversion were available, for a total of 148 sera included in the study. Of these 148 sera, 114 (77%) were collected within 180 days from the estimated seroconversion date and 34 were obtained later.

V3-based assays

The V3 synthetic peptides were chosen according to previously published data [11] and corresponded to the following HIV-1 clades: A (KSVHIGPGQAFYAT), B (KSIHIGPGRAFYTT), C (KSIRIGPGQTFYAT), D (RQRTHIGPGQALYTT),E(DTSITIGPGQVFYRT), F (DKSIHLGPGQAFYAT), O (CDIQEMRIGPM AWYSMGIGGTAGNS). Peptides were synthesized with a purity > 95% (Biomol International; Matford Court, UK). Two different V3-based indirect EIA assays, Antigen Limiting-EIA (AL-EIA) and Competitive-EIA (C-EIA), were performed [11, 12]. Each sample was tested at least twice with each method.

AL-EIA was carried out as previously described [11], and sero-subtype was defined according to the peptide that was associated with the highest antibody binding at the lowest V3 peptide concentration. C-EIA was slightly modified from the previously described procedure [12]. In particular; microplate coating was done with 200 µl of an equimolar mixture of V3 peptides, each peptide diluted in Blocking Buffer (BB) (PBS containing 0.05% Tween20 and 2% dry milk) to the final concentration of 0.05 µg/µl. The subtype was defined according to the V3 peptide showing the strongest competition, as reported in literature [12].

Anti-gp120 EIA

The presence and the titers of anti-HIV-1 gp120 IgG were evaluated by an in-house EIA. Briefly, 96-well microplates were coated with SF162 (clade B)-derived gp120 momomeric protein, at a concentration of 2 ng/µl in Carbonate Buffer, overnight at + 4 °C and then washed with PBS containing 0.05% Tween 20. After saturation with BB for 90 min at 37 °C, 100 µl of each sample, diluted in BB (minimal dilution: 1:100), were added to each well and incubated for 90 min at 37 °C. One known anti-gp120 IgG positive sample and three known anti-gp120 IgG negative samples were included in each run as positive and negative controls, respectively. After incubation, anti-human IgG horseradish peroxidase-conjugated secondary antibody, diluted in BB, was added to each well and incubated for 90 min at 37 °C. Antigen-bound antibodies were then revealed by the addition of ABTS solution. Absorbance was measured at 405 nm. The cut-off (CO) value was calculated as 3 Standard Deviations above the mean of the optical density (OD) value of the three negative controls. Samples were considered positive for the presence of anti-gp120 IgG when the difference (Δ) between the sample OD and the CO was a positive value.

Avidity Index assay

The AI assay with sera from both clade B and non-B HIV-1-infected individuals has been previously described and validated [13, 14, 10]. Testing was carried out as previously reported [13]. An optical density of 0.80 has been used as a CO to discriminate recent (≤ 180 days) from established (> 180 days) HIV infections, as previously reported [13, 10].

Statistical analysis

The Student's t-test was used to compare the days from seroconversion and the AI values between sample groups with different reactivity to V3-peptides.

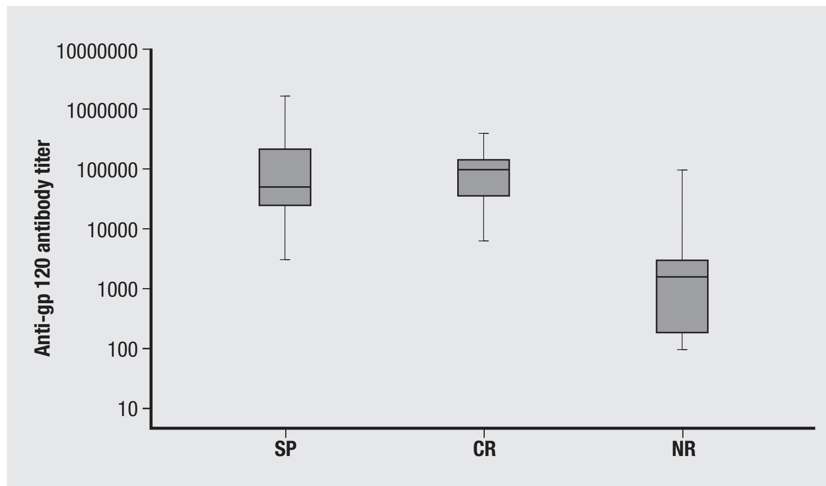

Fig. 1 | *Antibody binding to HIV-1 gp120 protein. Box-plot analysis of anti-gp120 IgG titers in the three sample groups. Groups are defined on the basis of their reactivity to V3-peptides: NR, non-reactive; CR, cross reactive; SP, specific.*

RESULTS

The 148 samples were tested using AL-EIA and C-EIA. The samples were classified according to their reactivity to the V3-peptides as following: non-reactive (NR), *i.e.* samples showing no reactivity to any of the V3-peptides in neither AL-EIA, nor C-EIA assays; cross-reactive (CR), *i.e.* samples showing reactivity to two or more V3 peptides or with discordant reactivity in the two tests; specific (SP), i.e. samples showing specific reactivity to a single V3 peptide with both methods. Surprisingly, according to the results obtained, a relevant number of 64 out of 148 (43.2%) samples were classified as NR, 16 (10.8%) as CR and only 68 (46.0%) as SP.

We have, therefore, investigated if the lack of reactivity to the V3-peptides of the 64 NR sera was due to the absence or to very low titers of anti-HIV-1 antibodies against the whole Env protein. To this aim, all 148 serum samples were tested for the presence of IgG antibodies against a monomeric, clade B-derived gp120 *(Figure 1)*. Six samples, all classified as NR in the V3 testing, showed no reactivity to the gp120. All the other 142 sera were positive for anti-gp120 IgG antibodies. The median titer of anti-gp120 antibodies in the NR samples was 1600 (range: 0-102 400), as compared to 102 400 (range: 6400-409 600) in the CR samples and 51 200 (range: 100-1 638 400) in the SP samples *(Figure 1)*.

Reactivity of sera against V3-peptides was then analyzed taking into account the estimated seroconversion dates *(Figure 2)*. Results shown in *Figure 2* indicate that most of the sera collected within 180 days from the estimated seroconversion date are classified as NR. Only a few of the NR sera were collected after 180 days from the seroconversion date and these sera were all obtained in the period from 181 to 240 days. Conversely, most of the SP and CR sera were obtained later after infection, the majority of them being collected after 180 days from the estimated seroconversion date.

In addition, for 14 individuals a couple of sera collected sequentially were also available: the first specimen was taken within 60 days from estimated seroconversion date and the second one after 60 days. All early collected sera were NR, whereas 43% of the latter specimens were classified as SP (data not shown).

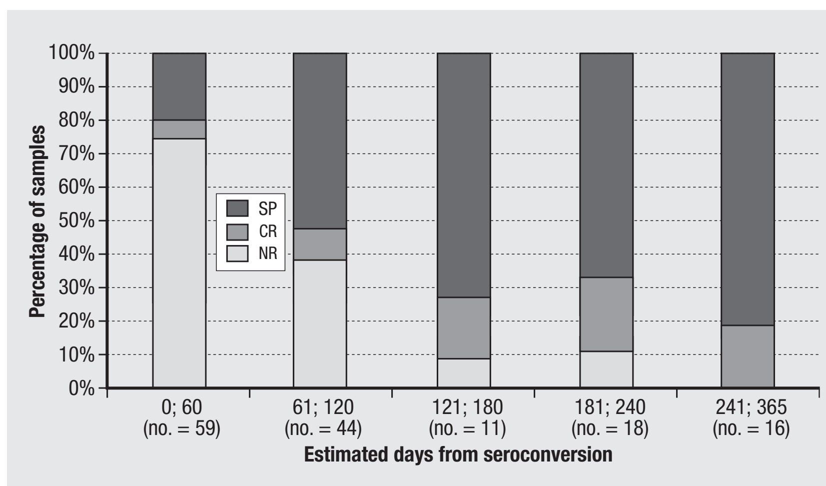

Fig. 2 | *Antibody reactivity to V3-peptides according to the estimated date of seroconversion. Sample groups are shown in the legend, where NR is the non reactive group, CR the cross-reactive group, and SP the specific group. Samples have been stratified on days of interval from seroconversion. Sample number in each interval is indicated in brackets.*

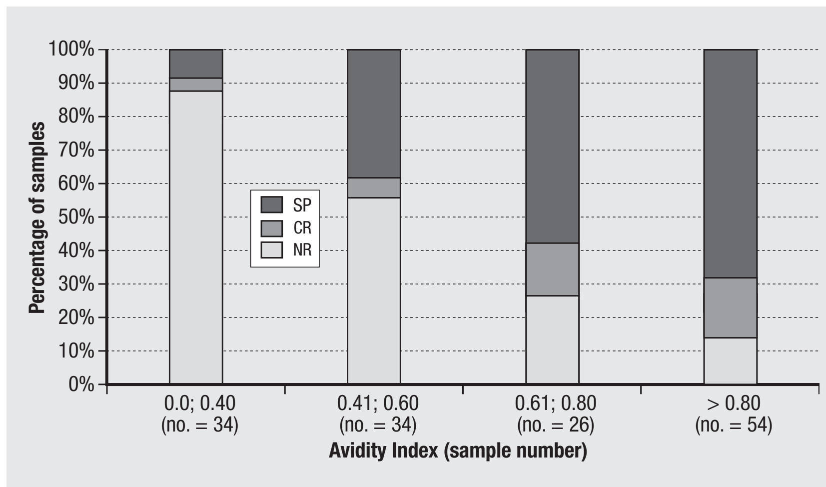

Fig. 3 | *Antibody reactivity to V3-peptides according to Avidity Index. Sample groups are shown in the legend, where NR is the non reactive group, CR cross-reactive group, and SP specific group. Samples have been stratified on Avidity Index values grouped in intervals, as shown. Sample number in each interval is indicated in brackets.*

To investigate whether the lack of V3 reactivity of NR sera could be explained by low avidity of antibodies to HIV antigens, all sera were tested for AI *(Figure 3)*. Almost all NR sera showed an lower than 0.80, indicating that these sera had a still low avidity for the HIV antigens. Conversely, SP and CR sera had a higher Avidity Index for HIV antigens (AI > 0.80).

As the trend of association between the days from seroconversion and the AI values was comparable in CR and SP samples, we grouped these samples into the reactive cluster (CR + SP). The mean of days from seroconversion of the NR samples was 60.4 (95% CI: 52.2; 68.6), whereas that one of the CR + SP samples was 150.0 (95% CI: 130.4; 169.3). The difference between the two means was statistically significant ($p < 0.0001$). Similarly, the mean of AI values of the NR samples was 0.49 (95% CI: 0.44; 0.54), whereas that one of the CR + SP samples was 0.79 (95% CI: 0.74; 0.84). The difference between the two means was, again, statistically significant ($p < 0.0001$).

DISCUSSION

In order to evaluate the performance of V3-based serological subtyping in highly HIV endemic geographical areas, we tested serum samples from Ugandan individuals with estimated date of seroconversion, using two of the most used V3-based serotyping methods, AL-EIA and C-EIA [11, 12]. A relevant aliquot (43.2%) of the tested samples did not react to any V3 peptide (NR sera) with both serotyping approaches. All but six sera had, instead, anti-gp120 IgG antibodies. These data indicate that antibody response maturation could be partially developed in the group of individuals with V3-non-reactive antibodies. This hypothesis is in line with data reported in literature indicating that anti-HIV antibodies, in particular anti-gp120 IgG, can grow up in titers until about 1 year from infection [15, 17].

To further confirm whether a still immature antibody response could influence V3 serotyping performance, we stratified the NR, CR and SP sera according to seroconversion dates and found that almost all NR sera derived from recently seroconverted patients.

Since almost all the sera had antibodies against gp120, the low performance of V3-based assays in the NR sera could be due to the absence of V3-specific antibodies or, if present, to a still low avidity against the V3 epitopes. In fact, it has been described that antibody avidity to HIV antigens is low in the first months after the seroconversion and increases afterwards, as a consequence of the maturation of the humoral immune response [5, 4]. Therefore, still immature antibodies may have a low avidity for the V3 region and hamper V3 serotyping performance. The AI assay confirmed that NR sera had low avidity against HIV antigens. This result is also strengthened by our observation that in individuals for whom more than one sequential serum sample was available, antibodies against V3 peptides were present only in the sample collected later (data not shown). Our data are in agreement with those from Zwart *et al.*, which show that anti-V3 domain antibodies become detectable even up to 13 months after antibodies against other HIV-1 proteins, including gp120, have been developed [18].

Certainly, the high heterogeneity of the circulating HIV variants plays also a role in the low ability of some sera to bind V3 peptides. In fact, our results show that a small portion of cross-reactive (CR) sera is persistently present, in all seroconversion date clusters *(Figure 2)* and AI clusters *(Figure 3)*. In addition, the fraction of CR sera is proportional to the quota of sera reacting to only one V3 peptide (SP sera).

These results indicate that V3-based assays performance can have, unavoidably, some application limits, due to virus heterogeneity. More importantly, the performance of the assay can be further lowered when sera from recently infected individuals are tested, due to still immature antibody response and low avidity against HIV antigens.

These observations should be taken into account when planning HIV subtype investigations using a serotyping approach in populations with high HIV incidence.

Acknowledgments

This work was supported by grants from the National AIDS Research Program, Ministry of Health, Italy, subproject "HIV/AIDS Epidemiology". We thank C. Rovetto and E. Salvi for the technical support and G. Fornari Luswergh for her excellent editorial assistance. We are indebted with Mario Falchi for his help on editing the figures.

Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

Received on 29 April 2011.
Accepted on 19 September 2011.

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