

Low avidity antibody: a reliable method to diagnose a recent HIV-1 infection

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SUMMARY

Standard serological tests (both EIA and Immunoblotting) have reached high levels of sensitivity and reproducibility, but do not indicate whether infection is recent or longstanding. Since many patients with HIV-1 infection are not usually diagnosed until symptom presentation, the possibility to distinguish between acute and chronic infection has become increasingly important for the purposes of therapeutic decision-making, partner notification and epidemiological surveillance.

We evaluated a guanidine-based-antibody-avidity assay in a selected group of recent (within six months from seroconversion) and chronic (more than forty eight months) HIV-1 infections in an attempt to shed more light on the significance of the avidity index in establishing the time of infection.

Sera from newly infected individuals showed a low mean avidity index (ranging from 0.35 to 0.60 with a standard deviation 0.09) at baseline and a clear increasing value at the following times of observation. Our data showed that an avidity index <0.70 might be presumptive of infection occurring within 9 months.

Avidity index levels might distinguish between acute and chronic infection. The method is semi-automated, inexpensive and easy to perform, and estimates the time elapsed from seroconversion, thereby identifying a recent infection.

KEY WORDS: HIV-1, Acute infection, Avidity index

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INTRODUCTION

HIV-1/2 diagnosis in adults is routinely performed using serological tests able to detect specific antibody to the virus (Kassutto and Rosenberg,

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2004). During recent years, serological tests have rapidly evolved in combined antigen and antibody tests reaching high levels of sensitivity for the detection of primary infection (Iweala OI, 2004; Hecht *et al.*, 2002). Despite the introduction of new generation assays able to reduce the "window-period" (Weber *et al.*, 2002; Bourlet *et al.*, 2005), the goal of most diagnostic tests remains the detection of HIV infection as early as possible.

It is well known that acute HIV infection is only sometimes characterized by a mononucleosis-like syndrome in addition to fever, pharyngitis, malaise, lethargy, maculopapular rash, mucous membrane ulceration, lymphadenopathy and headache (Schacker *et al.*, 1998; Busch and Satten, 1997; Tattevin *et al.*, 2007). Moreover since it has recognized that many patients with HIV-

1 infection are not usually diagnosed until symptom presentation or onset of opportunistic infections (Advancing HIV Prevention, 2003), distinguishing between acute and chronic infection might be crucial to establish the incubation period and to make decisions regarding drug treatment, partner notification and epidemiological surveillance (McFarland *et al.*, 1999; Suligoi *et al.*, 2003; Chawla *et al.*, 2007).

Although a high level of sensitivity and reproducibility has now been reached, standard serological tests (both EIA and Immunoblotting) do not indicate whether infection is recent or long-standing.

It is well known that in HIV-1 infection, as in several other viral diseases, antibody avidity increases over time after infection and is able to discriminate between recent and established infections in a single serum sample (Le Guillou *et al.*, 2001; Riou *et al.*, 2000; Suligoi *et al.*, 2002). Avidity is, in fact, a measure of the strength of the binding between IgG antibody and the corresponding antigen, a property that increases over time (Thomas *et al.*, 1996). On the other hand, few and contrasting data have been reported on the usefulness of specific IgM and or IgA detection during the early phase of infection (Hashida *et al.*, 2000; Wu and Jackson, 2002). In particular, assays attempting to reveal IgM and/or IgA are fairly insensitive because their serum concentration is often very low (Hecht *et al.* 2002). The identification of newly acquired human immunodeficiency virus type 1 (HIV-1) infection guides public health intervention programs, in addition to providing information on the dynamics of the epidemic, transmission networks and patterns of transmitted drug resistance (Chawla *et al.*, 2007). For these reasons this study evaluated a guanidine-based-antibody-avidity assay in a selected group of recent (within six months from seroconversion) and chronic (more than forty eight months) HIV-1 infections in an attempt to shed more light on the significance of avidity in establishing the time of infection.

MATERIALS AND METHODS

Patients and sera

In this study, we enrolled 72 HIV-1 positive individuals. Thirty individuals represented the HIV-1

acute infection group (Group A) because they satisfied the following parameters: seroconversion ascertained within the last 6 months by immunoenzymatic assay (Vironostika, BioMerieux, Boxtel, the Netherlands) and Immunoblot (Chiron RIBA, HIV-1/HIV-2, Chiron Co., Emerville, CA, USA) with a previous negative serum sample determined by the same diagnostic procedures. To avidity assay analysis, 90 sera were recovered from Group A patients allowing a longitudinal study.

As controls we included 42 infected patients (group B) with a long-standing infection (more than 48 months). Among these subjects, most (29 subjects) had been under antiretroviral therapy (HAART) for at least a year.

The two groups (newly and long-standing infected) were age and sex matched, sharing similar sex composition (23 men plus 7 women *versus* 31 men plus 11 women) and mean age (35 ± 9 versus 38 ± 9.5).

All samples were derived from patients who attended the infectious disease outpatients' clinic between January 2003 and November 2006, showed a HIV-1 B subtype infection and had lived in Italy at least in the last ten years.

An intensive medical evaluation excluded a history of drug abuse and transmission was established to be by sexual contact in all subjects. All the patients included in the study were enrolled after informed consent according the Helsinki declaration of 1975. All naïve patients had never been treated by HAART since they had declined drug treatment or did not meet the criteria for inclusion in any therapy regimen.

Detection of low avidity antibody to HIV-1/2

A guanidine-based avidity detection assay was used for the analysis of avidity antibodies in all sera. Each serum was divided into two aliquots and diluted 1:10 with phosphate buffered saline (PBS) or 1M guanidine (G) respectively. Samples were incubated for 10 minutes at room temperature and tested using the automated AxSYM HIV 1/2 assay (Abbot Diagnostics, Delkenheim, Germany) as indicated by the manufacturer (Suligoi *et al.*, 2003). Antibody avidity index (AI) was calculated analyzing absorbance/cut off (S/CO) of samples as follows:

$$AI = \frac{\text{Guanidine aliquot S/CO (11)}}{\text{PBS aliquot S/CO}}$$

All the sera were tested in duplicate and the mean value of optical density (OD) was considered. The coefficient of variation (CV) of all tested samples was always <10%.

Peripheral blood CD4 lymphocytes at baseline

Peripheral blood CD4 lymphocytes were counted by flow cytometry (FACScan, Becton and Dickinson, Mountain View, CA) using commercially available monoclonal antibody (Becton-Dickinson).

HIV-1 RNA quantification at baseline

The 30 individuals who recently seroconverted and 42 individuals with a long-standing infection were requested - within a couple of days - to give a heparinized sample to verify the presence of viral replication in peripheral blood. All the available whole blood samples were centrifuged at 2500 rpm for 20 min and plasma was stored at -80°C until use and analyzed for HIV-1 RNA viral using the Quantiplex HIV-RNA-3.0 assay (Chiron Corporation, Emeryville, CA, USA), according to the manufacturer's instructions. The amount of HIV RNA levels was expressed as copy number per ml of plasma and the lowest detection limit of the assay was 50 copies/ml.

Statistical analysis

Statistical analysis was performed by Student's T test. The data were expressed as mean \pm standard deviation (SD).

RESULTS

All the samples (groups A and B with similar epidemiological, age and sex characteristics as already cited in material and methods.) derived from the patients enrolled in the study were first analyzed for the presence of specific HIV-1 antibody by ELISA and Western Blotting (WB). Avidity index (AI) was established in all sequential sera (from first positive EIA/WB result afterwards) obtained from group A (newly infected patients) and sera from group B (people with a long-standing infection).

Baseline samples (first EIA IgG positive serum) were examined for the presence of viral replication (HIV-1 viral load) and for CD4 levels. In par-

ticular, avidity index (AI) was established in all sequential sera obtained from groups A and B. In Group A, we performed a longitudinal study through three sequential serum samples from each patient. The first serum is the baseline seroconversion serum (time 1; first EIA/WB reactive sample) followed by another two sera obtained after 3 (time 2) and 6 (time 3) months. On the other hand, we recovered a single serum sample for each patient belonging to control Group B.

Analysis of HIV-1 acute infected patients

ELISA and Western blotting. At first control, all the samples showed a reactivity by immunoenzymatic assay ranging from medium [sample's absorbance \geq from once to twice the cut-off value] to high [sample's absorbance \geq more than three or four times the cut-off value]. In particular all the high-reactive EIA (HRE) samples showed a clear positivity by WB according World Health Organization criteria (Centers for Disease Control, 1989) (Table 1).

Among the five serum samples with a medium reaction (MRE) EIA, two - showing the presence of antibody to two envelope proteins (gp41 and gp120) alone (n° 5) or associated with p24 and/or17 (n° 25) - were also classified as positive and three (n° 1, 4, 23) out of five showing antibody to only gp41, with no reactivity to core proteins, were classified as "indeterminate" on the basis of international criteria for HIV-1 positivity (Centers for Disease Control 1989; Lazzarotto *et al.* 2001).

RNA viral load and CD4 cell count. As shown in Table 1, all the patients enrolled in the study showed a moderate to high level of viral replication, ranging from 100 to 500,000 HIV-RNA copies/ml (mean 1.7×10^5 HIV-RNA copies/ml). CD4 cell count showed variable levels ranging from 27 to 890 cells (mean baseline value of 487 ± 240 cells/mm³).

In addition, as shown in Table 1, all the patients enrolled in the study showed a moderate to high level of viral replication, ranging from 100 to 500,000 HIV-RNA copies/ml (mean 1.7×10^5 HIV-RNA copies/ml). CD4 cell count showed variable levels ranging from 27 to 890 cells (mean baseline value of 487 ± 240 cells/mm³).

Avidity index

All these patients - with a documented negative HIV-1/2 test followed by a positive test within six

TABLE 1 - Antibody to HIV-1 (EIA and WB), RNA value and CD4 levels of the 35 newly infected patients at baseline.

Patients N°	First sample (EIAIgG)	WB reactivity to:	RNA value	CD4 levels
1.	MP	gp41	500000	200
2.	HP	gp120,gp41,p31	300000	279
3.	HP	all HIV-1 proteins	500000	691
4.	MP	gp41	100000	470
5.	MP	gp120,gp41	500000	341
6.	HP	gp120,gp41,p31,p24	64000	161
7.	HP	gp120,gp41,p24	41000	706
8.	HP	gp120,gp41,p31	98000	394
9.	HP	all HIV-1 proteins	500000	170
10.	HP	gp120,gp41,p24,p17	200000	394
11.	HP	gp120,gp41,p24,p17	340000	27
12.	HP	all HIV-1 proteins	300000	601
13.	HP	gp120,gp41,p24	200000	215
14.	HP	all HIV-1 proteins	480000	147
15.	HP	all HIV-1 proteins	120	785
16.	HP	all HIV-1 proteins	100	472
17.	HP	gp120,gp41,p24,p17	2500	707
18.	HP	all HIV-1 proteins	2800	690
19.	HP	gp120,gp41,p24,p17	480	559
20.	HP	all HIV-1 proteins	49000	710
21.	HP	gp120,gp41,p24,p17	6000	459
22.	HP	gp120,gp41,p24,p17	66000	890
23.	MP	gp41	50000	478
24.	HP	all HIV-1 proteins	26000	820
25.	MP	gp120,gp41,p24,p17	15000	569
26.	HP	all HIV-1 proteins	360000	374
27.	HP	all HIV-1 proteins	160000	401
28.	HP	all HIV-1 proteins	170000	265
29.	HP	all HIV-1 proteins	16000	850
30.	HP	all HIV-1 proteins	48000	793

High positive (HP): sample's absorbance > more than three or four time the cut off value value. Medium positive (MP): [sample's absorbance > from once to twice the cut off value]. WB: Western blotting. RNA value: Plasma HIV-1 RNA copies/ml. CD4 levels: CD4 count cells × 10⁶ per l.

months - were monitored at baseline (Time 1: first EIA/WB reactive sample) and subsequently [Time 2 (3 months later) and time 3 (6 months later)] for the presence of avidity index, performed as described in materials and methods.

At baseline (time 1), the avidity index showed OD values ranging from 0.35 to 0.64 (AI mean 0.48 ± 0.1) and a clear increasing value in the following times of observation (AI mean 0.70 ± 0.099 and 0.97 ± 0.04 at time 2 and 3 respectively as shown in Figure 1).

Taking into consideration that values obtained at baseline represent the mean avidity index of sera collected within 6 months from seroconversion, and that following values represent the avidity index of sera collected 3 and 6 months later, it is possible to establish a cut-off to identify an infection occurring in a time-range from 3 to 9 months.

Moreover, among sera analyzed at time 2, twenty samples still showed AI value ≤ 0.75 , correlated, as reported by other authors (Suligoi *et al.*, 2002; WHO Collaborating Group on Western Blotting, 1990) to a recent seroconversion. Finally, at last observation point, high AI values (from 0.88 to 1.0 OD), very similar to those obtained in long-lasting infection (see below), were obtained.

Analysis of long-term infected patients

As controls we included in our study of 42 serum samples (group B) from infected patients with a long-standing certified infection (more than 48 months). All these subjects were characterized by high reactivity to both EIA and WB and variable RNA levels (ranging from a mean value of 5.9×10^3 in treated patients to 2.4×10^4 in untreated subjects). Avidity index mean did not show any significant difference ($p=0.60$ Mann Whitney test) between treated and untreated patients (0.964 versus 0.963). Moreover virus replication in these patients [both treated (mean value 2.4×10^4) and untreated (5.9×10^3)] was significantly lower when compared with RNA levels (1.7×10^5) obtained in newly infected subjects at baseline [T test $p=0.007$ for untreated patients and $p=0.00$ for treated patients]. In contrast, there was no association with CD4 counts [T test= 0.840 (newly seroconverted *versus* never treated long-standing infected patients) and T test= 0.904 (newly seroconverted *versus* treated long-standing infected patients)].

DISCUSSION

Since the possibility to distinguish between early and established HIV infection may have viro-

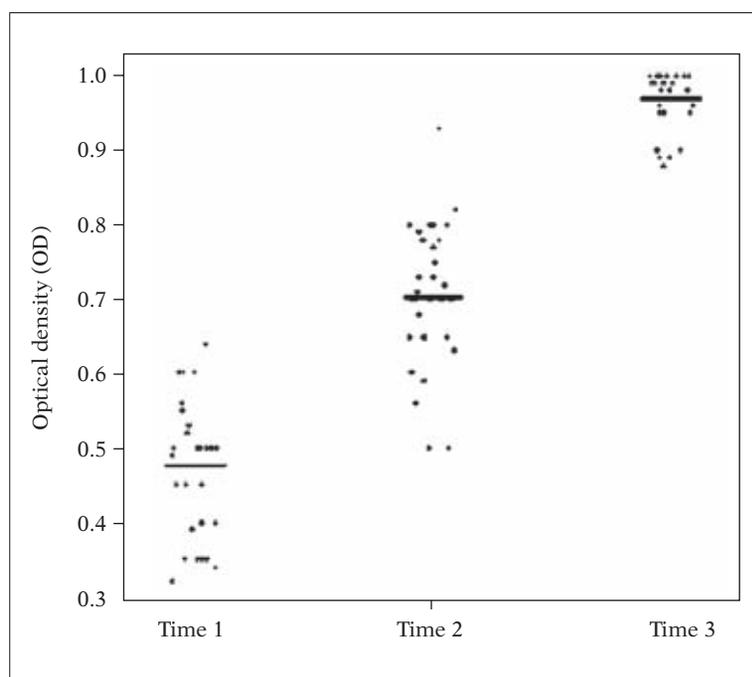


FIGURE 1 - Avidity index values observed with the 90 serum samples collected from the 30 newly seroconverted patients at different times of observation (from time 1 to time 3).

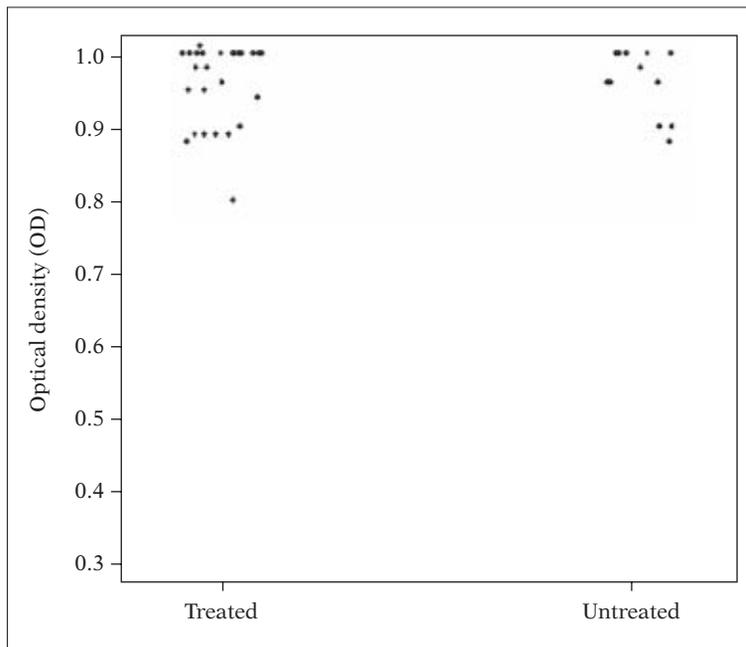


FIGURE 2 - Avidity index values in long-standing infected patients in the presence and absence of antiretroviral treatment.

logic, immunologic, and clinical benefits, we studied the course of antibody to HIV in recent seroconverters by a procedure based on the avidity index, proposed as a marker of recent infection (Hecht *et al.*, 2002, Suligoi *et al.*, 2003; Chawla *et al.*, 2007; Le Guillou *et al.*, 2001; Riou *et al.*, 2000). The AI, routinely used for several infectious diseases such as rubella, cytomegalovirus and *Toxoplasma gondii* infection (Lazzarotto *et al.* 2001; Mubareka *et al.*, 2007; Wilson *et al.*, 2006), is supported by the rationale that antibody produced in the first phase of infection shows a lower antigen avidity, that increases over time. This method is able to estimate the time elapsed from seroconversion, thereby identifying a recent infection. Even if other serological tests, such as IgM detection can be used, these assays are fairly intensive because the serum concentration of IgM is usually low (Hashida *et al.*, 2000; Wu and Jackson, 2002). In addition to serological assay, a diagnostic test directly detecting the virus (p24 antigen assay, HIV nucleic acid based assays and peripheral blood mononuclear cell culture) might represent a valid alternative to be applied in the first phase of infection (Iweala *et al.*, 2004; Re *et al.*, 2006). But the difficulty in obtaining a new heparinized blood sample favours the application of a sensitive serological test that can be performed in a single serum sample.

In our study we enrolled a group of recent seroconverters to obtain a detailed understanding of the time course of low avidity antibody. Even if an individual variability in the rapidity of avidity maturation exists, a cut-off of 0.8 has been proposed for antibody avidity assay (Suligoi *et al.*, 2003; Chawla *et al.*, 2007; Suligoi *et al.*, 2002) with an excellent agreement between guanidine-based avidity assay and detuned assay and a very low number of discrepant results (Chawla *et al.*, 2007). Previously Chawla *et al.* (2007) demonstrated that 24% of HIV-1 infections could be identified as "recent infection" using a conservative cut-off <0.75.

Our study followed up a group of newly infected patients whose known date of seroconversion was estimated within the last six months. In accordance with previous reports, we found low AI (ranging from 0.35 to 0.6 OD) at baseline and an increasing value over time. Taking into consideration that most recently seroconverted patients present a low index up to time 1 (time ranging 3-9 months from seroconversion) the possibility of routine application of such a test to identify persons in the early phase of the disease, could provide important indications to clinicians concerning the timing of infection, partner notification and the possibility to institute early antiretroviral treatment.

Our data are in agreement with previous papers (Suligoi *et al.*, 2003; Puchhammer-Stock *et al.*, 2005) where an avidity index less than 0.6 (Suligoi *et al.*, 2003) or 0.8 (Puchhammer-Stock *et al.*, 2005) was considered presumptive of a recent infection. In fact our results showed that all the sera from patients with an estimated seroconversion in the last six months (time 1) presented a low AI level never superior to 0.65. On the other hand, when the time elapsed from infection increased (time 3) all the sera showed high AI levels, comparable with that obtained from people with a long-standing infection.

Moreover, when we focused on RNA viral load detectable at baseline we found a positive association between low Avidity index and high virus replication observed in the early phase of HIV-1 infection, as previously observed and the lack of correlation between CD4 levels and avidity (Suligoi *et al.*, 2003).

However a high AI value (mean OD 0.98) might be suggestive of a chronic infection even if an antibody decrease during advanced disease (AIDS defining conditions) has been observed, supporting the idea that changes in qualitative and quantitative aspects of anti HIV-1 specific antibody response can be expected in the last phase of disease (Suligoi *et al.*, 2003).

In summary, our follow-up study of a group of sequential samples coming from newly infected subjects confirms previous investigations but also adds information on the maturation antibody's time using an inexpensive straightforward procedure. It provides useful indications to clinicians for partner notification and therapeutic decision-making, even if result interpretation should always occur in the context of several clinical and virological parameters.

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