Recent and long-lasting infections: the need for avidity testing in HIV-1 infected subjects

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—————— SUMMARY ——————

Standard serological tests have reached high levels of sensitivity and reproducibility but do not indicate whether infection is recent or long-standing. Among the 59960 sera analyzed for HIV positivity at the Retrovirus Laboratory, Operative Unit of Microbiology, Bologna, Italy, from January 2010 to July 2011, 134 samples showed an initial positive result.

Application of the avidity test, able to distinguish between recent or long-standing HIV infection, classified 59 subjects as recently infected and 75 as chronically infected.

Besides all the public health implications, the distinction between acute and chronic infection might serve to establish the time of infection and therefore reach any potential partners who might have been infected in a specific period of time. Although our results are limited to subjects referred to our laboratory and hence represent only a limited part of the problem, the routine application of methods able to distinguish recent from long-lasting infection could help monitor disease incidence, identify high-risk groups, and enhance epidemiological conclusions.

KEY WORDS: HIV diagnosis, Avidity test, Recent and long lasting infections.

INTRODUCTION

Although several efforts in the last decade have focused on HIV prevention of HIV-1/2 infection to reduce new transmissions, 27116 newly diagnosed HIV infections were reported by 28 countries of the European Union and European Economic Area (EU/EEA) in 2010, with evidence of continuing transmission and no clear signs of decline (Likatavicius G and van de Laar MJ, 2010). Moreover a high proportion of the total number of diagnosed HIV infections were among MSM followed by heterosexuals.

As epidemiological data can include individuals infected recently as well as those infected several years ago. the available information on newly diagnosed HIV infections does not reflect the incidence of infection. Nonetheless, serosurvey systems can offer an accurate description of the current transmission rates of HIV infection (Mammone *et al.,* 2012).

Since the early identification of newly acquired HIV-1 is crucial to yield information on the dynamics of the epidemic, transmission networks, patterns of transmitted drug resistance, and to obtain virologic, immunologic and clinical benefits, several data highlight the need to introduce routine laboratory tests able to distinguish recent from long-standing infections. To this end, the measurement of antibody avidity, which increases over time following seroconversion, might represent a useful tool to identify a new HIV infection, as already demonstrated for several infectious diseases such as rubella, cytomegalovirus and *toxoplasma gondii* infection (Lazzarotto *et al.,* 2001; Mubareka *et al.,* 2007; Wilson *et al.,* 2006).

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If an early diagnosis provides useful indications for an achievable immunological and virological follow-up, admission to a drug treatment protocol and prompt partner notification, a late diagnosis is frequently associated with adverse outcomes including an increased risk of clinical progression, blunted immune recovery on highly active anti-retroviral therapy and a greater risk of drug toxicity (Waters and Sabin, 2011). The proportion of subjects diagnosed late is largely unknown and varies according to geographical areas, setting and definitions (D'armino Monforte *et al.,* 2011). Although several HIV assays able to detect specific antibody are available, stigma prevents some people attending public health centers for testing and they discover they are HIV-positive late after infection. In view of this, the Centers for Disease Control and Prevention (CDC, 2003) has encouraged expanded HIV testing to identify the estimated 21% of persons living with HIV who remain undiagnosed (Campsmith *et al.,* 2010).

However, HIV counseling and testing are the mainstays of HIV prevention programs since the licensure of the first enzyme immunoassay to detect HIV antibodies in 1985. Since then testing technologies have improved, offering new possibilities to utilize HIV testing as a prevention approach (Heffelfinger *et al.,* 2008).

Because HIV testing represents secondary prevention for people who learn their HIV status, and primary prevention for the community, this report focuses on HIV serological results obtained during the last three years. To this end, among all the serum samples analyzed for HIV 1/2 antibody detection in our laboratory, we selected a restricted number of sera that resulted positive for the first time with any previous HIV test or with a previous negative result. Our aim was to distinguish recent from established infection by analysis of the avidity index, a robust method (Sweeting *et al.,* 2009) easily performed in a routine laboratory setting, in an attempt to shed more light on the importance of the early diagnosis of HIV infection.

MATERIALS AND METHODS

Patients and sera

From January 2010 to July 2012 (31 months), 59660 serum samples from hospitalized or laboratory admitted subjects were analyzed for the presence of antibody to HIV-1/2.

A total of 603 HIV positive samples were identified according to standard algorithms (CDC 1989; O'Brien *et al.,* 1992; Constantine *et al.* 2005; Masciotra *et al.,* 2011) for screening and confirmatory tests (Table 1).

Among this group, we selected 138 sera from patients who showed a positive result for the first time (Table 2). In particular, all selected patients met the following criteria:

1) a first-time indeterminate or positive enzyme-linked immunosorbent assay;
2) western blot profile positive or compatible with ongoing seroconversion;
3) HIV-1 RNA detectable plasma levels. We excluded from our database archives all samples from a known previous HIV serum positivity.

The baseline characteristics of the patients included in the study are shown in Table 3. All the subjects were enrolled after informed consent according the Helsinki declaration of 1975. Intensive medical evaluation established that transmission had occurred by sexual contact in all subjects excluding any drug abuse. All the samples were analyzed by western blotting assay, guanidine-based avidity assay, RNA viral load and CD4 count.

ELISA and Immunoblot assay

Each sample was tested by ELISA (CMIA Abbott, USA) and western blotting using INNO-LIA™ HIV-1/2 score assay (Innogenetics, Gent, Belgium) according to the manufacturer's instructions. Immunoblot strip was interpreted following the criteria of the INNO LIA HIV-1/II score test, read and interpreted by LIRAS™ software, providing scanner calibration to ensure accurate readings of results, as previously described (Re *et al.,* 2010).

HIV antibody avidity assay

For each serum sample, two aliquots were prepared by a 1/10 dilution with phosphate buffered saline (PBS) and 1M guanidine (G) as denaturing agent respectively. Both aliquots were shaken and incubated for ten minutes at room temperature and then tested as previously described (Re *et al.,* 2008, Suligoi *et al.,* 2002) using an ELISA platform from Vironostika, BioMerieux (Boxtel, the Netherlands). Sample/cutoff (S/CO) ratios

were calculated and the avidity index of HIV antibodies was computed as (S/CO ratio of the G aliquot)/(S/CO ratio of the PBS aliquot). A cut-off of 0.75 was selected according to previous observations (Selleri *et al.,* 2007, Re *et al.,* 2008).

TABLE 1 - *HIV 1/2 positivity from January 2010 to July 2012 observed in the Retrovirus Laboratory, Bologna, Italy.*

	Total number of serum samples assayed by EIA	Number of HIV positive by EIA and/or WB
January-December 2010	21960	186
January-December 2011	25045	207
January-July 2012	12655	210
Total	59660	603

TABLE 2 - *Serum samples with HIV positivity by EIA and/or WB and serum samples with an initial EIA and WB positivity, selected in the period considered (from January 2010 to July 2012).*

	Number of serum samples with an initial EIA and WB positivity
January-December 2010	46/186 (24.7%)
January-December 2011	49/207 (23.7%)
January-July 2012	39/210 (18%)
Total	134/603 (22.2%)

TABLE 3 - *Baseline characteristics of HIV-infected patients (134 subjects) with an unknown previous HIV serum positivity.*

Gender	*116 men; 18 women*
Age (mean years ± SD)	39.5+10 (men) 38.8±11 (women)
CD4 count cells × 10^6 per l (mean)	400+264
Plasma HIV-1 RNA copies/ml (mean)	$3.4 \times 10^5 \pm 2.9 \times 10^5$

HIV-1 RNA quantification

HIV RNA quantification was performed in all plasma samples obtained within one week after the first ELISA positive samples from all subjects who met the inclusion criteria. Samples were analyzed for HIV-1 RNA level using the COBAS AmpliPrep/Cobas TaqMan HIV-1 Test (Roche Diagnostics GmbH, Mannhein, Germany) according to the manufacturer's instructions. HIV RNA levels were expressed as copy number per ml of plasma and the lowest detection limit of the assay was 20 copies/ml.

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

Statistical analysis
Simple descriptive statistical measures for the avidity index were calculated (mean values and range). Fisher test and Wilcoxon Rank Sum test were used for immunoblot and avidity index results, respectively.

HIV-1 subtyping

HIV-1 subtyping was conducted by phylogenetic analysis using a neighbor-joining method with the Kimura 2-parameter distances (DNADIST and NEIGHBOUR modules of the PHYLIP software) [http://evolution.genetics.washington.edu/phylip.html]. All *pol* sequences, available for each sample, were used for alignment with reference strains of a known subtype derived from the Los Alamos database [www.hiv.lanl.gov] using ClustalX and edited manually by BioEdit.

RESULTS

Among the 59960 sera analyzed for HIV positivity from January 2010 to July 2012, 603 presented a specific level of HIV antibody by conventional tests.

HIV positivity by EIA and avidity index

By consulting our laboratory databases, we selected only positive samples without any previous record of EIA and/or WB HIV positive test.

TABLE 4 - *Serum samples with an initial EIA and WB positivity divided into recent (RI) and long-lasting infection (LLI) on the basis of the avidity index (<0.75 OD and > 0.76 OD respectively).*

	Recent infections	*Long-lasting infections*	*Total*
January-December 2010	27 (59%)	19 (41%)	46
January-December 2011	16 (30%)	37 (70%)	49
January-July 2012	16 (41%)	23 (59%)	39
Total	59 (44%)	75 (56%)	134

On this basis we enrolled only 134 HIV-1 positive samples (Table 2) with the following parameters: a first positive or indeterminate or enzyme-linked immunosorbent assay followed by a western blot profile positive or compatible with ongoing sero-conversion. Most of patients were men (116 men versus 18 women).

All the samples with a settled HIV positivity were analyzed for antibody avidity levels and divided into two groups on the basis of AI: 59 sera with low avidity levels (AI ≤0.75OD classified as sera from a presumed recent infection) and 75 with high avidity levels (AI ≥0.76 OD classified as sera from subjects with a presumed long-lasting infection) (Table 4). Results obtained by avidity test disclosed a recent infection in more than 40% of subjects enrolled in our study.

Western blotting assa
Antibody patterns were studied by western blotting analysis in all positive samples without any previous record of EIA and/or WB HIV positive test. As shown in material and methods we considered positive the antibody response to specific HIV protein when the intensity of the antigen line reaction was between 2+ and 4+ positive rating, validated by software comparison with positive and negative control strips. The specific HIV-1 proteins used as target in Inno Lia system are the gp120, gp41, p31, p24 and p17 viral proteins.

The percentage of serum reactivity to HIV-1 proteins differed widely in the two groups of serum samples (RI versus LLI). In particular, the WB analysis performed in sera from patients with a presumed old infection (more than six months from seroconversion) showed a complete pattern of antibody reactivity against all *gag*, *env* and *pol* proteins. Moreover, a significantly lower reactivity (p>0.001) to HIV-1 proteins has been found in sera from newly infected individuals (within six months from seroconversion). In this group on-
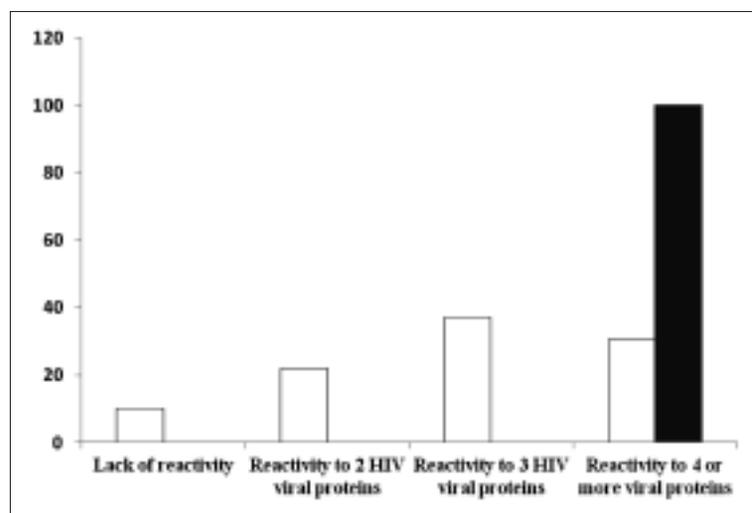

FIGURE 1 - *WB reactivity in serum samples from newly (white square) and chronically (black square) HIV-infected patients from 2010 to mid 2012.*

TABLE 5 - *Virological and immunological markers in detected new and long-lasting infections.*

	New HIV infections	*HIV RNA viral load*	*CD4*
2010	27	$2.0x10^5 \pm 1.8x10^5$	777 ± 360
2011	16	$1.1x10^5 \pm 2.3x10^5$	608.9 ± 572
2012	16	$9.0x10^5 \pm 2.8x10^5$	498.5 ± 545
Total	59	$4.1x10^5 \pm 2.3x10^5$	628.1 ± 492.3
	Long-lasting HIV infections	*HIV RNA viral load*	*CD4*
2010	19	$2.3X10^5 \pm 1.7x10^5$	188.9 ± 230
2011	37	$2.1x10^5 \pm 3.6x10^5$	172.7 ± 239
2012	23	$4.3x10^5 \pm 7.9x10^5$	160.6 ± 189
Total	79	$2.9x10^5 \pm 4.4x10^5$	174.0 ± 219

ly 18 sera out 59 (about 30%) showed a similar pattern of reactivity compared with the sera from long-lasting infected subjects, while most samples (70%) showed a reduced pattern of antibody reactivity (Figure 1), confirming an ongoing seroconversion model. In particular, 35 samples showed a reactivity against two or three proteins, while six sera did not present any reactivity by WB analysis despite a positive or indeterminate result by EIA.

HIV-1 RNA viral load and CD4 levels

Focusing our attention only on samples from patients with a first positive or indeterminate EIA, we performed HIV viral load on a plasma sample obtained from the same subject within one week of EIA/WB analysis. The overall viral load at baseline ranged from $1.8x10^3$ to $5x10^5$ copies/mL (new infections) and $1.8x10^4$ to $5x10^5$ copies/ml (long-lasting infections) with a mean value of $4.1x10^5$ and $2.9x10^5$ respectively, showing a higher but
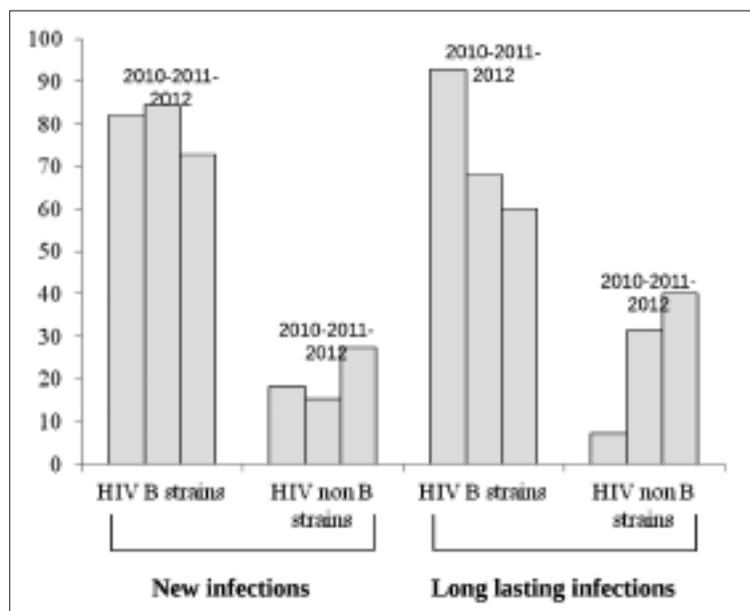

FIGURE 2 - *HIV strains in new infections and in long-lasting infections from 2010 to mid 2012.*

not significant (p=0.058) mean value in naïve individuals.

Consistently, the CD4 cell count (cell/ l) was clearly higher in newly infected in comparison with chronically infected patients (628.1±492.3 versus 174.0±219, p<0.0001) (Table 5).

HIV-1 subtyping

Viral strains were analyzed in most patients enrolled in this study. In particular, plasma samples were available for 46 out of 59 new infections diagnosed and 48 out of 75 long-lasting infections. Results showed (Figure 2) a major prevalence of B strains in both new and old infections. Focusing on viral strains isolated from plasma samples of recently HIV-infected subjects, we found a prevalence of B strains in a variable percentage from 80 and 70% (81.8%, 84.6% and 72.7% in 2010, 2011 and 2012 respectively) and non-B strains in a variable percentage from 18 to 27%.

On the other hand, the prevalence of B strains in long-lasting infected patients showed a decreasing trend in the years considered, and an increasing prevalence of HIV non-B (from 7% in 2010 up to 60% in 2012) in long-lasting infections. Moreover, recorded non-B strains were mainly represented by A1, F1 and G subtypes both in newly and chronically infected individuals, even if HIV recombinant forms (CRFs) were only observed in long-lasting infections (data not shown).

DISCUSSION

This study reports a retrospective analysis of serum samples with an initial positive HIV-1/2 serological result to distinguish recent and established infections.

As is known, an early diagnosis offers several advantages since it maximizes the benefit of HIV care, offers the opportunity for monitoring and timely initiation of therapy, and reduces morbidity and mortality (Heffelfinger *et al.,* 2008).

Serological results obtained at the Retrovirus Laboratory, Operative Unit of Microbiology, Bologna, Italy from January 2010 to July 2011 showed initial positive results in 134 samples. Further analysis disclosed a higher prevalence of men (86% men versus 14% women) with a similar mean age between genders (39.5 and 38.8 re-

spectively). None of the selected samples showed any previous positive HIV-1/2 results, as certified by our database archives.

To distinguish between recent or long-standing HIV infections, in addition to WB analysis we applied a homemade avidity test previously standardized in our and other laboratories (Re *et al.,* 2008, Suligoi *et al.,* 2003, 2011).

Among our subjects, 59 subjects could be classified as recently infected, in accordance with previous reports suggesting a presumed seroconversion time within the last six months, since the avidity test showed an OD ranging from 0.35 to 0.75.

Interestingly, WB analysis was less sensitive than immunoenzymatic assay routinely applied in our laboratory: 10% of sera with a positive or indeterminate ELISA test did not show any reactivity to HIV specific proteins, suggesting that immunoenzymatic assays (fourth generation tests) are often able to precede (from days to a couple of weeks) WB positivity (Re *et al.*, 2008,Cohen *et al.,* 2010; Daskalakis A. 2011).

In addition, all the plasma samples from both recently and long-lasting infected people showed high levels of RNA copies. After HIV infection, plasma HIV RNA levels begin to increase at about 1.5 weeks to two weeks, peaking at around three to six weeks after infection (Cohen *et al.,* 2010, Daskalakis A. 2011)

As expected, the CD4 cell count was significantly higher in newly infected subjects compared to patients with high avidity levels, emphasizing that despite an obvious viral replication an acceptable level of CD4 cells in HIV-infected subjects represents a positive indication to delay antiretroviral therapy. However, the main problem is people found to be HIV-infected but with an estimated seroconversion time exceeding six months at the time of study. In addition to a high avidity index, the medium-low levels of CD4 and high levels of viral replication could date these infections as long-lasting and open the scenario to *late presenters*, whose number is largely unknown worldwide.

Patients are commonly defined as HIV *late presenters* if they are first diagnosed with a CD4 count below 200/mm^3 or a clinical diagnosis of AIDS, but other definitions consider using CD4 + T cell count thresholds as low as 100/mm^3 (D'Arminio Monforte, 2011, Krentz *et al.,* 2004,

Antinori *et al.,* 2010 and 2011). According to several data, 25% of individuals are unaware of their HIV infection and are responsible for 54% of new infections (Daskalakis A. 2011).

Besides a major prevalence of B strains in both new and old infections, our results showed an increasing prevalence of non-B subtypes, at least in the three years considered. This phenomenon seems to be more prominent in long-lasting infected patients with an increasing circulation (from 7% in 2010 up to 60% in 2012) of HIV non-B subtypes (Ciccozzi *et al.,* 2010).

Although our data are strictly limited to a local survey, they demonstrate that a non-negligible number of subjects were found positive late after seroconversion with high levels of HIV RNA replication. In addition, a considerable number of subjects with a late diagnosis showed a CD4 cell count already below the threshold at which ART initiation is recommended (Antinori *et al.,* 2012). Since a late presentation to care is associated with a worse prognosis, the need to identify HIV infection as early is possible must be considered an absolute priority. However, in a complex scenario also characterized by the so-called "late presenters" the importance of early HV diagnosis is unquestionable and three main points must be considered: the introduction of HIV testing as a routine part of medical care, the implementation of new models for a rapid diagnosis outside the medical setting, and the routine application of avidity tests able to approximate the time of acquisition of HIV infection at in each first diagnosis.

Besides all the public health implications, the distinction between acute and chronic infection might serve to establish the time of infection and therefore reach any potential partners who might have been infected in a specific period of time. Although our results are limited to subjects referred to our laboratory and hence represent only a limited part of the problem, the routine application of methods able to distinguish recent from long-lasting infection could help monitor disease incidence, identify high-risk groups, and enhance epidemiological conclusions.

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