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Immunological markers for identifying recent HIV infection in North-West India

Chandar Kanta Chauhan[1], P.V.M. Lakshmi[1], Vivek Sagar[1], Aman Sharma[2], Sunil K. Arora[3] & Rajesh Kumar[1]

[1]Department of Community Medicine, School of Public Health, Departments of [2]Internal Medicine & [3]Immunopathology, Postgraduate Institute of Medical Education & Research, Chandigarh, India

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***Background & objectives*: Being more efficient and widely used, limiting antigen (LAg)-avidity enzyme immunoassay (EIA) based on the recent infection testing algorithm (RITA) has been developed for differentiating recent and established HIV-1 infection. So far, LAg-avidity EIA has not been validated among the Indian population. Hence, the present study was planned to identify recent HIV infections in high risk patients in the North-West region of India using modified LAg-avidity RITA.**

***Methods*: Four hundred HIV-positive high risk patients registered on pre-antiretroviral therapy (ART) programme in the last one year, from five ART centres in North-Western States of India, were included for identifying the recent HIV infections. One hundred HIV-positive cases registered for pre-ART for greater than two years in ART centres were included for estimating false recent rate (FRR). Single-well LAg-avidity EIA-based modified RITA was used to identify recent HIV infection cases.**

***Results*: Of the 400 HIV-1-positive samples, 64 (16%) were found to have been infected within the past 130 days. The proportion of recent HIV infections was 16.8 per cent (18/107) among female sex workers, 10.7 per cent (9/84) among men who have sex with men and 17.7 per cent (37/209) among injecting drug users. The FRR was one per cent (1/100).**

***Interpretation & conclusions*: LAg-avidity EIA-based modified RITA provided good discrimination between recent and non-recent HIV infection, hence, it could be considered suitable for estimating HIV incidence in sentinel surveillance system in India.**

Key words False recent rate - high risk groups - HIV - incidence - LAg-avidity - RITA

Human immunodeficiency virus (HIV) continues to be a major global public health issue. In 2019, with approximately 1.7 million new infections, an estimated 38 million people were living with HIV with a global HIV prevalence of 0.8 per cent among adults[1]. India has the third highest burden of HIV infection in the world[2].

The determination of HIV incidence in a population is important to monitor the epidemic, to identify the target population for HIV prevention and care services and to evaluate the effectiveness of HIV prevention and treatment programmes[3]. Several methods for incidence measurement have been used in the past, including longitudinal studies, back calculation, p24 antigen

enzyme immunoassay (EIA) and viral RNA testing. These methods are either difficult to perform or are costly or may not give accurate estimate[4]. Hence, avidity assays have been proposed to estimate HIV incidence. Limiting-antigen (LAg)-avidity EIA is commercially available single-well assay which is used to distinguish recent from long standing HIV infections for the purpose of estimating incidence in cross-sectional populations. Avidity measures the strength of the binding of immunoglobulin G antibodies with their corresponding antigen, a characteristic feature that enhances over a period of months in newly acquired infections[5]. Antibody avidity assays classically use urea or guanidine to extract low-avidity and low-affinity antibodies after antigen-antibody bonds are formed which can then be detected by a simple ELISA[6]. A new recombinant protein derived from immunodominant region of gp41 (rIDR-M) from all major subtypes and recombinants of HIV-1 group M has been developed[7]. This assay helps to identify sub-populations with high incidence for targeting resources and prevention efforts and to study avidity maturation of developing HIV antibodies in infected patients. There are some issues regarding identifying the recent HIV infection using LAg-avidity assay, as it cannot determine recent or long-term status of HIV-2-infected patients, because of lack of reactivity of rIDR-M with HIV-2 antibodies. In antiretroviral treatment, whether initiated early in infection or later is likely to interfere with normal maturation and/or persistence of HIV antibody, which may also affect its avidity[7]. Another alternative that has been proposed is based on the conclusion that in recent HIV infection there is an initial immunoglobulin isotype IgG3 response to p24. It takes about 120 days for isotype response to mature when isotype IgG3 is replaced by IgG1 isotype[8]. Although working efficiently with subtype B infections, the strength and efficacy of these assays are questionable with non-B subtypes such as the one predominant in India, sub-type C[8-10]. Hence, there is a need to develop algorithm for testing a combination of serological assays and clinical information for the detection of recent HIV infections for epidemiological investigations. Thus, the aim of this study was to use modified recent infection testing algorithm (RITA)[11] based on LAg-avidity EIA for the identification of recently acquired HIV-1 infections among high risk individuals in North-West region of India and to calculate the false recent rate (FRR) of the algorithm.

Material & Methods

This study was conducted mainly in the department of Community Medicine, Postgraduate Institute of Medical Education & Research, (PGIMER), Chandigarh, India. The study protocol was approved by the Institutional Ethic Committee and written informed consent was obtained from all participants.

Female sex worker (FSW), men having sex with men (MSM) and injecting drug user (IDU) were enrolled in the study as per the inclusion and exclusion criteria, from five antiretroviral therapy (ART) centres of North-West India (Chandigarh, Amritsar, Jalandhar, Ludhiana and Rohtak). Only newly diagnosed HIV-positive patients who visited ART centres for the evaluation of their CD4 count were consecutively recruited from September 2013 to June 2016. The sample size of 400 HIV-positive cases registered for pre-ART in the past one year (2012) was considered to be sufficient for estimating recent HIV infection rate of 10 per cent with 95 per cent confidence and three per cent absolute precision. The sample size of 100 HIV-positive cases registered for more than two years was considered to be sufficient for estimating false recent HIV-positive rate (FRR) of 10 per cent with 30 per cent co-efficient of variance.

For identifying new HIV infections using RITA algorithm, HIV-positive patients registered for pre-ART in the past one year (2012) at the five ART centres of North-West India, who were ≥18 yr of age, with CD4+ count ≥200 cells/µl and who had not received any kind of ART prior to enrolment in the study were included for identifying new HIV infections using RITA. For the study of FRR, HIV-positive patients registered for pre-ART for more than two years in the ART centres, who have not received any kind of ART, who were ≥18 yr of age with CD4+ count was ≥200 cells/µl were included. Patients on directly observed treatment, short course (DOTS) for tuberculosis were excluded.

Prior to sample collection, permission was obtained from the National AIDS Control Organisation (NACO) and respective State AIDS Control Societies (SACS). Written informed consent was obtained from all the participants before enrolment. The counsellor was explained about inclusion and exclusion criteria of participants. The study participants were interviewed using semi-structured questionnaire. The information about socio-demographic characteristics, history of sexual and other behavioural risk factors and treatment history especially intake of antiretroviral drugs, CD4 cell counts was collected.

From each selected individual, 5 ml of venous blood was collected; and 2 ml blood was used for CD4 count estimation at respective ART centres.

The remaining 3 ml blood was centrifuged to obtain plasma which was stored in separate vials at −80°C for determination of recent infection.

Estimation of recent HIV infection by RITA: In the avidity assay, anti-HIVgp41-specific antibodies interact with the recombinant immunodominant region of gp41 (rIDR-M) antigen. Modified RITA was used to identify recent HIV infections. The algorithm used in the study is presented in Figure 1.

Limiting antigen (LAg)-avidity enzyme immunoassay: Recent HIV infection was detected using commercially available LAg-avidity assay kit (Maxim Biomedical Corporation, Inc., USA)[7] as per the procedure described by manufacturer in the kit protocol. Assay controls [negative control (NC), calibrator (CAL), low-positive control (LPC) and high-positive control (HPC)] and HIV-positive specimens were diluted 1:101 (v/v) in specimen diluents. After dilution, 100 µl of the diluted specimens or controls were added to appropriate wells of antigen coated plates and incubated for 60 min at 37°C. NC was added in two wells, other controls (CAL, LPC and HPC) were added in three wells and specimens were added in singlet in the same plate. Plates were washed four times with 300 µl of phosphate-buffered saline Triton X-100 detergent (wash buffer) to remove unbound antibodies. A dissociation buffer (*p*H 3.0) was added to each well (100 µl/well) and incubated for 15 min at 37°C to dissociate low-avidity antibodies, if any. Following four washes, goat-anti-human IgG peroxidase (100 µl/well) was added to each well and incubated for 30 min at 37°C. Tetramethylbenzidine (TMB) substrate (100 µl/well) was added and incubated for 15 min at 25°C. Colour development was stopped by the addition of 1 N H_2SO_4 (100 µl/well). The optical density (OD) was read at 450 nm with 650 nm as a reference. HIV-positive infections were classified as recent if OD was ≤1.5.

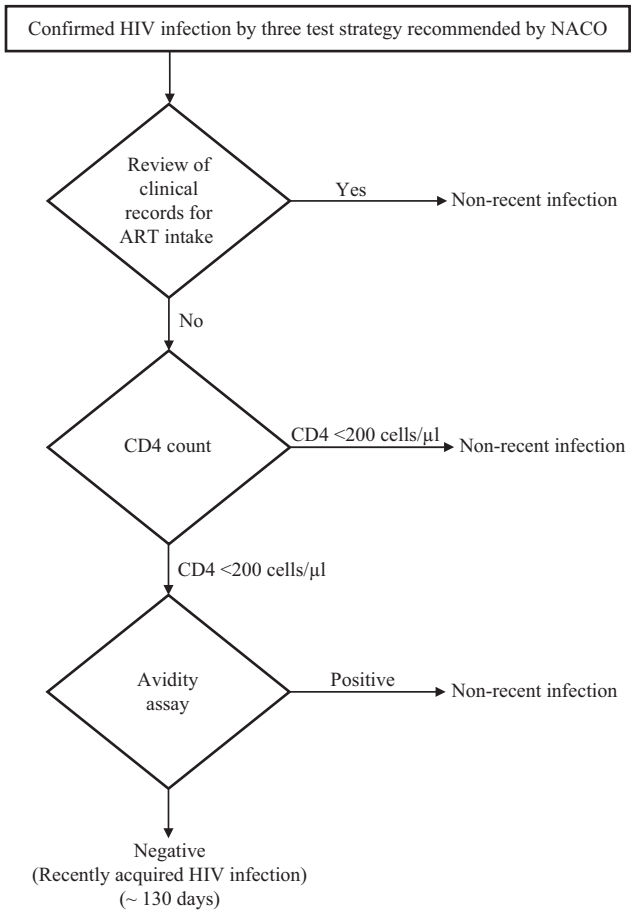

Fig. 1. Modified recent infection testing algorithm (RITA). NACO, National AIDS Control Organisation; ART, antiretroviral therapy.

Statistical analysis: Socio-demographic characteristics according to high risk group (HRG) were presented using frequency and percentage. The association between HIV infection (long-term infection and recent infection) and demographic characteristics was tested using Chi-square test. Relation between the initial and the confirmatory run of the LAg-avidity EIA was tested using Pearson's correlation coefficient. Binomial regression was done to find association of the characteristics of respondents with recency of HIV infection. All statistical analysis were done using SPSS version 21.0 (IBM Corp., Armonk, NY, USA) at 0.05 level of significance.

Results

Socio-demographic characteristics: Of the, 400 HIV-1-positive HRG patients, most were IDUs (n=209, 52%) followed by FSWs (n=107, 27%) and MSMs (n=84, 21%). The socio-demographic characteristics of the study participants are shown in Table I. The mean age was 34.8 ±10 years. Three-fourth of them (n=293) were male and two-third were married. Majority (53%) of the respondents were either illiterate or studied up to primary class. Most of the MSMs (99%) had travelled outside their district as compared to IDUs (91%) and FSWs (80%). Average frequency of travel outside the district was three in the past two years, and it did not differ in various HRGs. The average stay during travel outside the district was maximum among FSWs (3.3 days) as compared to MSMs (2.9 days) and IDUs (2.8 days).

Table I. Socio-demographic characteristics according to high-risk group (n=400)

Variables	IDU, n (%)	FSW, n (%)	MSM, n (%)	Total, n (%)
Age (yr)				
15-25	30 (15)	29 (27)	21 (25)	80 (20)
26-35	85 (40)	35 (33)	42 (50)	162 (41)
36-45	58 (28)	26 (24)	18 (21)	102 (26)
46-55	24 (11)	10 (9)	1 (1)	35 (9)
>55	12 (5.7)	7 (7)	2 (2)	17 (5)
Gender				
Male	209 (100)	0	84 (100)	293 (73)
Female	0	107 (100)	0	107 (27)
Marital status				
Never married	58 (28)	26 (24)	25 (30)	109 (27)
Married	130 (62)	72 (68)	54 (64)	256 (64)
Divorced/separated/widowed	21 (10)	9 (8)	5 (6.0)	35 (9)
Literacy status				
Illiterate	52 (25)	42 (20)	6 (7)	100 (25)
Literate and 5th standard	64 (31)	24 (22)	24 (29)	112 (28)
6th-10th standard	45 (21)	20 (19)	21 (25)	86 (22)
11th to graduation	41 (20)	21 (20)	28 (33)	90 (23)
Post-graduation	7 (3)	0	5 (6)	12 (3.0)
Travel history				
Travelled outside district in the past two years	190 (91)	86 (80)	83 (99)	359 (89)
CD4 count in recent infections (cells/µl)				
200-500	30 (15.5)	13 (14.I)	8 (9.9)	51 (13.9)
>500	7 (43.8)	5 (33.3)	1 (33.3)	13 (38.2)

FSW, female sex worker; MSM, men who have sex with men; IDU, injecting drug user

Standardization of limiting antigen (LAg)-avidity assay

Initial run: All 400 samples were initially run singularly on the LAg-avidity EIA. The CAL and controls (NC, LPC and HPC) were tested in triplicate on every plate and the median values were used to calculate the normalized OD (ODn). Samples with initial ODn >2.0 were considered long-term seroconversion but those with ODn value ≤2.0 were tested again in triplicate in the confirmatory run.

Confirmatory run: A total of 146 of the 400 samples with ODn ≤2.0 in the initial run were retested in triplicate by the LAg assay to confirm their status. The median values for the triplicate values were used to calculate ODn. All the samples with ODn of ≤1.5 were considered to be recently infected with HIV corresponding to mean seroconversion duration of 130 days. Of the 146 retested samples, 64 were found to be recent HIV infection. The correlation coefficient between the initial and the confirmatory runs was 0.97 (Fig. 2), demonstrating a high degree of reproducibility of the test procedure.

Recent HIV infection: Among the 400 respondents, 336 (84%) were identified as having long-term infection and the remaining 64 (16%) were identified as having recent HIV infections. The proportion of recent HIV infections was 16.8 (18/107) among FSWs, 10.7 (9/84) among MSM and 17.7 per cent (37/209) among IDU.

The characteristics of those having recent infection and long-term infection are presented in Table II. There was no significant difference in the socio-demographic characteristics between those having recent and long-term HIV infection. Among those who had recent HIV infection, the median CD4 count was 332.5

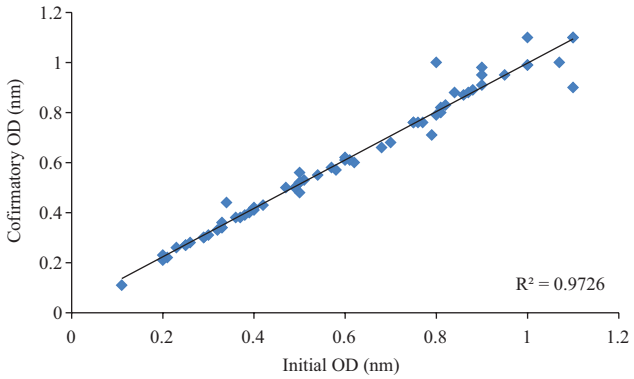

Fig. 2. Concordance of the initial versus the confirmatory run of the limiting antigen (LAg)-avidity EIA. OD, optical density.

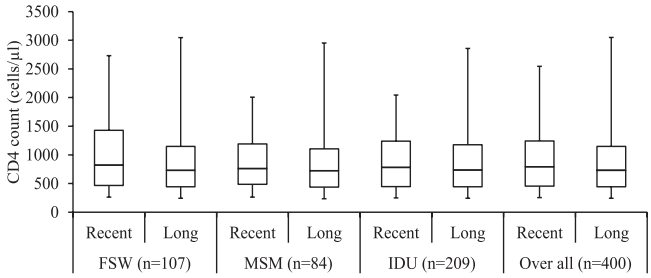

Fig. 3. Boxplot of CD4 count among patients with recent and long-term HIV infection according to high risk groups (n=400). FSW, female sex worker; MSM, men who have sex with men; IDU, injecting drug user.

cells/µl [interquartile range (IQR): 257-491] compared to 287 cells/µl (IQR: 243-420) among those with long-term infection (Fig. 3). Binomial logistic regression was used, keeping recent infection (recent infection=1 and long-term infection=0) as dependent variable and risk groups (1=MSM, 2=FSW, 3=IDU) and CD4 counts (1=200-500, 2=>500) as independent variables. Compared to MSM group the odd ratio for FSW and IDU was 1.4 and 1.7, respectively, but the association was found to be non-significant. Odd ratio for CD4 count of >500 cells/µl was found to be 3.7 compared to 200-500 cells/µl and this association was significant (P<0.01).

False recent rate (FRR): The median CD4 counts among these 100 samples were 374 cells/µl. One sample was found to be recent infection by LAg-avidity EIA, the proportion of samples misclassified as recent positive was found to be 0.01 (95% CI: 0.05-4.8).

Table II. Characteristic of those having recent and long-term human immunodeficiency virus infection detected by LAg-avidity assay

Characteristics	Long-term infection, n (%)	Recent infection, n (%)	*P*
Age (yr)			
15-25	68 (20.0)	11 (17.0)	0.2
26-35	136 (41.0)	26 (41.0)	
36-45	80 (23.0)	22 (34.0)	
46-55	30 (9.0)	4 (6.0)	
>55	22 (7.0)	1 (2.0)	
Gender			
Male	247 (74.0)	45 (70.0)	0.6
Female	89 (27)	19 (29.7)	
Marital status			
Never married	65 (19.0)	14 (21.9)	0.1
Married	264 (79.0)	46 (71.9)	
Divorced/separated/widowed	7 (2.0)	4 (6.3)	
Literacy status			
Illiterate	84 (25.0)	15 (23.0)	0.3
Literate and 5th standard	94 (28.0)	19 (30.0)	
6th-10th standard	74 (22.0)	16 (25)	
11th to graduation	82 (24.0)	12 (19.0)	
Post-graduation	2 (0.6)	2 (3.0)	
Travel history			
Travelled outside the district in the last two years	299 (89)	59 (92.0)	0.4

Discussion

The HIV-1 LAg-avidity EIA used in this study was developed by the Centers for Disease Control and Prevention **(**CDC), to address some of the shortcomings that existed in previous incidence assays. Lag-avidity exploits a multi-subtype recombinant gp41 protein, which broadens its application for determining HIV incidence for various sub-types including A, B, C, D and E[12].

In the present study, all the samples were found to be HIV-1, subtype C. This fact was corroborated by previous studies conducted in India[13,14], which reported that the most common subtype of HIV-1 in Indian subcontinent was subtype C[15]. In the present study, of the 400 HIV positive, treatment naive HRGs, 16 per cent were found to be recently infected within the past four months. The higher proportions of recent infections were seen among IDUs, followed by FSWs showing that active transmission was going in these key population groups. The recent infection was least in MSMs. The reason for higher proportion of recently infected cases among IDUs could be the study samples which were collected mainly from North-Western States (Punjab, Haryana and Chandigarh) of India where an increasing HIV prevalence among IDUs was reported. Integrated Biological Behavioral Surveillance (IBBS) 2014-2015 reported the prevalence of 9.7 per cent (95% CI: 6.6-14.2) among IDUs in Punjab and Chandigarh and 7.3 per cent (95% CI: 5.4-9.7) in Haryana and Himachal Pradesh[16].

The CD4 cell count may indicate the recency of HIV infection. In this study, the overall median CD4 count among recent HIV infection cases was 332.5 cells/µl. It is a common observation among HIV-infected people that CD4 counts halve within eight weeks of an initial count; about 25 per cent average variation is found from the mean over this period[17]. There is an ambiguity in the anticipated CD4 count within the first six months or year of infection, which may clarify why the CD4 count among likely recent HIV infection is not higher enough or similar to people who are HIV negative. It is established that CD4 counts may decrease during seroconversion[18]. According to the RITA used in this study, individuals who had CD4 count below 200 cells/µl were classified as having a long-standing infection and LAg-avidity assay was performed only on those having CD4 cell count more than or equal to 200 cells/µl. The purpose of including the additional information such as CD4 count in the RITA was to reduce the FRR by assisting in identifying cases with long-standing infection.

In the current study it was found that MSMs had travelled more often outside from their current district in the past two years as compared to IDUs and FSWs. Migration and mobility are more common among recent cases of HIV as compared to long-term infections. Mobility status, however, varies by districts, age at sexual debut with a male, sexual identities, marital status and main source of income[19,20]. In the study performed by Ramesh *et al*[21] in southern India, it was observed that of the 1608 MSMs, about 26 per cent were mobile. Of these, three-fourths had travelled to different districts of a State (56%), and one-fifth (20%) among different States. Higher proportion of mobile MSMs as compared to non-mobile MSM reported involvement in unprotected sex with any male partner. The FRR obtained in the present study was similar to the studies conducted elsewhere with larger sample size. A study conducted by Moyo *et al*[22] in Botswana utilizing the LAg-avidity assay with an assay cut-off of 1.5 ODn units, found FRR to be 0.97 per cent. A study by Shah *et al*[23] in Vietnam using BED-CEIA and the LAg-avidity EIA, the LAg-avidity EIA proportion FRR was 1.2 per cent and the BED-CEIA FRR was 1.7 per cent. They concluded that the LAg-avidity EIA FRR was lower than the BED-CEIA FRR. The FRR can be influenced by various factors such as HIV-1 subtype, geographical area, presence of long-term non-progresses and extent of the use of ARV in the area. The FRR being a part of the HIV recency algorithm affects the estimated HIV incidence. Hence, lack of local FRR estimates can be held accountable for existing uncertainty in the estimation of the HIV incidence. FRR and mean duration of recent infection (MDRI) are supposed to be crucial parameters for the evaluation of incidence assays[24]. Key requirements of an HIV incidence assay are high reproducibility and a low FRR when applied across different populations and viral clades. The MDRI should ideally be between 6 and 12 months.

In conclusion, the LAg-avidity EIA may be a potential assay with consistent mean duration of recency in different populations and subtypes.

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Conflicts of Interest**:** None.

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For correspondence: Dr Rajesh Kumar, Department of Community Medicine, School of Public Health, Postgraduate Institute of Medical Education & Research, Chandigarh 160 012, India
e-mail: dr.rajeshkumar@gmail.com