Application of human immunodeficiency virus type 1 BED enzyme immunoassay on dried blood spots in India

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Dried blood spots (DBSs) on filter paper are being used increasingly in population-based human immunodeficiency virus (HIV) studies. This study evaluated the application of a BED enzyme immunoassay (EIA) on DBSs to estimate HIV incidence in a population-based study in India. The Calypte HIV-1 BED Incidence EIA was performed on 224 HIV-1-positive DBS samples, after screening 12 617 individuals from a population-based sample in Guntur district in the southern Indian state of Andhra Pradesh. The number of recently infected HIV cases was identified using this BED assay and was used to estimate the annual HIV incidence rate based on calculations and adjustment formulae suggested by the Centers for Disease Control and Prevention (CDC). The updated BED data management software provided by the CDC was used for analyses. Of the 224 HIV-1 antibody-positive DBS samples, 29 (12.95 %) were estimated by the BED HIV-1 assay to have been infected within the past 155 days. After adjusting for age, gender and rural/urban distribution of the population, the annual incidence rate of HIV-1 infection was estimated to be 0.32 % (95 % confidence interval 0.20–0.44 %). This annual incidence was 18.6 % of the HIV prevalence of 1.72 % in this study. Thus, the BED assay revealed a higher incidence of HIV in this study than was expected from the prevalence. Correlation of the BED assay with panel testing and longitudinal incidence data in the Indian population is needed to calibrate it for use in India.

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INTRODUCTION

India is now estimated to have about 2.5 million people living with human immunodeficiency virus (HIV) infection (Dandona & Dandona, 2007; NACO, 2007; UNAIDS, 2007). HIV surveillance has mostly been limited to monitoring prevalence, as no direct measures of incidence have been available until recently (Wolday *et al.*, 2007). To understand the recent changes in the HIV epidemic, it is necessary to estimate the number of new infections, as changes in HIV prevalence may not reflect trends in incidence (Karita *et al.*, 2007). Laboratory methods for detecting recent HIV infections can enhance public health surveillance and prevention efforts. The data generated on

HIV incidence would be relevant in depicting the current dynamics and trends of the epidemic, and will have significant implications, including evaluation of prevention efforts and informed allocation of resources for HIV prevention programmes.

Several methods of incidence estimation, including longitudinal studies, back calculation, p24 antigen enzyme immunoassay (EIA) and viral RNA testing have been utilized in the past; however, these methods are tedious to perform, costly or problematic (Rutherford *et al.*, 2000). Increasing interest in estimating the incidence of HIV-1 infection has led to the development of a sensitive/less-sensitive serological strategy using a high dilution (1:20 000) of HIV-1-positive serum, called the Serological Testing Algorithm for Recent HIV Seroconversion (STARHS), in 1998 (Janssen *et al.*, 1998) at the Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA), which has been widely used (Gouws *et al.*, 2002; Rawal *et al.*, 2003). Antibody avidity has long

Abbreviations: A_n , normalized absorbance value; BED-CEIA, peptide-based BED capture enzyme immunoassay; CAL, calibrator; CDC, Centers for Disease Control and Prevention; CI, confidence interval; DBS, dried blood spot; EIA, enzyme immunoassay; HIV, human immunodeficiency virus; HPC, high positive control; LPC, low positive control; QA, quality assurance.

been used as a marker for recent infection based on the rationale that, in the early phase of infection, antibodies show a low antigen avidity that can be dissociated with the use of certain chemicals. The dissociated antibodies can then be detected by a simple two-well ELISA (Martró *et al.*, 2005; Chawla *et al.*, 2007). Recently, rapid HIV tests using the same principle of higher dilution have been modified to detect recent HIV-1 infections (Constantine *et al.*, 2003; Soroka *et al.*, 2005). Another approach that has been proposed takes advantage of the observation that, in recent HIV infection, there is an early immunoglobulin isotype IgG3 response to p24 and, as the isotype response matures after about 120 days, the major HIV-specific immunoglobulin isotype becomes IgG1 (Wilson *et al.*, 2004).

Although these assays work reasonably well with samples from HIV-1 subtype B-infected individuals, the validity and performance of these assays is questionable with non-B subtypes because they use an antigen derived from a single subtype (Parekh *et al.*, 2001; Kothe *et al.*, 2003; Young *et al.*, 2003). A peptide-based BED capture EIA (BED-CEIA), whose performance is independent of HIV strain specificity and which detects an increasing proportion of HIV IgG in the serum, was put into use in 2005 by the CDC. This second-generation assay uses a branched peptide with gp41 immunodominant sequences from HIV-1 subtypes B, E and D, but its use has also been validated for subtypes A and C (Parekh *et al.*, 2002). The BED-CEIA has been used to estimate HIV incidence in a number of cross-sectional population studies (Hu *et al.*, 2003; Bulterys *et al.*, 2004).

Whole blood collected as dried blood spots (DBSs) on filter paper is being used increasingly as a convenient tool in many HIV serosurveillance studies, as it simplifies blood collection, transportation and conservation (Cassol *et al.*, 1997; Johnstone *et al.*, 1998; Lakshmi *et al.*, 2007). Although several HIV incidence studies internationally have been used to understand the dynamics of HIV infection (Wilson *et al.*, 2004; Karita *et al.*, 2007; Wolday *et al.*, 2007), to the best of our knowledge no report of any large-scale HIV incidence study using DBSs is available from India. The present study was undertaken with the objective of evaluating the application of the BED-CEIA on DBS samples collected as part of a large population-based assessment of HIV in southern India.

METHODS

Sample. The DBS samples were obtained from 12 617 individuals aged 15–49 years from the Guntur district of Andhra Pradesh, India, as part of a population-based study of HIV. A stratified random sampling method was used to obtain a representative sample of these individuals from 66 rural and urban clusters (Dandona *et al.*, 2006). Of the originally eligible sample, 91.2% participated. Of the 12 617 participants in the study, 6382 (50.6%) were women. This sample obtained using standard epidemiological methods and with a very good participation rate was unlikely to have a selection bias (Dandona *et al.*, 2006). The Institutional Ethics Committees of the Nizam's Institute of Medical Sciences and Administrative Staff College of India approved the study.

The methods related to the DBS samples have been described elsewhere (Lakshmi *et al.*, 2007). Six drops of blood were obtained using a finger-prick method with a non-reusable lancet. After adequate drying, the DBS samples were stored in sealed polythene bags with desiccant at room temperature for no more than 1 week in the field study office and then under refrigeration at 2–8 °C until further testing. Previous reports have shown no loss of sensitivity in detecting HIV from DBSs stored under refrigeration for up to 20 weeks and 36 months for ELISA and PCR, respectively (Behets *et al.*, 1992; Panteleeff *et al.*, 1999).

Overall, 224 DBSs were detected as positive for antibodies to HIV-1 out of the 12 617 DBSs screened for HIV infection, and of these, 221 samples were antibody positive for HIV-1 and three were antibody positive HIV-1 and -2 (Dandona *et al.*, 2006). These 224 DBS samples were further processed using the Calypte HIV-1 BED Incidence EIA (Calypte Biomedical) to determine recent HIV infections (<155 days). This commercially available HIV-1 BED Incidence EIA is an *in vitro* quantitative EIA for the determination of the proportion of HIV-1-specific IgG in a given serum or plasma specimen with respect to total IgG as an aid in determining the elapsed time since HIV-1 infection occurred. The wells of the microtitre plate are coated with goat anti-human IgG as the ligand. When HIV-positive samples are added to the wells, anti-HIV IgG along with the rest of the non-specific IgG is captured by the goat anti-human IgG-coated wells. The relative amounts of anti-HIV IgG and non-anti-HIV IgG captured represent the IgG antibody populations found in the serum of the individual. A lower proportion of HIV-specific IgG is found in the serum in the early phase of seroconversion than in serum samples from patients with long-term infection. Studies have indicated that HIV-specific IgG titres may continue to increase for more than 2 years after seroconversion when tested by this assay (Parekh *et al.*, 2001).

BED-CEIA procedure. The standard optimized procedure as described by B. S. Parekh (CDC, Atlanta, GA, USA) (Parekh, 2005) and in the Calypte HIV-1 BED Incidence Assay instruction manual (Calypte Biomedical) was followed to carry out the incidence assay on DBSs (Phillips *et al.*, 2005). The calibrator (CAL) and test controls were prepared by applying 50 µl control sample to Whatman no. 3 filter paper, and drying and preparing the dried spots in a similar manner to the test DBSs. A 6 mm punch from each of the DBSs was placed into individual wells of a 96-well holder plate for elution of the controls, CAL and 224 reactive samples. Specimen diluent (200 µl; provided in the kit) was added to each of the punched DBSs. After gentle tapping of the plate and complete soaking of the punched DBS, the plate was incubated overnight at 4–8 °C for IgG elution. The following day, 50 µl specimen diluent, and 50 µl eluted controls, CAL or sample, were added to each of the test wells in the BED assay plate (provided in the kit) and gently mixed. The plate was incubated and all further steps of the assay were performed as for the regular ELISA protocol given in the kit instructions.

Initial run. Test sera were initially tested singly. The CAL and controls were tested in triplicate on every plate, and median values were used to calculate the normalized absorbance value (A_n). Samples with an A_n of <1.2 were retested in the confirmatory run to increase the reproducibility and also as a quality check for classifying those recently infected samples that were near the cut-off point.

Confirmatory run. A total of 59 of the 224 DBS samples with A_n values of ≤ 1.2 (in the initial run) were retested in triplicate by the BED assay to confirm their status. The median values for the triplicate values were used to calculate A_n . All the samples with an A_n of <0.8 were considered to be recently infected with HIV corresponding to a mean seroconversion duration of 155 days.

The evaluations and analyses using additional panels with other subtype infections suggested that an A_n of 0.8 corresponded to a mean

seroconversion duration of 155 days. Due to the absence of any published estimates for the window period from India, there is a need to evaluate the best window period estimate for the HIV-infected Indian population from empirical data.

Quality control for the incidence assay. Twenty per cent of the samples that were above the cut-off value (>1.2) were repeat tested using the BED assay as an initial run, for quality assurance (QA).

BED-CEIA on patients with advanced-stage HIV-1 infection.

Individuals with end-stage AIDS have been reported previously to be misdiagnosed as early infections with the BED-CEIA (Janssen *et al.*, 1998; Schwarcz *et al.*, 2001; UNAIDS, 2005). To assess this, we performed the Calypte HIV-1 BED Incidence EIA on 25 end-stage AIDS patients hospitalized in the Nizam's Institute of Medical Sciences, categorized by the World Health Organization guidelines as stage 4, with $CD4^+$ cell counts of <100 cells μl^{-1} . The Surveillance and Survey and Laboratory Working Groups on the use of the BED assay also recommend that the data from the BED assay be adjusted to account for the misclassification of individuals with long-term infection identified as recent infections (false recent) by the BED-CEIA (UNAIDS, 2005).

Recommendations of the Surveillance and Survey and Laboratory Working Groups.

Based on their review of the study results, the working groups on incidence assays have developed interim recommendations for organizations using the BED-CEIA for the estimation of HIV-1 incidence in cross-sectional serosurveys, intervention designs and case-based surveillance in USA government-supported activities. The working groups have reviewed two adjustment formulae and advise the use of both adjustments to develop separate incidence measures and to allow for cross-checking of results for consistency (UNAIDS, 2005; McDougal *et al.*, 2006; Hargrove *et al.*, 2008). The method described by McDougal *et al.* (2006) uses an adjustment for both short-term (≤ 310 days), false long-term and recent cases, as well as an adjustment for longer term (>310 days) false recent individuals. As we did not have any estimates of sensitivity and specificity values available from Indian studies using the BED assay, the McDougal's adjustment formula with a sensitivity value of 0.7682 for detecting recent (<155 days) infection and a short-term specificity value of 0.7231 and a long-term specificity value of 0.9443 were used for calculating the annualized probability of HIV infection (McDougal *et al.*, 2006). The method of Hargrove *et al.* (2008) uses an adjustment for long-term (>365 days) false recent cases. The false recent rate in individuals with long-term infection, indicated as ϵ , is not yet available for India and thus an ϵ value of 0.0557 as estimated by Hargrove *et al.* (2008) was used for calculating the annualized probability of HIV infection. As the two sets of adjustments are expected to give similar results in most settings, we used both of these formulae to calculate the adjusted HIV incidence in our study.

Statistical analysis. To avoid transcription errors, as commonly seen in paper-based data recording, all of the plots – concordance graphs, best-fit plot and quality-control graphs for CALs – were prepared and analysed using the updated BED data management software provided by the CDC. The annual HIV-1 incidence and 95 % confidence interval (CI) were calculated using the consensus formulae predetermined by the HIV-1 BED Incidence EIA manufacturer. Adjusted incidence was calculated by entering our results into the Excel spreadsheet provided by the CDC for calculating unadjusted incidence and incidence calculated with the two adjustments.

RESULTS AND DISCUSSION

A total of 59 of the 224 samples (26.3 %) from our study had A_n values of ≤ 1.2 in the initial test run and these were

further tested in triplicate for confirmation as recently infected. In the confirmatory run, 29/59 samples had an A_n value of ≤ 0.80 and all of these were considered to be recently infected with HIV-1 (i.e. seroconversion occurring within the last 155 days).

In the confirmatory run, 94.9 % of the specimens (56/59) retained the same classification of recent (29 samples) or established (27 samples) HIV infection. Confirmatory testing of the 59 samples indicated that the classification had changed for only one specimen (1.69 %) from the initial classification of recently infected to that of established infection. The correlation coefficient (R^2) between the initial and the confirmatory runs was 0.91 (Fig. 1), indicating a high degree of reproducibility of the test procedure. Thus, the overall performance of the BED assay on the DBS samples was excellent, with good concordance between the two runs, high R^2 values, best-fit plots and least variability, all indicating that the assay has very high reproducibility as also reported by Dobbs *et al.* (2004).

The median absorbance values for the negative control, CAL, low positive control (LPC) and high positive control (HPC) were 0.053, 0.595, 0.328 and 0.969, respectively. The CAL, by definition, had a mean A_n of 1.0, whilst the negative control, LPC and HPC had mean A_n values of 0.082, 0.551 and 1.642, respectively. The control plot generated using CAL, LPC and HPC had a value of $R^2=0.9997$, thus presenting the best-fit graph (Fig. 2). The absorbance and A_n values for the controls were consistent for all runs. The percentage correlation of variation of mean absorbance values of CAL, LPC and HPC were between 6.1 and 12.9 %, but the variability was further reduced as the correlation of variation for mean A_n values was below 7.2 and 7.3 % for LPC and HPC, respectively, demonstrating the high precision of the assay. Use of the median values for the controls and CAL was to ensure that an outlier result in any of these did not affect the results.

Fig. 1. Concordance of the initial versus the confirmatory run of the BED-CEIA.

Fig. 2. A best-fit plot for the controls used in the BED-CEIA. ●, A_n ; □, LPC; △, HPC.

With a calculated window period of 155 days from seroconversion (based on information in the manufacturer's instruction manual), the annual HIV incidence rate using the McDougal formula, after adjusting for age, gender and rural/urban distribution in the Guntur district, was estimated as 0.32 % (95 % CI 0.20–0.44 %), and by the Hargrove formula as 0.35 % (95 % CI 0.22–0.47 %). With a non-significant P value of 0.76 and a χ^2 value 0.092, the incidence results from the two formulae indicated that both sets of adjustments led to similar results. This annual incidence was 18.6 % of the HIV prevalence of 1.72 % in this study (Dandona *et al.*, 2006).

The HIV-1 BED Incidence EIA was developed by the CDC to address some of the shortcomings (e.g. 1:20 000 dilution, assay variability, subtype-dependent performance) of the less-sensitive EIAs. This assay extends the possibilities for determining HIV incidence worldwide in populations with varying subtypes that include the A, B, C, D and E subtypes (Parekh *et al.*, 2001, 2002). Indirectly, the assay measures the proportion of HIV-1-specific IgG in a given specimen with respect to total IgG, based on the principle that recently seroconverted individuals have a lower proportion of HIV-1-specific IgG than long-term seroconverters. In the present study, the information on the nature of the subtypes of HIV-1 was not available.

However, as all samples were from India, these were assumed to belong mainly to the subtype C, according to the geographical distribution of the different HIV-1 genotypes (Siddappa *et al.*, 2004; Ramalingam *et al.*, 2005). As the performance of the earlier incidence assays on HIV-1 subtype C-infected individuals was questionable, the HIV-1 BED Incidence EIA was adopted for our study on HIV incidence as it has been reported to work well with subtype C (Parekh *et al.*, 2001). We did not perform any validation of the BED assay for use with DBS samples as the Calypte HIV-1 BED assay is already validated with a high agreement of 94.6 % between DBS samples and corresponding serum samples, with a high correlation coefficient of 0.98 (Parekh, 2005).

A total of 20.51 % (40/195) of the samples with established infection was repeat tested using the BED-CEIA to rule out technical error (Fig. 3). There is no proof that the cases were truly established infections, as the rate of increase of absorbance in the BED assay is very variable and a reasonable proportion of people may have an A_n value of >1.2 before they are HIV positive over the previous 155 days. In our study, the initial and repeat runs recorded a BED A_n of >1.2, indicating good reproducibility of the assay (Fig. 3).

It has been observed that people with AIDS, especially in the terminal stages and those on highly active antiretroviral therapy, may be classified incorrectly as being recently infected due to their lower levels of HIV antibodies (Janssen *et al.*, 1998; Schwarcz *et al.*, 2001). Information on antiretroviral therapy was not available in the field survey, but antiretroviral therapy was very uncommon in this part of India at the time of data collection for this study.

When the BED assay was performed on DBS samples from 25 individuals with end-stage AIDS in a hospital setting, with mean CD4⁺ counts of 35.84 cells μl^{-1} (range 5–88 cells μl^{-1}) in their plasma, all were correctly diagnosed as established infection, indicating that advanced HIV infection did not lead to misdiagnosis of recent infection in this set of patients. However, using the recommended calculation method, the annual HIV incidence rate in this population was estimated to be 18.6 % of the 1.72 % prevalence rate (Dandona *et al.*, 2006), which is higher

Fig. 3. QA testing of long-term cases using BED-CEIA. ◆, Initial run A_n ; ▲, QA run A_n .

than would be expected over the past year. Data from sentinel surveillance in young pregnant women in the Guntur district has indicated that the rate of new HIV infections is not increasing in this population (NACO, 2007). Against this background, and assuming a duration of about 10 years from HIV infection to death from AIDS without antiretroviral treatment (Hira *et al.*, 2003), one would expect the annual HIV incidence to be 10% or less of the prevalence estimate. Further investigation is required to ascertain the reasons for the higher-than-expected incidence estimate with the BED-CEIA, possibly by validation of this assay on panels of sera from HIV-1-infected seroconverters representing different subtypes and by correlation of the results from this assay with longitudinal incidence data in India.

To the best of our knowledge, this is the first report using the HIV-1 BED-CEIA to estimate the incidence of HIV-1 infection in India carried out on population-based DBS samples. Our results suggest that the Calypte HIV-1 BED Incidence EIA can yield reproducible results when applied to DBS samples in India. However, calibration of the incidence calculation from this assay in India is needed, as the existing calculation method led to a higher-than-plausible incidence estimate. Once this is accomplished, this incidence assay can serve as a useful tool in more effective planning and monitoring of HIV control efforts in India.

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REFERENCES

- Behets, F., Kashamuka, M., Pappaioanou, M., Green, T. A., Ryder, R. W., Batter, V., George, J. R., Hannon, W. H. & Quinn, T. C. (1992). Stability of human immunodeficiency virus type 1 antibodies in whole blood dried on filter paper and stored under various tropical conditions in Kinshasa, Zaire. *J Clin Microbiol* **30**, 1179–1182.
- Bulterys, M., Chao, A., Dushimimana, A. & Parekh, B. S. (2004). Unsafe injections and transmission of HIV-1 in sub-Saharan Africa. *Lancet* **363**, 1650.
- Cassol, S., Gill, M. J., Pilon, R., Cormier, M., Voigt, R. F., Willoughby, B. & Forbes, J. (1997). Quantification of human immunodeficiency virus type 1 RNA from dried plasma spots collected on filter paper. *J Clin Microbiol* **35**, 2795–2801.
- Chawla, A., Murphy, G., Donnelly, C., Booth, C. L., Johnson, M., Parry, J. V., Phillips, A. & Geretti, A. M. (2007). Human immunodeficiency virus (HIV) antibody avidity testing to identify recent infection in newly diagnosed HIV type 1 (HIV-1)-seropositive persons infected with diverse HIV-1 subtypes. *J Clin Microbiol* **45**, 415–420.
- Constantine, N. T., Sill, A. M., Jack, N., Kreisel, K., Edwards, J., Cafarella, T., Smith, H., Bartholomew, C., Cleghorn, F. R. & Blattner, W. A. (2003). Improved classification of recent HIV-1 infection by employing a two-stage sensitive/less-sensitive test strategy. *J Acquir Immune Defic Syndr* **32**, 94–103.
- Dandona, L. & Dandona, R. (2007). Drop of HIV estimate for India to less than half. *Lancet* **370**, 1811–1813.
- Dandona, L., Lakshmi, V., Sudha, T., Kumar, G. A. & Dandona, R. (2006). A population-based study of human immunodeficiency virus in south India reveals major differences from sentinel surveillance based estimates. *BMC Med* **4**, 31.
- Dobbs, T., Kennedy, S., Pau, C. P., McDougal, J. S. & Parekh, B. S. (2004). Performance characteristics of the IgG-capture BED-enzyme immunoassay (BED-CEIA), an assay to detect recent HIV-1 seroconversion. *J Clin Microbiol* **42**, 2623–2628.
- Gouws, E., Williams, B. G., Sheppard, H. W., Enge, B. & Karim, S. A. (2002). High incidence of HIV-1 in South Africa using a standardized algorithm for recent HIV seroconversion. *J Acquir Immune Defic Syndr* **29**, 531–535.
- Hargrove, J. W., Humphrey, J. H., Mutasa, K., Parekh, B. S., McDougal, J. S., Ntozini, R., Chidawanyika, H., Moulton, L. H., Ward, B. & other authors (2008). Improved HIV-1 incidence estimates using the BED capture enzyme immunoassay. *AIDS* **22**, 511–518.
- Hira, S. K., Shroff, H. J., Lanjewar, D. N., Dholkia, Y. N., Bhatia, V. P. & Dupont, H. L. (2003). The natural history of human immunodeficiency virus infection among adults in Mumbai. *Natl Med J India* **16**, 126–131.
- Hu, D. J., Vanichseni, S., Mock, P. A., Young, N. L., Dobbs, T., Byers, R., Jr, Choopanya, K., van Griensven, F., Kitayaporn, D. & other authors (2003). HIV-1 incidence estimates by detection of recent infection from a cross-sectional sampling of injection drug users in Bangkok: use of the IgG Capture BED enzyme immunoassay. *AIDS Res Hum Retroviruses* **19**, 727–730.
- Janssen, R. S., Satten, G. A., Stramer, S. L., Rawal, B. D., O'Brien, T. R., Weiblen, B. J., Hecht, F. M., Jack, N., Cleghorn, F. R. & other authors (1998). New testing strategy to detect early HIV-1 infection for use in incidence estimates and for clinical and prevention purposes. *JAMA* **280**, 42–48.
- Johnstone, F., Goldberg, D., Tappin, D., Mathie, L., Cameron, S., Brown, A., Burns, S., Hamilton, B., Codere, G. & Girdwood, R. W. (1998). The incidence and prevalence of HIV infection among childbearing women living in Edinburgh city, 1985–1995. *AIDS* **12**, 911–918.
- Karita, E., Price, M., Hunter, E., Chomba, E., Allen, S., Fei, L., Kamali, A., Sanders, E. J., Anzala, O. & other authors (2007). Investigating the utility of the HIV-1 BED capture enzyme immunoassay using cross-sectional and longitudinal seroconverter specimens from Africa. *AIDS* **21**, 403–408.
- Kothe, D., Byers, R. H., Caudill, S. P., Satten, G. A., Janssen, R. S., Hannon, W. H. & Mei, J. V. (2003). Performance characteristics of a new less-sensitive HIV-1 EIA for use in estimating HIV seroincidence. *J Acquir Immune Defic Syndr* **33**, 625–634.
- Lakshmi, V., Sudha, T., Bhanurekha, M. & Dandona, L. (2007). Evaluation of Murex HIV Ag/Ab Combination assay on dried blood spots. *Clin Microbiol Infect* **13**, 1134–1136.
- Martró, E., Suligoi, B., González, V., Bossi, V., Esteve, A., Mei, J. & Ausina, V. (2005). Comparison of the avidity index method and the serologic testing algorithm for recent human immunodeficiency virus (HIV) seroconversion, two methods using a single serum sample for identification of recent HIV infections. *J Clin Microbiol* **43**, 6197–6199.
- McDougal, J. S., Parekh, B. S., Peterson, M. L., Branson, B. M., Dobbs, T., Ackers, M. & Gurwith, M. (2006). Comparison of HIV-1 incidence observed during longitudinal follow-up with incidence estimated by cross-sectional analysis using the BED capture enzyme immunoassay. *AIDS Res Hum Retroviruses* **22**, 945–952.

- NACO (2007).** *HIV Sentinel Surveillance and HIV Estimation, 2006*. New Delhi: National AIDS Control Organization, Ministry of Health and Family Welfare, Government of India. <http://www.nacoonline.org/upload/Publication/M&E%20Surveillance,%20Research/HIV%20Sentinel%20Surveillance%20and%20HIV%20Estimation,%202006.pdf>
- Panteleeff, D. D., John, G., Nduati, R., Mbori-Ngacha, D., Richardson, B., Kreiss, J. & Overbaugh, J. (1999).** Rapid method for screening dried blood samples on filter paper for human immunodeficiency virus type 1 DNA. *J Clin Microbiol* **37**, 350–353.
- Parekh, B. S. (2005).** Use of DBS with BED testing for recent infection. In *HIV Diagnostics: New Developments and Challenges*, 28 February–1 March 2005, Orlando, FL, USA. <http://www.hivtestingconference.org/hivtesting2005/Presentations.htm#posters>
- Parekh, B. S., Hu, D. J., Vanichseni, S., Satten, G. A., Candal, D., Young, N. L., Kitayaporn, D., Srisuwanvilai, L. O., Rakhtam, S. & other authors (2001).** Evaluation of a sensitive/less-sensitive testing algorithm using the 3A11-LS assay for detecting recent HIV seroconversion among individuals with HIV-1 subtype B or E infection in Thailand. *AIDS Res Hum Retroviruses* **17**, 453–458.
- Parekh, B. S., Kennedy, M. S., Dobbs, T., Pau, C. P., Byers, R., Green, T., Hu, D. J., Vanichseni, S., Young, N. L. & other authors (2002).** Quantitative detection of increasing HIV type 1 antibodies after seroconversion: a simple assay for detecting recent HIV infection and estimating incidence. *AIDS Res Hum Retroviruses* **18**, 295–307.
- Phillips, S., Dobbs, T., Kuehl, D. & Parekh, B. S. (2005).** Use of dried blood spot (DBS) or serum spot to detect recent HIV-1 seroconversion by HIV-1 BED Incidence Assay. In *HIV Diagnostics: New Developments and Challenges*, 28 February–1 March 2005, Orlando, FL, USA. <http://www.hivtestingconference.org/hivtesting2005/Presentations.htm#posters>
- Ramalingam, S., Kannangai, R., Vijayakumar, T. S., Mathai, D., Abraham, O. C., Subramanian, S., Rupali, P., Jesudason, M. V. & Sridharan, G. (2005).** Subtype & cytokine profiles of HIV infected individuals from south India. *Indian J Med Res* **121**, 226–234.
- Rawal, B. D., Degula, A., Lebedeva, L., Janssen, R. S., Hecht, F. M., Sheppard, H. W. & Busch, M. P. (2003).** Development of a new less-sensitive enzyme immunoassay for detection of early HIV-1 infection. *J Acquir Immune Defic Syndr* **33**, 349–355.
- Rutherford, G. W., Schwarcz, S. K. & McFarland, W. (2000).** Surveillance for incident HIV infection: new technology and new opportunities. *J Acquir Immune Defic Syndr* **25**, S115–S119.
- Schwarcz, S., Kellogg, T., McFarland, W., Louie, B., Kohn, R., Busch, M., Katz, M., Bolan, G., Klausner, J. & Weinstock, H. (2001).** Differences in the temporal trends of HIV seroincidence and seroprevalence among sexually transmitted disease clinic patients, 1989–1998: application of the serologic testing algorithm for recent HIV seroconversion. *Am J Epidemiol* **153**, 925–934.
- Siddappa, N. B., Dash, P. K., Mahadevan, A., Jayasuryan, N., Hu, F., Dice, B., Keefe, R., Satish, K. S., Satish, B. & other authors (2004).** Identification of subtype C human immunodeficiency virus type 1 by subtype-specific PCR and its use in the characterization of viruses circulating in the southern parts of India. *J Clin Microbiol* **42**, 2742–2751.
- Soroka, S. D., Granade, T. C., Candal, D. & Parekh, B. S. (2005).** Modification of rapid human immunodeficiency virus (HIV) antibody assay protocols for detecting recent HIV seroconversion. *Clin Diagn Lab Immunol* **12**, 918–921.
- UNAIDS (2005).** Statement on the use of the BED-assay for the estimation of HIV-1 incidence for surveillance or epidemic monitoring. In *Report of a Meeting of the UNAIDS Reference Group for Estimates, Modelling and Projections*, 13–15 December 2005, Athens, Greece. Geneva: UNAIDS. <http://www.epidem.org/Publications/BED%20statement.pdf>
- UNAIDS (2007).** *AIDS Epidemic Update: December 2007*. Geneva: UNAIDS. http://data.unaids.org/pub/EPISlides/2007/2007_epiupdate_en.pdf
- Wilson, K. M., Johnson, E. I., Croom, H. A., Richards, K. M., Doughty, L., Cunningham, P. H., Kemp, B. E., Branson, B. M. & Dax, E. M. (2004).** Incidence immunoassay for distinguishing recent from established HIV-1 infection in therapy-naïve populations. *AIDS* **18**, 2253–2259.
- Wolday, D., Meles, H., Hailu, E., Messele, T., Mengistu, Y., Fekadu, M., Parekh, B. S. & Wuhib, T. (2007).** Temporal trends in the incidence of HIV infection in antenatal clinic attendees in Addis Ababa, Ethiopia, 1995–2003. *J Intern Med* **261**, 132–137.
- Young, C. L., Hu, D. J., Byers, R., Vanichseni, S., Young, N. L., Nelson, R., Mock, P. A., Choopanya, K., Janssen, R. & other authors (2003).** Evaluation of a sensitive/less-sensitive testing algorithm using the bioMérieux Vironostika-LS assay for detecting recent HIV-1 subtype B' or E infection in Thailand. *AIDS Res Hum Retroviruses* **19**, 481–486.