

SEA-HLM-390
Distribution: General

Enumeration of CD4 T Lymphocytes

*Report of a Workshop
Bangkok, Thailand, 6-10 June 2006*

WHO Project: ICP BCT 001



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1. Introduction

The AIDS epidemic continues to spread across the South-East Asia (SEA) Region, which is the most affected part of the world after sub-Saharan Africa. To date, around 40 million people around the world have been infected with the human immunodeficiency virus (HIV), the virus that causes AIDS. Of them more than six million are in the SEA Region.

The “3 by 5” initiative has stimulated Member States to accelerate and strengthen mechanisms for providing antiretroviral therapy (ART) to needy people living with HIV. The availability of economical generic drugs has further boosted the process. Additional funds have also been made available by the Global Fund where ART therapy has been recognized as one of the priority areas.

With considerable efforts being initiated in providing ART, a close monitoring of the patient on these drugs is essential to initiate treatment and ascertain the response. Enumeration of CD4 T lymphocytes is the most sensitive and specific indicator for this assessment. This along with the estimation of viral load constitutes two universally accepted monitoring tools. Since viral load is not recommended by WHO, capacity building in CD4 T lymphocytes estimation is of great relevance.

Measurement of lymphocyte subsets is done by several methods of which flow cytometry is the gold standard. Flow cytometry is undergoing considerable evolution to make it more cost-effective and user-friendly with improved ease of performance. Several other low-cost technologies are becoming available and may be of greater use in many countries. Some of these have been evaluated and found to be useful. Ensuring quality has become a critical issue with increasing use of laboratory support for antiretroviral therapy.

To address these issues and to orient participants about these technologies and integration of a quality system in routine testing, an

intercountry workshop on CD4 T lymphocytes enumeration was organized at Siriraj Hospital, Bangkok from 6 to 10 June 2005. Sixteen laboratory professionals from Bangladesh, India, Indonesia, Myanmar, Nepal, Sri Lanka and Thailand participated in the workshop, which was facilitated by experts from Siriraj Hospital and the National AIDS Research Institute, Pune, India. The list of participants and the workplan are at Annex 1 and 2 respectively.

2. Objectives

The objectives of the workshop were to:

- (1) Review the status of enumeration of CD4 T lymphocytes in high HIV-burden Member countries of the SEA Region;
- (2) Orient on new, economical and field-friendly technologies for CD4 enumeration;
- (3) Provide hands-on training on selected technologies including the interpretation of results, and
- (4) Identify a mechanism for external quality assessment scheme.

3. Inaugural session

The Dean of the medical faculty at Siriraj Hospital welcomed the participants. In his inaugural remarks, he emphasized that CD4 enumeration technology is critical for effective implementation of any programme on antiretroviral therapy as well as for providing efficient care to the patient.

Acting WHO Representative, Thailand read the inaugural address of Dr Samlee Plianbangchang, Regional Director, WHO South-East Asia (SEA) Region. In his inaugural address, Dr Samlee said that the Millennium Development Goals (MDGs) have identified HIV as a disease of global importance and set a target of halting evolution of the pandemic and even reversing it by 2015. ART is an integral component of any strategy aimed at achieving the HIV-related MDGs.

Dr Samlee also informed that in countries of the SEA Region an estimated 800 000 persons needed treatment with ART, while only 20 per cent of these (164 000) were receiving it at present. Rational use of antiretroviral drugs is critical in patients with HIV because of the severity of illness and the potential of the virus to mutate and become resistant to the drugs. Initiation and monitoring of ART is possible only with accurate enumeration of CD4 T lymphocytes. To assure reliability, laboratories conducting CD4 measurements need to have the capacity to perform the tests, establish quality assurance methods and participate in the external quality assessment scheme.

Dr Samlee urged participants to make use of this opportunity to discuss various technologies and avail of the opportunity to gain hands-on experience. He assured them that the workshop would provide sufficient knowledge to choose the appropriate technology depending upon infrastructure and resources available.

4. Proceedings of the meeting

The workshop included a presentation of country reports to review the existing capacity of the countries; presentations and discussions to disseminate new information; group work to formulate a generic plan for establishment of external quality assessment scheme and hands-on experience in various technologies to enumerate CD4 T lymphocytes.

4.1 Country reports

Bangladesh

Bangladesh faces many risk factors for HIV infection but the burden of HIV/AIDS in the country is minimal. The first HIV case was reported in 1989 and the number of people living with HIV infection in 2006 was about 700. Recent national surveillance indicated a high prevalence (>4%) of HIV among intravenous drug users (IVDUs). Such infection has been reported from all over the country. Facilities for HIV diagnosis are available at the district level but the follow-up and management of the infection is not well organized. The

facilities for CD4 counting are available only with one international agency (the International Centre for Research in Diarrhoeal Diseases-Bangladesh) in Dhaka only.

Indonesia

Indonesia has reported 10 156 HIV infections by the end of March 2006. The number of estimated cases is, however, between 100 000 to 300 000. There has been a marked increase in the detection of HIV antibody among blood donors and IDUs. Nine flow cytometers are currently available in Indonesia, five of them are in Jakarta. In the past few years there has been a marked increase in CD4 testing requests in the country. Currently only two laboratories are participating in WHO-EQAS carried by Centre of Excellence, Siriraj Hospital, Bangkok. No national External Quality Assessment Scheme (EQAS) is operational in the country.

India

A total of 54 ART clinics and 51 CD4 testing laboratories are operational in the public sector in India. ART is provided free of cost by the Government of India. A total of 32 000 HIV- positive individuals in India are currently on ART. The ART is also provided in the private sector with the CD4 count estimation facility.

To cater to the government ART clinics, an additional 134 FACSCount machines are being installed at various locations by the National AIDS Control Organization (NACO), Government of India. Internal quality control measures are in place that include accuracy of pipetting, maintenance of equipment and use of laboratory controls.

The majority of centres are using FACSCount machines and reagents. Each centre carries out CD4 testing on an average for 500-2000 samples/year (both baseline and follow- up) depending on the number of patients attending the clinics. It has been observed that 30-40% of patients attending the clinics have CD4 counts <200/cumm. These patients are being followed up at an interval of four to six months. The mean CD4 count for normal healthy individuals was 765/cumm in the sample population tested in New Delhi.

EQAS in CD4 testing was started on a pilot basis in 2005 for 32 laboratories affiliated to ART clinics. The results have been satisfactory for 31 laboratories.

Some centres are carrying out studies to compare the utility of plasma viral load and CD4 counts in monitoring ART. Studies are also in progress to collect information on prevalent opportunistic infections in HIV cases in India.

Myanmar

AIDS is one of the three priority diseases of the National Health Plan in Myanmar. The first AIDS case in the country was reported in 1991 and sentinel surveillance of the disease was launched in 1992. ART for people living with HIV started in public sector in 2003. While the reported number of people living with HIV in 2003 was 53 015, the estimated number is pegged at 177 279. Till 2003, 7174 cases of AIDS have been detected and 3324 have died of the disease.

ART was launched in Myanmar in 2003 at the Specialist Hospital, Yangon and expanded to public hospitals in June 2005. Currently, it is available in six townships and four border towns. The total number of patients receiving ART is around 2500.

In Myanmar, CD4 count testing was started in 2001 at the National Health Laboratory. Every month around 20-40 specimens are tested at a cost of US\$ 15 per test. The first FACS Count machine was installed in April 2006.

Nepal

The number of reported HIV infections in Nepal is 6443 (4647 male and 1296 female). The number of AIDS cases is 1025 (male: 747, female: 278) with 311 deaths till date. The facility for CD4 count estimation is available at the National Public Health Laboratory, a government referral laboratory situated at Teku, Kathmandu.

Nepal has four ART centers in different parts of the country. In 2005, of the 300 CD4 estimations carried out more than 100 had CD4<200/cumm. In the year 2006, of 694 CD4 estimations, 266 had CD4<200/cumm. No EQAS is available in the country.

Sri Lanka

Sri Lanka reported its first HIV-reactive case in 1986. Since then there has been a steady increase in the number of HIV positives. By the end of March 2006, 771 HIV positive cases were reported. However, the estimated number of HIV-positives is 3500. Over 200 000 tests for HIV antibody detection are being performed in the country every year, the majority of which take place in the National Blood Transfusion Service, STD clinics and in the private sector.

CD4 enumeration for HIV-positive patients was initiated in 2001 with the installation of a flowcytometer at the Medical Research Institute (MRI) in Colombo with financial support from the World Bank. The number of tests performed at the MRI varied from 60 to 135 annually.

With the launching of the "3 by 5" initiative by WHO in 2003, the Government of Sri Lanka decided to provide anti-retroviral treatment to people living with HIV who were eligible to receive them. Consequently, ART was made available to patients at Government Hospital from December 2004. This endorsed the need for an easily accessible CD4 count test at the point of care.

To meet this requirement, in January 2005 a FACSCount was installed in the central laboratory of the National STD AIDS Control Programme (NSACP), enabling patients to obtain the services easily. In 2005, 188 tests were performed at the NSACP.

Thailand

It was estimated that till 2004, 1 074 155 persons were infected with HIV since the beginning of the epidemic in Thailand. Among these, 501 600 have died and 572 500 are currently living with HIV and AIDS. It is also estimated that 19 500 new infections would occur during this year compared to 143 000 new infections in 1990.

There are approximately 160 flow cytometers in Thailand. More than 80% of them are in hospitals that belong to the Ministry of Public Health. User training and refreshing courses are organized by the Centre of Excellence

(CoE), Siriraj Hospital, Bangkok. Since 2004, national training courses were also organized at the Bamrasnaradura Infectious Disease Institute (BIDI).

Several centres are participating in national and international external quality assessment schemes with satisfactory results.

4.2 Presentations and topics for discussions

Role of CD4 count in HIV infection

Dr Ramesh Paranjape, Director, National AIDS Research Institute (NARI), Pune, India, discussed the immunopathogenesis of HIV infection and the role of CD4 count estimation in HIV infection. He gave an account of the infection of CD4+ T cells by HIV and the co-receptor usage of HIV for entry in the cell. He elaborated the clinical course of HIV infection, different patterns of HIV disease progression and mechanisms of CD4 + T cell depletion in HIV infection. Dr Paranjape also explained about the use of CD4 counts in monitoring disease progression, initiation of ART and treatment of opportunistic infections and monitoring the success of ART.

Principles of flow cytometry

Dr Kovit Pattanapanyasat, Chief, Division of Instruments and Research, Centre of Excellence, Siriraj Hospital, Bangkok, explained the principle of flow cytometry, its comparison with fluorescence microscopy and blood smear examination. He gave an account of advantages of flow cytometry and explained different systems of flow cytometer - fluidics, optics, and electronics – and their principles and functions. The use of different filters in flow cytometers was explained with the help of suitable diagrams and examples. The creation of voltage pulse and its conversion to electronic signal for analysis was elaborated. Different types of fluorochromes with their excitation wavelength and respective use were also presented.

Low-cost technologies to enumerate CD4

Dr Madhuri Thakar, National AIDS Research Institute (NARI) gave an account of the low-cost methodologies available for CD4 count estimation and their

use in resource limited settings. Various technologies like modified FACSCount, Guava, Partec, Pointcare and manual methods such as Dyna beads and Cytosphere techniques were discussed. The factors to be considered while making the choice of methodology in individual laboratories were analyzed. Dr Kovit informed about a few more technologies that were still in the developmental stage. It was emphasized that before making the choice, information on multicentric validation studies on the technology (independent of manufacturer) should be undertaken.

Gating strategies and selection of reagents related to sub-set counting

The gating strategies that have been used and are currently in use in flowcytometry for CD4 count estimation were explained by Dr Kovit with their respective merits and demerits. He provided details of the FSC/SSC gating to identify lymphocyte population that was in use in the beginning of flow cytometry for about 20 years. The disadvantage of this gating strategy was contamination with non-lymphocytes in the gate. He described the evolution of gating strategies. The CD45 gating to count only lymphocytes (CD45 is the only marker expressed by all lymphocytes), and the combination of CD45, CD3 and CD4 or CD8 (Tritest reagents) were explained and discussed with the participants.

Panleucogating

Dr Kovit explained the novel approach of panleucogating which has a gating on CD45 expression and a further gate on CD4 T cells. This can reduce the cost of the testing, without compromising accuracy and the information obtained in the testing, he said. The panleucogating strategy can be combined with the Trucount or Flowcount tubes to get absolute CD4 counts in addition to CD4%. It, however, increases the costs as the Trucount or Flowcount tubes are expensive.

Hematology analyzers: TLC and differentials

Prof. Sathien Sukpanichnant, Department of Clinical Pathology, Siriraj Hospital, made a presentation on the principles of haematology analyzers to obtain total leucocyte count (TLC) and differential counts from whole blood. Prof. Sathien explained the electronic circuits and their use in haematology

analyzers in detail. He gave details of different haematology analyzers available, their performance and other aspects like cost etc. The quality systems required for TLC and differential counts were discussed with the participants.

External Quality Assessment Scheme (EQAS)

A comprehensive account of the EQAS in CD4 count estimation was provided by Dr Kovit. Issues that were discussed included objectives of EQAS, different agencies that organize EQAS for CD4 count estimation, the number of the EQAS runs per year and cost of this assessment. He also explained the importance of quality control charts in documenting the internal quality control process and its use in identifying the problem before hand.

Dr Kovit described the EQAS that is being carried out by his department for Thailand, Indonesia and India. It was emphasized that each CD4 estimating laboratory should participate in EQAS.

EQAS experience in India

Dr Thakar provided information on the EQAS exercise that was carried out in India in collaboration with WHO and the Center of Excellence, Thailand for some of the Indian laboratories. Four rounds of EQAS has been carried out so far and the results were found to be satisfactory. The reasons for the failure to submit the results of EQAS were discussed. The need for a national EQAS for Indian laboratories was strongly expressed during the discussion. Dr Thakar also gave an account of the internal quality control being carried out at NARI.

Selection and purchase of technology and equipment

Dr Kovit discussed the factors to be considered while selecting and purchasing a new technology and equipment for CD4 count estimation for the particular laboratory. The important factors are workload, availability of trained personnel, availability of service back- up and the system being a closed or open one.

He narrated his experiences in Thailand on procurement of equipment and use of open systems like FACSCalibur/FACScan with reagents from other

companies. He said that reagents from other systems work well with the open system, but the equipment manufacturer did not take responsibility for any problems of maintenance or with the results obtained using these reagents. He also discussed the procedures to be followed for procurement and outlined the advantages of centralized procurement.

Biosafety

Dr Paranjape made a presentation on biosafety guidelines to be followed in laboratories undertaking CD4 T lymphocyte enumeration. He explained the sources of risk in the laboratory and precautions to be taken to avoid them. He emphasized the need to have a dynamic biosafety committee in the laboratory to observe the biosafety guidelines and give appropriate directions. The need for a biosafety manual and its strict compliance was stressed. Dr Paranjape enumerated general biosafety steps to be followed in the laboratory, including those for the management of spills and hazard communication.

Role of stabilizers

The role and importance of stabilizers of specimens in CD4 count estimation was discussed. The stabilizers are used to stabilize whole blood samples for making them useful for CD4 count estimation after three or four days after collection. He gave details of available material, their stability in practical situations and cost. He also described two types of stabilizers: short-term (effective up to 10 days) and long-term (effective for more than 10 days). The short-term stabilizers can be used in the situation where the samples have to be shipped to the central laboratory for CD4 count estimation while long-term stabilizers can be used as internal quality control. Both the stabilizers have been used to prepare the samples for the external proficiency round. The long-term stabilizers, however, may pose a problem in the analysis due to faint staining of the cells.

Equipment maintenance

Dr Paranjape discussed the utility and value of equipment maintenance in CD4 estimation in the laboratory including flow cytometers and pipettes and other equipment. He explained the procedures for routine maintenance of flow cytometers that should be carried out in the laboratory as well as those

steps that should be followed by the service engineer. He explained the importance of a checklist and documentation of the performance of equipment. Use of standard operating procedures is critical for the proper functioning of equipment. The pipette calibration has to be carried out at least once in 6 months and the laboratory should keep records.

Comparison of methodologies

Dr Thakar provided a comparison of the available methodologies for CD4 count estimation. She discussed the flowcytometric methods, both conventional and modified, and alternate methodologies for their respective principles, gating strategies, number of samples that can be tested in a day and approximate cost of equipment as well as reagents. The analytical characteristics – about whether the method is single platform or double platform, various gating strategies and maintenance required for different methodologies – were discussed. It was emphasized that the choice of method should depend on the number of tests to be performed per day, the availability of trained manpower and support system and, finally, the cost. It was also mentioned that the methods should be validated in a multicentric evaluation exercise and should pass external proficiency panels before being adapted for routine use.

4.3 Group work

Participants developed a generic plan for the establishment of a national external quality assessment scheme for CD4 T lymphocytes enumeration. The draft plan emphasized on responsibility of the National Programme Officer to institutionalize national EQAS, development of infrastructure for EQAS, training of human resource, logistics for shipment of material with or without stabilizers, continuous collaboration between the organizer of the scheme as well as the participating units and a continuing endeavour towards quality improvement.

4.4 Hands-on enumeration of CD4 T Lymphocytes

All participants were demonstrated flow cytometry techniques using FACSCount and Tritest on FACSCalibur. The tests were also performed by the

participants themselves. The facilitators discussed various issues, commonly encountered problems and ways to obviate these.

An open session was organized with the participants on the last day of the workshop. All subjects that were discussed at the workshop were reviewed. Country-specific issues were also discussed and possible solutions to improve laboratory support to ART programmes in countries were suggested.

5. Recommendations

5.1 For WHO

The following actions were recommended for WHO:

- (1) WHO should develop guidelines and standard operating procedure for CD4 T lymphocytes enumeration.
- (2) WHO should support the expansion of Regional CD4 T lymphocytes EQAS to Sri Lanka, Myanmar, and Nepal in addition to India, Indonesia and Thailand where it is already in operation.
- (3) WHO should develop criteria for clinical application of CD4 counts in paediatric population.

5.2 For Member States

The following were recommended for Member countries:

- (1) Member countries should accord priority to establish infrastructure for CD4 T lymphocytes enumeration to provide effective support to ART programmes.
- (2) Member countries should initiate a national EQAS for CD4 T lymphocytes and selected laboratories should participate in WHO Regional EQAS.

- (3) Member countries should conduct studies to ascertain the baseline data of CD4 T lymphocytes in normal population to develop realistic country-specific cut off values for initiating ART
- (4) Member countries should ensure maintenance mechanism while selecting and procuring equipment for CD4 count technologies.

6. Concluding session

WHO's initiative in organizing this meeting was appreciated in the concluding session. The participants also felt that the deliberations shall go a long way in developing or improving the laboratory support to ART programmes. The meeting ended with a vote of thanks to officials who managed the meeting. Dr Rajesh Bhatia thanked the local organizers, Siriraj Hospital and the Ministry of Public Health, Thailand, for the conduct of this workshop. He also thanked the facilitators, local organizers as well as international experts who facilitated the workshop with their valuable contributions.

Annex 1

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Annex 2

Programme

Tuesday, 6 June 2006

0800 – 0930	Registration Inauguration Objectives Mechanism	
0930 – 1015	Pre-workshop test Overview of HIV, ART and CD4 enumeration	Dr Kovit
1015 – 1230	Country Reports	Moderator: Dr Rajesh Bhatia
1330 – 1430	Role of CD4 count: Discussions	
1430 – 1530	Principles of Flow cytometry and recent advances in its improvements	Dr Kovit
1530 – 1700	Low cost technologies to enumerate CD4 and their use in SEAR countries	Dr Madhuri Thakkar

Wednesday, 7 June 2006

0830 – 0930	Gating strategies and selection of reagents related to subset counting	Dr Kovit
0930 – 1015	Flow cytometer calibration	Dr Ramesh Paranjape
1015 – 1130	Haematology analyzers: TLC and differentials	Professor Sathien Sukpanichnant
1130 – 1230	Concept of pan-leucogating	Dr Kovit
1330 – 1430	Tour of the Institute	

1430 – 1530	Quality system and need in CD4 enumeration	Dr Kovit
1530 – 1700	Establishment of EQAS for CD4 enumeration	Dr Ramesh Paranjape

Thursday, 8 June 2006

0830 – 1230	Flow Cytometry Practical in Two Groups
1330 – 1430	Review of practical and trouble shooting
1430 – 1700	Group work 1 on haematology and flow cytometry

Friday, 9 June 2006

0830 – 1015	Presentation of group work 1 and discussion	
1015 – 1130	Selection and purchase of technology and equipment	Dr Kovit
1130 – 1230	Bio-safety	Dr Ramesh Paranjape
1330 – 1700	Practical on new technologies: Two Groups	

Saturday, 10 June 2006

0830 – 0930	Storage and shipment of material	Dr Kovit
0930 – 1015	Role of stabilizers	Dr Kovit
1015 – 1130	Equipment maintenance	Dr Madhuri Thakkar
1130 – 1230	Comparison of methods	Dr Kovit
1330 – 1430	Group work on requirements of CD4 lab and its functioning/management	
1430 – 1530	Post workshop assessment	
1530 – 1700	Recommendations and valedictory	Dr Rajesh Bhatia