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# CD4 T Lymphocytes Enumeration Techniques

*Report of a Regional Workshop  
Bangkok, Thailand, 24-27 November 2003*

WHO Project: ICP BCT 001



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## 1. INTRODUCTION

The AIDS epidemic continues to spread in the South-East Asia Region (SEAR), which is the second most affected Region in the world after sub-Saharan Africa. To date, close to 40 million people throughout the world have been infected with human immunodeficiency virus (HIV), the virus that causes AIDS. Of these, almost 6 million are in the SEA Region. Estimated population prevalence rates per 100 000 population range from less than 1 in DPR Korea to over 1200 in Thailand. Over 99 % of cases have been reported from three countries, Thailand, India and Myanmar.

The epidemic has affected persons in every country in the world. The majority of persons living with HIV infection and AIDS are found in the developing world where resources are poor and access to care and new treatments is limited. Despite the high prevalence rates, care for persons with HIV infection living in these countries was not given high priority, as a result of the high burden of disease due to other illnesses. In areas where the epidemic is most severe, the provision of care has been further compromised by the rapidly weakening economies and the costs of effective medications. Realizing the importance of anti-retroviral therapy, WHO has initiated various steps, the most notable being the commitment by the Director-General that within the next five years, at least 3 million HIV patients from developing countries shall be provided antiretroviral therapy (ART) by 2005 ("3 by 5" initiative).

The Global Fund for AIDS, Tuberculosis and Malaria has also identified ART therapy as one of the priority areas. The Regional Office is focusing attention on increasing access to treatment with antiretroviral drugs in preventing mother-to-child transmission and in the care of infected persons. With huge efforts being initiated in providing ART, a close monitoring of the patient who is on these drugs is also essential to ascertain the response. Enumeration of CD4 T lymphocytes is the most sensitive and specific indicator for this assessment. This indicator, together with the estimation of viral load constitutes two universally accepted monitoring tools. Since viral load has not been recommended by WHO, capacity building in CD4 T lymphocytes estimation is of great relevance.

The progressive depletion of CD4 T lymphocytes is the cardinal event in the pathogenesis of infection by the human immunodeficiency virus-1 (HIV). The number of these cells in the peripheral blood is the single most important parameter for monitoring the disease associated with HIV infection. Quantification of CD4 T lymphocytes is important in the staging and monitoring of patients infected with HIV. Throughout the course of the disease, the total T cell levels remain fairly constant despite a fall in CD4 T lymphocyte count, due to a concomitant rise in CD8 T lymphocytes. Therefore, the ratio of CD4 T lymphocytes to CD8 T lymphocytes is an additional important measure of disease progression. Measurement of lymphocyte subsets is done by flow cytometry which is the gold standard for enumeration of CD4 T lymphocytes. CD4 T lymphocytes enumeration is also utilized as a surrogate marker for HIV-induced damage; single CD4 T lymphocytes counts are used to assess the degree of immune deterioration; while repeated CD4 T lymphocytes tests define a declining slope of CD4 T lymphocytes counts, indicating the speed of progression towards AIDS. While on therapy, improvement in CD4 T lymphocytes counts is indicative of the success of therapy. In resource-poor countries, with the arrival of generic drugs for anti-retroviral therapy, the need for CD4 T lymphocytes counts has dramatically increased. CD4 T lymphocytes counts are the criteria for initiating ART as well as monitoring the therapeutic response in a patient.

Accordingly, a hands-on training course was organized at Siriraj Hospital, Bangkok, Thailand, from 24 to 27 November 2003 for enumeration of CD4 T lymphocytes through flow cytometry for those countries of the SEA Region where ART is expected to be taken up in a big way in the near future to facilitate effective utilization of scaled-up ART intervention. The detailed programme of the Workshop is placed as Annex 1. Fourteen participants from Bangladesh, India, Indonesia, Myanmar, Nepal, Sri Lanka and Thailand attended this Workshop. Five experts from India, Thailand and WHO facilitated the Workshop (see (1) List of Participants and (2) Programme at Annexes 1 and 2).

## **2. OBJECTIVES**

The objectives of the workshop were as follows:

- (1) To review the status of infrastructure available for enumeration of CD4 T lymphocytes in selected Member Countries;

- (2) To provide hands-on training on flow cytometry including the interpretation of results;
- (3) To discuss how to select and purchase the most appropriate technology;
- (4) To provide guidance on quality assurance, maintenance and biosafety aspects, and
- (5) To discuss the follow-up of strengthening national capacities for CD4 T lymphocytes estimation.

### **3. INAUGURAL SESSION**

The workshop was inaugurated by Prof. Piyasakol Sakolsatayatorn, Dean of the Siriraj Hospital. Dr Jai Narain, Coordinator, HIV/AIDS & TB, WHO SEARO, gave a brief introduction to the WHO '3X5' strategy and stressed the need for building laboratory capacity in the Region for meeting the needs of the HIV/AIDS patients who are likely to receive Anti-Retroviral Therapy (ART). Dr Sudarshan Kumari, Regional Adviser, Blood Safety and Clinical Technology, WHO SEARO, welcomed all the participants and outlined the objectives of the workshop.

The technical session was chaired by Dr Suniti Solomon, YRG Care, Chennai, India. It began with a presentation by Dr Jai Narain, who detailed the WHO 3X5 strategy as well as the current scenario of HIV/AIDS problem in the Member Countries of the SEARO region. He also provided the current scenario of ART in the SEARO Region and highlighted the gaps that existed currently between those in need of ART vis a vis those who were actually taking ART. In order to bridge this gap, one of the requirements was to build laboratory capacity in terms of CD4 enumeration technology. Dr. Gaby Vercauteren, BCT/HTP, WHO/HQ, Geneva, presented an overview of WHO-related CD4 activities. She outlined the various testing methods recommended by WHO for HIV testing, and CD4 enumeration. She also informed that WHO was in the process of developing uniform guidelines for CD testing across the world and stated that the draft guidelines would be posted on the Internet in January 2004 and requested all participants to view them and make suggestions, if any. She also stated that WHO was in the process of formulating an External Quality Assessment programme for CD4 estimation and making available reference reagents for this purpose to national reference laboratories in various Member Countries.

Country reports based on the WHO questionnaire were presented by Mrs Sirirat Likanonsakul from Thailand, Dr Ravi and Dr Balakrishnan from India and Dr Sondang Maryutka Sirait from Indonesia. It was observed that there was a need to evolve guidelines and /or Standard Operating procedures for CD4 testing in the Region, especially for specimen collection and transport.

Dr Vercautern, WHO/HQ, Geneva, made a presentation on the need for CD4 estimation in HIV/AIDS and the relevance of CD4 T cells in the pathogenesis of HIV/AIDS. This was followed by a presentation by Dr Kovit Pattanapanyasat of Siriraj Hospital, Bangkok, on the principles of flowcytometry, wherein he discussed the various physical principles involved in the technique of flowcytometry such as forward and side scattering of light; the basis of distinguishing the three cell populations viz. lymphocytes, monocytes and granulocytes; the principles of gating cells, the various types of flowcytometry such as single platform measurements using reference beads as controls; double and triple staining procedures, and their advantages and disadvantages. He also presented the various strategies for gating cells and the principles involved in selection of reagents and techniques for CD4 enumeration.

Dr.Sathien of Siriraj Hospital, Bangkok, made a presentation on haematology analyses: total leucocyte count, differentials and total lymphocyte counts. He explained the two principles of cell counting used in automated machines (electrical impedance and light scattering), the coefficient of variation between manual and automated methods, the principles of differential leucocyte enumeration (three part differential staining and the five part staining), and the factors that contribute to variation in differentials. Dr Sathien also explained the problems associated with automated differential counting such as lack of EQAS etc. Three part differential methods are considered adequate for use in the countries of the Region as the five part differential did not give any great advantage.

Dr Punneporn Wacinrapee of Siriraj Hospital, made a presentation wherein she discussed the principles, procedures, limitations and quality control issues involved in the FACS count technology. Although FACS Count provides absolute CD8 values and CD4/CD8 ratios, these two parameters do not add great value to clinicians in the management of HIV/AIDS patients. It is the absolute CD4 values that are used by clinicians. On the contrary, CD8

measurements only add to more costs of the assay. Therefore, it was felt that manufacturers should either reduce the cost of reagents, or do away with the CD8 tube and reagents to bring down the cost.

Dr Kovit of Siriraj Hospital, Bangkok, presented the findings of comparative evaluations of FACS scan and FACS count in Thailand and stated that there was excellent correlation between the two systems. He also presented the comparative evaluation of FACS scan, FaCS count and the single platform cyflow method. The Single platform Cyflow method showed good correlation with FACS scan and FACS count systems.

Two brief presentation were made by Dr Vijay Lakshmi of Nizam Institute of Medical Sciences from Hyderabad and Dr Balkrishnan from the Voluntary Health Service Hospital, Chennai on their experience of using FACS Count method in India. Dr Balakrishnan also presented the comparative evaluation of Coulter cytosphere method, which is a non-flowcytometric assay and uses a simple microscope. This assay showed good correlation with FACS count results but within a limited range of CD4 counts especially in the <200 cell range. He also highlighted the advantages (low investment cost) and limitations of this technique.

The participants attended a laboratory demonstration of four methods – FACS Count staining method, FACS scan staining method, the Coulter cytosphere method and the single platform cyflow method. They were shown how these methods were used, results obtained and interpreted as well as required quality control procedures.

Dr Ilesh Jani, Mozambique, Africa, delivered two talks on calibration procedures for flow cytometry and the concept of panleucogating. He also summarized the various practical considerations required for panleucogating.

Two group assignments on haematology and flowcytometry were undertaken by participants. Both groups presented their answers to the questions provided in the exercise. Dr Jani pointed out that the majority of the answers were correct, except that both the groups missed out the importance of pipetting errors contributing to the variations in the results pointed out in the exercise. He made a presentation on Quality Control (QC) and Quality Assurance (QA) in CD4 testing and long and short term stabilization of blood in CD4+ T cell counting. In the QC/QA lecture, he outlined the definitions and concepts related to quality and discussed two

major aspects of quality control - related to the instrument as well as the procedures. In the presentation on the use of stabilized whole blood, he outlined the need for stabilization, the use of these cells in quality control of CD4 testing and also the advantages and limitations of the short term and long term stabilization procedures. This was followed by a presentation on gating strategies and their comparative evaluation of non flow cytometric methods such as Dyna dynabeads and Coulter cytospheres.

Group work was also undertaken on how to select and purchase the most appropriate technology for CD4 enumeration. The two groups presented the various criteria to be considered for choosing a particular technology. Dr Gaby, WHO, Geneva, who facilitated the group work, pointed out that one of the most important criteria was the end use needs assessment and the policy-maker's considerations in choosing an appropriate technology. Dr Kovit of Siriraj Hospital, Thailand, made two presentations on the need for EQAS in CD4 testing and the Thailand experience on running an EQAS programme for CD4 testing for the past three years. He pointed the various advantages and limitations of running an EQAS programme and valuable lessons learnt in carrying out this exercise. Dr Kovit also presented a summary of practical issues in the management of immunophenotyping methods such as laboratory design, equipment maintenance, staffing pattern and laboratory QC/QA issues. Mr Pavan Behl of Guava Technologies, New Delhi, India, presented a new technology for CD4 estimation called the micro capillary flowthrough cytometry. He highlighted on the low cost and the novelty of this new technology which was currently being validated at several centres.

Dr Jani briefed participants on the statistical methods used for comparison. He explained the difference between correlation and agreement between two technologies and explained the limitations of using correlation coefficients,  $r$  values and  $p$  values. He subsequently detailed the currently available appropriate methods of agreement analysis and discussed the advantages of this new approach. Dr Gaby stressed on the importance and need for safety in the laboratory, the six safety considerations in any laboratory. He also underlined the need for evolving a safety policy for every laboratory and also fixing responsibility for safety by appointing a safety officer in the lab. Dr Gaby and Dr Kovit subsequently spoke of the importance of equipment maintenance, especially the aspects pertaining to the preventive maintenance.

The participants were given a group assignment to identify the constraints and the needs in their respective nations which were currently impeding the process of implementing quality testing in CD4 estimations and formulated recommendations.

#### **4. CONCLUDING SESSION**

The workshop ended with a concluding ceremony where the participants thanked the WHO for providing a very useful learning experience at the workshop and Dr Kovit and his team from Siriraj Hospital for their excellent arrangements and hospitality.

#### **5. RECOMMENDATIONS**

##### **For Member Countries**

- (1) SOPs should be developed and made available for the various CD4 technologies available currently.
- (2) A national EQAS programme for CD4 testing centres should be set up.
- (3) A concrete mechanism should be formulated for procurement of appropriate CD4 technologies based on their needs.
- (4) A needs assessment should be carried out by laboratory experts to assess the existing capacity in the Region and identify laboratory infrastructure support required to meet the key elements of the WHO "3X5" strategy.
- (5) With anti-retroviral therapy likely to be free of cost to HIV/AIDS patients, all Member Countries should work out a strategy with their respective national HIV/AIDS programme managers to ensure that CD4 testing also would be free of charge.

##### **For WHO**

- (1) WHO should assess the need for laboratory capacity including for CD4 enumeration and quality assurance within the context of WHO-led "3 by 5" initiative in the Region.
- (2) WHO should prepare a training plan on the basis of the need assessment in order to assist in rolling out ART in the SEA Region.

- (3) WHO should ensure capacity at the regional level to provide technical support on laboratory aspects of "3 by 5" initiative by including in the resource mobilization plan a staff with laboratory background and the requirements for laboratory capacity building at country level as stated in # 2 above. (Action: HIV/AIDS & TB)
- (4) WHO should develop uniform guidelines for the collection and transport of specimens for CD4 testing for use by all Member Countries in the SEA Region.

## Annex 1

### LIST OF PARTICIPANTS

#### India

Dr Amita Joshi  
Professor  
Department of Microbiology  
J.J. Group of Hospital  
Mumbai

Dr D.S. Chitnis  
Officer In-Charge  
VCTC  
Choitram Hospital  
Indore  
Madhya Pradesh

Dr V. Laxmi  
Additional Professor and Head  
Nizam Institute of Medical Sciences  
Hyderabad  
Andhra Pradesh

Dr. V. Ravi  
Additional Professor and  
Head of Neurovirology  
National Institute of Mental Health  
and Neuro Sciences (NMHANS)  
Bangalore

Dr L. Sharma  
Officer In-Charge  
CD4/CD8 Counts (Flowcytometer)  
Indira Gandhi Medical College  
Shimla  
Himachal Pradesh

#### Indonesia

Dr Sondang Maryutka Sirait  
SpPK from RSPH  
Infectious Disease Hospital  
Jakarta

Dr Nany Nursianti  
PHL  
Surabaya  
Dr Elly Santosa  
RSCM  
Cipto Hospital  
Jakarta

#### Nepal

Mr Purushottam Poudyal  
Dy. Chief  
Medical Technologist  
National Public Health Laboratory  
DHS, Ministry of Health  
Teku, Kathmandu

#### Sri Lanka

Miss D.N. Abeysinghe  
Medical Laboratory Technician  
National STD/AIDS Control Programme  
Ministry of Health  
De Saram Place  
Colombo – 10

#### Thailand

Mrs. Sirirat Likanonsakul  
Medical Technologist  
Chief of Laboratory of Immunology  
and Virology  
Barmrasnaradura Institute  
Department of Disease Control  
Bangkok  
Tel.: 0066-2590-3559-60  
Fax: 0066-2590-3561  
Email: siritlik@health.moph.go.th

Mr Wattana Auwanit  
Medical Scientist  
National Institute of Health  
Department of Medical Sciences  
Bangkok  
Tel.: 0066-2589-8701  
Fax: 0066-2591-5449  
Email: wattana@dmsc.moph.go.th

Miss Varipin Prasertsilpa  
Researcher  
The Research & Development Institute  
The Government Pharmaceutical  
Organization  
Bangkok  
Tel.: 0066-2203-8120  
Fax: 0066-2246-2134

#### **Temporary Advisers**

Prof. Sathien Sukpanichnant  
Department of Clinical Pathology  
Siriraj Hospital  
Bangkok 10700  
Thailand

Dr Suniti Solomon  
VHS-YRG CARE Infectious Disease Laboratory  
Centre for AIDS Research and Education  
IInd Floor, Main Building  
Voluntary Health Service Hospital Campus  
Taramani  
Chennai – 600 113, India  
Tel.: 0091-44-22542929  
Fax :0091-44-22542939

Dr. P. Balakrishnan  
Quality Manager  
VHS-YRG CARE Infectious Disease Laboratory  
Centre for AIDS Research and Education  
IInd Floor, Main Building  
Voluntary Health Service Hospital Campus  
Taramani  
Chennai – 600 113, India  
Tel.: 0091-44-22542929  
Fax :0091-44-22542939

Local Organizer

Prof. Kovit Pattanapanyasat  
Center for Excellence for Flow cytometer  
Office for Research and Development  
Faculty of Medicine  
Siriraj Hospital  
Bangkok 10700  
Tel.:0066-2-4197000 (Extn. 6644)  
Fax:0066-2-4181636  
Email: grkpy@mahidol.ac.th

#### **WHO Secretariat**

Dr Gaby Vercautern  
BCT/HTP  
WHO/HQ, Geneva

Dr Ilesh Jani  
WHO/HQ Geneva

Dr Sudarshan Kumari  
RA-BCT  
WHO, SEAR, New Delhi

Dr Jai Narain  
HIV/AIDS & TB  
WHO, SEAR, New Delhi

## Annex 2

### PROGRAMME

#### Monday, 24 November 2003

0830-0900 hrs	Registration	
0900-0930 hrs	Official opening ceremony	
1000-1015 hrs	Objectives	Dr Kumari
1015-1030 hrs	Overview of 3 x 5 strategy on ARV Therapy	Dr Jai Narain
1030-1045 hrs	Overview of the WHO CD4 related activities	Dr Vercauteren
1045-1130 hrs	Country reports: <ul style="list-style-type: none"><li>• India</li><li>• Indonesia</li><li>• Myanmar</li><li>• Thailand</li></ul>	All participants
1130-1145 hrs	Discussion	
1145-1200 hrs	Pre-workshop test	Participants
1200-1245 hrs	The role of CD4 T cell counting in HIV/AIDS	Dr Vercauteren
1245-1300 hrs	Discussion	
1400-1445 hrs	Overview of CD4 T cell enumeration technologies	Dr Jani
1445-1530	Principles of flow cytometry	Dr Pattanapanyasat
1530-1600 hrs	Discussion	
1615-1700 hrs	Gating strategies and selection of reagents related to lymphocyte subset counting	Dr Pattanapanyasat
1700-1730 hrs	Discussion	

1730-1745 hrs Review of day 1

**Tuesday, 25 November 2003**

0900-0920 hrs	Flow cytometer calibration	Dr Jani
0920-0930 hrs	Discussion	
0930-1015 hrs	Haematology analysers: total leucocyte, lymphocyte counts; differentials	Dr Sathien Sukpanichnant
1015-1030 hrs	Discussion	
1045-1130 hrs	The concept of PanLeucogating	Dr Jani
1130-1300 hrs	Tour of the Institute	
Practical exercises		
1400-1545 hrs	Flow cytometry: group 1 Alternative technologies: group 2	Dr Pattanapanyasat Indian Expert
1600-1745 hrs	Alternative technologies: group 1 Flow cytometry: group 2	

**Wednesday, 26 November 2003**

0830-0900 hrs	Review of practical experiments	
0900-0945 hrs	Group work: paper exercises on haematology and flow cytometry	Dr Jani
0940-1015 hrs	Presentation of group work and	
1015-1030 hrs	Discussion	
1045-1145 hrs	Group work: How to select and purchase the most appropriate technology	All facilitators
1145-1230 hrs	Presentation of group work discussion	
1400-1500 hrs	Quality assurance: How to produce reliable results	Dr Jani
1500-1515 hrs	Discussion	
1515-1545 hrs	Role of stabilizing reagents	Dr Jani
1600-1630 hrs	External quality assessment schemes	Dr Pattanapanyasat
1630-1700 hrs	Maintenance of equipment in the immunophenotyping laboratory	Dr Pattanapanyasat

1700-1715 hrs Discussion

1715-1730 hrs Review of day 3

**Thursday, 27 November 2003**

0900-0945 hrs Method comparison Dr Jani

0945-1000 hrs Discussion

1015-1045 hrs Safety in the immunophenotyping laboratory Dr Vercauteren

1045-1100 hrs Discussion

1100-1145 hrs Group work: identify the main problems/solutions in running a CD4 counting service

1145-1230 hrs Presentation group work and discussion

1230-1300 hrs Practical issues in the management of an immunophenotyping laboratory Dr Pattanapanyasat

1400-1415 hrs Post workshop test

1415-1530 hrs Recommendations

1530-1600 hrs Closure