

# Sentinel Surveillance for Drug Resistance in Leprosy

Report of the Workshop  
Paris, France, 26-27 October 2009



**World Health  
Organization**

Regional Office for South-East Asia

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## Contents

	<i>Page</i>
1. Background.....	1
2. Objectives.....	1
3. Opening session.....	2
4. Setting the scene.....	2
5. Country presentations.....	3
5.1 Brazil.....	3
5.2 China.....	4
5.3 Colombia.....	5
5.4 Indonesia.....	5
5.5 Myanmar.....	6
5.6 Pakistan.....	6
5.7 Philippines.....	7
5.8 Uganda.....	7
5.9 Viet Nam.....	7
6. Open conference.....	8
7. Technical forum.....	9
8. Presentations from collaborating reference laboratories on Drug Resistance Surveillance.....	11
9. Conclusions and recommendations.....	15
10. Proposed next steps.....	16

## Annexes

1. Agenda.....	17
2. Participants.....	18



## 1. Background

The Global Leprosy Programme (GLP) has initiated the establishment of a sentinel surveillance network for monitoring drug resistance (DR) in leprosy after consultations and deliberations at two workshops held in India (2006) and Viet Nam (2008). The network is currently operating in eight leprosy-endemic countries: Brazil, China, Colombia, India, Myanmar, Pakistan, Philippines and Viet Nam. The following countries have also shown interest in joining the network: Burkina Faso, Indonesia, Mali and Nigeria.

To standardize the technical and operational procedures and to maintain quality control, WHO has issued "Guidelines for Global Surveillance of Drug Resistance in Leprosy" (SEA-GLP-2009.2). Reference laboratories in Brazil, France, India, Japan, Korea, Switzerland and USA are collaborating with WHO. These laboratories are providing free testing services to support the initiative. The Global Health Institute, Lausanne, Switzerland (Professor Stewart T. Cole) has offered to provide support for quality control aspects of the surveillance system. A laboratory-based training workshop was also conducted in Brazil in 2009.

The workshop for Sentinel Surveillance for Drug Resistance in Leprosy was held in Paris, France on 26-27 October 2009. The National Reference Centre for Mycobacteria and mycobacterial resistance to anti-tuberculosis in Paris, France (University Paris Diderot, AP-HP) which is one of the participating reference laboratories hosted this workshop. This laboratory was instrumental in investigating the mechanism of drug resistance in *M. leprae* and is currently carrying out further research. The Association Francaise Raoul Follereau and the Association de l'Ordre de Malta which are members of the International Federation of Anti-Leprosy Associations (ILEP) agreed to support this workshop.

## 2. Objectives

The objectives of the workshop were:

- To review the drug resistance surveillance data from 2008-2009;
- To review trends in relapses reported by the national programmes;
- To update on recent advances in DNA sequencing technology; and
- To develop ways to improve the quality of operations.

### 3. Opening session

The workshop was held at “Le Musee des Moulages”, Saint Louis Hospital. This historic museum houses an extensive collection of wax models for various dermatological diseases. The two-day sessions were organized into ‘Setting the stage’, ‘Country presentations’, ‘Open Conference’, ‘Technical forum and workshops’, and ‘Presentations’ from collaborating reference laboratories on drug resistance (DR) surveillance.

The opening remarks were given by Dr Benoit Shlemmer, Dean of the Paris-Diderot University. He spoke on the history and importance of leprosy at the University Diderot and Saint Louis Hospital, a hospital specializing in dermatology since the 18<sup>th</sup> century, in basic and applied research.

Dr V. Pannikar, Team leader, WHO Global Leprosy Programme, SEARO, welcomed the participants, introduced the Chair, Dr Herman Joseph Kawuma from the German Leprosy Relief Association (GLRA), Uganda and the rapporteurs Dr Varalakshmi Vissa from Colorado State University and Dr Khin Saw Aye from the Department of Medical Research, Lower Myanmar following which the participants introduced themselves and the institutes they represent.

The meeting was attended by about 60 delegates from 19 countries, whose responsibilities and expertise encompassed aspects of global and national leprosy programmes on policy and control, patient care and services in the government and NGO domains, academic research (basic and translational), and commercial enterprise.

### 4. Setting the scene

Dr Myo Thet Htoon, Medical Officer, WHO Global Leprosy Programme, SEARO gave a succinct summary of WHO’s leprosy drug resistance surveillance initiative, recent findings of drug resistance in several regions (WHO, Weekly Epidemiological Record, No.26, 2009,84, 261-268), the impetus and continued global acceptance and expansion of the programme, and the gaps therein.

Professor Thomas P. Gillis from National Hansen’s Disease Programmes, USA further set the stage for the workshop by briefly reviewing the current drugs used in leprosy treatment, their mechanisms of action and the ability of *M. leprae* to become resistant to them, via proven mutations in drug resistance determining regions (DRDR) in target genes *rpoB*, *folP1* and *gyrA*. He also addressed unresolved issues, such as the unknown mechanism of clofazimine resistance, and resistance in leprosy patients that show no *M. leprae rpoB/folP/gyrA* mutations. He also reminded the participants that not all mutations in these genes may be related to DR. These issues call for banking of *M. leprae* isolates in the laboratory (in mouse foot pad infections) and the development and testing of new drug regimens, such as new drug candidates emerging from investigations in tuberculosis (e.g., diarylquinolone TMC207).

## 5. Country presentations

Country presentations on the leprosy situation and involvement in DR surveillance were given by Dr Maria Leide W. Oliveira from Brazil, Prof Zhang Guocheng and Dr Shen Jianping from China, Dr Nora Cardona-Castro from Colombia, Dr Prima Kartika Esti from Indonesia, Dr Kyaw Kyaw from Myanmar, Dr Christine Schmotzer from Pakistan, Dr Francesca Gajete from Philippines, Dr Kawuma from Uganda and Dr Nguyen Thi Hai Van from Viet Nam. Each country/organization discussed their proposed approach for monitoring drug resistance, including proposed sentinel sites, plans for drug resistance identification using PCR and/or mouse foot pad infections; many discussed current leprosy statistics in their country as well as the increased focus on identifying relapse patients.

### 5.1 Brazil

Dr Maria Leide W. Oliveira gave a presentation on a pilot project on relapse investigations developed in 2007-08 for evaluation of drug resistance in *M. leprae* with the use of DNA sequencing technology. She said the percentage of relapse cases among new entries for leprosy treatment in Brazil (2001 to 2008), increased from 2.4% (1,096 relapses) in 2001 to 4.0% in 2008 (1,541 relapses). Despite the percentage being considered high, she emphasized that this is secondary data as it has not been confirmed and probably many of these cases could be having reactions. Also, the increase is proportional to the larger contingent of cured cases and the longer monitoring period.

It was pointed out that in 2009, the Ministry of Health provided guidelines on relapse surveillance, especially for areas in and around the former leprosaria with a precedence of resistant cases. The use of a specific form was recommended by the national programme to collect data on clinical events after release from treatment with the objective of standardizing diagnostic criteria and collecting information on the registered relapse cases. In order to implement this guideline and to comply with the Global Surveillance of Drug Resistance in Leprosy/WHO, the following data collection instruments were proposed: a technical guideline from the National Hansen's Disease (Leprosy) Control Programme (PNCH/MoH) for establishment of relapse surveillance measures, including investigation of drug resistance; instructions for the management of suspected relapse cases, using an investigation form for the cases notified as relapse in the SINAN national information system; and an organizational chart and guidelines for the collection and processing of skin smears to be used in molecular biology testing at regional and national levels (FIOCRUZ, Rio de Janeiro), as well as for biopsy samples to be sent to the Lauro de Souza Lima Institute, Sao Paulo to carry out mouse footpad (MFP) studies in addition to DNA sequencing.

There is also a recommendation that there should be no re-treatment of leprosy cases without the patient first undergoing a full clinical and laboratory evaluation. Cases with incorrect classification (that are MB instead of PB) or having defaulted treatment in the past should not be considered relapses, but rather classification errors. Suspected

relapse cases should be referred to the closest reference centre or undergo sample collection (slit skin smears and biopsy) for laboratory examinations such as bacilloscopy and DNA tests.

Dr Oliveira explained that cases of relapse in leprosy are rare in patients treated regularly with multi-drug therapy. Generally these cases are observed during a timeframe of five to ten years after cure (average of 10 years in the 2008 pilot study). However, late reactions represent a difficult differential diagnosis and each state should identify reference centres to undertake technical discussions on these cases so as to help meet the needs of the primary care system. The forms are to be completed by the health unit that confirms the relapse diagnosis, preferably a reference centre. If the patient is not referred with a copy of the notification / treatment evaluation form, this should be requested from the primary care unit or the state/municipal health secretariat. The completed forms should be forwarded together with the relapse notification form to the state secretariat, where they will be analysed by qualified technicians in this area.

Dr Oliveira shared notes about cases that presented after a history of regular treatment with different therapeutic regimens, with clinical conditions suggestive of infiltrated lepromatous (Virchowian) lesions, a high bacteriological index and whole bacilli or those who have seen no lesion regression with the standard MDT-MB. Such cases should be considered as possible resistance cases. For these cases, she suggested that a special protocol be followed at reference centres for laboratory assessment for drug resistance.

## 5.2 China

Professor Wang Hongsheng gave a presentation on the case detection rate in China which has not decreased for 15 years with about 1,600 new cases and 150 relapse cases detected each year. The number of cases with positive skin smears accounted for 65% of the newly detected cases. He explained that MDT was introduced to China in 1982 and covered the whole country in 1986. The patients with positive skin smears before MDT is administered should have a skin smear test once a year until they are skin smear negative. Therefore the duration from start of treatment to release of patients is usually 3-6 years for MB and 1-3 years for PB patients. Relapse is confirmed by reappearance of new symptoms and signs of the disease, after successful completion of MDT treatment and with any one of following findings: (a) the skin smear is positive again after skin smear was negative and has an increase of at least 2 units of BI at any previously tested site or (b) biopsy shows specific histopathologic change of leprosy with strong acid-fast positivity and significant number of bacilli. If necessary, the suspected relapse patient would be carefully examined by at least two professional workers at county or district level by skin smear test and biopsy for confirmation of relapse.

Yunnan and Guizhou provinces were originally selected as the sentinel sites for surveillance for drug resistance in leprosy in 2009, but based on the situation of leprosy in the country, the programme was expanded to include a total of 15 key provinces in Southern China.

Professor Hongsheng pointed out few difficulties in conducting drug resistance surveillance such as transportation of samples from some provinces especially Yunnan and Guizhou due to a strict regulation that prohibits shipment of liquid and corroded material by post and the implementation of the WHO form for the collection of samples which is complicated for local health workers. Furthermore, most of the relapses in China have been successfully retreated with a new course of MDT. So the health workers lack interest to collect the samples. These issues make it difficult to collect enough samples.

### 5.3 Colombia

Speaking on behalf of Dr Ernesto M. Naranjo (National Programme Manager, Colombia) Dr Nora Cardona-Castro from the Instituto Colombiano de Medicina Tropical reviewed the national prevalence, incidence and relapse statistics on leprosy. MB cases constitute approximately 70% of the cases. Certain municipalities have a prevalence of more than 1 case per 10,000 inhabitants. Relapses since 2001 have ranged from 7 to 25 cases per year. She informed of the recent change in standard drug treatment for leprosy (24 months reduced to 12 months for MB, 12 months reduced to 6 months for PB). Use of ofloxacin in combination with minocycline instead of clofazimine is limited to patients who refused it due to skin pigmentation. Regarding molecular testing, she presented laboratory findings based on skin smear or biopsy samples collected from 117 patients (89 new or under treatment and 28 relapses) from different regions of Colombia, which revealed that six of the 28 relapsed cases had mutations in *M. leprae* DR target genes. These include a case with double mutation in *M. leprae* [*folP1* codon 55 CCC-CTC and *rpoB* codon 425 TCG-ATG] and two cases previously treated with DDS monotherapy [*rpoB* codon 425 TCG-TTG]. One other case with several relapses had the *rpoB* codon 410 GAT-TAT mutation. One case carried a previously unreported substitution at *folP1* in codon 91, while another had the known DR mutation in codon 53 ACC-GCC along with a mutation in codon 88. A patient with 2 months of MDT carried a typical *M. leprae* codon 53 *folP1* DR mutation. However, she expressed concern that not all 117 cases were PCR positive at all DR targets and relapses were not always associated with known target mutations. Points for discussion thus include relapse classification, re-infection and lack of detailed information on adherence to unsupervised components of MDT. A novel *gyrA* codon 99 mutation not related to DR was identified in a subset of the patient samples.

### 5.4 Indonesia

Dr Prima Kartika said that in Indonesia the definition of relapsed case is the same as in the WHO guidelines. The diagnosis is by national consultants and supervisors and patients are also treated as per WHO guidelines. The number of MB cases relapsed, diagnosed at Manado and tested for drug resistance (2000-2005) was 21. Results of DNA sequencing showed mutation in *folP1* (dapsone resistance) in one case and *rpoB* (rifampicin resistance) in two cases.

Other molecular-biology studies carried out at the Tropical Disease Center Laboratory on *M. leprae*, were presented by Prof. Indropo Agusni. These include establishment of new PCR primers for detection of *M. leprae*, for clinical and environmental specimens, *M. leprae* viability studies, genomic mapping, and dapsone, rifampicin and quinolone resistance studies.

## 5.5 Myanmar

Dr Kyaw Kyaw gave his presentation on the establishment of procedures for relapse identification and definition according to the WHO guidelines. The guidelines to differentiate between relapse and reactions are being provided to the team leaders of leprosy control programmes and a referral network for confirmation has been set up. He reported that there were 35 relapses in 2009; out of these skin specimens were collected from 20 (57.1%) cases. DNA sequencing from relapse showed mutation in *folP1* (DDS resistance) in one case (codon 55: CCC-CGC) and *rpoB* (rifampicin resistance) in one case (codon 526: CAC-CTC). Results of 18 randomly selected active MB cases showed two cases of DDS resistance (*folP1* codon 53: ACC-TCC and 55: CGG-TGG). He suggested that the surveillance coverage area should be increased for specimen collection and that specimens from some active MB cases should be included. He said that more sensitive tests in molecular detections for drug resistance are needed due to cases with highly positive skin smears that showed PCR negativity and that there should be a supply of alternative drugs for confirmed drug resistant patients.

## 5.6 Pakistan

Dr Christine Schmotzer informed that Pakistan is a low burden country for leprosy where prevalence is 0.52 per 10 000 population and the detection rate is 0.28 per 100 000 population. The leprosy control programme in Pakistan is partly integrated in primary health care (PHC) centres, run by specialized staff with strong support from NGOs since its inception. MDT according to WHO recommendations was introduced in the early 1980s. The leprosy elimination target was achieved at country level in 1996. The leprosy data show a slow but steady decline in new cases, a persistent high rate of Grade 2 disability and satisfactory figures for treatment completion.

Dr Schmotzer reported that relapse rates remain steady. However, over-diagnosis of relapse cannot be excluded as not all projects follow the stringent WHO criteria. The Rawalpindi Leprosy Hospital has been selected as a sentinel site for drug resistance surveillance in leprosy in 2009. Till now two relapse cases of leprosy have fulfilled the criteria for sample collection according to the WHO guidelines. For the future, she recommended organizing a country consensus consultation on leprosy management including relapse, to initiate validation of leprosy data in 2010, and to include other possible cases of drug resistant leprosy into the target group for testing.

## **5.7 Philippines**

Dr Francesca Gajete presented the trend and strategies for leprosy control in Philippines. She identified barriers to treatment as the existing healthcare services (referral centres) in the country cannot meet and provide for the leprosy patient needs: the prices of services (laboratory, surgical and physical therapy) do not fit the patients income and ability to pay; the inaccessibility of services capable of providing quality leprosy care to the leprosy patients; the organization of health care services that does not meet leprosy patients' expectations in most areas and the attitude of healthcare providers towards persons affected by leprosy. Operational research is being carried out to improve implementation of a sustainable national leprosy control strategy specifically on prevalence survey for the last 10 years particularly on relapse cases. She concluded that the focus of the National Leprosy Control Programme for the next 5 years will be on quality assured epidemiological data collection; evidence based care and operational research; establishment of functional sentinel surveillance system for drug resistance in collaboration with National Epidemiology Center (NEC), and Field Health Surveillance Information System (FHSIS).

## **5.8 Uganda**

The leprosy situation in Uganda was discussed by Dr Herman J. Kawuma. It was highlighted that 300-400 new cases are reported annually. In 2008, 345 new cases (80% MB) were reported by 50 out of 80 districts of which 70% of new cases came from just 13 districts. MDT was introduced in 1982, and achieved national coverage in 1994. In the last 26 years, about 17,000 people completed MDT. Information about new cases, relapses, transfers, treatment completion, defaulting and death are reported quarterly to the Ministry of Health by each of the 80 districts. Over 90% of expected reports are actually submitted. The reporting format has numerical data about relapses started on MDT as well as provisions for more detailed information on each case and the treatment outcome. Information about relapses has been collected since 1990; more systematically during the period 2000-2008. In nine years 139 suspected relapses were reported, 64 (46%) treated with non-MDT regimens (variety of rifampicin and clofazimine containing regimens). Suspected cases of relapse after WHO MDT were 68 and those continued and reported to WHO were 36 (53%). He mentioned the reasons for excluding cases from the list of relapses after MDT are: treatment with non-WHO regimens; regimens not recorded; classification errors (MB treated as PB); late reactions labelled as relapse; treatment after default called relapse; recording errors and wrong diagnosis of leprosy. He advised a revision of the national guidelines to define more clearly the criteria for suspecting and diagnosing relapse.

## **5.9 Viet Nam**

Dr Nguyen Thi Hai Van introduced the administrative set up of the national leprosy control programme and provided the figures for leprosy indicators in Viet Nam for the 2004-2008 period. With a steady prevalence rate of 0.1/10,000 population, the number

of new cases had decreased from 858 to 530 over the five-year duration. Sixteen provinces however have a prevalence rate of  $>1/10,000$ . One hundred and eight relapses were identified during 2000-2008. Relapse cases identified at the provincial level are confirmed at regional and national levels with histopathology tests in addition to the BI, MI and clinical exams. Dr Van presented results from 12 MB relapse cases that were recruited in 2009 for drug resistance surveillance. The samples were tested in Korea and Japan. Three cases showed DDS resistance (mutations in *folP1* codon 55: CCC-CTC, 53: ACC-ATC, and 53: ACC-GCC) and nine cases showed no mutation in *folP1* (silent mutations were not reported). There were no mutations in *rpoB* and *gyrA* in all relapse cases.

## 6. Open conference

In the evening session of the “Open conference” held at the university auditorium several presentations were made.

*A review of molecular research in Leprosy: Present-day needs* was given by Professor Patrick Brennan (Colorado State University, USA). He reviewed the history of leprosy research and clinical trials made possible through initiatives, leadership and sponsorship from WHO, government and various charitable institutions. He commended the prominent players in the past and present committees that have been responsible for landmark global programmes such as vaccine trials, *M. leprae* whole genome sequencing and the generation and distribution of research reagents such as antigens, antibodies and recombinant gene libraries to the research communities.

Dr Nicolas Veziris (National Reference Centre of Mycobacteria, France) discussed the *Chemotherapy research in leprosy: current use and future of mouse footpad models before clinical trials*. Current options for treating drug resistance as well as the need to find new drugs to treat those with resistance to all of the current drugs were reviewed. He mentioned several possible new drugs; moxifloxacin, diarylquinoline (R207910), nitroimidazole (PA-824), and oxazolidinone. However, he added that only moxifloxacin and diarylquinoline show real promise as possible new drugs for leprosy treatment. Nitroimidazole and oxazolidinone have low or no activity against *M. leprae*. He reviewed results from murine studies, in which he looked at the possibilities of new drug regimes for leprosy treatment from both existing second-line leprosy drugs and existing drugs not yet used for leprosy treatment. It was shown that clarithromycin and minocycline may be able to replace dapsone and clofazimine in daily standard regimens, and that rifampin, ofloxacin, and minocycline (ROM) after a two-year evaluation showed promise in treatment of MB leprosy. Some existing drugs that are not currently used in leprosy are showing some promise, moxifloxacin and rifapentine both have shown good activity against *M. leprae*. Diarylquinoline, a drug currently under development, may be a good candidate for treatment of drug-sensitive and drug-resistant leprosy.

Dr Stewart Cole (Ecole Polytechnique, Switzerland) gave a lecture on *Comparative Genomics and Phylogeography of M. leprae*. His investigations in these aspects originally stemmed from a need for genomic tools to differentiate relapses from re-infections. His

research efforts led to the completion of the whole genome sequence definition of an *M. leprae* strain, from an Indian patient. The striking features of the *M. leprae* genome, in particular the extensive gene loss and decay and their implications in slow doubling time and intracellularism were pointed out. Then, he discussed approaches for identifying and screening candidate markers for strain typing based on the known genome sequence. He described the discovery of a few SNPs based on partial genome sequence of representative strains and a model for the origin of leprosy and the global distribution of four major types (SNP type 1-4) through human migrations. Finally, he presented new data based on the complete elucidation of the genome sequences of three other reference armadillo-passaged human isolates genomes (Br4923, a Brazilian isolate), Thai-53 (from Thailand) and NHDP63 (of N. American origin).

Professor Emmanuelle Cambau gave her presentation on *Drug resistance in leprosy and recent advances in its detection*. She explained that molecular detection of drug resistance in leprosy was both easily implemented and well supported. She emphasized that gene mutations are not always predictive of antibiotic resistance and therefore assays to demonstrate this are required. She also discussed the current methods of genotypic detection of drug resistance, focusing on PCR, pointing out the *in vivo* mouse footpad susceptibility test has a lower efficiency for biopsies with low bacterial index compared to PCR method of target amplification. She then introduced the DNA STRIP technology which is based on hybridization of multiplexed PCR amplification products of DR targets to a strip coated with specific probes representing various nucleotide substitutions.

## 7. Technical forum

On the second day of the workshop, a series of presentations were made on the technical aspects of the WHO recommended methods for drug resistance surveillance and other recent and independent research developments.

The first presentation was by Dr Masanori Matsuoka (Leprosy Research Center, Tokyo) on *Quality Control: technology, logistics and reporting*. Dr Matsuoka also reviewed previous published research on the conventional methods for testing drug susceptibility. With regard to the ongoing WHO surveillance initiative, as part of quality control exercises, he had distributed nine coded vials (mouse foot pad derived *M. leprae*) of bacillary suspensions in 70% ethanol to eight reference centres. However, only three centres reported back with results. A second batch of coded samples was sent. Again only three centres reported results. At the meeting, he revealed the sample codes and the concentrations (bacilli/ $\mu$ l). The nine samples (0.1ml each) comprised four reference isolates in two concentrations,  $\sim 10^5/5 \mu$ l and  $10^3/5 \mu$ l; one sample was a negative control. Poor sensitivity of PCR detection was noted in the testing laboratories; nested PCR was applied in one laboratory for detecting amplification. Regarding reporting of data, Dr Matsuoka proposed that the current WHO form be modified so that when samples are sent to reference centres for testing, the sample ID information is included.

Dr Khin Saw Aye discussed trials in Myanmar based on *the use of FTA elute cards for collection of skin smear samples* for PCR testing. She reviewed examples of current use of

the FTA elute card in other clinical applications. The FTA elute card is based on the principle that the cells are lysed and proteins denatured to protect nucleic acids from microbial and fungal attack, allowing the cards to be spotted with sample, collected, stored, shipped, and archived at room temperature and for the DNA to be purified later in a laboratory setting. A study was undertaken to determine if the FTA elute cards worked as well as 70% ethanol for the storage of slit skin smears and subsequent PCR of *M. leprae*. This hospital and laboratory-based cross-sectional study was held in three areas of Myanmar. Slit skin smear (SSS) samples were collected from the same site on the same patient, the 1<sup>st</sup> was spotted on the FTA elute card and the 2<sup>nd</sup> was rinsed into a vial containing 70% ethanol. DNA from FTA elute card was obtained by punching out the specimen spot with a sterile disposable biopsy punch into a tube containing water, briefly vortexing the tube to rinse the matrix, transferring the punched out disc to a fresh tube containing 0.03 ml of water. The DNA was released by heating the sample at 95°C for 30 minutes and repetitive vortexing. The disc was discarded and 5-10 µl of the extract was used as template in nested PCR for the amplification of a multicopy *M. leprae* specific target RLEP. After performing the PCR on the samples and comparing the positive and negative results it was shown that the PCR positivity for the samples collected on FTA elute cards (52%) and the ethanol in tubes (60%) were not significantly different. The FTA elute card has several advantages over the ethanol tubes, including their small size, easy storage, and easy DNA elution. However, more studies are needed to determine the relationship between the sample collection method and bacillary index and the efficiency of PCR amplification of drug resistance surveillance relevant to *M. leprae* targets.

Dr Masanori Kai (Leprosy Research Centre, Japan) discussed his concerns on *DNA extraction and PCR Sequencing* aspects, and proposed a simple technical amendment. He highlighted the need to conduct PCR amplification for the high copy *M. leprae* specific targets RLEP, prior to that for the drug resistance genes to make sure that the samples contain *M. leprae*. This was based on findings that in some samples, drug resistance targets amplified while RLEP did not, and that sequencing of the amplification from such samples revealed that the DNAs were not of *M. leprae*, but that of other mycobacterial species.

In her presentation on the techniques involved in conventional drug resistance testing in leprosy by *mouse footpad inoculation*, Professor Emmanuelle Cambau reminded the participants of the challenging minimal requirements and the practical considerations with regard to maintenance of the mouse colonies and the execution of the assays for investigations in leprosy, particularly for diagnosis of drug resistance and for evaluation of new drugs. These include:

- the need for large numbers of animals for controls and tests,
- specialized facilities and equipment,
- rapid transport of fresh biopsy specimens to the laboratory that yield inoculum of at least 5000 bacilli/30µl,
- trained animal housekeeping and technical staff,

- lengthy assay durations with no interim indicators regarding progress of experiment, and
- compliance with administrative and regulatory policies for animal and infectious agent use.

However, for the banking of reference and other clinical *M. leprae* strains for the research community, it would be of interest to survey the number of laboratories globally with previous, current or future interest/experience in mouse foot pad inoculation methodologies and to discuss the cost-benefit aspects of such services.

In this context, Dr Cambau described once again the *DNA strip technology* (Hain Lifescience) which carries oligonucleotide probes for wild type and a panel of known mutations in target genes *folP1*, *rpoB* and *gyrA* predictive of drug resistance to dapsone, rifampicin and fluoroquinolone. The technology was evaluated with DNA from 101 skin biopsies, some of which carried mutations in *rpoB* (n=14), *folP1* (n=18) and *gyrA* (n=1). The results were highly concordant with sequence based (99/101 = 98%) and mouse foot pad susceptibility assays (91/91=100%) thus suggesting that it can replace DNA sequencing in the current work flow for molecular detection of drug resistance (for patients with BI>2).

Referring to *DNA data banks and analysis of mutations*, Dr Stewart Cole briefly showcased a new web interface version of the Mycobrowser which contains updated annotated data for all genes of *M. leprae* and *M. tuberculosis* H37Rv genomes. Besides providing access to information about genes, their DNA and protein sequences, features, functions, genome context and allowing other advanced searches, Mycobrowser is aimed at enabling future storage and analysis of new data such as genome sequences of other *M. leprae* strains and drug targets.

## 8. Presentations from collaborating reference laboratories on Drug Resistance Surveillance

During the course of the two-day sessions new molecular data collected from skin smears or biopsy specimens from patients in Asia (China, India, Indonesia, Korea, Myanmar, Philippines, Thailand and Viet Nam), Africa (Mali), South America (Bolivia, Brazil, Colombia, Uruguay, Venezuela) and Europe (France and French territories) in the various reference laboratories was introduced. The total number of patients (new and relapsed) identified at the sentinel sites, the number of patient samples processed by PCR amplification, and mutation if seen were reported. Overall, the presentations emphasized the need to monitor dapsone and rifampicin resistance and its causes, as well as the need for an agreed standard definition as to what is a relapse case.

Dr Euzenir Sarno (Oswaldo Cruz Foundation, Brazil), reported on the findings of a long-term relapse study (1986-2006 and 2006-2009) conducted by her institute. Characteristics of relapse patients identified at FioCruz and elsewhere were investigated and salient findings were summarized. The different duration of treatment regimens were

MDT of 24, 12 and 6 months. A total of 110 suspected 'relapse' patients presented during the study period, 66 of these were diagnosed at FioCruz. Of the 66 patients, 32 had received their first treatment at FioCruz and 17 (9 MB, 8PB) completed a full course of MDT. Four of the PB cases relapsed as MB. The incidence rate (per 10,000 person year) based on the 17 cases are 8.1, 3.1 and 5.5 for the 24, 12 and 6-month regimens respectively. None of the 66 patient specimens showed DR mutations.

In general, the relapses presented 4-23 years after completion of treatment. Of concern to Dr Sarno was the possibility of relapse with new skin lesions, or as BI + reversal reactions five years after MDT, and worsening nerve lesions with acid fast bacilli in nerve biopsy. She also recommended revision of diagnostic criteria of the indeterminate form. SSS sampling instead of biopsy and use of FTA cards were proposed as practical alternatives in the DR surveillance process. The importance of requiring national coordination, with decentralized administration and the resources to collect, bank and validate data was stated. Dissemination of standard protocols and training was offered by FioCruz. These activities require budgets for equipment, supplies, travel and meetings.

Dr Philip Suffys (Oswaldo Cruz Foundation, Brazil) elaborated on the ongoing molecular testing performed in connection with relapse surveillance in five states (Amazonia, Para, Ceara, Espirito Santos and Rio de Janeiro) in Brazil. DNA extraction (using an in-house sephaglass binding method or the commercial Qiagen DNeasy kit) from 230 skin biopsies stored in 70% ethanol were subjected to a four gene PCR-sequence screen. The PCR products were excised from agarose gels and purified using the Charge Switch Kit (Invitrogen USA), sequenced using the institution's ABI3730 Genetic Analyzer based on the ABI Big Dye 3.1 reaction system. Results were tabulated for 145 samples drawn from a collection of 135 MB and 66 PB relapse cases. From the PCR positive data (46/127 for *folP1*, 52/155 for *rpoB*, 73/145 for *gyrA*), the DR rates were estimated as 8% for rifampicin and 4% for both dapsone and rifampicin, these cases coming from Amazonia and Para only. Philip Suffys highlighted the following molecular testing problems faced in this study: specificity of *gyrA* primer that yields product with *M. tuberculosis* DNA, sensitivity with regard to MB and PB cases (60-70% of MB cases produce PCR products that can be sequenced, although some genes perform better than others; PCR amplification from PB patient specimens is poor and sensitivity of detection of mutant (resistant) alleles in the presence of wild type (susceptible) DNA by sequencing methods. He pointed that out that five cases showed possibility of mixed infection based on *folP1* sequences. In this context of detection of mixed populations, he suggested that alternatives such as reverse line blots (RLB), SNPlex, DNA chip and other assays are needed.

He presented evidence of re-infection rather than reactivation as a cause of 'relapse' on the basis of findings of a SNP in *gyrA* (synonymous T to C transition, not related to DR). Interestingly, relapses were of 'C' allele in Brazilian states where the predominant allele of general cases is 'T'. These tentative observations of whether a new strain is selected over background strains in relapses require further strain typing for validation, although testing of the *gyrA* SNP in paired specimens (first and relapse diagnosis) in 8

cases showed that T to C conversion occurred in 4/7 cases (data was not available for the first event in one case).

The South Korean investigations in the area of drug resistance surveillance were presented by Dr Sang Nae Cho from Yonsei University, Korea. The number of relapses in S. Korea during 1999 to 2008 was 339, yielding a cumulative risk of relapse of 2%. The relapse numbers have steadily declined from 23 in 1999 to three in 2008. In 2008, 51 new cases were detected. The cases tend to belong to the >50 year age group. He discussed results from a recent unpublished study (26 new and 17 relapses) and that from a published 2005 study (104 patients with BI>3+, including 16 relapses). Mutations in *folP1* DRDR in both new and relapses are evident, though more frequent amongst relapse cases (6/17 in relapse versus 3/26 in new cases) in the recent work as seen previously (in 4 new and 16 relapses). However, *rpoB* mutations have been restricted to relapses. Moreover, combining both studies, five relapsed patients appear to be resistant to both DDS and Rifampicin. Thus, in this region DDS resistance is at least 10% amongst new cases, and higher in relapses. The Yonsei laboratory also provides technical assistance to the National Institute of Dermatology and Venereology, Hanoi, Viet Nam and to the Korea Institute of Leprosy Research (KILR), in particular for development of sequence-alternative platforms for rapid drug susceptibility testing (DST), drawing from experience with tuberculosis (TB) specimens. In TB, detection of as little as 3% of mutant alleles in the presence of excess wild type is possible if a radioisotope labeled probe is utilized in reverse blot hybridization, while at least 20% mutant DNA is required when using the commercial INNO-LiPA (Innogenetics, Belgium) detection method. One-tube nested PCR compared to single PCR significantly enhances detection of DR targets in leprosy skin biopsies with BIs of 0 (84 vs 24%), 1-3 (91 vs 17%) and 4-6 (92 vs 32%).

Dr Ray Cho also presented exciting preliminary feasibility results of a zebra fish model for *M. leprae* infection as an alternative to armadillo and mouse footpad systems for the study of leprosy, and propagation of *M. leprae*.

Dr Rupendra Jadhav (The Leprosy Mission, India) summarized the goals of TLM ('World without leprosy'), the services, clinical and research capabilities of the 18 hospitals in 10 states that cater to 5000 new patients registering for MDT and nearly 10,000 for general leprosy admissions per year. TLM has taken up relapse DR surveillance in 2009 as per the WHO initiative and created a work flow plan. TLM network has thus far identified 20 relapse cases during May-Oct, of which 14 (at the time of reporting) were evaluated for molecular tests performed at the Stanley Browne research laboratory in Shahdara, New Delhi. All 14 SSS samples were PCR positive for three targets. Three *folP1* and two *gyrA* DR mutations were identified amongst these cases (one case with both *folP1* and *gyrA* mutations). Mutations of *rpoB* predictive of rifampicin resistance were not seen. Other mutations in *folP1* and *gyrA*, not related to drug resistance were seen in some sequences. Dr Jadhav suggested that *gyrA* mutations are of concern since ofloxacin is one of the second line drugs of choice in leprosy.

Dr Ram Das (National JALMA Institute for Leprosy and other Mycobacterial Diseases, Agra, India) has coordinated a multi-centric study in India in which biopsy specimens from a total of 80 patients with treatment failure, irregular treatment, and

treatment with unconventional regimens or dapsone monotherapy were taken and investigated for dapsone as well as rifampicin resistance by MFP and molecular methods. Growth in MFP was observed only in 35 specimens and drug resistance was not observed. This showed that MFP was not appropriate for this purpose. All 80 *M. leprae* samples were tested for mutations in *rpoB* gene and *folP1* gene loci. Out of these 80 samples *rpoB* gene and *folP1* gene were amplified in 67 using primers and assays described previously by Maeda *et al.* whereas these could not be amplified in the remaining 13 samples. The sequencing results of 67 samples for *rpoB* gene did not show any mutation. However, the sequencing of *folP1* gene showed 2 point mutations (one sample from Mumbai was mutated at codon 53 AAC-ATC (Thr53Ile) and another from Karigiri in codon 55 CCC-CTC (Pro55Leu). This study based on a three year duration suggested that drug resistance is very low in the MDT era and the findings also indicated the need to analyze more samples for detection of relevant mutations for deriving any meaningful data and conclusions.

Dr Masanori Kai (Leprosy Research Centre, National Institute of Infectious Diseases, Tokyo, Japan), in the capacity of the reference laboratory investigator for the Myanmar and Viet Nam surveillance efforts presented their joint data. The participating clinics are the Central Special Skin Clinic (CSCC), Yangon, Myanmar and Quyhoa National Leprosy Dermatology Hospital (NLDH), Quynhon, Viet Nam. Twenty relapses (Oct 2008 through July 2009) were reported from Myanmar and four (Feb-Mar 2009) from Viet Nam. The samples were transferred to Tokyo for molecular tests. Dr Kai conducted the RLEP *M. leprae* specific PCR test in all samples. Of the 17 RLEP+ samples from Myanmar, 14 were PCR positive for the DR targets. One case had a codon 55 *folP1* mutation while another had a codon 526 *rpoB* mutation. Two of the three Viet Nam PCR positive cases had mutations in *folP1* (codon 53), but none in the *rpoB* and *gyrA* targets. A larger study (2004-2009) in Viet Nam identified 19 relapses and 404 non-relapse cases (new, under MDT or observation). In both these patient groupings, *folP1* DR associated mutations were seen [9/13 and 11/167 *folP1* respectively], while a silent *rpoB* mutation was observed in one and five cases respectively, which need to be verified. *GyrA* was unaffected. With regard to correlation of mutation detection and type of treatment, DDS resistance was shown in 6/9 patients who received DDS monotherapy and 2/2 after prior treatment with DDS followed by full 24 month MDT. However, 2/3 cases on 24 month-MDT relapsed with mutations in *folP1*. These numbers warrant continued molecular surveillance for DR in this region in both relapse and other cases, particularly concerning the role of DDS in MDT.

Dr Emmanuelle Cambau, responsible for monitoring drug resistance in France and French territories on behalf of the National Reference Centre, France, summarized findings for the 1989-2009 period. A total of 199 cases (102 new, 74 relapses, and 23 under post-treatment observation) have been investigated. Rifampicin resistance was seen in 13 cases, the majority from the French territories of West Indies, Mali and Cameroon. All were confirmed via mouse foot pad infection assays. They had a variety of prior treatment regimens including DDS monotherapy. DDS resistance was associated with rifampicin resistance in 8 cases.

Findings from drug resistance surveillance by molecular tests gathered by Dr Varalakshmi Vissa from Colorado State University (CSU), USA and collaborators in China (Beijing Tropical Medicine Research Institute), Colombia (Instituto Colombiano de Medicina Tropical), Philippines (Leonard Wood Memorial, Cebu Skin Clinic), Thailand (National Institute of Health) and India (Blue Peter Research Institute) on new and relapsed cases were summarized. The drug resistance surveillance was initiated due to the continued leprosy incidence in several countries despite the widespread application of WHO recommended MDT in leprosy control. Of the clinical samples being collected and which yielded drug resistance target amplifications, mutations in *rpoB* were only seen in Colombian patients, as was presented earlier by Nora Cardona-Castro. A few cases of *folP1* mutations were detected in Colombia, Thailand and India. Dr Vissa commented that the current method in her laboratory is based on a multiplex PCR of four drug resistance targets using the Qiagen Multiplex PCR enzyme system. After PCR, the products are precipitated with ethanol prior to DNA sequencing of individual targets. In the majority of the samples, DNA sequence results can be acquired without primer interference. The sensitivity of PCR is affected by the bacteriological index (BI) and whether the samples are obtained from patients under MDT. The multiplex PCR is efficient for samples (slit skin smears and skin biopsies stored in 70% ethanol) when the pre-MDT BI is  $>1$ . The Qiagen DNeasy kit based extraction which entails a column-based purification for skin biopsies is practiced in CSU and in several of the collaborating laboratories. For slit skin smears, the simple method of overnight proteinase K digestion that generates crude total DNA extracts is also suitable. She showed preliminary tests with nasal swab DNA that may also be suitable for PCR of drug resistance targets in high BI patients and proposed that nasal swab could serve as an alternative sample to monitor the evolution of drug resistance in patients before, during and at the end of MDT since PCR positivity is correlated with BI. She presented a snapshot of the strain typing efforts, another research objective within the collaborative international molecular epidemiology leprosy programme. She showed the potential of these studies in the detection of regional variable number of tandem repeat signature patterns at one or more genomic loci and the ability to monitor shared strain types within multi-case in endemic communities.

Dr Stewart Cole from Ecole Polytechnique, Switzerland who is the DR surveillance collaborator for South American countries - Venezuela, Uruguay and Bolivia - received biopsy samples stored in 70% ethanol and slit skin smears on slides from new cases. DNA released by freeze boiling method has been used for PCR and sequencing of DR targets *rpoB*, *folP1* and *gyrA* (for over 100 samples). Mutations were not detected in any samples for any target thus far. His laboratory has also developed allele specific qPCR assays which were tested on a subset of the samples. The results were concordant with the sequence-based assays. He proposed that the laboratory can accept and test more samples (from Asia and Africa) to increase sampling size for the global DR surveillance programme.

## 9. Conclusions and recommendations

- The group acknowledged and appreciated the support provided by various partners especially Saint Louis Hospital, Paris in making the meeting possible.

- There is further evidence in favour of the potential threat to the achievements made thus far in leprosy control posed by the emergence of drug-resistant strains of *M. leprae*.
- There is wide disparity between countries and even different regions of the same country in the approaches to investigation, management and collection of data on leprosy relapses. There is need to standardize the operational definition of a “relapse case” as stated in the Operational Guidelines.
- All collaborating centres demonstrate in-house efforts to perfect existing molecular biological tools for detecting drug resistance. Nested PCR can be used as a supplement to Single PCR Amplification for amplifying DRDR for purposes of increasing specificity and sensitivity of sequencing analysis.
- Concern was expressed regarding the sustainability of mouse- foot-pad (MFP) laboratories. It was agreed that some MFP laboratories should be kept as at the moment there is no other technique for testing new drugs. Alternative methodologies need to be developed in view of emerging ethical issues relating to use of animals for research purposes.
- Although some promising observations have been made of possible advantages of FTA card as an alternative to transporting specimens in 70% alcohol, it was agreed that it was still too early to adopt it for application.
- Network Banking for genetic data (according to a numbering system consistent with the genomic data bases) is recommended for resistant reference strains conserved in the mouse footpad.

## 10. Proposed next steps

Measures will be taken to speed up on-going efforts to:

- Scale-up collection and documentation of information on relapses.
- Improve diagnosis and management of relapses in referral centres.
- Investigate and test promising new antimycobacterial agents for leprosy.

## **Annex 1**

### **Agenda**

1. Opening Session
2. Update on current situation of drug resistance
3. Country presentation on relapses : current situation, trends and case management from: Brazil, China, Democratic Republic of Congo, India, Indonesia, Mozambique and Philippines
4. Lessons from the genome deciphering of *M. Leprae*
5. Recent advances in DNA sequencing for the detection of drug resistance
6. Use of FTA cards for collection of skin smear
7. Current experiences on the treatment of dapsone and rifampicin resistance cases with second line regimen
8. Development of new drugs for use in non-rifampicin containing regimens
9. Quality control: technology, logistics and reporting
10. Country presentations on drug resistance surveillance data: current practices for diagnosis, referral, investigations and results from: Brazil, Democratic Republic of Congo, India, Myanmar, Columbia, China, Mozambique, Pakistan, Philippines and Viet Nam
11. Mouse footpad labs: current use and plans for their role in future research for chemotherapy research
12. Expanding surveillance network
13. Conclusion and Recommendations
14. Closing

## Annex 2

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The workshop on Sentinel Surveillance for Drug Resistance in Leprosy was held at the Faculty of Medicine Diderot, Saint Louis Hospital, Paris, France on 26-27 October 2009. Reports on relapses from 10 countries from the African, American, Eastern Mediterranean, South-East Asian and Western Pacific Regions were presented. The drug resistance surveillance work carried out by the seven reference laboratories in Brazil, France, India, Japan, South Korea, Switzerland and the United States of America were presented.

Topics on molecular research, chemotherapy research, comparative genomics and phylogeography of *M. leprae*, drug resistance in leprosy and recent advances, quality control and the use of FTA cards for collection of skin smear samples were presented and discussed.

The workshop concluded that there is further evidence of the potential threat of emergence of drug-resistance strains of *M. leprae* and that surveillance of drug resistance is an important activity.



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