

Meeting on Sentinel Surveillance for Drug Resistance in Leprosy

A Report
9-10 November 2010, Tokyo, Japan



**World Health
Organization**

Regional Office for South-East Asia

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1. Background

The WHO Global Leprosy Programme (GLP) in 2008 initiated a Sentinel Surveillance Network to monitor drug resistance in leprosy. Drug resistance is a potential threat to the success of the current leprosy control efforts and this surveillance will help to monitor the development of drug resistance especially to rifampicin which is the main component of the multidrug therapy (MDT).

The sentinel network is currently operating in nine leprosy-endemic countries: Brazil, China, Colombia, India, Myanmar, Pakistan, Philippines, Viet Nam and Yemen. To standardize the technical and operational procedures and to maintain quality control, WHO has developed “Guidelines for Global Surveillance of Drug Resistance in Leprosy” (SEA-GLP-2009.2).

Ten reference laboratories from Brazil, India, France, Japan, Korea, Switzerland and USA are collaborating with WHO in this exercise. These laboratories are providing free testing of samples in support of this initiative. In addition, the Leprosy Research Centre, National Institute of Infectious Diseases, Tokyo, Japan is providing support to the quality control aspects of the surveillance system.

To discuss these and related issues, a meeting on “Sentinel Surveillance for Drug Resistance in Leprosy” was held at the National Institute of Infectious Diseases in Tokyo, Japan from 9-10 November 2010.

2. Objectives

The objectives of the meeting were:

- to review the drug resistance surveillance data,
- to review trends in relapses reported by the national programmes, and
- to review and update recent advances in DNA sequencing technology.

3. Opening session

Opening remarks were given by Dr Yoshio Nanba, Director of Specific Diseases Control Division, Ministry of Health, Labour and Welfare, Dr Haruo Watanabe, Director-General, National Institute of Infectious Diseases, Ministry of Health, Labour and Welfare and Dr Myo Thet Htoon, Team Leader, WHO Global Leprosy Programme.

Professor Stewart Cole from the Global Health Institute, Ecole Polytechnique Federal de Lausanne, Switzerland was nominated as chairperson and Dr Norihisa Ishii, Director, Leprosy Research Centre, National Institute of Infectious Diseases was nominated as co-chairperson. Dr Abdul Rahim Al-Samie and Dr Roch Christian Johnson were nominated as rapporteurs.

A total of 43 participants including experts from the sentinel sites (Brazil, Burkina Faso, China, Colombia, India, Mali, Madagascar, Mozambique Myanmar, Pakistan, Philippines, Viet Nam and Yemen), researchers from the reference laboratories and partner organizations attended the meeting. The programme and list of participants are provided in Annex 1 and Annex 2 respectively.

4. Updates: 2009 surveillance activities

Dr Myo Thet Htoon explained about the surveillance system which consists of two components. One component is the systematic collection of tissue samples from multibacillary (MB) relapse cases at the sentinel sites. This includes accurate identification of a case of relapse, collection of samples (slit skin smear tissue or skin biopsy) and the transportation of samples to reference laboratories along with all relevant information about the relapse case. The sentinel sites have been selected based on the capacity to detect a significant number of relapse cases per year and the presence of an expert along with facilities to perform the necessary laboratory examinations to confirm a case of relapse. The second component comprises the test for drug resistance that is carried out in the reference laboratories and returning the result to the sentinel sites. At present there are 10 collaborating laboratories (Annex 3).

In 2009, reports were received from six countries (Brazil, China, Colombia, India, Myanmar and Viet Nam). A total of 213 MB relapse cases with a bacteriological index (BI) of two and more were recruited into the surveillance system. Among these 213 relapses cases, 209 presented with new skin lesions and 188 cases had a BI increase of +2 and more. A majority of the relapse cases were between 34 to 59 years and only 18% of them were females.

Of these 213 relapse cases tested for drug resistance, 12 were found to be dapsone resistant and nine were rifampicin resistant. Two relapse cases from Brazil and Colombia were resistant to both dapsone and rifampicin. Ofloxacin resistance was found in two relapse cases from India.

Out of 213 samples tested for drug resistance, in over 50% of the samples DNA amplification was not possible. This could either be due to insufficient tissue scrapings in the sample collected or problems in the amplification of DNA.

5. Country presentations on relapse cases

5.1 Brazil

Dr Maria Aparecida de Faria Grossi presented the situation of relapse cases from 1990 to 2009 in Brazil. The country has had a progressive decrease in the number of Hansen's Disease cases under treatment. New cases detected increased from 28,765 in 1990 to 51,941 in 2003. With steady decrease year by year, 37,610 new cases were diagnosed in 2009. The prevalence rate decreased from 9.79 per 10 000 population in 1990 to 1.02 in 2009 showing that the prevalence rate has been declining along with the detection rate in the last four years.

In general, the detection rates from 1994 to 2003 increased from 21.61 to 29.37 per 100 000 population. At the same time the proportion of new cases under 15 years increased from 5.74% to 7.98%. In 2009, the new case detection rate decreased to 19.64 per 100 000 population and the new case under 15 years proportion also decreased to 5.43%.

In the Brazilian Information System for Notifiable Diseases – SINAN, there is a specific entry for reporting of relapses by health facilities in the primary health care system. From 2001 to 2009, a total of 12 691 cases of relapse were reported. However, not all were confirmed by the referral services. The percentage of relapse cases among all entries (old and new registering for treatment) ranges from 2.3% in 2001 to 3.6% in 2009 in all the states. The highest percentage is reported in the southern region that usually reports a smaller number of new cases annually compared to other regions during the above mentioned period. In 2009, 1 483 relapse cases were reported in Brazil.

Various studies in Brazil have shown that the rate of relapse after various treatment regimens has been very low. Facilities for conducting mouse foot-pad inoculation are available in Brazil and PCR techniques are being standardized in the various laboratories that are participating in the surveillance of drug resistance.

5.2 China

The situation of relapse cases in China was presented by Dr Shen Jianping. China has reported about 150 relapse cases annually in the past 10 years. In 2009, there were 148 relapse cases reported by the provinces. Among them, 79 relapsed after DDS mono-therapy and 69 after MDT. In the first half of 2010, seventy one relapse cases were reported. Fifty two relapsed after DDS mono-therapy and 19 after MDT. MB cases are currently being treated for 24 months and 65% of the new MB cases are skin-smear positive. The trend of relapse after MDT seems to be increasing. However, the programme has not carried out supervision on the quality of diagnosis of a case of relapse in all the provinces. Some relapse cases could be misdiagnosed with reactions.

At the operational level, the local health workers are not keen to collect the samples from suspected cases because this may not be a problem in dealing with the patient's

treatment. All relapses have been successfully retreated with a new course of MDT. However, the local health workers are interested in checking patients with a persisting high bacillary index (BI) which does not seem to be declining within a normal time interval after MDT. Such cases are being referred by the health workers as a case of suspected drug resistance.

5.3 Mali

Dr Mamadou Sidibe presented the situation of relapse in Mali. At present Mali uses the WHO definition of a case of relapse. At the district level there are one or more community health centres where a nurse has been trained to diagnose and treat a case of leprosy. These centres will be suspecting relapse cases and referring them for confirmation. A total of 346 new cases were detected in 2009 and six cases of suspected relapse cases were reported. These suspected cases need to be confirmed.

5.4 Madagascar

Dr Emerantien Benoit Ramarolahy presented the leprosy situation in Madagascar. The country has 111 health districts, each with several health centres where leprosy is being diagnosed and treated. The number of new cases detected in 2009 was 1 588 cases and the trend is declining. WHO's definition of a case of relapse is being used in the national guidelines. However, no accurate data are available on relapse cases although some health workers have reported such unconfirmed cases.

5.5 Yemen

Dr Abdul Rahim Al-Samie gave a presentation on the situation of relapse cases in Yemen. The country is a low burden country for leprosy with a registered prevalence rate of 0.19 per 10000 population and the new case detection rate of 1.7 per 100000 population. The number of new cases detected in 2009 was 387. The national leprosy elimination programme in Yemen is completely integrated in the primary health care (PHC) centres. MDT according to WHO recommendations was introduced in the early 1980s. The leprosy data shows a slow but steady decline in new cases. However, a persistently high rate of Grade 2 disabilities is seen. The treatment completion rate is over 90%.

All suspected relapse cases are identified by the programme at the primary health care centres and clinically confirmed by medical supervisors who regularly visit the clinics every quarter. The confirmed relapse case is then referred to the Skin and Venereal Diseases Hospital in Taiz which is the national referral centre for further laboratory testing.

In 2009, a total of six relapse cases were diagnosed in the referral hospital in Taiz. The number of relapse cases has been fluctuating each year due to factors such as over-diagnosis and also lack of awareness among peripheral health workers. Of the six relapse cases, three were found to be skin-smear negative and as such have not been included in the surveillance report.

5.6 Pakistan

Information on relapse cases in Pakistan was presented by Dr(Mrs) Christine Schmotzer. Pakistan is a low-burden country for leprosy with a prevalence rate of 0.54 per 100 000 population and the detection rate is 0.33 per 100 000 population. The leprosy control programme in Pakistan is partly integrated into the primary health care system and, at the same time, it is run as a combined vertical programme with the blindness control programme. It has received strong support from NGOs since its inception. MDT according to WHO recommendations was introduced in the early 1980s. The leprosy data shows a steady number of around 500 new cases being detected annually with a comparatively high rate of Grade 2 disabilities. The treatment completion rates are over 95% for both PB and MB cases.

Eleven relapse cases were detected in 2009. In 2010, seven cases of relapse were diagnosed and have been included in the drug resistance surveillance activity. The number of reported relapses remains low. However, over-diagnosis of relapse cannot be excluded as not all projects follow the WHO criteria and the national guidelines. The programme will be refreshing the knowledge about relapse among the leprosy staff and also create awareness about relapse among dermatologists.

6. Technical discussion

6.1 Improving quality control: technology, logistics and reporting

Dr Masanori Matsuoka and Dr Masanori Kai gave presentations on improving direct sequencing. Bacterial suspension of four strains, wild type or resistant, with two different concentrations and negative control samples were provided to 11 reference laboratories in the second quality control study. Of the 11 laboratories, eight reported the results.

Sensitivity of PCR was found to be a little low in three laboratories. DNA sequencing results were not good in three laboratories, because of low yield of PCR products. Consequently, improvements of PCR condition are required. A proposal was made to apply the highly sensitive PCR such as Nested-PCR or high cycles.

Discordant sequences with provided strain were reported from one laboratory. A proper sequencing procedure was advised to the laboratory. No false-positive results were shown for the negative control.

Dr Kai reported some non-specific amplification from clinical samples and proposed applying PCR for RLEP sequence first, followed by PCR for *folP1*, *rpoB* and *gyrA* to exclude positive PCR from *Mycobacteria* other than *M. leprae*. Application of FTA card for collecting and transporting samples was suggested, since sending samples in 70% ethanol is not allowed in some countries.

The following seven laboratories participated in the quality control exercise: Institute of Dermatology and National Centre for Leprosy Control, China; National Reference Centre for Mycobacteria and resistance to anti-tuberculosis drug, France; Stanley Brown Laboratory, India; Yonsei University, College of Medicine, South Korea; Colorado State University, USA; Leprosy Research Center, Japan and National Hansen's Disease Programs at LSU School of Veterinary Medicine, USA.

6.2 Recent advances in DNA techniques for the detection of drug resistance

Dr Wei Li discussed recent advances in DNA techniques for the detection of drug resistance. The development of real-time PCR assay is based on high resolution melt curve analysis (HRM) and allele-specific PCR methods for the detection of single nucleotide polymorphisms (SNP) including drug resistance mutations in *Mycobacterium leprae*. HRM is a post-PCR melt analysis method that is sensitive enough to discriminate differences in double stranded DNA with a single base difference between strands. Preliminary results showed that HRM can classify DNA from bacterial strains with and without a mutation into different clusters for several genetic loci: drug targets *gyrA*, *rpoB*, *folP1* and a strain typing SNP locus. HRM is a useful method for high throughput screening of multiple samples and for identifying new genetic variations, and does not require allele specific primers. The candidate mutated samples can then be further processed to verify mutations in the DNA sequences.

Allele-specific PCR on the other hand can be used to screen for previously characterized mutations and has fewer requirements on template quality and more stable results. Since these two methods make use of real-time PCR, the whole amplification process can be monitored as it occurs, and the template in the reaction can also be quantified to compare with the clinical bacillary index (BI) data. If real-time PCR (RT-PCR) followed by HRM is used instead of the traditional PCR and sequencing steps, the mutated strains can be identified as separate from the wild-type strains without further sample manipulation. In this way, far fewer samples require confirmatory sequencing. The RT-PCR and HRM method provides a rapid, robust, and inexpensive way to detect mutations in *M. leprae* strains but generates much less information than the currently recommended direct DNA sequencing methods for analysing drug resistance loci.

6.3 Collection of samples: use of FTA card, skin smears and punch biopsy for polymerase chain reaction (PCR) testing

Professor Emmanuelle Cambau gave a presentation on the use of FTA card, skin smears and punch biopsy for PCR testing. The rationale for using skin-smear samples rinsed in 70% ethanol for detection of resistance was based on the knowledge that enough DNA is obtained in the slit-skin- smear sample to obtain a positive PCR test and that live bacilli are not required unlike mouse footpad inoculation. This technique also allows simple shipment of samples as normal mail can be used since no live bacilli are being shipped. In addition, samples can be stored for longer periods before actual shipment.

Scraping slit-skin smears onto a FTA Elute Card has been shown to be as good as collecting samples in a tube containing 70% ethanol solution. This type of sample collection make shipment of samples simpler as it does not involve a liquid medium.

Drug resistance testing on skin biopsies by mouse footpad inoculation was also carried out if the samples were received in the laboratory within less than four days. Over the years, out of 86 biopsies received in the National Reference Centre for Mycobacteria and Resistance to Anti-tuberculosis Drugs, 55% of the samples were successfully grown in mouse footpad. In general, most of the participating laboratories in the surveillance network handled more biopsies in the past.

The challenge for the laboratories is to improve the DNA extraction method. Based on the surveillance data of 2008, out of 59 samples from relapse cases tested PCR was negative in 40% of the samples and in 2009, out of 209 tested PCR was negative in 49% of the samples. Based on the current extraction methods, it was seen that PCR results were better in samples with a BI of 3 to 4+. This finding was also seen when comparing the use of FTA card with slit-skin smear samples rinsed in 70% ethanol. Biopsies have been found to provide better PCR results compared to slit-skin smears.

To improve PCR results, it is suggested that samples be collected from skin lesions with the highest BI score. The use of either slit-skin smears or skin biopsies for collection of samples from relapse cases will very much depend on the method that the reference laboratory is familiar with. The use of quantitative real time PCR will also help in improving the DNA extraction protocol from samples.

6.4 Surveillance of drug resistance in leprosy

Professor Stewart Cole presented the topic on surveillance of drug resistance in leprosy. An update on the surveys carried out in South America was presented. Limited drug resistance was reported in the past in patients receiving inadequate therapy and in relapse cases it is usually dapsons resistance that was reported. The tests were either based on *in vivo* mouse inoculation techniques or *in vitro* by radiometric method. Both these methods require viable bacilli and are costly, time consuming and the expertise to carry out such tests is disappearing. The above-mentioned methods of detecting drug resistance are being replaced by molecular detection methods of mutations in *rpoB* gene for rifampicin resistance, *folP1* for dapsons resistance and *gyrA* for ofloxacin resistance.

Biopsies and slit-skin smears were collected from 219 old and new cases (11 cases from Bolivia, 14 from Brazil, 192 from Venezuela and two from Uruguay) and DNA sequencing was performed. Only two cases were observed to have mutations in *folP1* (dapsons resistance).

A case history of a German national who was first diagnosed as an MB case and who had failed to respond to MDT was also tested for drug resistance. This patient had a past history of living in Brazil and has also visited this country frequently. No mutation was observed in any of the target loci. The *M. leprae* strain was found to be subtype 3I which

is known to be most prevalent in Brazil. Upon carefully checking the history of the patient, it was found that the patient was not complying with treatment due to side effects.

In 2010, DNA sequencing was done on six samples sent from Yemen of which three were from relapse cases. PCR amplification was not successful in the non-relapse cases. No mutation was seen in the three relapse cases. The three strains belong to subtype 1B, 1D and 2E. The presence of type 1 strains may reflect immigration while type 2 is consistent with its presence in Turkey and Iran.

Molecular methods offer a rapid, sensitive, reliable and inexpensive way of testing for drug resistance and no other methods have equivalent capacity. Surveillance of drug resistance is a valuable adjunct to control programmes.

7. Country presentations of drug resistance surveillance data

7.1 Brazil

Dr Philip Suffys presented the data on surveillance carried out in Brazil. Data on results obtained so far includes 34 patients with lymph slit-skin smears and 23 cases with biopsy samples. Samples were stored in 70% ethanol and DNA extraction performed by using the DNeasy kit. So far, PCR positivity was observed in lymph samples in 73.5% (*folP1*), 58.8% (*rpoB*) and 82.3% (*gyrA*), while for biopsy samples, PCR positivity for these three genes was 74%, 70% and 74%, respectively. Sequencing of these fragments is underway.

Data on some experimental procedures were also presented where it was observed that the sensitivity of PCR amplification was lower than expected in the MB biopsy samples and this could be due to PCR conditions. A low number of PCR cycles and the use of only one primer set could be the reason. Further experiments to optimize PCR conditions will be tested. Nonetheless, a statistically significant association was observed between PCR positivity and bacterial load.

Additional data were presented on the genotyping performed on skin biopsy samples that were collected between 2007 and 2008 from relapse cases. Among 135 confirmed relapse cases, four cases with mutations in one or more resistant genes were observed, two from the state of Amazonas, one from Espirito Santo and one from Para State. All four had mutation in *rpoB*, three of these also presented mutations in codon 55 of *folP1*. No mutations associated with drug resistance were observed in the DNA gyrase genes but a SNP *gyrA*-297 (C->T) was observed in *gyrA*. This SNP, together with short tandem repeat (STR) analysis suggested that re-infection may occur in a considerable number of relapse cases.

The current flow chart for monitoring drug resistance in Brazil was also presented which includes the sampling of clinical material from confirmed relapse cases in 12 referral clinical centres. Samples are to be sent to five institutes, including ILSL (Sao

Paulo), FUAM (Amazonas), CMC (Para), CREDESH/UFU (Uberlandia) and Fiocruz (Rio de Janeiro). Fiocruz will be receiving the final results of the DNA sequencing data for organising and distribution. Quality control measures are being taken in the different sites independently.

7.2 Colombia

On behalf of Dr Nora Cardona-Castro from the Instituto Colombiano de Medicina Tropical and its collaborators, Dr Wei Li gave a presentation on the results of the surveillance data carried out during 2010. A total of 95 leprosy patients including 5 relapse cases were tested for drug resistance during 2010 in Colombia. Among the 95 patients, 35 were on MDT from the departments of Bolivar (30 cases), Antioquia (three cases) and Choco (two cases). The remaining 60 were post-treatment cases of which 56 were from Bolivar and four from Antioquia.

Laboratory examinations were performed on 73 cases that include 38 post-treatment patients and 35 patients during MDT treatment, from which lymph, skin biopsy, and blood samples were collected. In these 73 cases, 37 patients were BI positive and 36 were BI negative. Molecular tests for detection of drug mutations were carried out on the 37 BI positive patient samples. After DNA was extracted from lymph and skin biopsies, PCRs for these samples were done in Colombia. Of the 37 samples, 29 patients were PCR positive for at least one DR (drug resistance) target. PCR positive products, diluted and mixed with sequencing primers, were sent to Colorado State University for DNA sequencing. In all, 13 *rpoB*, 18 *folP1*, 26 *gyrA* and 21 *gyrB* PCR products were successfully sequenced. One case from Chigorodo-Antioquia with a BI=1 after finishing 24-months of MDT in July 2010 (the initial BI was 0.2) had an *rpoB* silent mutation at codon 521 [CTG-CTA]. Another case from Simiti-Bolivar, also with an increasing BI from 1.0 to 1.4 after 19 months of MDT, had a *gyrA* substitution mutation at codon 107 [CGG-CTG] which had not been reported before. A subset of patient samples from Santander, Antioquia and Cundinamarca department showed silent (C->T) *gyrA* mutations at codon 99. This polymorphism, better known as SNP7614, is a useful marker for molecular epidemiology and is associated with *M. leprae* of SNP genotype 31.

7.3 China

The data on surveillance carried out in China was presented by Dr Shen Jiaping and Professor Wang Hongsheng. The 15 Provinces in the south of China were covered for sentinel surveillance of drug resistance in leprosy in 2010. Patients monitored for drug resistance were classified into two categories. The first was relapsed patients with a positive skin smear after clinical cure that was usually more than two years after stopping MDT. The second category included patients with persisting and high BI during the monitoring phase after completion of MDT. Samples were collected mainly by biopsy kept in a small bottle containing 70 % ethanol and sent to the Mycobacterium Laboratory in the National Centre for Leprosy Control, for testing.

A total of 14 patients were tested for the presence of drug resistant mutations. Among them, eight were relapses after MDT, and six with persisting high BI after MDT. All samples were positive for PCR amplification of *M.leprae* DNA, but all samples were negative for *rpoB* and *gyrA* mutations. Only two patients with persistently high BI displayed *folP* gene mutation.

7.4 India

Dr Rupendra Jadhav and Dr Vijayalakshmi each presented the results of the drug resistance surveillance carried out by the Stanley Browne Laboratory in Delhi and Blue Peter Public Health & Research Centre (BPHRC) in Hyderabad, Andhra Pradesh State.

The Leprosy Mission is currently operating several hospitals for persons affected by leprosy in India. Nearly 5 200 new cases were diagnosed and treated in these facilities during 2009. In these facilities, 38 relapse cases were reported during 2009. Samples from these relapse cases were sent to the Stanley Browne Laboratory for DNA sequencing. The definition of relapse is based on the WHO guidelines. From January to October 2010, 21 relapse cases were reported in the Leprosy Mission hospitals of which 16 were tested for drug resistance. DNA amplification was not successful in two of the 16 cases tested. No mutation was observed in the 14 samples tested for drug resistance.

Blue Peter Public Health & Research Centre (BPHRC) receives samples from the states of Andhra Pradesh, Orissa, Bihar and Madhya Pradesh. Nine relapse cases were recruited for the drug resistance surveillance system. DNA was extracted from the sample using the DNeasy Kit. Multiplex PCR was performed to detect mutations in the *folP1*, *rpoB*, *gyrA* and *gyrB* genes. For some samples, DNA sequencing was done at Colorado State University and other samples were out-sourced locally.

7.5 Myanmar

Dr Mya Thida presented the results of the surveillance activities that were carried out in Myanmar. MDT was first introduced in the country in 1988 and at present over 3,000 new cases are being detected annually. Around 20 to 30 relapse cases are being diagnosed every year. Two sentinel sites, one in Yangon and another in Mandalay are participating in the surveillance network. As of October 2010, 26 relapse cases have been diagnosed of which 21 cases were recruited for the sentinel surveillance and slit-skin smear samples were sent for molecular testing for drug resistance. Out of 21 samples tested so far, only three cases of mutation have been reported for dapson resistance. PCR amplification was not successful in three relapse cases for dapson, eight cases for rifampicin and 10 cases for quinolone.

7.6 Pakistan

Dr(Mrs) Christine Schmotzer presented the data on the surveillance activities carried out in 2010. Rawalpindi Leprosy Hospital was selected as a site for sample collection for

sentinel drug resistance surveillance in leprosy in 2009. It is collaborating with Laboratoire de Microbiologie, Hospital Saint Louis, Paris, France. In 2010, seven relapse cases were recruited into the surveillance system from among the relapse cases diagnosed at the Rawalpindi Leprosy Hospital and samples were sent for DNA sequencing. Amplification was not successful in three samples and in the remaining four samples no mutations found for either dapsone or rifampicin resistance.

7.7 Philippines

Surveillance for rifampicin resistance in the Philippines was presented by Dr Paul Saunderson, representing the Leonard Wood Memorial Research Centre, which is the designated sentinel site. The Philippines has a population of 92 million and 1,795 new cases of leprosy were reported in 2009, with 12 relapses being reported nationwide. In the island of Cebu, approximately 200 new cases are reported per year; in 2010, three relapse cases were detected and examined for drug resistance by Professor Thomas Gillis of the National Hansen's Disease Programs in Baton Rouge, United States of America. DNA was successfully amplified in all three cases.

Inclusion criteria for relapse cases entering the surveillance programme are: previously well-documented MB leprosy; completion of at least 12 doses of MB-MDT; current signs of MB relapse; exclusion of lepra reaction (by means of clinical features and timing); patient consent. The definition of relapse used in Cebu is the re-occurrence of disease after a full course of treatment, diagnosed by the appearance of new skin lesions, associated with an increase in the BI of at least 2+ at any site. Dapsone resistance was found in one case, but no resistance to rifampicin or the quinolones. Most MB relapses occur between six and 13 years after stopping treatment.

7.8 Viet Nam

Dr Tran Hau Khang gave a presentation on the results of the drug resistance surveillance carried out in the country. Leprosy services in Viet Nam are part of the dermatology and venereology programme. During 2009, 413 new cases were detected with a MB proportion of 71% and child proportion of 2.9%. The annual new case detection rate has been declining steadily since 2001. In 2009, five MB relapse cases were diagnosed and slit-skin smear samples were sent for DNA sequencing. Only two cases of dapsone resistance were reported among these five cases.

7.9 Yemen

Dr Abdul Rahim Al-Samie presented the data on surveillance of drug resistance conducted in Yemen in 2010. The Skin and Venereal Diseases Hospital in Taiz is the national referral centre and is the site for drug resistance surveillance in leprosy in 2010. During 2010, three relapse cases fulfilled the criteria for sample collection according to the WHO guidelines. Samples were sent to the Global Health Institute in Lausanne, Switzerland for DNA sequencing. No mutation was observed in the three samples tested for drug resistance.

8. General discussion

8.1 Status of mouse foot-pad labs, inventory and their future role in laboratory research

The status of mouse foot-pad (MFP) labs, inventory and their future role in leprosy research was presented by Dr Paul Saunderson. He identified laboratories currently doing leprosy work using the MFP model, through papers published in the last 10 years and by personal communication. There are two groups of MFP labs. Firstly, academic centres in low or non-endemic areas. These are: National Hansen's Disease Programs in Baton Rouge, USA; National Reference Centre for Mycobacteria in Paris, France and the National Institute for Infectious Diseases in Tokyo, Japan.

The second group of research laboratories in high-endemic areas are: National JALMA Institute of Leprosy and other Mycobacterial Diseases, Agra, India; Foundation for Medical Research, Mumbai, India; Schieffelin Institute of Health – Research and Leprosy Centre, Karigiri, Tamil Nadu, India and the Central Leprosy Teaching and Research Institute, Chengalpattu, Tamil Nadu, India; Mycobacterial Research Laboratory, Anandaban Hospital, Kathmandu, Nepal; and Instituto Lauro de Souza Lima in Bauru, Sao Paulo, Brazil.

While the MFP model remains useful for certain types of research, sustainability will be an increasing challenge for laboratories in endemic areas in future. There are a number of issues relating to sustainability to consider, especially in resource-poor settings:

- The availability of staff trained in Shepard's technique, which requires a consistently high degree of technical skill, as well as the capacity for study design, analysis and interpretation of results.
- The standard of care of animals at the animal facilities in the research institutes are coming under increased scrutiny, and the standards are generally being raised. In order to attract funding, facilities increasingly need to meet the accreditation requirements of bodies such as Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC), which may involve significant expense.
- More sophisticated management systems now need to be in place to achieve best practice standards in current research, including sentinel mice to monitor inter-current infection, as well as more demanding and more expensive animals, such as nude and knock-out mice.
- Increasing funding is needed simply to maintain and upgrade the facility, as well as carry out research.
- Transport of specimens is now more feasible and it should be possible to send frozen samples by air to another centre, rather than carry out MFP work in the endemic area.

- The future role of such labs is in more sophisticated research, rather than routine testing of drug resistance.

8.2 Current status of dapsone, clofazimine and quinolone resistance in leprosy

Professor Thomas Gillis gave a presentation on the current status of dapsone, clofazimine and quinolone resistance in leprosy. Experience has shown that treating chronic mycobacterial infections requires long-term regimens using multiple, complementary antibiotics. Leprosy is no exception and current treatment regimens include rifampicin, dapsone and clofazimine for 12 months or longer for multibacillary cases with high BI. Protracted treatment schemes of this duration can result in intermittent treatment often due to noncompliance by the patient. In turn, incomplete treatment can lead to selection of drug resistant mutants of *M. leprae* within patients that becomes more difficult to treat upon relapse. Second line drugs (e.g., minocycline, fluoroquinolones and clarithromycin) are available to treat leprosy but they are much more expensive than current drugs used in MDT and the outcome of using these drug combinations for treating drug-resistant strains of *M. leprae* have not been fully reported from field programmes.

While dapsone resistance became widespread during the “dapsone monotherapy era” for leprosy, MDT with dapsone, rifampicin and clofazimine has appeared to hold further resistance in check. Surveys for dapsone, rifampicin, clofazimine and fluoroquinolone resistance continue to demonstrate the presence of mostly mono-resistant strains. However, a few multi-resistant strains have been reported recently. Moreover, an accurate assessment of the frequency of mono-resistant and multi-resistant strains around the world is not available. Therefore, the threat of drug resistance in leprosy remains undefined.

Pre-genomic era work on drug resistance in leprosy required resource and labour-intensive studies using Shepard’s mouse foot-pad model. Advances in mutational analysis of gene targets involved in rifampicin, dapsone and fluorquinolone resistance have moved the field forward allowing for direct DNA testing for appropriate mutations. This is particularly helpful in leprosy work as cultivable bacteria from lesions are not required for the analysis.

Of the three drugs used in MDT for leprosy, missense mutations in genes encoding key enzymes related to bacterial folate metabolism (*folP1*) and the DNA-dependent RNA polymerase (*rpoB*) account for most cases for drug resistance in *M. leprae*. These genes have been well characterized and in some cases mutations have been shown to be associated with altered enzyme function.

Resistance to clofazimine, the third drug used in MDT for leprosy, has not been understood. In fact clofazimine-resistant mutants have been very rare or difficult to demonstrate in both *M. leprae* and *M. tuberculosis*. Research over the last 30 years has suggested that clofazimine acts by inhibiting respiration, disrupting cell membranes, binding GC-rich DNA and, most recently, by blocking potassium uptake in *M.*

tuberculosis. None of these presumed activities have been attributed to a specific gene product or pathway. Therefore, assigning a mechanism of resistance to clofazimine, as has been done with dapsone and rifampicin, has not been accomplished. Further research is needed.

Because clofazimine is an inexpensive, effective anti-mycobacterial drug which also demonstrates desirable therapeutic effects against erythema nodosum leprosum (ENL), studies should be encouraged to understand its interaction with the immune system and the resultant immuno-modulatory effects. A recent study has identified clofazimine as a potent modulator of calcium flux in T-lymphocytes resulting from the blockade of potassium uptake by these cells through their Kv 1.3 receptor. This is an interesting finding that should be investigated further with reference to clofazimine's effect on ENL.

8.3 Review of reporting forms from sentinel sites

The review of the existing relapse case history forms was presented by Dr Myo Thet Htoon. The sections on reporting details of the sentinel sites, demographic details of the relapse case, clinical presentations at the time of relapse, past clinical history and code numbering of the samples were discussed.

Specific clinical features of the skin lesions were added in the current form along with the address of the relapse case so that the geographic distribution of the cases can be analysed. A revised case report form was agreed upon (Annex 4).

9. Conclusions and recommendations

- The participants acknowledged and appreciated the support provided by various partners and especially the National Institute of Infectious Diseases, Ministry of Health, Labour and Welfare, Japan in hosting this meeting.
- Although the results presented so far do not indicate a significant problem of rifampicin resistance, it is recommended that the sentinel surveillance be continued based on the agreed guidelines and reporting forms in order to collect data systematically and monitor this situation.
- Samples to be sent from the sentinel sites to the reference laboratories could be either skin smear scrapings placed in 70% ethanol tube or skin smear scrapings placed on a FTA card or skin biopsies.
- Participating laboratories in the surveillance network are encouraged to collaborate in creating a repository of the DNA sequencing results with the aim to share information for future research. The Global Health Institute, Ecole Polytechnique Federal de Lausanne, Switzerland will host this repository.
- The group acknowledged the continuing importance of maintaining expertise in the existing mouse foot-pad laboratories in view of their important role in testing new drugs.

- Measures should be taken to include as many relapse cases as possible in the surveillance programme from each of the participating sentinel sites.
- It is recommended that further research should be carried out by the participating laboratories to improve the sensitivity of polymerase chain reaction (PCR) in order to reduce the number of failures in amplification.

Annex 1

Programme

Tuesday, 9 November 2010	
09.00 - 09.30 hrs	<p>Opening session</p> <ul style="list-style-type: none"> - Opening speech by <i>Dr Yoshio Nanba</i> - Opening remarks by <i>Dr Haruo Watanabe</i> - Opening address by <i>Dr Myo Thet Htoon</i> - Introduction of participants - Nomination of Chairperson, Co-chairperson and Rapporteur
10:00- 10:30 hrs	<p>Drug Resistance: updates on current situation (<i>Dr Myo Thet Htoon</i>)</p> <ul style="list-style-type: none"> - <i>Discussion</i>
10:30 – 12:30 hrs	<p>Country presentation on relapses: current situation, trends and case management (10 minutes each)</p> <ul style="list-style-type: none"> - Brazil (<i>Dr Maria Aparecida de Faria Grossi</i>) - China (<i>Dr Shen Jianping</i>) - India - Mali (<i>Dr Mamadou Sidibe</i>) - Yemen (<i>Dr Abdul Rahim Al-Samie</i>) - Pakistan (<i>Dr Christine Schmotzer</i>) - Burkino Faso (<i>Dr Kafando Christophe</i>) - <i>Discussion</i>
14:00 – 14:30 hrs	<p>Improving quality control: technology, logistics and reporting (<i>Dr Masanori Matsuoka</i>)</p> <ul style="list-style-type: none"> - <i>Discussion</i>
14:30 – 15:00 hrs	<p>Recent advances in DNA sequencing techniques for the detection of drug resistance (<i>Dr Wei Li</i>)</p> <ul style="list-style-type: none"> - <i>Discussion</i>
15:00 – 15:30 hrs	<p>Collection of samples: use of FTA card, skin smears and punch biopsy for PCR (<i>Professor Emmanuelle Cambau</i>)</p> <ul style="list-style-type: none"> - <i>Discussion</i>
16:00 - 16:30 hrs	<p>Recent studies on drug resistance in other (non-relapse) cases (Professor Stewart Cole)</p> <ul style="list-style-type: none"> - <i>Discussion</i>

Wednesday, 10 November 2010	
	Country presentations on drug resistance surveillance data: current practices for diagnosis, referral, investigations and results (20 minutes per sentinel site)
09:00 - 10:00 hrs	<ul style="list-style-type: none"> - Brazil (<i>Dr Philip Suffys</i>) - Myanmar (<i>Dr Mya Thida</i>) - India (<i>Dr Rupendra Jadhav and Dr Vijayalakshmi</i>) - <i>Discussions</i>
10:30 – 13:00 hrs	Continuation of country presentations <ul style="list-style-type: none"> - Columbia (<i>Dr Wei Yi - on behalf of Dr Nora</i>) - China (<i>Dr Shen Jianping</i>) - Pakistan (<i>Dr Christine Schmotzer</i>) - Philippines (<i>Dr Paul Saunderson</i>) - Viet Nam (<i>Dr Tran Hau Khang</i>) - Yemen (<i>Dr Abdul Rahim Al-Samie</i>) - <i>Discussions</i>
	General discussion
14:00 - 14:30 hrs	Status of mouse footpad labs, inventory and their future role in leprosy research (<i>Dr Paul Saunderson</i>) - <i>Discussions</i>
14:30 - 15:00 hrs	Literature review on current status of dapsone, clofazimine and quinolone resistance (<i>Professor Thomas Gillis</i>) - <i>Discussions</i>
15:00 - 15:30 hrs	Review of reporting forms from sentinel sites to reference laboratory (<i>Dr Myo Thet Htoon</i>) - <i>Discussions</i>
16:00 - 16:30 hrs	Conclusion and recommendations
16:30 hrs	Closing

Annex 2

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Annex 4

Form 1. Case Report Form
(MB relapse cases only)

1. Reporting details

- 1.1 Case identification number: _____ (given by national authorities)
1.2 Name of country _____
1.3 Date of report __/__/____ (dd/mm/yy)
1.4 Name of Sentinel Centre sending specimen _____
Address: _____
Tel: _____ Fax: _____ Email: _____

2. Demographic details of case

- 2.1 Year of Birth: _____ 2.2 Sex – Male Female
2.3 Address/Location of the relapse case _____

3. Clinical presentation at time of Relapse

Clinical features

- 3.1.1 Beginning of symptoms : _____ Months
3.1.2 Type of lesion(s): Maculae; Plaques; Papules; Diffuse infiltration;
 Nodules; Anaesthetic area.
3.1.3 Colour of lesion: Hypo-pigmented; Erythematous; Hyper-pigmented; Normal
3.1.4 Loss of sensation – Definite Doubtful Normal
3.2 Number of old skin lesions _____
3.3 Number of new skin lesions _____
3.4 Patient consent to participate: Yes; No (If No, skip steps 3.5, 3.6 and 3.7)
Date of Consent: _____
3.5 Skin smear results from specific sites (If any along with the date of test)
1. Site BI Date of Smear.....
2. Site BI Date of Smear.....
3. Site BI Date of Smear.....
4. Site BI Date of Smear.....
5. Site BI Date of Smear.....
6. Site BI Date of Smear.....

3.6 Biopsy – Yes; No

If 'YES' Date: _____ Sites _____

Main findings
Epidermis: <input type="checkbox"/> Confirmed leprosy; <input type="checkbox"/> Compatible with leprosy; <input type="checkbox"/> Non specific; <input type="checkbox"/> Indicative another disease;
Dermis: Inflammatory Infiltration:
Neural Involvement:
Vasculitis:
Panniculitis:
BAAR:
Clinical Form: <input type="checkbox"/> I ; <input type="checkbox"/> TT ; <input type="checkbox"/> BT; <input type="checkbox"/> BB; <input type="checkbox"/> BL; <input type="checkbox"/> LL;

3.7 Present classification (MB/PB and Ridley Jopling if available)

PB (< 5 cutaneous lesions); MB (> 6 cutaneous lesions)

Indeterminate; Tuberculoid; Lepromatous; Borderline Tuberculoid;

Borderline Borderline; Borderline Lepromatous

4. Past clinical history

4.1 Date of diagnosis _____ (dd/mm/yy)

4.2 Past clinical history

4.2.1 Beginning of symptoms _____ Months

4.2.2 Type of lesion(s) – Maculae; Plaques; Papules; Diffuse infiltration;
 Nodules; Anaesthetic area.

4.2.3 Colour of lesion – Hypo-pigmented; Erythematous; Hyper-pigmented; Normal

4.2.4 Loss of sensation – Definite loss Doubtful Normal

4.3 Classification at Diagnosis PB (< 5 cutaneous lesions); MB (> 6 cutaneous lesions)

Indeterminate; Tuberculoid; Lepromatous; Borderline Tuberculoid; Borderline Borderline; Borderline Lepromatous

4.4 MDT taken or not: Yes; No

4.5 If "YES": a) Date when treatment first started ___/___/___ (dd/mm/yy)

b) What type (PB or MB blister packs) _____

c) How many months MDT was taken _____

d) Date when treatment was completed ___/___/___ (dd/mm/yy)

4.6 Skin smear results at Diagnosis

1. Site BI Date of Smear.....

2. Site BI Date of Smear.....

- 3. Site BI Date of Smear.....
- 4. Site BI Date of Smear.....
- 5. Site BI Date of Smear.....
- 6. Site BI Date of Smear.....
- 4.7 Past change in classification (If any during the course of treatment) Yes; No
- 4.8 If yes, date of change in classification ___/___/___ (dd/mm/yy)
- 5. Sample(s) sent for DNA sequencing** (please write code numbers on the tube)
- 5.1 Date of collection of sample ___/___/___(dd/mm/yy)
- 5.2 Date of shipment of sample ___/___/___ (dd/mm/yy)
- 5.3 No: _____ Site _____ Remarks _____
- 5.4 No: _____ Site _____ Remarks _____

The WHO Global Leprosy Programme (GLP) organized a meeting on “Sentinel Surveillance for Drug Resistance in Leprosy” at the National Institute of Infectious Diseases in Tokyo, Japan, from 9-10 November 2010. The surveillance network to study drug resistance in leprosy was established in 2008 to monitor the development of drug resistance, especially to rifampicin, which is the main component of multidrug therapy (MDT) for leprosy.

Representatives from sentinel sites from 13 countries along with scientists from 10 collaborating laboratories participated in this meeting. Situations of relapses in select countries were reviewed along with the results of surveillance activities carried out during the year 2010. Recent developments to improve sensitivity of the polymerase chain reaction (PCR) test and issues relating to quality control were also discussed. This is the report of the said meeting.



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