Pre-Congress Workshop I – 'New Diagnostics and Molecular Epidemiology', 17th International Leprosy Congress, January 29, 2008 (organisers Drs. John Spencer, Thomas Gillis, and Vara Vissa)

Morning Session: New Diagnostics (morning session, 0900–1300)

Organiser: Dr. John S. Spencer, Colorado State University, CO, USA
Diagnostics moderators: Linda Oskam, KIT/Annemiek Geluk, LUMC/Malcolm Duthie, IDRI
Rapporteur: Diagnostics: Professor Warwick Britton, Head, Mycobacterial Research Group, Centenary Institute, Australia

Afternoon Session: Molecular Epidemiology (afternoon session, 1400–1700)

Organiser: Dr. Varalakshmi Vissa, Colorado State University, CO, USA
Molecular Epidemiology moderators: Drs. Thomas Gillis, NHDP/Vara Vissa, CSU

Morning Session: New Diagnostics

9:00	Welcome
Annemiek Geluk LUMC	'Development of novel T cell assays for detection of *M. leprae* infection'
Linda Oskam, KIT, The Netherlands	'New and improved opportunities for serological KIT, testing in leprosy'
John Spencer, CSU, USA	'Use of Western blot, ELISA and protein microarrays to define disease-state-specific antigenic profiles in leprosy patients'
Sang-Nae Cho, YUMC, Korea	'Enzyme immunoassays for PGL-I antigen and antibodies'
Yumi Maeda, LCR, Tokyo, Japan	'Recent findings of the use of the MMP-II protein as a potential diagnostic for leprosy detection using serology'
Juan Periche Fernandez, IDCP-Dr. HBD, Dominican Republic	'Difficulties in leprosy diagnosis'
Om Parkash, JALMA, India	'Serological detection of leprosy: Recent developments at our laboratory'
Malcolm Duthie, IDRI, USA	'Evaluation of the LID-1 fusion protein for the serological detection of leprosy'
Murdo Macdonald, NLM, Nepal	'Field testing of two new leprosy skin test antigens: A Phase II clinical trial in Nepal'

Morning Session: New Diagnostics

Dr. Annemiek Geluk: 'Development of novel T cell assays for detection of *M. leprae* infection'

Unique proteins and peptides diagnostics are being concurrently evaluated for specificity and sensitivity with the additional constraints of being intuitive to utilise for clinicians without the additional burden(s) of specific laboratory equipment for either procedure or interpretation. To that end, investigation is ongoing for a test in a 'lateral flow type' platform which can be used with whole blood (WBA) as opposed to peripheral mononuclear cells (PBMCs). The difficulty with the WBA is the loss of some specificity and the dilution of the cytokine ratios of responsivity. Recombinant ML2283 and other *M. leprae*-specific proteins are being investigated as a means to differentiate immune responses for healthy household contacts (HHC), which typically do not show a response for PGL-I. Initial results are promising, but require further study and resolution to ensure increased reliability of immune/cytokine profiling. As a further adjunct diagnostic, a new assay using U-Cytech (UPT converting phosphor technology) was added to the WBA. In this assay, light of a lower energy is converted to light of a higher energy. UPT reporter particles then absorb infrared light and emit a detectable signal.

The combination of the U-Cytech Ab of IFN-gamma ELISA were applied in a lateral flow 'immuno-sandwich' to combine both technologies in a field-friendly format, called the UPT-LF for IFN-gamma detection. The measurement is a ratio of control versus outcome; each strip is then its own control. UPT has a portable reader which can do either one flow strip at a time or multiple strips.

Research is ongoing to identify proteins or other target antigens to improve sensitivity and specificity, particularly for TT/BT individuals.

Dr. Linda Oskam: 'New and improved opportunities for a serological kit and use of serology in leprosy control to aid classification and predict nerve function impairment'

The ML Flow test was evaluated as an additional, serological, tool for the classification of new leprosy patients in Brazil, Nepal and Nigeria. 2632 leprosy patients were classified using three methods: (1) counting the number of skin lesions, (2) slit skin smear examination, (3) serology using the ML Flow test. In Brazil and Nepal about 1/3 of the patients were MB against 1/5 in Nepal. Seropositivity was 63% in Nigeria, 51% in Brazil and 36% in Nepal. ML Flow test results and smears were negative in the vast majority of PB patients and in 16% of Brazilian and 38% of Nepali MB patients. Testing all PB patients with the ML Flow test to prevent under-treatment would increase the MB group by 18%, 11% and 46% for Brazil, Nepal and Nigeria. Using the ML Flow test as the sole criterion for classification would result in an increase of 11% and 44% of patients requiring MB treatment in Brazil and Nigeria and a decrease of 4% for Nepal. The ML Flow test could be used to strengthen classification, reduce the risk of under-treatment and minimise the need for slit skin smears.

Anti-PGL-I IgM seropositivity reflects the systemic bacterial load of leprosy patients. It aids the diagnosis and classification of leprosy patients and can be used to predict nerve function impairment (NFI). Despite this, serology is still not used in routine practice, possibly due to perceived lack of applicability and costs. Here we provide strong evidence that serology is valuable for and can be used in routine leprosy control.

1037 new leprosy patients were included in a prospective cohort. The relation between clinical and demographical variables and seropositivity was calculated and the test potential

to predict NFI was determined using survival analysis. The number and extent of clinical signs as well as sex, age, disability grade, bacterial index and classification all correlated with seropositivity. The size of skin lesions was positively correlated with seropositivity. No difference in levels of seropositivity among patients with one or two skin lesions was observed, nor were there different levels among patients with or without satellite lesions. A NFI prediction rule was proposed using classification and serology results, which could predict 80% of all NFI events in the cohort.

Dr. Sang-Nae Cho: 'Enzyme immunoassays for PGL-I antigen and antibodies'

Dr. Cho described his independently developed lateral flow kit and the pending comparison with the work developed by Drs. Oskam and Bührer. He described other forms of ELISA based on 35kD, 36kD, and 45kD protein antigens. In particular the combination of 45kD and PGL-I based serology adds considerably to the power of the latter. In particular, he described finger-prick PGL-I based serological screening of populations from endemic regions throughout Vietnam as the basis for the subsequent chemoprophylactic intervention.

Dr. Om Parkash: 'Serological detection of leprosy: Recent developments at JALMA, India, our laboratory'

Dr. Parkash described independent serological studies on the use of the 45kD serine rich protein, further supporting the added value of this form of serology.

Dr. Yumi Maeda: 'Recent findings of the use of the MMP-II protein as a potential diagnostic for leprosy detection using serology'

The major membrane protein II (MMP II; 28kD bacterioferritin) was generated as a fusion protein, affinity purified and applied as an additional serological tool in the context of conventional ELISA and compared with the PGL-I based particle agglutination test. Evidence was presented that this combination provides an additional means for the detection of leprosy.

Dr. Malcolm Duthie: 'Evaluation of the LID-1 fusion protein for the serological detection of *M. leprae*'

The aim of the work is to provide an early case detection/diagnosis serologically based test which would lend itself to widespread, early and intuitive application. The crux of the current work is the utilisation of select antigens from the known repertoire of recombinant antigens in stock. Serological expression cloning using select engineered combinations of these protein-based antigens (fusion proteins containing pieces of several proteins) are under investigation. The work presented focused on the utilisation of LID-1, a synthetic combination of ML0405 and ML2331. LID-1 utilised in combination with PGL-1 has generated intriguing results worthy of further investigation and refinement. This line of research holds promise for leprosy diagnosis.

Dr. John Spencer: 'Use of Western blot, ELISA and protein microarrays to define disease-state-specific antigenic profiles in leprosy'

The current serodiagnostic test for individuals with leprosy is based on antibody responses to the *M. leprae*-specific phenolic glycolipid-I (PGL-I). To further understand the relationship of antibody responses to disease state, the reactivity patterns of 50 lepromatous and 20 tuberculoid sera to PGL-I, lipoarabinomannan (LAM) and six recombinant *M. leprae*

proteins, was examined. The response to Ag85 was consistently high in both patient groups, while responses to CFP-10 and GroES showed the most variability, from strongly positive to completely negative. The tuberculoid patient sera showed overall lower responses to all of the recombinant proteins, particularly in the case of GroES, where none in this group showed any reactivity to this protein. Only 25% of these same patients showed a weak response to CFP-10, while 76% of lepromatous patients showed a positive reaction to this protein. This analysis has given a clearer understanding of some of the differences in the responses, both between individuals at opposite ends of the disease spectrum, as well as illustrating the heterogeneity of antibody responses towards protein, carbohydrate, and glycolipid antigens within a group. Attempts will be made in future to determine if some of these response patterns can be correlated with a particular disease state or outcome.

Dr. Juan Periche Fernandez: 'Difficulties in leprosy diagnosis'

Dr. Juan Periche presented the paramount need for laboratory-based testing which can be readily applied in the field to assist the clinician with the accurate diagnosis of leprosy. Clinical diagnosis based solely on physical examination is often difficult. Data was presented on the importance of active and passive leprosy case discovery. Utilisation of active leprosy case discovery through the interview process of healthy household contacts, in combination with experienced clinical examination, yielded the greatest benefit and was the most efficacious for cumulative resource utilisation. Definitive serological/cell immunity-based tests would add greatly to this effort.

Major Recommendations:

- PGL-I based serology, particularly in the kit format, continues to play a greater role in leprosy control in the following manner:
    as a confirmatory marker
    a predictor of disease outcome
    an indication of nerve damage and exacerbation
    as a tool for pre-clinical intervention.
- Protein based serology in the microarray format adds to diagnostic resolution and may aid in early clinical detection once reduced to a 'field-friendly format'.
- Assays based on cellular immune responses, and the facile detection of gamma interferon; may assist in pre-clinical detection allowing for early diagnosis and appropriate treatment.

Afternoon Session: Molecular Epidemiology

The afternoon portion of the Pre-Congress Workshop was devoted to topics in the general area of the molecular biology of *M. leprae*, organised and chaired by Dr. Vara Vissa and Thomas Gillis. Discussions centered on two major themes, 'Diagnostic and Environmental', and 'Clinical'. The former focused firstly and briefly on the *Diagnostic Potential of PCR in Comparison to Immunological Approaches*. There were three presentations, by Drs. Xiaomen Weng, Benjawan Phetsuksiri and Malcolm Duthie. Drs. Weng and Duthie described experiences with protein-based antigens, particularly the chimeric fusion protein, LID-1, in serological and cellular immune formats with different populations, whereas Dr. Phetsuksiri described her applications of a very sensitive version of RT-PCR, capable of detecting as few as 3–4 bacilli. However, the conclusion was that PCR, even in its most

sensitive format, is most suitable for purposes of confirmatory diagnosis in light of reliance on biopsy specimens, and for the continuing examination of the relationship between nasal carriage and true infection. Discussions on the topic of *M. leprae in the Environment* were wide-ranging led by brief presentations by Drs. Ramanesh, Amare, Izumi, Rupendra, Gillis and Jadhav. Beyond the well-documented presence of *M. leprae* in wild armadillos in the south of the USA, there were now reports, as yet not adequately documented, of its presence in Brazilian and Columbian armadillos, besides in water and soils. However, most of these reportings were from areas of high endemicity suggestive of human origin. Questions of whether merely DNA rather than viable *M. leprae* was being observed, and significance in terms of human transmission, were raised. The consensus was that no meaningful hypothesis could be posed at this time, but rather continue to observe and report.

The second major theme of this molecular biology portion of the Pre-Congress Workshop, namely Clinical aspects, was discussed firstly under the sub-heading of *Molecular Markers*. Dr. Vissa summarised the diverse features of the present well known ~ 20 VNTRs in terms of stability and applicability to different epidemiological questions (global vs. community vs. household transmission), different geographical settings, etc., indicating that the goal was to arrive at a set of markers of different properties to address different questions. She emphasised that the search for suitable diverse markers will continue, particularly now with the availability of a second *M. leprae* sequenced genome. Dr. Gillis laid out some statistical rules for the analysis of data to arrive at meaningful conclusions, indicating that $\sim 20-25$ VNTRs with a boot-strap value of $>80\%$ will be needed. Dr. Cole produced data on the lack of robustness of some of the markers used to date to truly address the questions posed, particularly some AT-rich micro- and mini-VNTR satellites. He emphasised the power of SNPs to address the historical origins of leprosy; however, these were too stable to address more immediate transmission questions. Dr. Vishwa Katoch described his experience of the application of VNTR-based molecular epidemiology in high transmission areas of Northern India, areas where evidence of *M. leprae* in the soil was well established. At this stage, the emphasis was on collection of samples, standardisation of techniques and selection and optimisation of markers. Embedded in this very productive session were discussions on the *Practicalities of Molecular Epidemiological Studies*. Dr. Belagon contributed to this discussion with her experience in Cebu. The need for properly documented cohorts, training, SOPs, suitable equipment and materials, databases, planning such as that conducted by the IDEAL Consortium, were all emphasised by Dr. Vissa and amplified by the attendees.

The final topic of the afternoon session was *Drug Resistance*. This was led by Dr. Matsuoka and his studies in several global settings (much of it published). The need for well-planned surveys led by the Leprosy Control Programme at WHO were emphasised. The recommendations of an earlier WHO workshop at JALMA were seconded in that the collection of data on molecular evidence of RIF and Dapsone resistance should continue with haste. The question of the need and availability of mouse footpad colonies was discussed. There is no doubt that $1-2$ well run, busy facilities are required and several of those in existence do not meet minimal specifications. WHO and the community should consider this issue. This topic recurred often throughout the various segments of the Congress, at Guest Lectures, Plenary Sessions, Multiple Free Paper Sessions, and Posters.

Pre-Congress Workshop I continued the following morning of January 30th with a free-ranging, well attended vigorous discussion on the topics of the previous day. This was a very productive, timely and successful Workshop.

The conclusions were:

- PCR is now an extremely sensitive and specific tool for diagnostic confirmation; it is not a useful diagnostic tool.
- *M. leprae* in the environment (soil, water) probably arises from human shedding; role in leprosy transmission is not known; the acquisition of data should continue.
- Molecular Markers: there is consensus that certain markers, particularly AT-rich, are devoid of sufficient robustness to answer pressing epidemiological and transmission questions. Others do have potential, supported by published work; the search should continue governed by statistical 'bootstrapping' rules; there is need for input from the corresponding research communities in other infectious diseases viz. TB.
- Practicalities: The need for properly documented cohorts, training, SOPs, suitable equipment and materials, databases, cooperation planning such as that conducted by the IDEAL Consortium, was agreed. The need for shared databases, both clinical and research, was emphasised; the imposition of GPS technology in certain epidemiological settings was emphasised; the need for the imposition of classical, population-type epidemiology was emphasised.
- Drug resistance: focus should be on RIF resistance and it can be combined with strain typing; the issue of how genetic evidence for RIF and/or Dapsone resistance might affect chemotherapy regimens should be addressed by control programmes; the implementation of new simple technology such as the dot-blot hybdrisation protocol was emphasised; the need for 1–2 well run, reliable mouse foot-pad colonies was emphasised; the question of the need/sustainability of sub-par facilities should be addressed by funding agencies/administrators; there are no other alternatives to MFP on the horizon.