Challenges in the post genomic era for the development of tests for leprosy diagnosis

Desafios na era pós genômica para o desenvolvimento de testes laboratoriais para o diagnóstico da hanseníase

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ABSTRACT

Leprosy diagnosis is based mainly on clinical manifestations and no laboratory test is available to diagnose asymptomatic disease or to predict disease progression among exposed individuals. Novel comparative genomic *in silico* analyses and molecular biology tools have discovered unique *Mycobacterium leprae* proteins with potential diagnostic application. Tuberculoid paucibacillary leprosy (PB) shows low antibodies titers and strong Th1 type/ IFN-γ specific cell mediated immunity (CMI), while lepromatous multibacillary patients (MB) show high antibody titers and low CMI. Therefore, laboratory tests for PB and MB leprosy diagnosis will require CMI and antibody based assays. Serologically reactive recombinant *Mycobacterium leprae* proteins were identified and may be used in conjunction with PGL-I to improve MB patient diagnosis. *Mycobacterium leprae* recombinant proteins and synthetic peptides have been tested for CMI-interferon gamma based assays for PB diagnosis. Modified PGL-I serology incorporating new *Mycobacterium leprae* antigens and CMI tests based on IFN-γ γproduction may permit the detection of leprosy PB and MB forms in endemic countries.

Key-words: Diagnosis. Leprosy. Post genomic approach.

RESUMO

O diagnóstico da hanseníase se baseia em manifestações clínicas e não existe teste laboratorial para diagnosticar casos assintomáticos ou para prever progressão da doença entre indivíduos expostos. Novas análises genômicas comparativas *in silico* e ferramentas de biologia molecular têm sido empregadas para revelar proteínas exclusivas do *Mycobacterium leprae* que apresentem potencial aplicação diagnóstica. A hanseníase tuberculóide paucibacilar (PB) apresenta baixo nível de anticorpos e forte resposta imune celular (RIC) tipo Th1/interferon gamma (IFN-γ). A doença lepromatosa multibacilar (MB) apresenta sorologia positiva e fraca RIC. Portanto, testes laboratoriais para diagnosticar hanseníase PB e MB devem contemplar testes de RIC e sorologia. Proteínas recombinantes do *Mycobacterium leprae* sorologicamente reativas podem ser incorporadas ao antígeno PGLI para melhorar o diagnóstico sorológico de pacientes MB. Proteínas recombinantes e peptídeos sintéticos do *Mycobacterium leprae* têm sido testados em ensaios de RIC/IFN-γ para diagnosticar casos PB. Sorologia anti-PGLI modificada incorporando novos antígenos do *Mycobacterium leprae* e ensaios baseados na RIC/produção de IFN-γ devem permitir a detecção precoce de casos MB e PB em países endêmicos.

Palavras-chaves: Diagnóstico. Hanseníase. Abordagem pós-genômica.

Leprosy, one of the oldest human diseases, is still a major public health problem in many endemic countries, such as Brazil. The causative agent of leprosy, *Mycobacterium leprae*, was the first bacterial pathogen to be identified as the cause of a human infectious disease more than 130 years ago. Nevertheless, up to the conclusion of its entire genome sequence published in 2001, *Mycobacterium leprae* has remained a puzzle, mainly due to its incompatibility with in vitro culture growth[9]. One exclusive feature of *Mycobacterium leprae* is its tropism to Schwann cells, which provides the basis of severe neuropathies that underlie the sensorimotor loss responsible for most of the deformities and disabilities associated with leprosy[29].

The World Health Organization's effort to eliminate leprosy by the year 2000 was based on important advances in antimycobacterial therapy in the 1980s. Despite the dramatic decline in prevalence observed in most endemic countries over the last decade, detection of new cases has remained high[34]. Given that the disease has not been eliminated, the development of a field applicable diagnostic test is considered a research priority and is urgently needed. In this context, the IDEAL consortium *Initiative for Diagnostic and Epidemiological Assays for Leprosy*, established in 2004, represents an international task-force that brings together researchers from endemic and non-endemic countries aimed at the concerted development of diagnostic tests for the early diagnosis of leprosy (infection) and

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for the identification of molecular markers to improve current understanding of the epidemiology and transmission of leprosy with the ultimate aim of designing rational interventions to prevent leprosy[3]. Our research group is part of the IDEAL consortium and, as such, has been involved in this leprosy diagnostic initiative.

Leprosy diagnosis is based mainly on clinical manifestations and the scarcity of symptoms in early disease can contribute to misdiagnosis or to underdiagnosis. Early diagnosis permits specific chemotherapy, which is important to reduce possible sources of transmission and to prevent more severe disease with disabilities and handicaps. Until the *Mycobacterium leprae* genome was deciphered, the availability of new antigens was impaired, mainly because the bacillus has never been cultivated in axenic culture.

Comparisons of the genome and proteome of *Mycobacterium tuberculosis* and *Mycobacterium leprae* revealed that the latter has undergone reductive evolution with a genome of 3.3Mb compared with 4.4Mb for *Mycobacterium tuberculosis*[8 9]. The *Mycobacterium leprae* genome contains 1,133 pseudogenes and less than 50% of its genome encodes functional genes, whereas 90% of the *Mycobacterium tuberculosis* genome is functional. *Mycobacterium leprae* annotated genome contains 1,614 open reading frames potentially encoding functional proteins compared with 3.993 for *Mycobacterium tuberculosis*. The reduction in the *Mycobacterium leprae* genome resulted in the elimination of several important metabolic pathways, which explains its intracellular habitat and its inability to be cultivated *in vitro*.

Comparative genomics and proteomics indicated that among 1,614 genes encoding proteins in *Mycobacterium leprae,* 1,439 are common to *Mycobacterium tuberculosis*, leaving 165 genes/proteins that appear to be *Mycobacterium leprae*-specific and potentially more suitable for laboratorial diagnosis of leprosy. Currently, novel mycobacterial genome information and molecular biology tools have renewed interest in new approaches to diagnostics based on comparative bioinformatic analysis for data mining to discover unique proteins of *Mycobacterium leprae* with potential diagnostic application[20 22]. These antigens could provide improved diagnostic tools to aid classical clinical methods.

The immunological features of spectral forms of leprosy include tuberculoid paucibacillary (PB) patients, who present low *Mycobacterium leprae* antibodies titers and strong Th1 type specific cell mediated immunity (CMI) characterized by interferon gamma (IFN-γ) production. Lepromatous multibacillary disease (MB) presents high antibody titers and low or absent T cell specific immunity[29]. Therefore, laboratory tests for PB and MB leprosy diagnosis will most probably require both T cell and antibody based assays. Recently, several new *Mycobacterium leprae* antigens have been tested as diagnostic reagents[1 2 11 12 14 15 16 17 28 31].

Laboratory tests for leprosy diagnosis

The state of the art of laboratory tests for leprosy include bacilloscopy, which relies on the detection of acid fast bacilli in skin slit smears and biopsies of skin lesions. The results are expressed as the bacilloscopic index (BI) but it lacks specificity and sensitivity, especially for PB forms of the disease. Histopathology of skin lesions can assist clinical diagnosis, especially if neural aggression and bacilli are identified. However, both these tests are not routine in public health services and are not part of most leprosy control programs in endemic countries. Currently, no specific and sensitive test is available for the diagnosis of asymptomatic *Mycobacterium leprae* infection or for predicting progression to leprosy among exposed individuals.

Serology

In the serodiagnosis field, detection of IgM antibodies against phenolic glicolipid-1 (PGL-I), an immunodominant *Mycobacterium leprae* antigen, remains the best standardized and most evaluated test in leprosy. Several formats have been developed for the detection of anti PGL-I antibodies: ELISA, particle agglutination, dipstick and rapid lateral flow test. Anti PGL-I serology is highly specific and the presence of serum antibodies correlates with the bacterial index (BI) of MB patients. However, anti PGL-I serology has limited diagnostic value for PB leprosy, as this category has low or undetectable BI and is characterized by cellular rather than humoral immune response. Moreover, in endemic settings a significant proportion of healthy individuals may be anti-PGL-I positive[25]. The current consensus is that anti-PGL-I tests should be used together with clinical parameters to assist in the classification of MB and PB leprosy for treatment decisions, providing better specificity than lesion counts[6 7].

Immunological and molecular markers of disease progression were investigated by our group among a Brazilian multicentric cohort of single skin lesion paucibacillary (SSL-PB) patients, who are considered the earliest diagnostic form of leprosy. In this cohort, around 30% had detectable IgM anti-PGL-I antibodies, corroborating the low sensitivity of anti-PGL-I serology for PB disease[23 33]. As part of the *IDEAL* activities, our group and the group from Anandaban Hospital (Nepal) recently compared two anti-PGL-I lateral flow rapid tests: ML-Flow test (KIT, Netherlands), which detects IgM antibodies anti-PGL-I natural disaccharide-octil-BSA (ND-O-BSA), and ML-ICA (Yonsei University, South Korea), which detects IgM, IgG and IgA antibodies against PGL-I antigens in the forms of natural trisaccharide- phenyl- BSA (NT-P-BSA), natural disaccharide-octil-human serum albumin (ND-O-HAS) and ND-O-BSA. Whole blood and serum from newly diagnosed, untreated MB and PB leprosy patients, MB household contacts (HHC) and healthy endemic controls (EC) were tested. In Brazil ML-Flow positivity in serum was higher than in whole blood and seropositivity of ML-Flow was higher than ML-ICA among PB, MB leprosy patients and HHC[18]. The results obtained in the Brazilian population indicate that in addition to IgM, the detection of IgG, IgA antibodies in anti-PGL-I rapid tests did not enhance the sensitivity or influenced the performance of the test for PB and MB leprosy. Comparative analysis of the results obtained in Brazil and Nepal are under way.

Recently, several studies based on genomic sequences have identified new *Mycobacterium leprae*-specific proteins or peptides that could be suitable for leprosy serodiagnosis. The rational is to find additional *Mycobacterium leprae* antigens that could be used with the PGL-I antigen to enhance its specificity and, particularly, its sensitivity for PB leprosy, providing an improved diagnostic tool for leprosy control programs. Different studies have evaluated the immunoreactivity of several *Mycobacterium leprae* recombinant

proteins in leprosy patients and controls. *Mycobacterium leprae* recombinant proteins ML0308, ML1553, ML2177, ML2498, ML0410, ML1053, ML1055 and ML1056 were tested with sera from Korean leprosy patients and healthy endemic controls and ML0308 showed the best reactivity[5]. A panel of *Mycobacterium leprae* recombinant proteins, including a fusion protein ML0050-ML0049, ML0091, ML0317, ML0405, ML2028, ML0568, ML1213, ML2055, ML2655, ML0097, ML1812, ML2331 and ML2496 was tested among Filipino MB leprosy patients, tuberculosis patients (TB) and endemic controls (EC): ML0405 and ML2331 were the most reactive[28]. ML0678, ML0757, ML2177, ML2244 and ML2498 were demonstrated to be strong B cell epitopes, when tested among tuberculosis patients, household contacts and leprosy patients from Mali and Bangladesh[1].

In collaboration with IDRI-Infectious Disease Research Institute (Seattle, USA), our research group tested the seropositivity of ML0091, ML0405TR (without transmembrane region), ML1633, ML2055, ML2331, ML2346 and ML1556 among PB and MB Brazilian leprosy patients, TB, HHC and EC. These antigens were also tested in Filipino leprosy patients and controls. ML2331, ML0405TR, ML2055 and ML0091 were the best immunoreactive antigens identified among Brazilian leprosy patients[11]. In general, the results indicated that MB leprosy showed the highest seropositivity and that few PB patients were seropositive. Different serological patterns were observed among patients from Brazil and the Philippines, suggesting that differences in HLA or in *Mycobacterium leprae* strain antigens may play a role in immunogenicity. However, independent of the geographical location, leprosy patients serologically recognized ML0405 and ML2331. LID-1, a new single fusion molecule with ML0405 and ML2331 constructed by IDRI, retained immunoreactivity, suggesting potential diagnostic application, especially for early disease[11]. In order to improve assessment of the diagnostic potential of new *Mycobacterium leprae* proteins, it will be necessary to test them in multicentric, multicountry studies.

Microarray technology represents another strategy for identifying novel diagnostic antigens. Arrays constructed with proteins isolated from either the cell wall or membrane of *Mycobacterium leprae* or with unique *Mycobacterium leprae* recombinant proteins (ML0008, ML0957, ML1419, ML1157, ML1877, ML1829, ML0126, ML0396, ML1915, ML0050) were constructed. This approach identified different humoral immune response patterns among tuberculoid and lepromatous patients[19].

Overall, the results of post-genomic serological studies with several *Mycobacterium leprae* recombinant proteins reflect the leprosy spectrum: high antibody levels at the lepromatous pole and low antibody levels at the tuberculoid pole. Moreover, new recombinant *Mycobacterium leprae* proteins have been identified and these antigens may be used in conjunction with PGL-I to improve the serological diagnosis of PB and MB patients. Additional constructs of new fusion proteins, including the most immunogenic *Mycobacterium leprae* antigens, such as the LID-1 antigen, open the possibility to produce new chimeric antigens that may have greater sensitivity for the detection of MB and possibly PB patients. In partnership with IDRI, our group is currently screening the immunoreactivity of *Mycobacterium leprae* proteins to identify new antigens to improve anti-PGL-I serology. The development of a new serological assay, ideally in a rapid test format, is considered a research priority[2 11 28].

T cell based tests

Mycobacterial infections, such as tuberculosis and leprosy, are characterized by cell mediated immunity (CMI) and delayed type hypersensitivity is considered a manifestation of CMI. Since the description of the Mitsuda skin test in 1919, several attempts have been made to develop skin tests for leprosy[5]. Currently CMI to *Mycobacterium leprae* has been assessed by in vitro T cell based tests based on IFN-γ production. The development of a simple, field-applicable T cell-based diagnostic test that can be used within large-scale leprosy monitoring and intervention programs may contribute to leprosy control in endemic countries.

Since PB patients develop strong CMI to *Mycobacterium leprae* and low antibody production, a laboratory diagnostic test for early PB disease needs to rely on the detection of T cell immunity. Several CMI *Mycobacterium leprae* immunogenic antigens and peptides have been described. However, T cell cross-reactivity with *Mycobacterium tuberculosis*, *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) or with other nonpathogenic environmental mycobacteria has restricted the diagnostic application of new antigens, especially in countries with high TB incidence and routine BCG vaccination, like Brazil[4 16 32].

The *Mycobacterium leprae* genome is known to encode 1.614 *Mycobacterium leprae* proteins and, in principle, all of them would need to be screened for diagnostic application. To avoid this, the selection of antigens with diagnostic potential has been based on bioinformatic *in silico* comparative genomic analyses with all currently available mycobacterial genome public databases. This approach aims to identify unique *Mycobacterium leprae* genes or proteins that may be selectively recognized by leprosy patients, thus reducing the chances of cross-reactivity. Antigen selection has also been guided by HLA based bioinformatic softwares to predict T cell immunodominant promiscuous epitopes capable of binding to various HLA class II alleles among *Mycobacterium leprae* proteins, favoring their recognition by genetically diverse populations[21].

Production of IFN-γ has been considered a marker of specific memory or effector T cell response. Two commercially available IFN-γ based assays using *Mycobacterium tuberculosis* specific antigens have been developed for the diagnosis of *Mycobacterium tuberculosis* infection[13]. We hope that T cell assays with a similar format may be available to contribute to the diagnosis and research of leprosy[12 16]. Based on IFN-γ response, new *Mycobacterium leprae* specific proteins and peptides have been tested for the laboratory diagnosis of PB leprosy. Candidate antigens for a leprosy T cell based assay have been tested as recombinant proteins, individual 15mer, 9mer and combined 20mer overlapping synthetic peptides[12 16 17 31].

Evaluation of immunogenicity has been based on IFN-γ responses in 24 hours diluted or undiluted heparinized whole

blood assays (WBA) and peripheral blood mononuclear cells (PBMC) usually including control responses to medium alone, phytohemaglutinin (PHA), *Mycobacterium leprae* cell wall antigens and PPD. In general, the screening tests have been conducted in following study groups: PB and MB leprosy patients (usually untreated), household contacts of MB leprosy patients (HHC), healthy endemic (EC) and non-endemic controls (NEC) and tuberculosis patients (TB). IFN-γ production to recombinant proteins or peptides has been estimated in WBA plasma and culture supernatant from stimulated PBMCs. Although IFN-γ has been broadly used as an indicator of protective immunity, the identification of other *surrogate markers* of protective immunity is another important research area and is one of our group's and IDEAL's current research activities.

The list of *Mycobacterium leprae* recombinant proteins shown by different groups to induce CMI/ IFN-γ production in PB leprosy patients and HHC is increasing. CMI to 17 *M.leprae* unique recombinant proteins, among which ML1989, ML1990, ML2283, ML2567, ML0576 recombinant proteins were assessed in PB, MB leprosy patients and controls (HHC, TB, EC) from Rio de Janeiro and non-endemic Dutch controls[17]. ML2244c, ML1553, ML2177, ML2498, ML0410, ML1053, ML1829, ML0410, ML1057, ML1056 and ML0308 were shown to be reactive among leprosy patients and controls recruited in Mali and Bangladesh and non-endemic controls from Paris[2]. ML2177, ML2498, ML0410 and ML1053 were identified as *Mycobacterium leprae* CMI stimulators among Korean leprosy patients[1].

CMI to *Mycobacterium leprae* recombinant proteins ML0091, ML0276, ML0398, ML0541, ML0543, ML0840, ML0953, ML1011, ML1213, ML1623, ML2044, ML46f, ML56f were assessed by our group, in partnership with IDRI, among PB and MB leprosy patients, TB, HHC, EC and NEC and ML0276, ML1623, ML0840, ML2044 e ML46f were identified as the best candidates[12]. In this study, some *Mycobacterium leprae* proteins with high homology with *Mycobacterium tuberculosis* proteins showed specific responses in leprosy patients without cross-reaction. On the other hand, proteins showing no known homologue in *Mycobacterium tuberculosis* and other mycobacterial genomes/proteomes were recognized by leprosy and tuberculosis patients, HHC and EC. These results, together with other studies, indicate that the prediction of so called unique *Mycobacterium leprae* antigens by comparative genomic bioinformatic analyses requires validation by field investigations in endemic and non-endemic settings.

Besides recombinant proteins, several synthetic peptides derived from the most immunogenic and specific *Mycobacterium leprae* proteins were also tested in leprosy patients and controls[16 17 31]. The peptide strategy is based on the finding that recombinant proteins induce higher levels of IFN-γ, while increasing the chances of T cell cross-reactive responses even within low homology sequences[14]. Therefore, more specific CMI responses were observed when peptides derived from most immunogenic *Mycobacterium leprae* proteins were tested[17 31]. As part of the T cell studies for leprosy diagnostics conducted by the IDEAL consortium, our group participated in the study that tested IFN-γ production to *Mycobacterium leprae* recombinant proteins and several selected peptides using whole blood and PBMC from leprosy patients and controls. Besides Brazil, the experiments using the same, standardized research protocol was conducted in Ethiopia, Bangladesh, Pakistan and Nepal. In this study, T cell responses specific for leprosy patients and healthy household contacts were observed for ML2283 and ML0126 derived peptides, indicating their potential as diagnostic tools [16].

In general, T cell based studies for leprosy have shown that whole blood and PBMCs stimulated with selected antigens induce higher IFN-γ production among household contacts of MB and tuberculoid leprosy patients. The lowest production was observed among healthy non-endemic controls and MB patients, whereas variable results were observed among TB patients and healthy endemic controls, which may indicate exposure to *Mycobacterium leprae* in endemic areas[12 16 17 31]. The high IFN-γ responses among household contacts of MB patients may indicate protective immunity upon exposure or subclinical infection. Further follow-up studies are needed to clarify the meaning of CMI reactivity among household contacts of MB patients.

In conclusion, T cell based studies for leprosy have determined several candidate antigens, either recombinant proteins or peptides, with potential application for a T cell diagnostic test based on IFN-γ detection. Although the number of recombinant *Mycobacterium leprae* proteins tested has increased, a long list of untested candidate proteins needs to be evaluated before choosing the best antigen and its best presentation for a T cell based diagnostic test for PB leprosy. Both PBMCs and whole blood have been successfully used, however simple 24 hours whole blood assays may be a preferable, cost effective and field friendly format for a T cell based assay for endemic countries.

Mycobacterium leprae PCR

The revelation of species-specific nucleotide sequences in the *Mycobacterium leprae* genome has introduced DNA and RNA based detection methods, such as polymerase chain reaction (PCR) for the sensitive and specific detection of the bacilli. Several target antigens have been used and the reported sensitivity of *Mycobacterium leprae* PCR for PB leprosy has increased, but is still below 80%. The study on Brazilian single skin lesion leprosy cases showed 44.4% *Mycobacterium leprae* DNA-PCR positivity in skin biopsies and this positivity was associated with a higher frequency of type 1 reaction[30]. Even the *relatively* low sensitivity of ML- PCR among PB patients can already be considered an advance when compared to the very low detection rate of bacilli in tissue sections and with the inability to cultivate the bacilli *in vitro*.

TaqMan real-time PCR assay was shown to be more sensitive than conventional real-time PCR to detect *Mycobacterium leprae* DNA in clinical specimens with undetectable bacilli by conventional histological staining[24]. A reverse transcriptase PCR RNA-based assay method, based on *Mycobacterium leprae* ribosomal RNA genes was proposed for the molecular diagnosis of leprosy and to determine the viability of leprosy bacilli[27]. Despite the amount of new information regarding *Mycobacterium leprae*-PCR, this methodology remains a research and not a diagnostic

tool. Up to now, *Mycobacterium leprae*-PCR has not had much impact on routine diagnostic practice, as it remains dependent on specialized equipment, is not yet cost-effective and still presents relative limited sensitivity for PB cases.

Final considerations

Ideally the use of *Mycobacterium leprae* peptide or recombinant protein in T cell- IFN-γ assays in combination with a modified version of anti-PGL-I serology tests would permit the detection of most PB and MB forms of leprosy. T cell based tests may enable the detection of subclinical infection and the introduction of multidrug therapy at an early stage. In this context, the development of simple and rapid field tests, such as agglutination tests, that could be applied directly on a drop of blood or saliva would be of enormous value for leprosy diagnosis and control. A sensitive, specific, simple, field and user friendly test, heat stable, with a long shelf life and that is affordable is likely to promote a significant impact on leprosy control programs in endemic countries[10 26]. New *Mycobacterium leprae* antigens discovered in the post genomic era may provide diagnostic tests for early infection, tests with potential prognostic value or tests suitable for leprosy classification[10]. In addition to the possibility of identifying subclinical infection, new laboratory tests could improve current understanding regarding transmission patterns and surveillance at the population level.

REFERENCES

1. Araoz R, Honore N, Banu S, Demangel C, Cissoko Y, Arama C, Uddin MK, Hadi SK, Monot M, Cho SN, Ji B, Brennan PJ, Sow S, Cole ST. Towards an immunodiagnostic test for leprosy. Microbes and Infection 8: 2270-2276, 2006.
2. Araoz R, Honore N, Cho S, Kim JP, Cho SN, Monot M, Demangel C, Brennan PJ, Cole ST. Antigen discovery: a postgenomic approach to leprosy diagnosis. Infection and Immunity 74: 175-182, 2006.
3. Aseffa A, Brennan P, Dockrell H, Gillis T, Hussain R, Oskam L, Richardus JH. Report on the first meeting of the IDEAL (Initiative for Diagnostic and Epidemiological Assays for Leprosy) consortium held at Armauer Hansen Research Institute, ALERT, Addis Ababa, Ethiopia on 24-27 October 2004. Leprosy Review 76: 147-159. 2005.
4. Booth RJ, Williams DL, Moudgil KD, Noonan LC, Grandison PM, McKee JJ, Prestidge RL, Watson JD. Homologs of *Mycobacterium leprae* 18-kilodalton and *Mycobacterium tuberculosi*s 19-kilodalton antigens in other mycobacteria. Infection and Immunity 61: 1509-1515, 1993.
5. Brennan PJ. Skin test development in leprosy: progress with first-generation skin test antigens, and an approach to the second generation. Leprosy Review 71 (suppl): S50-S54, 2000.
6. Buhrer-Sekula S, Smits HL, Gussenhoven GC, van Leeuwen J, Amador S, Fujiwara T, Klatser PR, Oskam L. Simple and fast lateral flow test for classification of leprosy patients and identification of contacts with high risk of developing leprosy. Journal of Clinical Microbiology 41: 1991-1995, 2003.
7. Buhrer-Sekula S, Visschedijk J, Grossi MA, Dhakal KP, Namadi AU, Klatser PR, Oskam L. The ML flow test as a point of care test for leprosy control programmes: potential effects on classification of leprosy patients. Leprosy Review 78:70-79, 2007.
8. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry CE, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver K, Osborne J, Quail MA, Rajandream M A, Rogers J, Rutter S, Seeger K, Skelton J, Squares R, Squares S, Sulston JE, Taylor K, Whitehead S, Barrell BG. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393(6685): 537-544, 1998.
9. Cole ST, Eiglmeier K, Parkhill J, James KD, Thomson NR, Wheeler PR, Honore N, Garnier T, Churcher C, Harris D, Mungall K, Basham D, Brown D, Chillingworth T, Connor R, Davies RM, Devlin K, Duthoy S, Feltwell T, Fraser A, Hamlin N, Holroyd S, Hornsby T, Jagels K, Lacroix C, Maclean J, Moule S, Murphy L, Oliver K, Quail MA, Rajandream MA, Rutherford KM, Rutter S, Seeger K, Simon S, Simmonds M, Skelton J, Squares R, Squares S, Stevens K, Taylor K, Whitehead S, Woodward JR, Barrell BG. Massive gene decay in the leprosy bacillus. Nature 409: 1007-1011, 2001.
10. Corstjens PL, Zuiderwijk M, Tanke HJ, van der Ploeg-van Schip JJ, Ottenhoff TH, Geluk A. A user friendly, highly sensitive assay to dectect the IFN-γ secretion by T cells. Clinical Biochemistry 41: 440-444. 2008.
11. Duthie MS, Goto W, Ireton GC, Reece ST, Cardoso LP, Martelli CM, Stefani MM, Nakatani M, de Jesus RC, Netto EM, Balagon MV, Tan E, Gelber RH, Maeda Y, Makino M, Hoft D, Reed SG. Use of protein antigens for early serological diagnosis of leprosy. Clinical and Vaccine Immunology 14: 1400-1408, 2007.
12. Duthie MS, Goto W, Ireton GC, Reece ST, Sampaio LH, Grassi AB, Sousa ALM, Martelli CM, Balagon MVF., Tan E, Stefani MM, Reed SG. Antigen-specific T cell responses of leprosy patients. Clinical and Vaccine Immunology 15:1659-1665, 2008.
13. Ferrara G, Losi M, D'Amico R, Roversi P, Piro R, Meacci M, Meccugni B, Dori IM, Andreani A, Bergamini BM, Mussini C, Rumpianesi F, Fabbri LM, Richeldi L. Use in routine clinical practice of two commercial blood tests for diagnosis of infection with *Mycobacterium tuberculosis*: a prospective study. Lancet. 367: 1328-1334, 2006.
14. Geluk A, Klein MR, Franken KL, van Meijgaarden KE, Wieles B, Pereira KC, Buhrer-Sekula S, Klatser PR, Brennan PJ, Spencer JS, Williams DL, Pessolani MC, Sampaio EP, Ottenhoff TH. Postgenomic approach to identify novel *Mycobacterium leprae* antigens with potential to improve immunodiagnosis of infection. Infection and Immunity 73: 5636-5644, 2005.
15. Geluk A, Ottenhoff TH. HLA and leprosy in the pre and postgenomic eras. Human Immunology 67: 439-445, 2006.
16. Geluk A, Spencer JS, Pessolani MCV, Pereira GMB, Banu S, Honoré N, Reece S, MacDonald M, Ranjit C, Sapkota B, Bobosha K, Zewdie M, Aseffa A, Hussain R, Stefani M, Cho R, Oskam L, Brennan PJ, Dockrell HM. From Genome to Leprosy Diagnostics: from *in silico* predictions to *ex vivo* verification. Clinical and Vaccine Immunology. in press.
17. Geluk A, van der Ploeg J, Teles RO, Franken KL, Prins C, Drijfhout JW, Sarno EN, Sampaio EP, Ottenhoff TH. Rational combination of peptides derived from different Mycobacterium leprae proteins improves sensitivity for immunodiagnosis of *M. leprae* infection. Clinical and Vaccine Immunology 15: 522-533, 2008.
18. Grassi AB, Sampaio LH, Martelli CM, Cho R, Oskam L, Buhrer-Sékula S. Comparison Between two Rapid Tests for Anti PGL-I Serology. Proceedings of the 17th International Leprosy Congress, Hyderad, India. 206, 2008.
19. Groathouse NA, Amin A, Marques MA, Spencer JS, Gelber R, Knudson DL, Belisle JT, Brennan PJ, Slayden RA. Use of protein microarrays to define the humoral immune response in leprosy patients and identification of disease-state-specific antigenic profiles. Infection and Immunity 74: 6458-6466, 2006.
20. http://www.sanger.ac.uk/Projects/M_tuberculosis/. 2007.
21. http://www.imtech.res.in/raghava/propred. 2008.
22. http://genolist.pasteur.fr/Leproma. Leproma World-Wide Web Server, 2004.
23. Martelli CM, Stefani MM, Gomes MK, Rebello PF, Peninni S, Narahashi K, Maroclo AL, Costa MB, Silva SA, Sacchetim SC, Nery JA, Salles AM, Gillis TP, Krahenbuhl JL, Andrade AL. Single lesion paucibacillary leprosy: baseline profile of the Brazilian Multicenter Cohort Study. International Journal of Leprosy and Other Mycobacterial Diseases 68: 247-257, 2000.
24. Martinez AN, Britto CF, Nery JA, Sampaio EP, Jardim MR, Sarno EN, Moraes MO. Evaluation of real-time and conventional PCR targeting complex 85 genes for detection of *Mycobacterium leprae* DNA in skin biopsy samples from patients diagnosed with leprosy. Jornal of Clinical Microbiology 44: 3154-3159, 2006.
25. Oskam L, Slim E, Buhrer-Sekula S. Serology: recent developments, strengths, limitations and prospects: a state of the art overview. Leprosy Review 74: 196-205. 2003.

26. Peeling RW, Smith PG, Bossuyt PM. A guide for diagnostic evaluations. Nature Reviews Microbiology 4(supl 12): S2-S6, 2006.

27. Phetsuksiri B, Rudeeaneksin J, Supapkul P, Wachapong S, Mahotarn K, Brennan PJ. A simplified reverse transcriptase PCR for rapid detection of *Mycobacterium leprae* in skin specimens. FEMS Immunology and Medical Microbiology 48: 319-328. 2006.

28. Reece ST, Ireton G, Mohamath R, Guderian J, Goto W, Gelber R, Groathouse N, Spencer J, Brennan P, Reed SG. ML0405 and ML2331 are antigens of *Mycobacterium leprae* with potential for diagnosis of leprosy. Clinical and Vaccine Immunology 13: 333-340, 2006.

29. Scollard DM, Adams LB, Gillis TP, Krahenbuhl JL, Truman RW, Williams DL. The continuing challenges of leprosy. Clinical Microbiology Reviews 19: 338-381, 2006.

30. Sousa AL, Stefani MM, Pereira GA, Costa MB, Rebello PF, Gomes MK, Narahashi K, Gillis TP, Krahenbuhl JL, Martelli CM. *Mycobacterium leprae* DNA associated with type 1 reactions in single lesion paucibacillary leprosy treated with single dose rifampin, ofloxacin, and minocycline. American Journal of Tropical Medicine and Hygiene 77: 829-833, 2007.

31. Spencer JS, Dockrell HM, Kim HJ, Marques MA, Williams DL, Martins MV, Martins ML, Lima MC, Sarno EN, Pereira GM, Matos H, Fonseca LS, Sampaio EP, Ottenhoff TH, Geluk A, Cho SN, Stoker NG, Cole ST, Brennan PJ, Pessolani MC. Identification of specific proteins and peptides in *Mycobacterium leprae* suitable for the selective diagnosis of leprosy. Journal of Immunology 175: 7930-7938, 2005.

32. Spencer JS, Marques MA, Lima MC, Junqueira-Kipnis AP, Gregory BC, Truman RW, Brennan PJ. Antigenic specificity of the *Mycobacterium leprae* homologue of ESAT-6. Infection and Immunity 70: 1010-1013, 2002.

33. Stefani MM, Martelli CM, Gillis TP, Krahenbuhl JL. *In situ* type 1 cytokine gene expression and mechanisms associated with early leprosy progression. Journal of Infectious Diseases 188: 1024-1031, 2003.

34. World Health Organization. Global leprosy situation, 2006. Weekly Epidemiological Record. 81: 309-316, 2006.