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NEWS and NOTES

This department furnishes information concerning institutions, organizations, and individuals engaged in work on leprosy and other mycobacterial diseases, and makes note of scientific meetings and other matters of interest.

US-Japan Meeting, 2004

The 40th Anniversary Meeting of all of the panels of the US-Japan Cooperative Medical Sciences Program (USJCMSP) took place in Kyoto in December, 2004. The Joint Tuberculosis and Leprosy Panels organized two half-day sessions dedicated to TB and leprosy, and co-sponsored two half-day sessions with other panels—one with the AIDS Panel on TB/HIV interactions, and the other with the Acute Respiratory Infections Panel on antibiotic resistance. The Joint Committee of the USJCMSP announced that new guidelines for panel activities would be implemented in the coming year. The Joint Tuberculosis and Leprosy Panels were encouraged to develop high priority scientific programmatic goals, and identify implementable research objectives for the next five years. Examples might include TB vaccine and drug development, management of latent TB, and the development of molecular tools to better characterize leprosy transmission and incidence. A strong emphasis will be placed on strengthening the research capacity for both diseases in high-burden countries in the Pacific Rim, including training activities and technology transfer. The Joint TB and Leprosy Panels were also encouraged to develop strategies for interacting effectively with other relevant Panels in the coming years.

The 40th Annual US-Japan Conference on Tuberculosis and Leprosy will take place in Seattle from 28–30 July, 2005. The main conference will be preceeded by a half-day session co-sponsored with the Immunology Board which will focus on the definition of immunological determinants of protection induced by TB vaccines. The final day of the conference will consist of a Leprosy Workshop with invited speakers which will focus on the immunology and pathology of the disease, animal models, and challenges in leprosy research.

Modulation of the TH1 response to *Mycobacterium leprae* in experimental leprosy

Introduction. In leprosy, a disease caused by the obligate intracellular pathogen, *Mycobacterium leprae*, an array of symptoms are presented which are largely determined by the host's response, ranging from a high level of cell mediated immunity (CMI) in tuberculoid leprosy (TT) to absence of CMI in lepromatous leprosy (LL). Animal models for leprosy are limited. Armadillos exhibit a disease spectrum similar to man, but they are restrictively expensive and immunological reagents are scarce. The murine system, while well-characterized and armed with a plethora of immunological reagents, is essentially restricted to the foot pad for evaluating growth of *M. leprae* and exhibits limited nerve involvement by the bacilli. Nevertheless, murine models for leprosy, especially with the introduction of gene knockout (KO) strains, have shown promise for immunological studies of the leprosy spectrum. *M. leprae*-induced granuloma formation and maintenance depends heavily upon T cell and macrophage (MΦ) populations and their respective cytokines. Mice deficient in inducible nitric oxide synthase (iNOS), an important MΦ effector mechanism, have shown promise as a model for borderline tuberculoid leprosy in that intense granuloma formation rapidly appears without exacerbating *M. leprae* growth. IL-12, a key regulatory cytokine of the immune system, induces the production of IFN-γ by T cells and NK cells and promotes the development of a Th1 type cell mediated immune response. IL-10 is generated by T cells and MΦ and is an inhibitor of IFN-γ production.

Methods. *M. leprae* infection was evaluated in iNOS KO ($NOS2^{-/-}$), IL-10 KO ($IL10^{-/-}$) and IL-12 KO ($IL12^{-/-}$) mice using low dose (LD) and high dose (HD) infection models. C57Bl/6 (B6) control mice and

KO mice were infected in both hind foot pads with 6×10^3 (LD) viable *M. leprae* and growth, lymph node cell profiles and histology were monitored for 18 months. B6 and KO mice were also inoculated with 3×10^7 (HD) viable *M. leprae* in both hind footpads, with or without treatment with iNOS inhibitors such as LNil (L-N6-(1-iminoethyl) lysine hydrochloride) or aminoguanidine (AG). Foot pad induration, cell profiles, and cytokine expression were analyzed in these foot pads.

Results. In the LD model, growth of *M. leprae* was controlled in the $NOS2^{-/-}$ and $IL10^{-/-}$ mice, similarly to B6 control mice. In contrast, there was augmented growth of the bacilli in the $IL12^{-/-}$ mice. Flow cytometric analysis of the draining popliteal lymph nodes showed a sharp decrease in the level of T lymphocytes in B6 mice at 6 months post infection which corresponds with the peak of *M. leprae* growth. A similar decrease was seen in $IL10^{-/-}$ mice. In contrast, this decrease was not seen in $IL12^{-/-}$ mice. In the HD model, a large granulomatous response occurred in the $NOS2^{-/-}$ mice compared to B6 mice which consisted primarily of $CD11b^+$ MΦ and $CD4^+$ lymphocytes and, to a smaller extent, $CD8^+$ cells. The level of $CD4^+$ and $CD8^+$ cells expressing activation markers was significantly higher in $NOS2^{-/-}$ mice than B6 mice. Concomitant with foot pad induration was an augmented expression of IFNγ, TNFα, and IL-10 as well as MIP-1α, MIP-1β, and MCP-1. A similar induration occurred in *M. leprae*-infected B6 mice treated with L-NIL. Interestingly, the induration subsided if the iNOS inhibitor was removed; conversely, if the iNOS inhibitor was added 1 month post infection, enhanced induration ensued, thus emphasizing the dynamic nature of the foot pad lesion. Upon infection with HD *M. leprae*, $IL10^{-/-}$ mice also exhibited greater induration than control mice. Like B6 and $iNOS^{-/-}$ mice, the T lymphocyte infiltration was primarily $CD4^+$. Addition of LNil to the $IL10^{-/-}$ diet resulted in greater induration of the *M. leprae*-infected foot pad than either individual model of deficiency. If LNil administration began 1 month post infection, induration rapidly exceeded that of the $IL10^{-/-}$ mice and was similar to mice that were iNOS and IL10 deficient from the beginning of infection. In $IL12^{-/-}$ mice, HD infection with *M. leprae* induced little induration in the foot pad and the T lymphocyte infiltration was equally $CD4^+$ and $CD8^+$.

Discussion. These findings suggest that KO mice infected with *M. leprae* can provide insight into the subtle nuances of cell mediated immunity toward *M. leprae* infections as well as contribute to the overall understanding of the various processes underlying the broad host response to infection and, in particular, the unstable nature of the borderline area of the leprosy spectrum.

—Deanna A. Hagge, Nashone A. Ray, Vilma Tulagan and Linda B. Adams

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Initiative for Diagnostic and Epidemiological Assays for Leprosy (IDEAL)

In 1977, the World Health Organization (WHO) Expert Committee on Leprosy estimated the global number of leprosy cases to be over 12 million. In 1981, WHO convened the Study Group on Chemotherapy for Leprosy Control, which recommended combined-drug regimens based on supervised intermittent administration of rifampicin for both multibacillary (MB) and paucibacillary (PB) leprosy. Thanks to the implementation of this multidrug therapy (MDT), substantial progress in leprosy control has been achieved, and over 12 million cases had been cured by 2002. Thus, the WHO Leprosy Programme set a target for the elimination of leprosy (less than 1 case per 10,000) by the year 2000. With the failure to achieve this goal, more recently, WHO formed a Global Alliance for the Elimination of Leprosy (GAEL) with the aim of reaching the elimination target (less than 1 case per 10,000) in all countries by 2005. However, to date, there is no clear evidence of an impact of introduction of MDT on the rate of detection of new cases. While global prevalence has dropped from the millions in the 1970s to less than 650,000 cases in 2002, new case detection has remained steady over the years at over 700,000 per annum. Approaches to address

this problem are impeded by a lack of fundamental knowledge about the epidemiology of leprosy, the sources of infection, its precise mode of transmission, and the importance of contact patterns.

The "Initiative for Diagnostic and Epidemiological Assays for Leprosy" (IDEAL) resulted from two workshops under the auspices of the TDR program of WHO. The first workshop, held in Geneva in November 2002, identified two fields of leprosy research in which advances are needed in order to eliminate leprosy. These are:

1) Nerve damage and 2) Early diagnosis and transmission.

In October 2003 a second workshop was organized in Amsterdam, in which the research needs in the field of early diagnosis and transmission were further identified and made explicit in a proposal for a comprehensive leprosy research program. This research program aims at the application of new developments in the fields of molecular typing of *M. leprae* and specific antigen/epitope definition to field studies towards better understanding of the epidemiology and transmission of leprosy, and the improved diagnosis of leprosy infection. The three main areas of research in this program are:

- Assays for molecular epidemiology
- Immunology-based diagnostic assays
- Field studies related to transmission and diagnosis

An Interim Steering Committee (Drs. Brennan, Dockrell, Engers, Klatser, Oskam, Richardus) was appointed to coordinate efforts to obtain funding and to invite partners (both research institutes and field programs) to join the consortium. The partners and sites chosen all have a proven track record in leprosy research, providing access to sufficient leprosy patients and their contacts within a functional leprosy control programme, with well-equipped laboratories and/or with experience in capacity building and technology transfer. The specific aims of the IDEAL research program are to:

1. Identify and develop *M. leprae*-specific proteins and peptides suitable for use in T cell assays, to enable specific immune responses to be identified in paucibacillary leprosy patients or contacts.
2. Dissect biomarkers identifying protective and non-protective immune responses in groups of leprosy contacts, that could be used to develop simple assays to identify infected subjects without protective immunity in leprosy-endemic countries.
3. Identify and assess the full range of polymorphisms at short tandem repeat (STR) and single nucleotides in the *M.leprae* genome with sufficient genetic variability to define sources of infection, transmission patterns and distinguish between new and reactivated cases of clinical leprosy.
4. Apply these new tools in field settings with different population characteristics and levels of endemicity.
5. Form a global platform for research groups so that research can be implemented in a coordinated manner, thus speeding up the quest for solutions to the issues of leprosy.

The research developments underlying these aspirations will be explained as also will the convening of the first meeting of IDEAL patners in Addis Ababa, Ethopia, October 25–27, 2004 will be described.

—Patrick J. Brennan

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Regulation by clofazimine of cytokine production in *M. leprae*-infected macrophages

Anti-mycobacterial drug, clofazimine or B663, has been used for the treatment of leprosy and some of the mycobacterial infections for the purpose of killing of the causative bacilli, moreover, the drug is reported to be useful in several immunologically mediated skin disorders. The mechanism of action by clofazimine is still unclear, although several studies have suggested its modulatory effects on immune response. In leprosy, clofazimine is also used for the suppression of leprosy reaction. To investigate the mechanism of immunomodulation by

clofazimine, the macrophage, one of the immune cells which play a very important role in leprosy as a host cell of *M. leprae*, was studied on the basis of the effect of the drug on cytokine production in response to *M. leprae*. By *in vitro* study it was found that B663 enhanced TNF production in *M. leprae*-stimulated mouse macrophages, moreover, the drug suppressed IL-10 and PGE2 production in the cells. The suppressive effect on IL-10 production could be due to the suppression of PGE2 production, since PGE2 was required to induce IL-10 by elevation of intracellular cAMP level through the stimulation of adenylate cyclase. PGE2 is a well-known inflammatory factor, therefore, anti-inflammatory activity of the drug could be due to the suppressive effect on PGE2 production. TNF is known as the coactivator of macrophages with IFN gamma, suggesting that B663 could enhance antibacterial activity of the host through the enhancement of TNF.

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Nerve damage by bacteria causing Buruli ulcer—ultrastructure of mouse inoculated with *Mycobacterium ulcerans*

Buruli ulcer is an intractable skin disease caused by *Mycobacterium ulcerans*. It is observed in tropical area such as Africa and Australia. Large, necrotizing, relatively painless, deep skin ulcers are formed mainly in the extremities. Because of chronic course and occasional complication of severe deformities, socioeconomic handicap is a great problem.

Recent study demonstrated that Phenolic Glycolipid-I (PGL-I), a *Mycobacterium leprae*-specific membranous antigen responsible for Schwann cell invasion, is present in Buruli ulcer. Thus, we hypothesized that not only *M. leprae* but also *M. ulcerans* may invade peripheral nerve.

MATERIALS AND METHODS

Bacterial suspension of *M. ulcerans* colony 97-107 cultured at 32°C in 7H9 culture medium (CFU = 1.3×10^6/ml, 25μl) was inoculated into the bilateral footpads of female BALB/c mice. Local swelling and redness were observed at day 33 after the inoculation, and sequential histopathological examination was performed since then.

Perfusion fixation by 10% formalin was done, and hind limbs were histopathologically examined by HE, acid-fast staining and immunohistochemistry using anti-PGL-I antibody. Also in selected cases, perfusion fixation by 2% glutaraldehyde was done, and hind limbs embedded in Epon, cut into 1μm were examined. When nerve damage is observed, electron microscopic examination was performed.

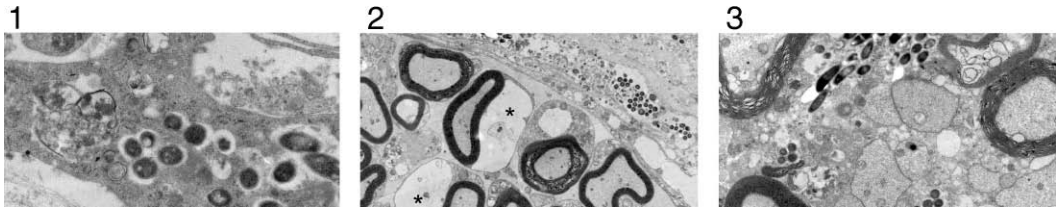
RESULTS

Day 33 after the inoculation of M. ulcerans: Dermal erosion and extensive edema of subcutaneous tissue were associated with infiltration of small number of neutrophils and monocyte. Granuloma formation was absent. Small clusters of long acid-fast bacilli were noted mainly in the stroma and in the cytoplasm of monocytes (Fig. 1). Peripheral nerves were well preserved even in the edematous lesion.

Day 55 after the inoculation of M. ulcerans: Remarkable deep skin ulcer and extensive subcutaneous edema were observed. Large number of acid-fast bacilli formed clusters in the edematous stroma. Many nerve bundles were well preserved, but some showed vacuolar change of Schwann cells (Fig. 2), and others were invaded by numerous acid-fast bacilli with massive nerve damage. Ultrastructurally, the bacilli were mainly in the endoneurium, and Schwann cells were spare (Fig. 3). PGL-I immunohistochemistry was negative.

DISCUSSION

Among the mycobacterial species, only *M. leprae* is known to show neurotropism and causes nerve damage. In the previous studies, mild degenerative change with thickening of Schwann cell basal lamina and vacuolar change of axons were reported



FIGS. 1–3. **1.** Day 33. Macrophage contains numerous bacilli. **2.** Day 55. Vacuolar change of Schwann cells (*). **3.** Nerve damage with endoneurial invasion of bacilli.

{Mwanatambwe, 2000}, but direct nerve invasion by the acid-fast bacilli has not been found. Our study first demonstrated that nerve bundles are damaged by numerous *M. ulcerans*. This finding raises a new possibility of pathogenesis of "painlessness" of Buruli ulcer.

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Chemotherapy and Drug Resistance in Leprosy

The chemotherapy of leprosy, which is caused by *Mycobacterium leprae*, was launched in 1943 and dapsone was introduced as standard chemotherapy for leprosy in the 1950s. Between the 1960s and 1970s, other anti-leprosy agents such as clofazimine and rifampin were introduced due to the emergence of dapsone resistance resulted from long-term monotherapy with dapsone. The first dapsone resistant case was proved by mouse foot-pad method in 1964. To conquer the increasingly worldwide spread of dapsone resistance and control leprosy, the World Health Organization recommended multidrug therapy in 1981. Regimens included dapsone, rifampin and clofazimine in different doses and durations for multibacillary case (MB) and paucibacillary case (PB). Recently, ofloxacin and minocycline have been added for treating single lesion paucibacillary case (SPB).

Dapsone targets dihydropteroate synthase (DHPS) encoded by the *folP* and inhibits folic acid biosynthesis by acting as a competitive inhibitor of *p*-aminobenzoic acid (PABA). The target for rifampin is beta subunit of the RNA polymerase, encoded by the *ropB*, and transcription is inhibited. The mechanisms of antimicrobial activity for clofazimine has not been fully elucidated, however, the drug appears preference to bind to GC-rich sequences of mycobacteria. Similarly, the mechanism of bactericidal activity of minocycline against *M.leprae* is unknown, but thought to inhibit protein synthesis by blocking the binding of aminoacyl transfer RNA to the messenger RNA. Ofloxacin, one of new quinolones, it is likely to inhibit DNA replication by binding to A-subunits (GyrA) of DNA gyrase, a type II topoisomerase.

In spite of discovery of the genetic background for understanding of drug resistance in dapsone, rifampin and ofloxacin, only a limited number of mutations responsible for resistance have been reported in *M. leprae* to date since drug susceptibility of *M.leprae* to anti-leprosy drug has been tested by mouse foot-pad method. Almost all mutations in relevant genes conferring resistance to each drug were point mutations. Responsible point mutations, at 513 (1 case), 516 (1 case), 526 (3 cases), 531 (24 cases) and 533 (1 case) in the *rpoB*, were observed from 30 isolates and a 6-bp insertion was shown between 514 and 515 in one isolate. No mutation was detected in rifmapin resistance determining region of 65 wild type isolates. Meanwhile, mutations at 53 (8 cases) and 55 (10 cases) in the *folP* were detected in dapson resistant isolates and 12 susceptible isolates showed no mutations in the gene. Three isolates resistant to ofloxacin harbored mutation at 91 in the *gyrA*.

The prevalence of drug resistance deduced by the mutation detection among re-

lapsed, intractable and new cases will be discussed. A method for rapid detection of resistant isolates and the significance of relapse by the persistence of susceptible bacilli will be also considered.

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Loop-Mediated Isothermal Amplification of the *dna*A sequence for rapid detection of *Mycobacterium leprae*

For the establishment of differential diagnosis of mycobacterial species, a part of the nucleotide sequences of the mycobacterial DnaA protein gene was determined by PCR based sequencing. Clinically relevant 27 mycobacterial species and 46 clinical isolates of *Mycobacterium avium, M. intracellulare* and *M. kansasii* were analyzed. Although *dnaA* partial sequences of *M. tuberculosis* complex were identical to each other, all of the nontuberculous mycobacterial (NTM) laboratory strains and clinical isolates tested, were easily identified as the respective species. The partial *dnaA* sequence similarity between *M. avium* and *M. intracellulare* was 78.3%, and that of *M. kansasii* and non-pathogenic *M. gastri* was 83.6%. Rapidly growing groups of mycobacteria were clearly separated from other species in unrooted phylogenetic tree. Based on the amplified DNA sequences, species specific-primers were successfully designed for the target mycobacterium species, *M. avium, M. intracellulare, M. kansasii,* and *M. gastri*. These results demonstrate that the variable sequence in DnaA coding gene were species-specific and were potent for the development of accurate and rapid diagnosis of *Mycobacterium* species. To develop rapid and simple identification method, we used loop-mediated isothermal amplification (LAMP) for detection of *Mycobacterium leprae*, *M. kansasii* and *M. gastri*. LAMP method is a novel nucleic acid amplification method in which reagent reacts under isothermal conditions with high specificity, efficiency and rapidity. The whole procedure was quite simple, starting with mixing of reagents in a singe tube, followed by an isothermal reaction during the reaction mixture is held at 63°C. The resulting amplicons are load to agarose gel electrophoresis. The only equipment needed for the amplification reaction is a heat block that furnishes a constant temperature. Species-specific primers for *M. leprae* were designed by targeting the *dna*A gene and specificity were validated with 27 mycobacteria species and 8 clinical isolates of *M. leprae*. The assay had a detection limit 5pg of purified DNA with 60 min. incubation time. The sensitivity and reaction time for LAMP methods to detect of *M. kansasii* and *M. gastri* purified DNA were 10 pg, 30 min. and 1 ng, 60 min., respectively. The results demonstrate the variable sequence in *dna*A gene was species-specific. Application on LAMP method was potent for the rapid diagnosis method of mycobactrerial species and especially useful in development country.

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Polymorphism on the 5′ Flanking Region of *IL12R2* Affects Establishment of Clinical Type of Leprosy.

The intensity of cell-mediated immune (CMI) responses in mycobacterial infection, determines individual differences in susceptibility to the diseases. These differences might be clarified from the viewpoint of T cell responsiveness against IL-12 in patients with leprosy since the disease shows the wide clinical spectrum due to the effect of their inherited factors. Leprosy, a chronic disease caused by the infection of *Mycobacterium leprae* (*M. leprae*), shows a wide spectrum of clinical features. Tuberculoid type of leprosy (T-lep) patients show high level of CMI responses against *M. leprae*, which results in the resistance to infection, whereas lepromatous type of leprosy (L-lep) patients show poor CMI responses (instead, rich antibody responses) against the pathogen which results

in the progressive form of the disease. Recently, it was reported that the IL-12Rβ2 was more highly expressed in tuberculoid lesions compared with lepromatous lesions, whereas IL-12Rβ1 expression was similar in both lesions. Then, we analyzed the polymorphisms on the 5′ flanking region of *IL12RB2* to determine possible immunogenetical factors that affect CMI responses, by employing leprosy as model.

The polymorphisms were examined by using direct sequencing technique to compare the allele frequencies between 129 L-lep patients and 46 T-lep patients. Several SNPs, including –1035A>G, –1023A>G, –650delG and –465A>G SNPs, were detected on the 5′ flanking region of *IL12RB2*. Frequency of haplotype 1 (–1035A, –1023A, –650G, –464A), which exhibited the highest frequency in the general Japanese population, was significantly lower in L-lep patients as compared with findings in T-lep patients and healthy controls. Reporter gene assays using Jurkat T cells revealed that all haplotypes carrying one or more SNPs exhibited lower transcriptional activity as compared with haplotype 1. Moreover, it was also elucidated that activated T cells derived from the donors carrying haplotype 1 showed higher expression of IL-12Rβ2 mRNA in the presence of IL-12 by employing real-time PCR method.

These results suggest that SNPs on the 5′ flanking region of *IL12RB2* affect the expression level of IL-12Rβ2 molecules, which may be implicated in individual differences in CMI responsiveness against mycobacterial antigens, thereby leading to the lepromatous and tuberculoid types of leprosy.

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Genotypic variation of *M. leprae* within a high endemic community.

Mycobacterium leprae is an obligate intracellular pathogen that is widely distributed around the globe. There are no recognized patho-vars or sub-types and the bacillus exhibits little genetic diversity. The only documented highly variable sequences are associated with variable number tandem repeat (VNTR) sequences distributed throughout the genome. We recently showed that VNTR polymorphisms could be used effectively to discriminate geographically diverse *M. leprae* strains used in the laboratory. Similar polymorphisms have been used with other bacteria to suggest phylogenetic relationships among worldwide isolates and to examine the dissemination dynamics of disease agents in populations. However, the sensitivity, specificity and predicative value of VNTR polymorphisms for describing these relationships among *M. leprae* are not yet known. To better understand these relationships we examined the utility for VNTR genotyping to discriminate *M. leprae* strain types in high endemic communities.

Using a battery of 7 VNTR loci we examined the genetic diversity of *M. leprae* strains recovered from 58 unrelated leprosy cases presenting in Karigiri, India over a ten year period. The alleles for microsatellites (GAA (21), GTA (9), AT (17), and TA (18)) were determined by direct sequencing off of PCR products using forward and reverse primers. The alleles for minisatellites (12-5, 21-3, and 27-5) were determined by electrophoresis of fluorescently labeled PCR products in agarose gels. The various VNTR exhibited diversity in alleles ranging from 0.3 to 0.9 in patient samples and successfully discriminated a total of 58 different VNTR genotypes among the 58 Karigiri cases tested. No epidemiologically significant relationships were discerned. To better understand the likelihood of chance influencing the remarkable discrimination seen hereobserved, we used the same battery of VNTR to examine *M. leprae* from multiple tissue samples recovered from naturally infected wild nine-banded armadillos.

M. leprae is intensely transmitted within armadillo communities in Louisiana. Inci-

dence density rates exceeding 3.5 cases/1000 animal-days have been measured and the disease may approximate an outbreak situation for *M. leprae* among armadillos. We recovered a total of 8 naturally infected wild armadillos from 2 Louisiana research sites during a 3 month period in 2004. *M. leprae* were harvested and the genotype of the bacilli determined from each of 8 different tissue samples for each animal. VNTR alleles showed markedly less diversity among armadillos than among the Karigiri patient samples. GAA remained the most highly diverse locus with other loci exhibiting greater stability. Analysis of *M. leprae* from the different tissues of each animal showed nearly identical VNTR genotypes and consistency in genotypes discerned for animals from each location.

VNTR genotyping can have high discriminatory power for differentiating *M. leprae* with high specificity. Additional studies addressing the appropriate mixture of alleles to be used and their combined sensitivity, specificity and predicative value are warranted.

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