Title of the project. A study on endemicity of leprosy and utilization of health services in selected areas of Uttar Pradesh, Chhattisgarh and Tamil Nadu.

Objectives:

Assessment of disease burden in selected districts of these states to

1. To study the disease by standard method.
2. To identify sources of transmission by classical epidemiological and molecular epidemiological methods
3. To promote utilization of health services by leprosy patients and participation of the community

Summary of the proposed research

This study is proposed to be carried out in selected districts of UP Tamil Nadu and Chhattisgarh to estimate actual burden of leprosy, its clinical profile, to understand the dynamics of transmission and to promote utilization of health services. About six districts of {2 each from Uttar Pradesh, Tamil Nadu and Chhattisgarh} are to be identified using the available NLEP data . Annual new case detection rates {ANCDR} are available on the website as well as with the Leprosy Division, Department of Health, Government of India . One district of high endemicity {ANCDR of > 10/100,000 }, and 1 districts of low endemicity {ANCDR of < 10/100,000 } will be included from each of the states in the study using Neyman's Optimum Allocation. Rural and urban cluster sampling of the areas will be done by using standardized population proportional statistical procedures. ASHA workers and other health personnel of the identified area will be trained for preliminary identification of suspected cases of leprosy using audio-visual tools and disease charts. The suspected cases will be examined and diagnosis of leprosy will be made by a medical doctor. Patient identified will be treated with the respective treatment. The total disease burden will include the newly diagnosed patient as per the above methodology as well as by observing and recording the data entered in the health registers of local PHC's and CHC's. reported for the period

The profile of the disease will be studies by making the lesions and preparation of patient chart. Besides the clinical classifications, special emphasis will be done to find the deformity / disability rates in untreated cases and also after release from treatment. The final diagnosis of the leprosy cases are to be made by the experienced doctor, classification of the disease will be based on the number of skin patches and the nerve involvement (PB/MB) as well as by clinical diagnosis based on the IAL classification. This data is expected to be useful in assessing the services to be provided for such/similarly placed population skin smears from at least two sites will be collected (ear lobule and site of patch) from cases occurring in the same family and neighborhood, for the transmission dynamics part of the study, after obtaining their informed written consent.. Slit skin smears will be stained by Zihl Nelson method and examined and recorded. In addition the smear blade to be put in TE buffer/RNA ladder prefilled tubes and transported to NJIL & OMD for RLEP- PCR detection, genotyping by satellite markers and recoded.

The investigation on sources will focus on the nature of the contact, presentation of primary/secondary cases. DNA fingerprints of M.leprae identified and detected in leprosy patients and their contacts by established methods will be undertaken for this purpose. The presence of viable M.leprae,in the environment (soil and water), will be studied by RT-PCR/Real time PCR. These tests will be done by the methods already established in the institute and found to be useful in our epidemiological studies carried out at Ghatampur. DNA fingerprinting of M.leprae from the patients and environment will be carried out to establish links between sources and the transmission chain. Genetic polymorphism in cases and their contacts in defined groups will also be carried out if epidemiological linkages become apparent. Long term follow-up of the population in selected areas will be carried out to link the findings with outcome in a prospective manner. Our earlier studies on nasal smear in contacts and patients at MRHRU, Ghatampur did not correlate with the occurrence of clinical disease as these were positive in contacts who did not have clinical evidence of disease. However, incorporating the suggestions of the reviewers these will be done in a proportion of patients and their contacts using established protocols.

Utilization of health services by leprosy patients, establishment of referral chains, assessing the usefulness of providing expert counselors, identification of the knowledge gaps and assessing the impact of intervention will be noted. This will be assessed by comparing the proportion of new cases detected and treated before and after the IEC activity undertaken as a part of the study. The information is likely to become available from the study is expected to be relevant to strengthen our national programme.

Present knowledge and relevant bibliography including full titles of articles relating to the project.

This is a very important study from the point of view of understanding the reasons for presenting endemicity of leprosy in some pockets, despite major success at the national level. We have experiences and basic infrastructure for investigating the clinical aspects, epidemiology, and molecular epidemiology and have developed sensitive and discriminatory methods for genotyping while investigating the problem earlier. This will be done in 2 districts of UP, 2 in Chhattisgarh as well as 2 in Tamil Nadu which have reported such pockets of varying endemicity (one each from districts with ANCDR less than 10 per one lakh population and one in district with. More than 10 per one lakh population, from each state). The districts will be selected by Neyman Optimum Allocation. In the district both the rural as well as the urban population will be covered which are detailed in the detailed work plan. The study is planned to include diverse climatic, ecology, cultural and geographical areas in the country and will provide wealth of information relevant to the programme. Establishing the same facilities for molecular epidemiology in these two areas will also help in net working and resource development in these areas. Although these methods are and have been used for leprosy they can be adapted for molecular epidemiology of other mycobacterium and bacterial diseases and can be used to assess the problems. The main focus will also be on utilization the activities of National Rural Health Mission (NRHM) so as to improve the public health activities, to identify, subsequent treatment and ultimately in prevention of the diseases. The list of recent publications on these aspects in leprosy is given below.

In order to understand the high occurrence of leprosy in some areas, it is necessary to identify the natural reservoir(s) of M.leprae, the route of infection and the mode of transmission. The exact mode of transmission is not fully known. During the last 20 years, rapid molecular assays have been developed for detection of M.leprae directly from patient specimens. These assays have been primarily based on the amplification of M.leprae specific sequences using PCR and identification of M.leprae DNA fragment. In addition different genotyping methods have been/ are being developed. These molecular biological techniques can help in devising suitable methods for understanding the epidemiology of leprosy and identifying sources as well as causes of persisting foci of disease (Katoch et al 2007).

With the publication in 2001 of the complete genome sequence of an isolate from Tamil Nadu, India, called the TN strain (Code et al 2001), selection of potential polymorphic genomic markers for strain typing became feasible. The first genetic markers that showed polymorphism were short tandem repeats (STRs)in the M.leprae genome. One was a 6-bp intragenic sequence in the ropT gene, and the second, a trinucleotide (TTC) repeat element upstream of a pseudogene (Matsuoka et al 2000, Shin et al 2000). These sequences exhibit variable numbers of tandem repeats (VNTRs) when sequenced in different isolates. Based on these observations, we short-listed 44 loci (including the rpoT and TTC loci by in silico analyses of the M.leprae genome and accomplished the screening of 11 STR loci, of which 9 were polymorphic when tested in a small panel of four human isolates derived from passage through armadillos (Groathouse et al 2004). Five were minisatellites (6- to 50-bp repeat units), and four were microsatellites (1-to 5-bp repeat units). Since then, others have also shown that VNTR loci exist in M.leprae isolates (Truman et al 2004, Young et al 2004, Zhang et al 2005). Three single-nucleotide polymorphisms have also been discovered by comparing sequences of a limited number of strains (monot et al 2005). These techniques have been used in patients from India and appear to be promising (Lavania et al 2005, 2007, 2008 and unpublished data). However, information about the genetic diversity among M.leprae from different parts of India is still small. Using these targates, new molecular system/schemes have been developed. These methods need to be investigated for identifying the genotypes and mode of transmission in these pockets of endemicity existing in parts of India.

Preliminary work already done by the Investigator on this problem, e.g. selection of subjects, standardization of methods, with results,if any. The preliminary work has already been accomplished and partially published as indicated in the list of publications above. A house to house re-survey was conducted in the population of Ghatampur tehsil of Kanpur Nagar district. The period prevalence of leprosy in the area was observed to be (178/100,000 of the examined population). About half of these cases were really new incident cases during the reporting period and the rest were the left out cases of the previous survey who had not taken treatment. The deformities were visible in 11/614 leprosy cases and in all of them they had occurred prior to MDT. None of the patients developed deformities during or after stoppage of therapy. Strain variation in M.leprae were identified by analyzing the number of TTC repeats as well as rpoT gene. The strains from Ghatampur were predominantly of 3 repeats as that of the Asian lineage. Other

microsatellite markers having potential molecular epidemiology have also been identified (Lavania et al under publication).
Protocol for transportation of biopsy samples and isolation of RNA from the samples enabled us to demonstrate viable bacilli in the tissues using micro array which has also been applied for patent. Using sequencing of TTC repeat from biopsy sample we could demonstrate strain differences of M.leprae. these differences could be demonstrated in the inhabitants of the same village and at times within the contact of the same family.
DNA as well as RNA of M.leprae could also be isolated from the environment (soil and water) of this area. This could possibly be a continued source of transmission in the area.

Links with other ICMR projects (ad-hoc, task force or collaborative).
With collaboration with the Dept of Transplant Immunology and Immunogenetics,All India Institute of Medical Sciences, molecular analysis of cytokine gene polymorphism in leprosy was undertaken and differences identified. It is proposed to undertake further studies using these markers if epidemiological associations become apparent.
We are co-investigators in ICMR Task Force Project on "Post elimination Leprosy Situation in India (PELSI) and are covering parts of Uttar Pradesh.
The good rapport and confidence of the local inhabitants has lead to the funding of several other projects by DBT, WHO, and Central TB division of Government of India and a establishment of a Model Rural Health Research Unit at Ghatampur with a satellite centre at Banda
List of important publications of last 5 years of the all the investigators in the relevant fields (enclose reprints, if available)

Reference 1-13 listed aboves, equipments and

Detailed research plan. (give here the design of study, indicating the total number of cases/samples/animals to be studied, the mode of selection of subjects specially in experiments involving human beings, equipments and other materials to be used, methodology/techniques to be employed for evaluating the results including statistical methods any potential to obtain patents etc.)

Assessment of disease burden in selected districts of these states

India has successfully achieved the elimination target at the national level, however, pockets of endemicity do remain and this needs to be investigated. Several districts in Uttar Pradesh and Chhattisgarh report annual new case incidence of leprosy of more than 1 per 10,000, while other districts in the same state report a much lesser incidence. The living conditions, cultural habits, personal hygiene may be playing a role but other factors like reservoirs of infection, transmission dynamics etc need to be investigated. For computation of sample size it is assumed that the new cases that may be identified (P) are likely to be 0.07/1000, with a permissible margin of error (D) of 0.001 and desired level of confidence of 95%. The sample size is calculated using the formula n=27804. It is proposed to use multistage stratified cluster survey design with design effect of 3. With this the sample size works out as n=83412 and this is rounded to 100000 which is on the higher side.

Sampling Design

Multistage stratified cluster sampling procedure will be adapted to select a sample of 100,000 individuals from 50 clusters with average number of persons per cluster as 2000. All districts of UP, Chhattisgarh and Tamil Nadu will be stratified in two strata according to ANCDR reported to NLEP as under :

Stratum – 1: Districts with high prevalence (ANCDR>10/100000)

Stratum- 2 : Districts with low prevalence (ANCDR<10/100000)

Two districts will be selected using Neyman proportional allocation random sampling method one each from group of districts stratified. As indicated . from each selected district, under assumption that proportion of population is 20% in urban area and 80% is in rural area. 50 clusters will be allocated to urban and rural areas as 10 & 40 respectively. The selection from urban and rural areas will be as under:

In urban areas- Municipal ward will be treated as urban clusters and 10 wards will be selected with population proportional sampling from the urban area of the district.

In rural areas- Village will be treated as a cluster and 40 villages will be selected in the following manner.

The sample is proposed to be selected using multistage design.

In stage 1: two tehsil will be selected using simple random sampling from all tehsils of the selected district.

In stage 2 : two blocks will be selected withsimple random sampling from the selected tehsils. In stage 3 : ten villages will be selected with PPS from each selected block. Importantly, this method will take care of the varied distribution of leprosy cases in the community and the spread of the sample will be from 4 blocks which is for operational convenience

Assessment of disease burden and disease profile; This will be done in the selected districts of these states (UP, TN and Chhattisgarh) as detailed above. This part of study will be carried out after proper training of ASHA workers and other health workers by NJIL & OMD and model Rural Health Research Unit staff using audio-visual projection and patient cards available for the purpose. Besides the routine demographic details of age, sex, number of members in the family etc, these trained and empowered ASHA workers will initially identify suspected cases and these will be confirmed by an experienced medical officer. Profile of disease will be studied by classical clinical methods including classification by the number of patches and nerve involvement (PB/MB) as well as clinical IAL classification (Ind, TT,BT,BB, BL,LL and pure neuritis cases). All these cases will be treated and remuneration to ASHA workers will be made as per NRHM guidelines.

Transmission dynamics:

This aspect will be studies by genotyping of M.leprae from patients, detection of M.leprae (live by molecular methods targeting RNA) and linking to case clusters by classical epidemiological approach etc.

Patients-

For Studying the genomic diversity, about 200 slit smear scrapings from different types of leprosy cases will be taken from each district of UP, Tamil Nadu and Chhattisgarh (150 from endemic district ANCDR >10/10000 and 50 from low endemic district ANCD of <10/10000). Genotypes will be investigated in relation to family and other village contacts.

While the genotypes likely to be discerned by various techniques cannot be predicted, arbitrary number of about 50 from each area will be critically analyzed and polymorphism will be observed. This number is arbitrary in the beginning, as polymorphism in M.leprae is not known in the group being proposed for analysis.
Information about village panchayats, other cases of leprosy in the household and vicinity, age of cases, treatment status, relapses will be collected and recorded. In addition in a proportion of these cases (50%), nasal smears will be collected and analysed using the standardized protocol available and tested for detection of M.leprae DNA using PCR based standard method.
Collection of specimens- Specimens from all types of leprosy patients partially treated or untreated patients defined in the patient section will be taken after informed cosent specimens will be collected in TE buffer and RNA ladder.

Preparation of genomic DNA of M.leprae from specimens – M.leprae DNA from specimens will be isolated using physiochemical procedures of freeze thawing, treatment with lysozyme-proteinase K, de-proteinization as described previously and fractionation of nucleic acids into DNA/RNA will be done, as being done by procedure already in use at the laboratory.
PCR detection of M.leprae DNA : Along with smear staining for AFB and detection andrecordin by ZN method, detection of M.leprae DNA will be done by targeting RLEP sequences.
PCR amplification and analysis of the targets genes (VNTRs and SNPs)- PCR amplification of fragments encoding for the VNTRs will be carried out with the help of earlier published primers (Groathhouse et al 2004, Zhang et al) and also primers designed at NJILOMD (Lavania et al, submitted for publication). New primers will be designed, if needed, for the amplification of any other relevant genes. Amplification and detection of STRs will be done using multiplex PCR. Multiplex PCR will be performed using the multiplex-PCR kit (Qiagen/ equivalent). Sequencing will be performed using a Big Dye Terminator Cycle Sequencing FS Ready Reaction kit (Perkin Elmer).
Fragment Length Analysis: After the multiplex PCR, 1ul of the PCR product will be diluted 30- to 60-fold, and 1ul of the diluted PCR product will be combined with 12ul deionized formamide (Applied Biosystems) and 0.3 ul of the LIZ-500 DNA standard (Applied Biosystems). The sample will be denatured at 94 C for min and subjected to capillary electrophoresis on the Applied Biosystems genetic analyzer 3130 at the Molecular Biology Laboratory at JALMA. The electropherograms will be visualized and analyzed using GeneMapper version 3.7 software (Applied Biosystems) to determine the major allele for each VNTR locus in each multiplex-PCR combination.
Single Nucleotide Polymorphism Genotype analysis: Variation in M.leprae genomic DNA will be done by specially focusing on SNPs (Cole et al 2001; Monot et al 2005) and will be determined by direct PCR/ DNA sequencing/Real Time PCR.
Analysis-