

# DISTINGUISHING GENOTYPIC VARIANTS OF *Mycobacterium leprae*

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"Being dependant entirely upon an in vivo supply of *M. leprae*... it was impossible to establish a "seeded" IMMLEP strain... To exclude the possibility of variants arising in strains serially passaged in armadillos, IMMLEP specified that all *M. leprae*-injected armadillos must be inoculated with strains obtained directly from patients, or... first-passaged, armadillo derived bacteria."

R.J.W. Rees; *Int. J. Leprosy*, 1983.

## Abstract:

Genotyping has practical application in outbreak investigations and variant classification of microorganisms. Until the sequence of the genome was completed, however, remarkably little variability had been noted among *M. leprae*. Recently, a few loci for allelic diversity have been identified, including small insertion sequences and tandem repeating elements. At least one of these, the TTC triplet occurring in a non-coding region of the putative sugar transporter pseudogene of *M. leprae*, also has been found to occur at variable copy numbers in different clinical isolates. To better understand the suitability of this and other VNTR markers in differentiating variant strains of *M. leprae*, we examined a battery of 12 *M. leprae* isolates derived from leprosy patients in different regions of the United States, Brazil, Mexico, and the Philippines, as well as from wild nine-banded armadillos and the Sooty Mangaby Monkey. The stability of the TTC VNTR was compared among the individual isolates as well as to those from bacilli obtained on subsequent passage in nude mice and armadillos. Copy numbers for the TTC repeat ranged from 10-15 among the isolates tested. No regional clustering was noted and all of the U.S. isolates showed a wide number of different repeats. Strains derived from different wild animals also were not identical. Greatest variability was seen over long term passage with the Thai-53 strain which has been maintained continuously in nude mice for many years. Thai-53 TTC copy number varied markedly over 8 passage intervals. However, the TTC VNTR genotype of most individual strains remained relatively constant for isolates passaged outside man for fewer than 12 generations. In addition, the TTC VNTR genotype of these strains tended to remain constant when passaged through an alternate animal host, experimentally infected nine-banded armadillos. Future studies with these and other allelic markers may evolve suitable variant typing schemes for *M. leprae* that will aid in assessing specimens harboring mixed strains, determining transmission patterns among endemic populations and provide a means to standardize reference strains of this organism.

Table

Diversity in TTC VNTR copy number between different isolates of *M. leprae* from animals and patients in different geographic regions.

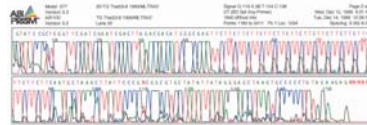
ID	Source	Region	Isolated	VNTR #
SMML	Monkey	Louisiana	1992	15
W260	Armadillo	Louisiana	1980	11
W415	Armadillo	Louisiana	1984	10
W508	Armadillo	Louisiana	1986	10
LWM	Human	Philippine	1998	15
1192	Human	Louisiana	1992	15
4089	Human	Texas	1996	10
4267	Human	Texas	2000	12
4281	Human	Louisiana	1996	11
4923	Human	Brazil	1998	12
T-53	Human	Thailand	?	12,13,14

## Introduction:

*M. leprae* is not cultivable on artificial media and most laboratories lack the capacity to grow the bacillus in animal models. Investigators usually obtain aliquots of *M. leprae* from only a few central support laboratories that prepare leprosy bacilli and distribute them for research purposes. To limit variation of bacilli from support laboratories, propagation programs have severely limited selection of laboratory strains, and today there are no established 'type' or 'reference' strains of *M. leprae*, or known variant cultivars. Recently the genome of *M. leprae* was fully sequenced (1). Uniform reference strains and reliable methods to assess variation and drift among different isolates will be essential to realize the full benefits of modern molecular technology in work with *M. leprae*.

Genomic variation in bacterial isolates can be useful in describing the evolution of bacterial strains and the clinical source of infection. However, *Mycobacterium leprae* appears to have a highly conserved genome. No significant variations have been detected among different *M. leprae* isolates from humans and animals of different geographic regions using routine Restriction Fragment Length Polymorphism analysis (RFLP), or in examining variations in the sequence of 16S RNA. While other species of Mycobacteria are easily typed according to variation in insertion sequences, among *M. leprae* insertion sequences also are conserved. The only reported variation with insertion sequences (78 bp) among *M. leprae* differentiates just a single isolate found on one island in Japan from all other *M. leprae* isolates all over the world (2).

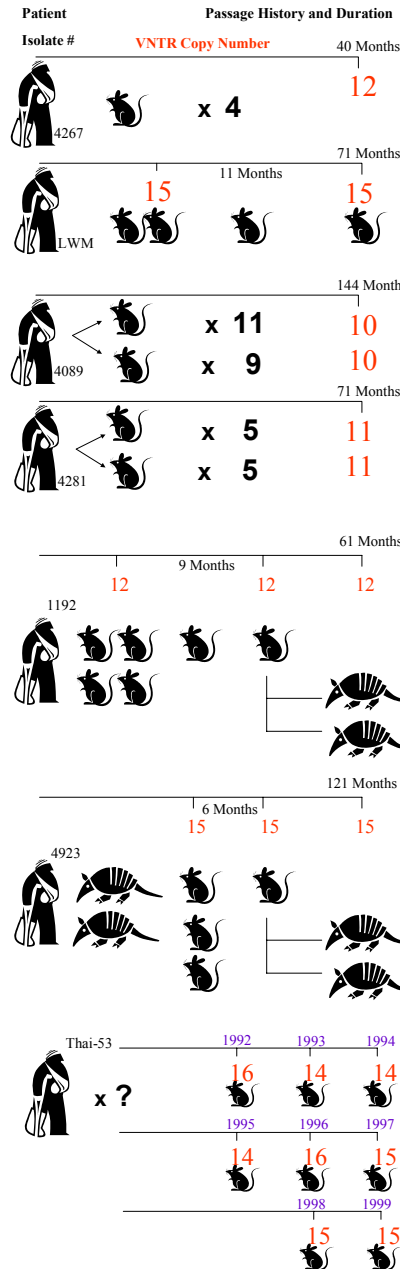
Various repeating elements recently have been shown to be useful in genotyping other highly conserved bacterial species like *Bacillus anthracis*. The *M. leprae* genome contains a few repeating elements and limited variation between different isolates has already been reported for some of them. Variations in copy number (3-4) are noted for the GACATC repeat in the *rpm7* gene (2). Recently a higher degree of diversity (10-37) has been reported in the TTC repeat within the putative sugar transporter pseudogene (3). Along with a few other repeating elements of unproven diversity, the significance of these markers and their utility for epidemiological study has not yet been described. We examined the diversity of TTC repeats within a battery of clinical isolates obtained from human patients and wild animals in different geographic regions and assessed the stability of the TTC repeat as a potential marker for drift with passage of these isolates in laboratory animals.



## Materials and Methods:

- M. leprae* was obtained fresh or frozen from nude mouse or armadillo tissues which were homogenized in buffer and washed extensively to purify bacilli.
- To obtain DNA, purified bacilli were subjected to Freeze/Thaw (-180C/95C) and treatment with protease K (60C/18hrs). Extraction of DNA was achieved with phenol/chloroform/isoamyl alcohol.
- In the known genome sequence, PCR of the TTC VNTR results in a predicted 201 bp Amplicon using 2 primers flanking TTC repeat in
  - Forward 5'-GGACCTAAACCATCCCCTTT-3'
  - Reverse 5'-CTACAGGGGGCACTTAGCTC-3'
- The actual VNTR copy number of each isolate was determined by sequencing the amplicon and counting the repetitive units as shown above

Figure: Stability of the TTC VNTR



## Results:

*M. leprae* presents several unique challenges for genotyping. Few strains have been well characterized and most are relatively recent clinical isolates. Patient samples of *M. leprae* are usually of poor quality. They typically show low percent viability, are frequently contaminated with other cultivable agents and often come from individuals with an unknown treatment history. In addition, because *M. leprae* must be propagated in animals, isolation involves interspecies transfer and selection by a different host. The resultant infection is quite slow, requiring months to years to manifest, and does not arise from a single colony or clone of bacteria. There is relatively little known about the adaptation of *M. leprae* to long term passage.

In examining the variation in copy number of the TTC repeating element within the sugar transporter pseudogene of *M. leprae*, we found diversity among different isolates taken from animals and human patients from different geographic regions (Table). Even isolates obtained from different animals and animal species varied. Copy numbers ranged from 10-15 among the isolates tested and no regional clustering of copy number type was noted. Among the tissues examined only a single VNTR copy number was detected for each isolate. Copy numbers did not vary between different tissues harvested and multiple copy numbers for a single isolate were not detected even among infections that lasted multiple years.

To determine the stability of the TTC VNTR we examined the samples taken from isolates in serial passage (Figure). Strains were isolated and passaged in nude mice and/or armadillos. Interspecies transfer did not influence the TTC copy number over the short term passage. Parallel samples of given isolates carried in long term passage also showed no divergence in copy number. Variability was seen in the TTC copy number among the Thai-53 passages. This strain was isolated more than 20 years ago and has been carried in continuous passage in nude mice since that time. It is likely the oldest known *M. leprae* isolate. Thai-53 samples examined from over an 8 year period showed marked variation in TTC copy number ranging from 14 to 16. In each passage only a single copy number was detected and it is possible that TTC copy number may become less stable as a result of continuous passage.

## Conclusions:

- We found marked diversity in TTC VNTR copy number among the isolates tested
  - Copy numbers varied both between different isolates and between isolates from different geographic regions
  - There was diversity in copy number between isolates obtained from human patients and in those obtained from animals
    - Diverse between different animal species
    - Diverse among isolates from different animals of the same species
- Most isolates show good short term stability in TTC VNTR
- No problems inherent to *M. leprae*'s unique culture requirements and challenges
  - Interspecies transfer (Multiple)
  - Non-clonal inocula and samples
  - Slow Chronic Infection
    - Only a single VNTR genotype in each passage
- In combination with other alleles such as repeating elements may be useful to mark strain drift and in epidemiological investigations on individual patients

## Literature Cited

- Cole ST, Eigmeier K, Parkhill J, et al: Massive gene decay in the leprosy bacillus. *Nature* 2001;409:1007-11.
- Cole ST, Eigmeier K, Parkhill J, et al: Massive gene decay in the leprosy bacillus. *Nature*. 2001;409:1007-1011.
- Shin YC, Lee H, Lee H, Walsh GP, Kim JD, Cho SN: Variable numbers of TTC repeats in *Mycobacterium leprae* DNA from leprosy patients and use in strain differentiation. *J. Clin. Microbiol.* 2000;38:4535-4538.