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Assessment of the Viability of Mycobacterium Leprae by 16S rRNA Using Different Methods for Rapid Extraction of rRNA

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Abstract – Leprosy, a chronic disease caused by Mycobacterium leprae is a general wellbeing worry in specific nations, including India. Despite the fact that the pervasiveness of the disease has fallen definitely after some time, new cases keep on happening at almost a similar rate in numerous locales. A few endemic pockets have been seen in India and somewhere else. The exact elements of infection transmission are as yet not unmistakably comprehended. Both live bacilli just as M. leprae DNA have been recognized in the dirt and water of endemic zones; they potentially assume a significant job in illness transmission. Nucleotide arrangement information for bacterial 16s ribosomal RNA was utilized to distinguish oligodeoxyribonucleotide groundworks appropriate for testing the rRNA quality of mycobacteria and related life forms, with the polymerase chain response. The strategy empowered us to separate mycobacteria from other firmly related genera, and to separate among moderate and fast growing mycobacteria. Mycobacterium leprae fell inside the moderate developing gathering of mycobacteria yet there are critical contrasts between the succession of the M. leprae 16s rRNA quality and that of other moderate developing mycobacteria. These distinctions were utilized to devise a quick, non-radioactive strategy for recognizing M. leprae in contaminated tissue. This paper is designed to evaluate the viability of Mycobacterium leprae by different methods for diagnosis.

Keywords: Mycobacterium Leprae, 16s rRNA, Viability, Lab Diagnosis, RNA

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INTRODUCTION

It is a chronic systemic infection disease, caused by the rod-shaped bacterium *Mycobacterium leprae,* first described by G. Armauer Hansen (so that this disease is also known as Hansen's disease) a Norwegian physician, in 1874. The word leprosy derives from the ancient Greek words "lepros", scale and "lepein", which mean "Defilement". Historically the term Tazaarth" from the Hebrew Bible is commonly translated as leprosy (Hansen 1874).

Leprosy is a fully treated disease with worldwide use of WHO recommended multi drug treatment (MDT) there is major decline in prevalence of disease. Hence the mode of transmission is not fully understood. It is thought to be transmitted by nasal discharges and skin sores, possibly also by contaminated objects, by direct skin it skin contact, mother to child through placenta, biting insects and arthropods (Euzeby JP;2008). Leprosy or infectious disease, is a chronic, mildly infectious malady capable of producing, when untreated, various deformities and disfigurements. Leprosy is

basically a unwellness of peripheral nerves however it's additionally affects the skin and generally sure alternative tissues notably, the eyes, the mucosa of the upper respiratory tract muscle, bone and testis. (Bratschi, M. W:2015, Katoch V.M.; 2007).

The first known leprosy hospitals were established by Christians in Rome and Caesarea in 4th century. Leprosy affected humanity at least 600 B.C and was well recognized in the civilization of ancient China, Egypt, and India also located in tropical and subtropical zone (Katoch V.M.; 2007).

M.leprae has never been grown on any acceptable media but can be maintained in axenic cultures. As a result propagation of M.leprae has been restricted in animal models, including the armadillo and normal, athymic and gene knockout mouse. Growth of M.leprae on mouse footpads also provides a tool for accessing viability of preparation of bacteria and testing the drug susceptibility of clinical isolate. M.leprae stored at 33oC in 7H12 media has been shown to remain viable for weeks. Other than human the bacteria affects armadillos, mangable monkeys, Rabbies, mice, Chimanzees and cynomologous Macaques (Araujo, S;2016).

The disease is portrayed by unusual changes of the skin. These changes, called injuries, are at first level and red. After developing, they have sporadic shapes and a trademark appearance. The sores are regularly darker in shading around the edges with stained pale focuses. Since the life form develops best at lower temperatures the Mycobacterium leprae highlights an inclination for the skin, the mucous films and the nerves. Contamination in and devastation of the nerves results in tactile misfortune. The loss of sensation inside the fingers and toes will expand the opportunity of damage. Insufficient consideration causes contamination of open wounds. Gangrene can likewise pursue, perpetrating body tissue to pass on and become deformed.

HISTORY OF LEPROSY

In **1886** Lehman and Neuman used the term Mycobacterium Hansen discovered lepra bacillus and **Robert Koch (1882)** isolated mammalian tubercle bacillus. Johne **(1895**) described *M. paratuberculosis* (**Johne's bacillus**). Mycobacterium is a bacterial genus containing 130 different species **(Euzeby 2008**) and includes *M.tuberculosis* and *M.leprae* the causative agents of tuberculosis and leprosy respectively. The genus includes obligate parasites genes as well as saprophytes, which are atypical nontuberculous mycobacteria (NTM).

Atypical mycobacteria cause pulmonary and generalized infections in immuno compromised individuals and other conditions like lymphadenitis in children **(Good 1985**). In immunocompromised individuals the infections due to non tuberculous mycobacteria (NTM) have been observed to major cause of mortality and morbidity in western countries **(Wallace et al 1990).**

Important species of NTM associated with disease in human are *M.scrofulaceum, M.malmoense, M. kansasii, Mxenopi, M.ulcerans, M.avium, M. intra cellulare* complex (MAIG) and *M.simiae.* One important category person with NTM diseases is patient with AIDS and disseminated disease *M.avium* is now recognized as one of the most common cause of opportunistic infection in patient with AIDS in the United States of opportunistic infection inpatient with AIDS in the United States (**Wallace et al 1990).**

Structure of *Mycobacterium Leprae*

M.leprae is a straight rod about 1-8 um long and 0.3 um in diameter. In infected tissue the rods are often stacked or clumped together in globi. Electron microscopy shows an ultra-structure common to all mycobacteria:

Capsule-Around the organism is an electron transparent zone of foamy or vesicular material, produced by and structurally unique to *M.leprae.* It is composed of two lipids, phthioceroldimycoserosate, which is thought to play a protective role,and a phenolic glycolipid, which is composed of three methylated sugar molecules linked through phenol molecule to fa(phthiocerol). The trisaccharide renders it chemically unique and antigenically specific to *M.leprae.*

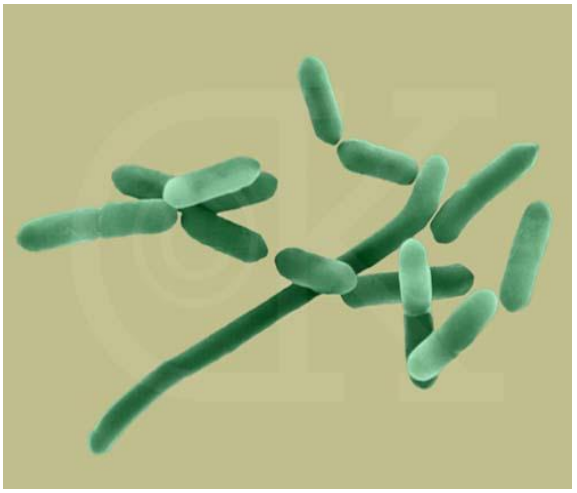

Figure- *Mycobacterium leprae*

Cell Wall- This is composed of two layers:

i) **Outer Layer:** It is electron transparent and contains lipopolysaccharide composed of branching chains of arabinogalactan esterified with long chain mycolic acids.

ii) **Inner Layer:** It is composed of peptidoglycan: carbohydrate-linked by peptides whose amino acid sequence may be specific to *M.leprae* although the peptide is too scanty to be used as a diagnostic antigen.

iii) **Plasma Membrane:** It is composed of lipid and proteins.The proteins are mostly enzymes and in theory constitute good targets for chemotherapy.They may also constitute the 'surface protein antigens' that have been extracted from the cell walls of disrupted *M.leprae* and extensively analysed.

iv) **Cytoplasm:** The inner contents of the cell contain storage granules, DNA, ribosomes. DNA analysis has been useful in confirming the identity as *M.leprae* of mycobacteria isolated from wild armadillos, and has shown that *M.leprae*, though genetically distinct, is closely related to *M.tuberculosis* and *M.scrofulaceum.*

***Mycobacterium leprae* genome**

M.leprae appears to have smaller genome than other eubacteria. *M.tuberculosis* genome is 4.4 Mb of DNA encoding approx.4000 genes. **(Cole et al,1998).** *M.leprae* genome size is 3.27 Mb.Sequencing of *M. leprae* cosmid covering the Rif-str regions revealed that approx. 50% of the potential coding sequence was used **(Honore et al, 1993).**

Unusual genetic structure and repetitive elements.

Unusual genetic elements which appears to be over-represented in *M. leprae* genome is protein splicing element, called "intein" which is initially found in the recA gene of *M.tuberculosis* **(Davis et el, 1994).** Subsequently two other protein splicing inteins have been identified in *M.leprae* genes, one is gyrA **(Fsihi et al, 1996)** and other in a gene of unknown function **(Pietrokovski, 1994).**

METHODS FOR ASSESSMENT OF VIABILITY

As *M.leprae* is not cultivable in any acceptable medium, several methods for assessment of viability have been developed **(Arnoldi et al 1992).**

Viability Methods: Sensitive techniques for determination of viable units of an organism in the lesions are needed to monitor the effect of any antimicrobial treatment. A variety of *in-vivo* and *in-vitro* techniques have been developed.

Methods for assessment of viability of *M.leprae*

Sensitive techniques for determination of viable units of an organism in the lesions are needed to monitor the effect of any anti-microbial treatment. Chemotherapy's effect is first on viability and much later on the above parameters pertaining to the total bacillary load. These methods are also relevant to detect the viable *M.leprae* in the environment. Even though there is no acceptable in vitro method for the cultivation of *M.leprae*, a variety of in vivo and in vitro techniques have been developed and used for its measuring viability.

Mouse food pad In vivo methods Animal models for assessing viability

The demonstration of limited multiplication in the normal mouse footpad was a major break-through in leprosy research. Normal mice after inoculation with 10^3 to 10^4 organisms in their foot pad do not develop the disease like humans but show limited multiplication **(Shepard 1960).** This model has been extensively used for drug susceptibility testing **(Colston et al 1978, Shepard 1967).** It has also been used to monitor viability of M. leprae; multiplication is seen with about 5 or more viable organisms **(Levy 1987).** This technique has some drawbacks. In leprosy samples from patients who have had a few months of multi-drug therapy (MDT), viable organisms are difficult to detect in the normal mouse foot pad as the few viable organisms are diluted by the very large number of dead organisms. It also takes a very long time (6 to 12 months) for the results to be available.

(b) Immunocompromised mouse/rat models

Immunodeficient animals have been used for the demonstration of multiplication of M.leprae. Neonatally Thymectomized Rats (NTR) and thymectomized irradiated mice (T900R) are normal laboratory bred animals that are made immunologically more susceptible and develop systemic infection after inoculation with M.leprae. They are much more sensitive than the normal mouse foot pad model, with inoculation of as few as 10^2 to 10^3 organisms possible **(Rees 1966, Gelber & Levy 1987, Hastings & Chehl 1991).** NTR are slightly more sensitive because a larger inoculum can be injected because of the bigger foot of the rat **(Fieldsteel & McIntosh 1971, Gelber & Levy 1987).**

These models are thus especially useful for studies on persisters. Another model widely used for leprosy research is the nude mouse. Nude mice are the main draw back with different types congenitally athymic hairless mutants with total absence of an immune system. This model is more sensitive than NTR and T900R mice for

detecting persisting viable bacilli and is capable of supporting multiplication of *M.leprae* to levels approaching 10^{10} bacilli/g in the tissue **(Levy 1987, Hastings & Chehl 1991).** It has been used to reassess the generation time of leprosy bacillus which has been calculated as one day **(Hastings & Morales 1982).** Nude mice are a good source for the supply of *M.leprae* for experimental purposes and to study the effect of therapy of immuno-compromised animals is that they are expensive, difficult to maintain and require 6 to 8 months' time for results to be obtained after inoculation.

(c) Biege mice

Biege mice, a strain of mice congenitally deficient in macrophages and natural killer cells, are useful for studying the multiplication of *M.leprae* for viability testing **(Gupta & Katoch 1995, Gangadharam & Dhople 1992).** Multiplication of *M.leprae* has been observed in several other animals including armadillos, monkeys, chimpanzees, Indian pangolin, and slender loris **(Gupta & Katoch 1995, Kirchheimer & Storrs 1971, Gormus et al 1993).** While armadillos have been used to generate large quantities of *M.leprae* for research and vaccine purposes, other models are being investigated for understanding the pathobiology of disease.

While the above models serve limited purposes of studying the viability from leprosy cases, their suitability to grow leprosy bacillus from environment is questionable as these will require 10^4 bacilli.

In Vitro viability methods

A variety of in vitro alternative techniques have been developed to estimate the viability of *M.leprae*. These include techniques based on morphology (morphological index) and biochemical processes elicited in cell free or macrophage cultures.

(a) The morphological index (MI)

It is based on the concept that solid staining bacilli are viable organisms. This has been shown to correlate with infectivity in mice and with bacillary ATP content **(Katoch et al 1989**). However, zero MI values have been recorded from untreated cases with positive mouse uptake **(Desikan 1976)** and ATP levels probably because of clumping and sampling errors. Another limitation that is common with other techniques is that besides the skin, bacilli are also lodged in other tissues like nerves, skeletal and smooth muscles, internal organs, etc. that cannot be bacteriologically examined routinely. However, the fall of MI is an important parameter for monitoring the progress of chemotherapy.

(b) Fluorescent diacetate-ethidium bromide (FDA-EB) staining

The FDA-AB method is based on the concept that viable cells possess enzymes (esterases) for splitting FDA so that they fluoresce green in UV light, whereas dead ones are unable to do so and appear orange-red by taking up the counter-stain EB **(Katoch et al 1989, Kvach et al 1984, Odinsen et al 1986,).** The proportion of green staining bacilli decreases progressively with chemotherapy. This technique could thus be used in individual cases for monitoring chemotherapy (**Katoch et al 1989, Odinsen et al 1986)** and as an index of viability to study the effect of drugs on *M.leprae* within macrophages **(Bhagaria & Mahadevan 1987).** However, the green staining bacillary population in case of *M.leprae* does not exactly correlate with ATP levels **(Katoch et al 1989).** It is possible that the enzymes retain functional activity for some more time after cellular death.

(c) Macrophage based viability assays

Infection of macrophages with live *M.leprae* produces some alteration on the surface and on the metabolism of macrophages. This fact has been used to develop various viability assays (**Nath et al 1982, Nair & Mahadevan 1984**). Infection with *M.leprae* induces changes in Fc receptors (assessed by EA rosetting and radioactive labeling) on the surface of human and mouse derived macrophages **(Nair & Mahadevan 1984).** The changes in the ratio of cholesterol to cholesterol esters within macrophages have been used for drug testing **(Nair & Mahadevan 1984).** These systems need approximately a million bacilli/test tube. Their lower limits of sensitivity are not clear.

(d) Uptake of various substrates for metabolic pathways

Using nucleic acid synthesis as an indicator of viability of *M.leprae* within differentiated macrophages, several viability testing systems have been developed **(Nath et al 1982, Harshan et al 1990).** Cultures of live *M.leprae* incorporate significant levels of ^{3}H thymidine for DNA synthesis as compared to parallel cultures of heat killed bacilli inside the macrophages. This information has been used for the evaluation of anti-leprosy drugs, the diagnosis of dapsone resistance, and for measuring the viability of *M.leprae* in cell-free systems **(Ambrose et al 1978,),** ^{3}H adenosine (**Harshan et al 1990)** and ^{3}H uracil have been used in place of ^{3}H thymidine but the latter is slightly less specific in macrophages because of its incorporation in the RNA of macrophages. While investigating the purine metabolism in *M.leprae*, it was found that incorporation of radioactive hypoxanthine was more rapid than that of

radioactive thymidine**.** Further, several steps in the catabolic pathways**,** and oxidation of carbon through the TCA cycle **(Wheeler 1986)** have been suggested as targets for developing viability methods.

A few viability assays for drug screening have been proposed based on these biochemical pathways Uptake of DOPA **(Ambrose et al 1978, Prabhakaran 1997),** acetate **(Ambrose et al 1978)** and ^{14}C palmitic acid into phenolic glycolipid of *M.leprae* have been reported to be useful for viability testing and drug screening against *M.leprae*. While most of these methods are technically sound, their usefulness has gradually decreased over the years due to decline in the number of bacilliferous leprosy cases.

(e) Radio-respirometery

Techniques based on the measurement of oxidation of ^{14}C-palmitic acid $^{14}CO_2$ by *M.leprae* using Buddemeyer type counting system or BACTEC 400 system **(Franzblau 1988,1989)** have been described for screening of potential anti-leprosy compounds.

(f) ATP estimation by bioluminescent assay

ATP is present in fairly constant amounts in each cell type and is rapidly lost after cell death. Hence measurement of the ATP content can be used for determining the viability of *M.leprae* (**Katoch 1989, Sharma et al 1992, Katoch et al 1991, 2004,).** Around 100 colony forming units of cultivable mycobacteria can be detected with this technique, which is sensitive enough to detect the small number of viable bacilli even after 2 years of MDT. ATP assays are useful for monitoring the effect of chemotherapy/immunotherapy and also for confirming relapses **(Katoch 1989, Katoch et al 1991, 2004,).** Bacillary ATP decay profiles of *M.leprae* when incubated with different drugs are useful for drug screening **(Katoch et al 1989).** For the purpose of drug screening there is limited application potential due to requirement for at least a million bacteria. Overall measurement of bacillary bioluminescence is a cheap and rapid technology for monitoring the response to chemotherapy and for drug screening.

(h) Lipase assay

The lipase content of purified *M.leprae* has been proposed as another indicator of viability. Although many unconventional assays for determining the viability of *M.leprae* have been developed, experience with most of them is limited. At present ATP estimation, single cell spectra of Na/K with LAMMA and rRNA targeting gene probes/PCR are among the most sensitive in vitro techniques available for monitoring the viability of *M.leprae*. Apart from their sensitivity and specificity, economic feasibility and reproducibility are other important issues that need to be addressed before these methods can be used in research or clinical practice. Other techniques appear more promising for drug sensitivity screening. Animal models like the mouse food pad (normal or immunocompromised mice/rats) will continue to be used as comparative standards. For studying the chain of transmission of live bacilli in the environment gene amplification targeting rRNA/mRNA would be better option.

Molecular biological approaches for Viability Detection

Advances in understanding the molecular genetic structure of *M.leprae* have provided a wealth of information which has led to the development of techniques to detect and quantify specific gene sequences in lesions and or other specimens (eg nasal scrapings) of epidemiological interest. Demonstration could be done in the clinical specimens directly or in the isolated organisms with DNA or RNA as target molecules. With the help of polymerase chain reaction (PCR) these techniques are applicable even to specimens having very low bacterial load.

(a) RNA based drug-screening assays

A rapid microassay for viability and drug sensitivity of *M.leprae* based on estimation of RNA levels has been reported **(Butcher et al 1993).** rRNA based methods have also been found to be useful for monitoring the response to therapy and detection of persisters (**Katoch 1998).**

(b) DNA targeting probes

In leprosy, probes targeting DNA have been reported to have the sensitivity of detection upto 10^4 to 10^5 organisms **(Williams et al 1990).** Experience of using such DNA targeting probes shows that these are not likely to be very useful both because of poor sensitivity in relapses in patients with PB leprosy and the difficulty of persistence of signals for quite some time after bacterial death **(Katoch et al 1998).**

(c) RNA targeting probes

RNA is a much more unstable molecule than DNA. After death RNAs degrade faster than DNA, therefore their demonstration and/or quantitation is likely to correlate better with the presence of live bacteria in the lesions. It is known that messenger RNA (mRNA) has the shortest half - life and thus would be an ideal targeting system for development of probes for viability determination. Probes targeting mRNA have not been very successful because of the very short half-life and technical difficulties of purification and detection of mRNA. Ribosomal RNA (rRNA) is another target molecule which is present as several copy

numbers (2,000-5,000) per live mycobacterial cell. Because of evolutionary conserved as well as variable regions, presence of large copy numbers, and correlation with viability, rRNA has attracted the attention of many scientists and a number of rRNA targeting probes have been developed and have been observed to be sensitive enough to detect 100-1,000 live *M.leprae* directly without any amplification (Sharma et al 1996). Further, an assay for quantitative measurement of these signals by microdensitometric scanning have been developed **(Sharma et al 1997)** and observed to be useful for monitoring the course of treatment and also for diagnosing relapses **(Katoch et al 1998).**

Real time PCR

The appearance of continuous PCR and ongoing converse interpretation PCR (constant RT-PCR) has significantly changed the field of estimating quality articulation. Ongoing PCR is the strategy of gathering information all through the PCR procedure as it happens, in this manner consolidating intensification and identification into a solitary advance. This is accomplished utilizing a wide range of fluorescent sciences that correspond PCR item fixation to fluorescence power (Parashar et al 2005). Responses are described by the point in time (or PCR cycle) where the objective intensification is first distinguished. This esteem is generally alluded to as cycle edge Ct (In ABI System) or Crossing over point (In Roche Applied Biosystem) the time at which fluorescence force is more prominent than foundation fluorescence. Conse¬quently, the more prominent the amount of target DNA in the beginning material, the quicker a noteworthy increment in fluorescent sign will show up.

Ongoing PCR innovation has improved atomic diagnostics of numerous pathogens. One of the benefits of this technique is the evaluation of bacterial DNA content in clinical examples. Finding of sickness can be founded on clinical examination, yet it might be hard to separate from infections that reason comparative side effects, particularly in zones where its rate is low (Naafs 1990). Research facility affirmation is attractive in such settings and can likewise be useful in separating a speculated reinfection or backslide from an extreme touchiness response (inversion responses) (Naafs 1990). Uses of PCR might be improved or upgraded by the utilization of novel ongoing recognition strategies.

Theory of Real Time PCR

PCR can be broken into four noteworthy stages: the direct ground stage, early exponential stage, log-straight (otherwise called exponential stage), and level stage (Tichopad et al 2003). Amid the straight ground stage (typically the initial 10–15 cycles), PCR is simply starting and fluorescence discharge at each cycle has not yet transcended foundation. Standard fluorescence is determined right now. At the early exponential stage, the measure of fluorescence has achieved an edge where it is fundamentally higher (generally multiple times the standard deviation of the pattern) than foundation levels. The cycle at which this happens is known as Ct in ABI Prism® writing (Applied Biosystems, Foster City, CA, USA) or intersection point (CP) in LightCycler® writing (Roche Applied Science, Indianapolis, IN, USA). This esteem is illustrative of the beginning duplicate number in the first format and is utilized to ascertain exploratory outcomes (Heid et al 1996). Amid the log-straight stage, PCR achieves its ideal intensification period with the PCR item multiplying after each cycle in perfect response conditions. At last, the level stage is achieved when response segments become restricted and the fluorescence power is never again valuable for information estimation (Bustin, 2000).

Experience of applications of real time PCR in leprosy

Kramme et al (2004) used real time PCR for the detection and quantification for *Mycobacterium leprae* from clinical specimens by Real Time PCR. They proposed that this technique help to monitor the effect of therapy in patients. When performed in untreated leprosy patients, the clinical diagnosis could be confirmed by PCR in 88.9% of MB and 33.3% of the AFB negative (PB) cases. However, Real-time detection did not increase the clinical sensitivity of PCR, despite its evidently high analytical sensitivity.

In another study by TaqMan real-time PCR was used for rapidly detecting and quantifying *M.leprae* DNA in clinical specimens in which bacilli were undetectable by conventional histological staining. Depending upon the availability of infrastructure and resources real time PCR can be used for various effect of leprosy.

CONCLUSION

PCR analysis provides a sensitive and specific means to detect microorganisms in complex environmental samples. Soil contain abundant humic and fulvic acids that are inhibitory to Taq DNA polymerase and other enzymes used in PCR assay Soil is one of the most challenging environmental matrices from which to obtain microbial DNA that will be useful for PCR detection.

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