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IN VITRO METHODS FOR RAPID MONITORING OF DRUG THERAPY AND DRUG RESISTANCE IN LEPROSY

Though bacterial etiology of leprosy was suggested in 1873 by Hansen, the progress in understanding the biology of the leprosy bacillus has been very slow. *Mycobacterium leprae* has not been cultivated *in vitro* in any acceptable medium system. Growth of *M.laprae* in animal experiments is useful only at the research level. However, for assessment of the efficacy of drug therapy, repeated monitoring of the load and viability of the bacillus is required which is not possible in the case of leprosy. Nevertheless the progress made in understanding the structure and function of the leprosy bacillus has helped in the development of various morphological, chemical/ biochemical and more recently molecular biological approaches for monitoring the effect of treatment. In this write-up the major advances in this area are discussed under the broad headings: assessment of bacterial load; techniques for *in vitro* determination of viability; and techniques for *in vitro* drug sensitivity screening and detection of drug resistance.

Assessment of Bacterial Load

Bacterial load refers to the total load (viable and non-viable) of bacilli infecting a host. In leprosy this can be estimated by four methods:

The bacteriological index

The bacteriological index (BI) is a semi-quantitative estimation of the density of acid fast bacilli (AFB) present in skin smears and can be measured on Dharmendra[1] and Ridley[2] scales. This is easy to perform and can be done under field conditions. Though there are mathematical problems of calculation of average BI , it continues to be a popular method for monitoring the responses to chemotherapy.

Bacillary counts per gram tissue

The bacillary load can also be measured by counting bacilli per gram tissue in biopsy specimens. It measures the total bacilli present in the specimen which includes the skin and the deeper tissues. It is perhaps a little more accurate than BI but is time consuming and its use is restricted to laboratories involved in research.

Bacteraemia/bacillaemia

The release of bacilli into the blood stream is an intermittent process and therefore estimation of bacillaemia has inherent drawbacks. In leprosy, bacillaemia has been shown to be present in both paucibacillary (PB) and

multibacillary (MB) leprosy. There are various methods for estimating the number of bacilli in the blood and the haemolysis method is considered to be most sensitive[3]. Bacillaemia has been shown to decrease with chemotherapy till it eventually disappears and can be used to monitor the response of chemotherapy[4].

Detection and estimation of M.leprae specific lipids

M. leprae has several characteristic lipids such as phenolic glycolipid type 1 (PGL-1), pthiocerol dimycoserosate (PDIM) and mycolic acids. These lipids can be demonstrated in skin biopsies and also quantified by TLC, HPTLC, HPLC and GLC. While the components like PGL-1 are highly specific for *M.leprae*, the detection of PDIM is more sensitive. A quantitative relationship between lipid content and BI has been demonstrated. These measurements can be used for confirmation of diagnosis and assessment of bacillary load in leprosy patients[5].

***In Vitro* Determination of Viability**

For monitoring the effect of any anti-microbial treatment, it is very important to have sensitive techniques for determination of viable numbers/infection units of that organism in the lesions. Chemotherapy first affects the viability and later on the other parameters pertaining to total bacillary load. Though there is no acceptable *in vitro* method for cultivation of *M.leprae*, a variety of *in vivo* and *in vitro* techniques have been developed for measuring viability[6]. Besides the limited multiplication of *M. leprae* in the mouse foot-pad, its growth has been observed in several other animals which include armadillos, monkeys, chimpanzees, Indian pangolin, slender loris, *etc*[7]. However, animal models have inherent draw backs such as the requirement of an inhouse animal facility, low sensitivity and at least 6-9 months are required for getting a result which limit their application in clinical practice. Lack of success in achieving *in vitro* cultivation of *M.leprae* has been partly compensated by development of different *in vitro* viability methods. Important methods are:

Morphological index

Solid staining AFB have been considered to represent viable bacillary populations[8]. The estimation of solid staining bacilli has been used for a long time for assessing the effect of treatment in leprosy patients[8,9]. Although the concept has some technical limitations of subjectivity and that of screening a very small sample from a large pool[9], it can still be used as one of the parameters along with others to monitor the treatment and for diagnosing relapse. This method again has a potential application in MB cases only as at least a few hundred bacilli should be demonstrable in the smears.

Fluorescein diacetate-ethidium bromide staining

Live cells possess the capacity to split fluorescein diacetate (FDA) to fluoresce green, while dead cells will take the red ethidium bromide (EB) stain The proportion of stained green cells by this staining method has been observed to correlate with viable populations in several eukaryotic and prokaryotic cells. These techniques have been standardized for mycobacteria including *M.leprae*[9-11] for use in direct clinical specimens as well as for assessing the growth/viability in macrophages. Their application to lepromatous leprosy patients shows that this parameter is good for monitoring the trends of chemotherapy responses[9]. This technique can therefore be used to confirm relapse provided sequential samples are studied and statistical limits defined. The persistence of green staining signals for some time after death[9] and lack of applicability to patients with paucibacillary leprosy are the main limitations of this method.

Laser microprobe mass analysis

This laser based technique has been reported to be a sensitive method for the determination of viability provided sufficient number of bacilli (more than 100) are present in the clinical specimens[12]. In this method the determination of intracellular sodium/ potassium ratio is done using laser pulse mass spectrometry (LAMMS). This technique can be used for viability determination of *M.leprae*. This method, however, requires expensive equipment.

Measurement of adenylate energy charge

This method is based on the determination of adenylate energy charge (AEC) of bacterial cells and using this approach a new technique for the determining the viability of mycobacteria (including *M. leprae)* has been described[13]. The need for specialized expensive equipment is the main limitation of this method.

Determination of bacillary ATP biomass

Estimation of adenosine triphosphate (ATP) content has been established as an important parameter for determination of viable biomass of different mammalian, other eukaryotic and bacterial species. Techniques for ATP assays for mycobacteria including *M. leprae* have

been developed[14-16]. By optimising the techniques for extraction and assay conditions, ATP assay has been observed to detect even 100 viable mycobacterial cells [15]. Studies in India and abroad confirm the usefulness of this approach for estimating the viable populations of leprosy bacilli as ATP contents in the same range have been observed by various workers[15]. This technique has been successfully applied to monitor the trends of responses to chemotherapy[9,14,17] as well as to demonstrate persisters[9,17,18]. As the technique is based on the determination of total biomass, the problem of viable bacilli getting diluted as in case of any inoculum does not occur. This technique is thus quite sensitive and its application to confirm relapse in patients with PB leprosy needs to be investigated.

Molecular biological approaches

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.

DNA targeting probes

In leprosy, probes targeting DNA have been reported to have the sensitivity of detection upto 10^4 to 10^5 organisms[19]. 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 sometime after bacterial death[6].

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[6]. 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[20 - 22] and have been observed to be sensitive enough to detect 100-1,000 live *M. leprae* directly without any amplification[23]. Further, an assay for quantitative measurement of these signals by microdensitometric scanning have been developed[24] and observed to be useful for monitoring the course of treatment and also for diagnosing relapses[6].

Gene amplification – PCR methods

Various PCR techniques to amplify different genes of *M. leprae* have been developed during the last decade[6]. These assays have been observed to be sensitive up to 1-10 organisms and are reported to be positive in 60-75% of the smear negative paucibacillary specimens[6]. When the PCR technology was introduced, it was reported that this may be useful both for diagnosis as well as for monitoring of viability[25]. These trends were confirmed in the subsequent studies[26, 27]. However, persistence of weak signals a long time after effective treatment has been reported[6,27]. This persistence could be due to residual DNA or some live organisms. Because of such findings, it appears that DNA based PCR assays may have limited application in monitoring situations like late reactions and relapses. Unless sequential specimens are investigated and some type of quantitative/semi-quantitative assessment is made[28], DNA based PCR assays would be difficult to apply to cases where a definite answer about viability is desired.

RNA based gene amplification assays

The sensitivity of RNA detection can be improved by combining with amplification steps by reverse transcription based RT-PCR[29] or nucleic acid sequence based amplification – NASBA[30]. RNA based reverse-transcription PCR (RT-PCR) which involves the initial reverse transcription of target RNA to DNA followed by amplification has been used in leprosy[29]. In a study 80% of specimens from patients with PB leprosy in relapse and about 25% of late reaction specimens were positive by RT-PCR assay targeting rRNA[6]. NASBA is based on the intrinsic properties of RNA amplification and is well suited for small number of RNA templates. This technique targeting rRNA has been shown to correlate well with

viability[30]. Such approaches could also be useful for monitoring the responses to therapy and investigating conditions like late reactions and relapses for detection of viable organisms for individualized patient care. Patients with late reaction with such positivity should be considered for chemotherapy cover when being treated with steroids. The availability of such technique(s) would thus be very useful as it might be unwise to treat such patients with steroids alone.

Techniques for *In Vitro* Drug Sensitivity Screening and Detection of Drug Resistance

Determination of susceptibility to drugs is another important goal relevant to management of patiants and drug development. Several methods based on measurement of limited multiplication inside macrophages, uptake/ utilization/incorporation of metabolites/substrates in *M. leprae* in *in vitro* conditions and ATP synthesis have been developed for this purpose[31-50]. While the investigations assessing multiplication or demonstration of metabolic activity need about a million bacterial cells, ATP synthesis based assays are more sensitive requiring 100-1000 cells. More recently molecular methods, for detection of drug resistant mutants have been developed which have the sensitivity of 1-10 organisms. Important methods are:

Macrophage based assays

M. leprae is an intracellular pathogen and limited multiplication of *M.leprae* in human and murine macrophages has been reported[11,32-39]. Viability within the macrophages has been assessed by different markers such as DNA synthesis measured by uptake of ^{3}H thymidine[32,33], radiolabeled amino acids[34], incorporation of purines and pyrimidines[35], measuring the alterations in Fc receptors[36,37], FDA-EB staining[11,38], and alterations in cholesterol metabolism of macrophages[39], *etc*. As all these techniques require a large number of bacilli, they are not applicable to patients with paucibacillary leprosy and have limited application in MB cases.

Assays using cell free media

Several *in vitro* methods based on incorporation/ utilization of various substrates in cell free media conditions have been published[40 – 44]. These assays are based on uptake of labeled DOPA, thymidine[40], incorporation of ^{14}C palmitic acid into phenolic glycolipid of *M. leprae*[41], measurement of oxidation of ^{14}C-palmitic acid to $^{14}CO_2$ by *M. leprae* using Buddemeyer type counting system or BACTEC 400 system[42], incorporation of radioactive purines and pyrimidines[43] and uptake of radiolabeled acetate[44]. Lipase activity of *M. leprae* harvested from patients has also been proposed as a marker of assessment of viability and drug screening [45]. These assays have been reported to be useful for drug sensitivity screening using bacilli harvested from patients and experimental animals.

Drug screening based on reduction of alamar blue

The property of reduction of alamar blue, an indicator of oxidation-reduction potential by live cells has been translated into a new drug screening format for *M.leprae*[46]. Using *M.leprae* grown in nude mice or armadillos or even those isolated from highly bacillated cases, drug screening for new compounds seems to be possible with this simple method.

Energy synthesis/ decay profiles for drug screening

Over the years several media and conditions have been identified in which the metabolic status of *M. leprae* can be maintained *in vitro*[47] or limited ATP synthesis can be demonstrated *in vitro*[48,49]. In these specialized media conditions accelerated ATP decay has been demonstrated by incubating the organisms with various anti-leprosy drugs[47-50]. These assay systems have been observed to be useful for *in vitro* drug sensitivity screening for *M.leprae* harvested from patients/armadillos[48,49]. These systems have been found to be of value in identification of new promising anti-leprosy compounds[47,48,50].

Molecular detection of drug resistance

Molecular techniques have been used for analysis of the mechanisms of drug resistance in leprosy as well as tuberculosis. Mutations in the target genes can be detected by various techniques like PCR-sequencing, PCR-SSCP, PCR-RFLP, PCR and hybridization with probes[51]. While like in *M.tuberculosis* the mutations in *rpo*B locus have been shown to be responsible for rifampicin resistance[52,53], the situation appears to be too complex for dapsone where no mutations in *folP* locus have been found which indicates the need to identify other targets[54]. As these techniques can directly be applicable to clinical specimens these can be incorporated into the surveillance programmes to determine the exact magnitude of drug resistant mutants to rifampicin present. After the target loci for other drugs are identified this strategy should be applicable to other drugs as well. For application in small laboratory settings easy to use approaches like PCR – probe hybridization would be preferable.

To summarize, several rapid techniques to monitor the responses to chemotherapy in leprosy are available. These techniques show good correlation with mouse foot-pad which is regarded as the gold standard. The availability of such a large number of methods provides a cafeteria choice to different type of workers such as researchers/ clinical laboratory personnel. Most of these methods are, however, applicable to MB cases only. The ongoing programmes for technique development particularly in the area of gene probe technology are very promising as the techniques will be easily applicable to PB cases also. Use of micro-array based approaches is likely to be useful in the identification of novel targets which would be useful for development of techniques for clinical application and field programmes.

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