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# Analysis of Quantitative Relationship Between Viability Determination in Leprosy by MFP, ATP Bioluminescence and Gene Amplification Assay[1]

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The demonstration of limited multiplication of *Mycobacterium leprae* in the mouse foot pad (MFP) was a major breakthrough in leprosy research ([20]). This model has been extensively used for the determination of *M. leprae* viability, for monitoring of chemotherapy, as well as for drug screening ([17, 18]). Because of the long time required for detection of growth and certain other limitations, such as the requirement of a minimum 5000 to 10,000 organisms, other alternative model systems for viability determination have been developed. The measurement of bacillary adenosine triphosphate (ATP) content for determination of *M. leprae* viability has been done successfully by several workers ([4, 5, 14, 15]). Detection of nucleic acid also has been considered for monitoring the therapeutic responses ([25]), while using the modified ATP assay technique developed at this institute, it has been possible to detect around 100 colony forming units (CFU) of cultivable mycobacteria ([14]). It is well known that by the polymerase chain reaction (PCR) technique it is possible to get positive signals from specimens with less than 10 organisms. ATP and PCR thus offer considerably higher sensitivities than any other techniques. It would thus be necessary to compare the efficiency of these methods by using some quantitative criteria as is possible with measurement of ATP.

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## MATERIALS AND METHODS

In the present study, a total of 221 biopsies from patients who were part of various therapeutic trials have been investigated for viability by MFP, ATP and PCR. Biopsies at the beginning of and 12/24 months after treatment were collected under standard aseptic precautions. The treatment groups included: a) immunotherapy (BCG/Mw) + MDT regimen. Treatment comprised of chemotherapy with monthly doses of rifampin, daily clofazimine and dapsone ([9, 11]) along with 6 monthly administrations of BCG or *Mycobacterium w* until smear negativity, b) patients on MDT + pyrazinamide at 0 and 24 months. This regimen also included the above MDT regimen along with pyrazinamide daily for 1 year. c) controls (MDT same as above regimen) at 0, 12 and 24 months. d) patients on MDT + minocycline 100 mg once a month supervised + ofloxacin 400 mg once a month supervised (0, 12 months) ([12]).

Biopsies were divided into three different portions for use in the MFP, molecular and ATP investigations which were carried out in a blind manner. Results were compared retrospectively.

### Mouse foot pad

One part of the biopsy was processed for inoculation into the MFP. The bacterial enumeration was done as described by D'Arcy Hart and Rees ([1]). A batch of five random bred BALB/C mice were inoculated in each of the hind foot pads with a 0.03-ml suspension containing up to 10,000 bacilli. The mice were housed at 25°C in an air-conditioned room. The mice were harvested 6 months after inoculation and acid-fast bacilli (AFB) were

counted as per Desikan and Venkataramanaiah ([2]).

### ATP estimation

Bacillary ATP measurements also were made from the skin biopsy specimen at the respective time duration as per the technique standardized earlier ([15]). Briefly, biopsy suspensions was treated with 2% NaOH, trypsin and ATPase to remove the host ATP. From the final preparation, smears were prepared, stained by the Ziehl-Neelson method, and assayed in a LKB 1251 luminometer (Perkin Elmer-Wallac, Gaithersburg, Maryland, U.S.A.) using an ATP monitoring kit (Bio orbit, Oy, Finland). ATP levels were estimated as pg/million acid-fast bacilli (AFB) ([15]). Cultures were set up to rule out any cultivable mycobacteria or any other organisms in the final preparation.

### Polymerase chain reaction

**Extraction of nucleic acids.** DNA was extracted by using lysozyme and proteinase K treatment followed by removal of proteins. DNA was precipitated with isopropanol ([20]).

**PCR for 18-kDa gene.** The PCR assay for detection of the *M. leprae* 18-kDa gene sequence was done using the primers and methods of Williams, *et al.* ([24]), and as used in our earlier study ([19]). Primers/probes were synthesized in a Pharmacia Gene Assembler Plus. Briefly, two 25-mer primers were used for the synthesis of a 360-bp fragment of the 18-kDa protein gene of *M. leprae.* The PCR was performed using recombinant Ampli Taq Polymerase with the Gene Amp Kit (Perkin-Elmer-Cetus, Norwalk, Connecticut, U.S.A.). Temperature cycling was performed in a programmable thermal cycler (Coy Lab Products, Greenlake, Michigan, U.S.A. and MJ Research, Watertown, Massachusetts, U.S.A.) with three temperature shifts of 94°C (denaturing, 1 min) 60°C (primer annealing, 1 min) and 72°C (extension, 2 min). In all cases, 45 cycles were done for the assay. The amplified product was analyzed by dot-blot analysis and/or by Southern transfer and detection of an amplified fragment of 360 bp by using a 212-bp probe for intron of the amplified fragment as recommended by developers of the assay.

**PCR for 36-kDa gene.** Primers and the gene amplification method of Hartskeerl, *et al.* ([7]) and as used by de Wit, *et al.* ([3]) were followed for the PCR. The probe used for hybridization was 530-bp fragment prepared after amplification. Primers were synthesized in a Pharmacia Gene Assembler Plus by following the procedure as detailed in the manual for the instrument. Reagents used were from Pharmacia, Stockholm, Sweden or were more generally obtained from Rama Biotechnologies India Pvt. Ltd., Hyderabad, India. Briefly, 18 mer and 19 mer primers directed the synthesis of a 530-bp fragment, approximately 80% of the 36-kDa protein gene of *M. leprae.* The PCR was performed using recombinant amplitaq polymerase with gene amp kit (Perkin-Elmer-Cetus Instruments, Norwalk, Connecticut, U.S.A.). Temperature cycling was performed in a programmable Thermal Cycler (MJ Research PTC-100) with three temperature shifts of 94°C (denaturing 2 min), 60°C (primer annealing 2 min) and 72°C (extension 3 min). In all cases 35 cycles were used for the assay and confirmation was done by Southern blot analysis with random aDIG-labeled PCR-amplified 36-kDa gene fragment of *M. leprae* as the probe. Detection was done using a kit from Boehringer Mannheim/Roche Diagnostics, Basil, Switzerland.

### Statistical analysis

For the purpose of analysis the biopsies from different regimens were grouped at 0 (untreated), 12 months and 24 months duration since the study focuses on viability indicators and not the type of treatment. Some of the data pertaining to the effects of treatment have been published elsewhere ([6, 9, 10, 21]). Statistical analysis was done by Fischer's exact test, Yates' uncorrected and Yates' corrected tests.

## RESULTS

### Overall trends

**Untreated patients.** There was a general agreement among all three techniques (Table 1) and the viability ranged from 48.2% by MFP to 100% by the ATP and the PCR assays. The differences between MFP versus ATP, MFP versus PCR (18-kDa) and MFP versus PCR (36-kDa) were statistically significant, while other differences were not (Table 1).

TABLE 1. *Comparison of viability by MFP, ATP and gene amplification assays at different stages of treatment.*

| Treatment stage | No. of samples | MFP (+) | ATP (+) | PCR 18-kDa (+) | PCR 36-kDa (+) |
|---|---|---|---|---|---|
| Pre-treatment[a] | 114 | 55 (48.2%) | 114 (100%) | 114 (100%) | 114 (100%) |
| 1 year after treatment[b] | 82 | 1 (1.2%) | 2 (2.4%) | 6 (7.3%) | 8 (9.7%) |
| 2 years after treatment[c] | 25 | 0 (0%) | 1 (4%) | 1 (4%) | 2 (8%) |
| Total | 221 | 56 (25.3%) | 117 (52.9%) | 121 (54.7%) | 124 (56.1%) |

[a] In untreated patients MFP vs ATP = highly significant (p <0.0001), MFP vs PCR (18-kDa and 36-kDa) = highly significant (p <0.0001), ATP vs PCR (18-kDa and 36-kDa) = Test not valid; PCR (18-kDa) vs PCR (36-kDa) = test not valid.

[b] In patients after 1 year of treatment ATP vs PCR (36-kDa) = significant (p <0.05); remaining comparisons were not significant.

[c] In patients after 2 years of treatment, all of above comparisons were not significant.

At 1 year after treatment, the trends again correlated except for some differences where MFP did not detect growth but the PCR and the ATP assays were still positive in a few cases. Only the difference between ATP versus PCR (36-kDa) was found to be significant.

At 2 years after treatment, it is clear that the PCR and the ATP assays showed higher positivity as compared to MFP which was negative in all the specimens studied. However, the differences were not statistically significant (Table 1).

**Quantitative relationship**

A total of 221 specimens were included in the comparison. Viable numbers were estimated by taking $3.5 \times 10^{-15}$ g as the estimated average weight of one viable unit of *M. leprae* ([15]). It was observed that a) When the bacillary ATP levels were high (i.e., 3.6 pg/million or higher), there was 100% concordance among all techniques (Table 2). b) For ATP levels between 0.04 to 3.59 pg/million bacterial cells (viable proportion 0.001% to 0.1%), there was 50% concordance between MFP and ATP; whereas, there was 90% concordance between PR and ATP. The differences between detection rates by MFP and ATP as well as MFP and PCR were statistically significant (p = 0.024). c) For very low ATP levels, i.e., 0.02 to 0.04 pg/million cells (viable proportion 0.0005% to 0.0001%), only 1/53 (2%) specimens showed growth in MFP; whereas, 51/53 (96%) were positive by PCR. The differences between MFP and ATP and MFP and PCR were highly significant (Table 1). d) For specimens with undetectable ATP, there was growth in 1/104 (1%) by MFP while PCR assays were positive in 7/104 (6%) for the 18-kDa and 10/104 (9%) for the 36-kDa targeted systems. The differences between MFP and PCR and MFP and ATP were not significant.

## DISCUSSION

The mouse foot pad technique continues to be the one most commonly used for monitoring the effect of treatment as well as for screening of antileprosy compounds. This model has provided valuable information on the above aspects, including the detection of viable persisters ([17, 18, 22]). Over the years several other *in vivo* (nude mouse, thymectomized mice/rats) and *in vitro* methods (using metabolic substrates macrophage based methods, fluorescent staining, morphological index, LAMMA, ATP, DNA, and r-RNA probes, etc.) have been developed and used for this purpose. The ATP bioluminescence and PCR assay have emerged as important alternate methods for assessment of *M. leprae* viability ([4, 6, 15, 25]).

While the experience with ATP has been wide ([4, 6, 15]), the published data on PCR is limited ([25]). With the ATP assay, published most of the work, showed similar trends ([4, 6, 15]). Besides the confirmation of diagnosis, PCR assays have another potential benefit of monitoring the therapeutic responses ([21]). Even though the PCR assay and ATP bioluminescence were carried out in stored biopsies in different groups of patients, these patients were comparable since they belonged to similar bacteriological

TABLE 2. *Quantitative relationships among MFP, ATP and PCR assays.*

| ATP levels (pg/million) | Total | Positive by MFP | Positive by 18-kDa PCR | Positive by 36-kDa PCR |
|---|---|---|---|---|
| >36 | 32 | 32 | 32 | 32 |
| 3.6–35.9 | 10 | 10 | 10 | 10 |
| 0.4–3.59[a] | 22 | 12 | 21 | 21 |
| 0.02–0.039[b] | 53 | 1 | 51 | 51 |
| Not measurable[c] | 107 | 1 | 7 | 10 |
| Total | 221 | 56 | 121 | 124 |

[a] MFP vs ATP, MFP vs PCR (18-kDa and 36-kDa) = significant, p = 0.024; ATP vs PCR (18-kDa and 36-kDa) = not significant.
[b] MFP vs ATP, MFP vs PCR (18-kDa and 36-kDa) = highly significant, p <0.0001; ATP vs PCR (18-kDa and 36-kDa) = not significant.
[c] MFP vs PCR (18-kDa and 36-kDa) = not significant, p = 0.065; PCR (18-kDa) vs PCR (36-kDa) = not significant, p = 0.613.

profiles and were harboring high positivity in the mouse foot pad.

The present study shows that there is a good overall correlation among the viable biomass estimated by bacillary ATP levels, PCR assay and growth in the mouse foot pad. Since data at 1 year and 2 years were less and restricted to regimens with good effects on persisters, the figures do not reflect the persister rates. The purpose of this analysis was to focus on the quantitative relationships. In this study, specimens were positive when the ATP content was >3.6 pg/million (approximately 2–5 live organisms per inoculum). However, positive takes in the mouse foot pad decreased when the content decreased below 3.5 pg/million (<1 viable cell/inoculum) but the PCR positivity correlated with the ATP bioluminescence. When the ATP content was further lowered, the positivity in the mouse foot pad was possibly a matter of chance, while PCR positivity was observed in 96% of the cases. For specimens with undetectable ATP, positivity was seen in 1% of the MFP cases and 6% of the PCR (18-kDa) cases and 9% of the PCR (36-kDa) cases. While the positivity by the mouse foot pad in 1% of the cases shows the inability of the ATP bioluminescence method to measure (differentiate/detect) against the low background due to host ATP, positive PCR signals in some could be due to higher sensitivity of the method or persistence of DNA after bacterial death in some cases.

That issue can be decided by using RNA targeting systems nucleic acid sequenced-based amplification (NASBA) ([13, 16, 25]). It would be necessary to undertake future prospective studies on different types of amplification techniques targeting DNA or RNA. It may be worthwhile to quantify PCR assays (Gabrielle, M. E. V., de Wit, M. Y. L. and Klatser, P. R. Quantification of *M. leprae* template DNA after PCR by a colorimetric assay. In: Proceedings Workshop on PCR Technology for the Detection of *M. leprae* Infection, Nonthaburi, April 8–19, 1991, pp. 27–31.) and study relationships with MFP, ATP and other methods because such studies would be important to determine intra-observation and interlaboratory variation, if any, among different labs before making final recommendations for the use of these alternate methods in clinical practice. To conclude, all three assays have been established to correlate with a viable load as quantified by bacillary ATP and correlated with results in MFP and PCR. Depending upon the need, correlation of any of the two methods can be used in follow-up studies for monitoring the trends of therapeutic responses.

## SUMMARY

Two hundred twenty-one untreated, borderline lepromatous/lepromatous (BL/LL) leprosy patients have been investigated for viability by the mouse foot pad method (MFP), adenosine triphosphate (ATP) and polymerase chain reaction (PCR). The biopsies were collected at the beginning of and 12/24 months after treatment. The patient group was treated with a) immunotherapy (BCG/Mw) + MDT; b) MDT + pyrazinamide; c) control MDT; d) MDT + minocycline 100 mg once a month supervised + ofloxacin 400 mg once a month su-

pervised. Biopsies were divided in three parts for use in the mouse foot pad, molecular and ATP investigations. In untreated and treated patients (at 12 and 24 months), there was a general agreement among all three techniques, and PCR and ATP showed higher positivity as compared to MFP. Further, there was good correlation among the viable biomass estimated by bacillary ATP levels, PCR assay and growth in mouse foot pads. The positivity was observed by MFP as well as PCR assay (18-kDa and 36-kDa) from all of the specimens when the ATP content was more than 3.6 pg/million. When the ATP content was below 3.5 pg/million, the positive takes in MFP decreased but the PCR positivity correlated with ATP bioluminescence up to 0.04 pg/million. When the ATP content was even lower, the uptake in the MFP was possibly a matter of chance, while PCR positivity was observed in 96% of the cases. For specimens with undetectable ATP, positivity was seen in 1% of the cases, showing the inability of ATP bioluminescence method to detect low background due to host ATP. PCR signals in some cases could be due to the higher sensitivity of the method or persistence of DNA after bacterial death in some cases. On the whole, the PCR methods even though targeting DNA have shown good correlations with biomass which confirm their usefulness in monitoring therapeutic responses in leprosy.

## RESUMEN

Se investigó la viabilidad de *Mycobacterium leprae* en 221 pacientes con lepra lepromatosa/lepromatosa subpolar (BL/BL) sin tratamiento, usando las técnicas de la almohadilla plantar del ratón, del adenosin trifosfato (ATP) y la reacción en cadena de la polimerasa (PCR). Las biopsias se colectaron al principio y al final de 12–24 meses de tratamiento. El grupo de pacientes se trató a) con inmunoterapia (BCG/Mw) + PQT, b) con PQT + pirazinamida, c) con PQT sola, y d) con PQT + minociclina (100 mg por mes) + ofloxacina (400 mg por mes) con supervisión. Las biopsias se dividieron en 3 partes para su uso en los 3 métodos de investigación. En los pacientes no tratados y en los tratados (a los 12 y 24 meses), hubo una correlación general entre los tres métodos, con mayor grado de positividad en las técnicas de PCR y ATP. Además, hubo una buena correlación entre la biomasa viable estimada por las tres técnicas. Cuando el contenido en ATP fue superior a 3.6 picogramos/millón, las pruebas en la almohadilla plantar del ratón y la PCR resultaron positivas; sin embargo, cuando el contenido en ATP fue menor a 3.5 picogramos/millón, la positividad por el método de la almohadilla plantar del ratón disminuyó, aunque la positividad por PCR correlacionó con la bioluminiscencia del ATP hasta 0.04 picogramos/millón. Cuando el contenido en ATP fue aun menor, la prueba en la almohadilla plantar fue azarosa, mientras que la PCR fue positiva en el 96% de los casos. Para los especimenes con niveles no detectables de ATP, la positividad se observó en el 1% de los casos, demostrando la incapacidad del método de la bioluminiscencia del ATP para discriminar entre los niveles bajos de ATP de la bacteria y del huésped. Las señales de PCR en algunos casos pudieron deberse a la mayor sensibilidad del método o a la persistencia de DNA después de la muerte bacteriana en algunos casos. En conjunto, los métodos de PCR, dirigidos al DNA, han mostrado buena correlación con la biomasa, lo cual confirm su utilidad en la evaluación de las respuestas terapéuticas en la lepra.

## RÉSUMÉ

Deux cent vingt et un cas de lèpre lépromateuse et lépromateuse borderline (LL/BL) furent biopsiés au début et 12/24 mois après traitement, afin de tester la viabilité bacillaire au moyen de trois méthodes: l'inoculation à la patte de souris (MFP), la production d'ATP et la réaction de polymérase en chaîne (PCR). Les patients furent regroupés par traitements: a) immunothérapie (BCG/Mw) + polychimiothérapie (PCT); b) PCT + pyrazinamide; c) groupe témoin (PCT seule); d) PCT + minocycline 100 mg une fois par mois prise surveillée + orofloxacine 400 mg une fois par mois prise surveillée. Les biopsies furent divisées en trois parties, afin qu'une partie soit utilisée dans chaque méthode. Parmi les patients traités et non traités (à 12 et 24 mois), les résultats issus des trois techniques concordaient globalement entre eux, avec le PCR et l'ATP montrant un plus fort taux de positivité par rapport au MFP. De plus, il existait une bonne corrélation entre la biomasse viable estimée par les niveaux d'ATP, le PCR et le développement de masses dans les pattes de souris. Tous les échantillons étaient positifs pour le MFP et le PCR (amplifiant les gènes des protéines 18 kDa et 36 kDa) lorsque la teneur en ATP était supérieure à 3.6 pg/million. Lorsque la teneur en ATP était en dessous de 3,5 pg/million, le taux de positivité de la méthode MFP décroissait, tandis que les résultats positifs au PCR étaient corrélés à la bioluminescence de l'ATP jusqu'à 0,04 pg/million. Lorsque la teneur en ATP était plus basse encore, la croissance après inoculation à la patte de souris était le plus souvent une question de chance, tandis qu'un résultat positif au PCR était présent dans 96% des cas. Concernant les échantillons sans ATP détectable, le taux de positivité était de 1%, montrant l'incapacité de la méthode de détection de l'ATP par bioluminescence à détecter un faible bruit de fond associé à la présence d'ATP provenant de l'hôte. Ces PCR positifs pourraient être dus à une plus grande sensibilité de cette méthode ou bien résulter de la persistence d'ADN après mort bac-

térienne dans certains cas. Globalement, les méthodes par PCR, bien qu'elles ciblent l'ADN, ont montré de bonnes corrélations avec la biomasse, ce qui confirme leur valeur pour la surveillance des réponses aux traitements contre la lèpre.

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