

Comparison of the mouse foot pad test with a Buddemeyer type radiorespirometric assay in detecting viable *Mycobacterium leprae* in human lesional biopsies

Vinay P. Agrawal, Vanaja P. Shetty

Foundation of Medical Research, Mumbai, India

Address for correspondence: Dr. Vanaja Shetty, The Foundation for Medical Research, 84-A, R. G. Thadani Marg, Worli, Mumbai - 400 018, India.
E-mail: fmr@vsnl.net

ABSTRACT

Aim: This study assesses the utility of a Buddemeyer type radiorespirometric (RR) assay in detecting viable *Mycobacterium leprae* in clinical samples taking the mouse foot pad (MFP) test as gold standard. **Methods:** A total of 131 skin biopsy specimens comprising of 56 untreated, smear-positive, borderline lepromatous and lepromatous leprosy (BL-LL) and 75 untreated, smear-negative, borderline tuberculoid and mid-borderline (BT-BB) specimens were processed by both the methods. The cut-off value (in counts per minute, cpm) for test samples in the RR assay was determined using nonleprosy (normal) skin biopsy specimens. **Results:** In the untreated BL-LL and BT-BB groups, 86 and 56% of the cases tested positive in the RR assay respectively, which was comparable to the results of the MFP test (80 and 45% respectively). The overall concordance between the two tests was 74.79%, whereas the sensitivity and specificity were 75 and 74.3% respectively. A *Kappa* value of 0.459 indicated a fairly good agreement between the two methods. There was no linear relationship observed between the bacteriological index (BI) and the morphological index (MI) with the counts per minute (cpm) output. **Conclusion:** The results indicate the ability of this RR assay to detect viability; however a comparison with another sensitive method would further validate the assay system.

Key Words: *In Vitro* method to, *Mycobacterium leprae*, Mouse foot pad test, Radiorespirometric assay, Viability

There is a need for a simple, rapid, reproducible and cost-effective test for the detection of viable *Mycobacterium leprae* in tissue samples from leprosy patients. So far, the only established means to assess the viability of *M. leprae* has been its relative growth in the foot pads of conventional mice.^[1] The mouse foot pad (MFP) test is technically difficult, takes several months to yield results and more importantly, uses a large number of animals, which are major ethical and financial concerns.

Mycobacteria are known to metabolize a variety of fatty acids.^[2] *Mycobacterium leprae* in particular has been shown to rapidly oxidize palmitate to carbon dioxide.^[3] This has been used effectively as a basis for the detection of viable *Mycobacterium leprae* and inhibition of such activity as an

indicator of drug action in clinical samples.^[4-9] A study by Truman and Krahenbuhl^[10] further showed that the substrate is used rapidly and reliable activity is detected with as few as 10^5 – 10^6 *Mycobacterium leprae* cells. The current study therefore was aimed at assessing the use of a Buddemeyer type radiorespirometric assay based on $^{14}\text{CO}_2$ evolution from ^{14}C -labeled palmitate by *Mycobacterium leprae* in an axenic culture as an index of viability. The results obtained were compared with the conventional MFP test serving as gold standard.

METHODS

The study was cleared by the institutional ethics committee. All the biopsies were done using local anesthesia with informed consent from the patients before recruitment.

How to cite this article: Agrawal VP, Shetty VP. Comparison of the mouse foot pad test with a Buddemeyer type radiorespirometric assay in detecting viable *Mycobacterium leprae* in human lesional biopsies. *Indian J Dermatol Venereol Leprol* 2007;384-8.

Received: April, 2007. **Accepted:** July, 2007. **Source of Support:** Grant (5/8/3(6)/2001-ECD-1) from The Indian Council of Medical Research.

Conflict of Interest: None declared.

The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) cleared the use of animals in the study. The Foundation for Medical Research is registered with CPCSEA (registration number 424/01/a/CPCSEA, June 20, 2001).

The following positive and negative controls were used:

Positive controls

M. smegmatis and *M. tuberculosis* H37Rv cultures grown in Dubos broth (DB) supplemented with 10% fetal calf serum (FCS) were harvested in the logarithmic (log) phase and used as positive controls in this assay system.

The following three sets of experiments were carried out with *M. smegmatis*: a) heat killed (HK), formalin-treated and rifampicin-treated suspensions containing 10^6 , 10^4 and 10^2 cells/ml were used to observe the metabolic activity with killed cells, b) serial tenfold dilutions in the range of 10^1 to 10^6 cells/ml were used as viable inocula and c) Suspension containing 106, 104 & 102 viable cells/ml was treated with three different concentrations viz, 0.01, 0.05 and 0.1% of Tween-20 to prevent clumping of bacilli. The following two sets of experiments were carried out with *M. tuberculosis* H37Rv: a) HK and formalin-treated cell suspensions (10^5 and 10^2) were used as negative controls and b) serial tenfold dilutions in the range of 10^1 to 10^6 cells/ml were used as viable inocula.

Negative controls

Fifteen samples each of nonleprous human skin biopsies (NLS) and normal mouse foot pads (NMFP) were used as negative controls. A blank (DB containing 0.1 μ Ci/ml of 14 C-labeled palmitate) was used as an internal control in all the experiments.

Test samples

A total of 131 skin biopsy specimens freshly obtained from untreated cases of leprosy and classified according to the Ridley and Jopling scale^[11] were divided into two groups as follows:

Group 1 consisted of 56 untreated, smear-positive, borderline lepromatous and lepromatous leprosy (BL-LL) cases.

Group 2 was comprised of 75 untreated, smear-negative, BT-BB cases including 68 Borderline tuberculoid (BT), three mid-borderline (BB) and four indeterminate cases.

In each case, approximately 0.15 g of the biopsy tissue

was homogenized using a fixed weight to volume ratio of 0.1 g/ml. Bacterial load/g wt, bacteriological index (BI) and morphological index (MI) were determined using the standard protocol.^[12] The homogenate thus obtained was used for both the MFP test and the Buddemeyer assay within 48 hours. No purification steps were applied to avoid loss of bacteria.

Radiorespirometric Buddemeyer assay

All skin biopsy homogenates obtained from leprosy cases and other negative / positive controls were processed for palmitate oxidation by the method described by Buddemeyer et al.^[13] The readings of 14 CO₂ evolution were recorded at 24 h intervals for three weeks using a liquid scintillation counter (Wallac 1409 DSA). All the vials were checked for sterility in the end, using Nutrient broth, Sabouraud's broth and Loweinstein Jensen slants. If found contaminated, those results were not considered for the study.

Mouse foot pad inoculation and harvesting of

M. leprae

The hind foot pads of a minimum of ten Swiss White (S/W) mice per sample were inoculated with 0.03 ml of appropriately diluted human biopsy tissue homogenates containing $\leq 1 \times 10^4$ *M. leprae* cells. All the mice were fed a standard mouse diet and exposed to a 12 h light-dark cycle in an air-conditioned room. Foot pads were harvested at 6, 7 and/or 8 and 12 months postinoculation using Shepherd's method.^[11] Briefly, foot pads were disinfected, cut and collected in sterile cups containing sterile saline (0.5 ml/foot pad). This was then electrically homogenized and the bacterial counts were taken by Ziehl Neelsen Carbolfuchsin (ZNCF) staining on a spot slide. A minimum of 200 microscopic fields / sample were covered for counting acid-fast bacilli (AFB). The lower limit of detectability by this method is 1×10^4 *M. leprae* per foot pad. A minimum of two counts per foot pad per interval was obtained at 6, 7 and 8 months. The remaining mice were harvested at the end of the 12th month. Any foot pad count exceeding 1×10^5 was considered as a significant fold-increase, and hence, as a positive yield.

Statistical methods

The means of triplicate cpm readings of test samples assayed at the peak were compared with mean \pm 2SE (standard error) value of NLS also assayed using the Buddemeyer (RR) assay. The statistical significance of the difference between the results of the Buddemeyer assay and the MFP test was determined using the *Chi-square test*. Overall sensitivity and specificity of the Buddemeyer assay system were determined using MFP results as a gold standard. The relationship, if any, between BI, MI and the quantum of CO₂ evolution

was assessed using a linear correlation method. All these statistical tests were performed using the program InStat 3.06 (GraphPad®, Inc.). $P \leq 0.05$ was considered to be statistically significant. The *Kappa* value was calculated to determine the agreement between the two methods.

RESULTS

Bacteriological findings in the lesional biopsies in groups 1 and 2

All the cases in group-1 (BL-LL) were positive in the smears as well as in the biopsy homogenates with BI ranging between 1+ to 5+. In group-2 (BT-BB), all the cases were smear-negative, however six of these-two BB and four indeterminate cases scored positive in the homogenate with BI ranging between 1+ to 2+.

Determination of cut-off values and metabolic activity in the Buddemeyer assay

Viable cells metabolize ^{14}C -Palmitate to $^{14}\text{CO}_2$; the initial lag phase is followed by a log phase (a steady increase and peak cpm output) followed by a plateau. The system measures the cumulative output of evolved $^{14}\text{CO}_2$ and the viability is determined on the basis of the total cpm output at the peak interval.

It was noted that the average cpm values of NLS, NMFP and the blank were comparable. Since the test samples comprised of human skin biopsies, the NLS readings were used to determine the cut-off value. The average cpm value for NLS was 300.17 with SD of ± 175.09 . Applying 2SE, any value above 390.1 cpm was considered as an indication of positive metabolic activity and the presence of viable *M. leprae*.

Findings with *M. smegmatis* and *M. tuberculosis* H37Rv

The HK, formalin- and rifampicin-treated cells of both *M. smegmatis* and *M. tuberculosis* H37Rv showed average cpm values that were comparable to those of NLS, NMFP and blank readings (i.e., <390). In contrast, the viable *M. smegmatis* and *M. tuberculosis* H37Rv cells showed good metabolic activity. It was noted that the metabolic activity peaked by days 5-6 in case of higher cell densities ($> 10^5$), by days 7-8 for lower cell densities (10^2 and 10^3) and a plateau thereafter. In both cases, the serial dilution experiments revealed a significant cpm value for even 10 cells, indicating a good sensitivity.

Use of 0.1 and 0.05% of Tween-20 showed suppression in the metabolic activity indicating toxicity, while with 0.01%, the triplicate cpm readings with and without Tween-20 were

comparable (results not shown). These results confirm the validity of our assay system.

Findings with leprosy lesional biopsies

The average peak cpm outputs observed in cases from the two groups are depicted in Figure 1. It was noted that unlike *M. tuberculosis* H37Rv and *M. smegmatis*, *M. leprae* showed an extended lag phase of about seven days. In 80% of cases, the peak metabolic activity was observed between 11 and 14 days [Figure 1]. This is in variance from the earlier findings in the RR assay wherein the peak was recorded at \sim day 7.^[3,6,7] However, this earlier peak can be explained by the use of semipurified *M. leprae* in the earlier study obtained from tissues of armadillos and nude mice while the current study uses whole skin tissue homogenate.

Comparison of Buddemeyer and MFP test results [Table 1]

Table 1 depicts the overall number and % of cases that were positive in the Buddemeyer assay (considering 390.1 cpm as the cut-off) and the corresponding MFP test results. Results of eight cases (6%) were not considered due to contamination and other technical problems.

Buddemeyer assay and MFP test results were comparable for both the groups. Notably, of the six cases that scored positive in homogenates among the smear-negative BT-BB cases, two (33%) were positive in the MFP test while four (66%) were positive in the Buddemeyer assay (data not shown). Of the remaining 67 cases that were both smear-negative and homogenatenegative (two cases were not considered due to contamination), 30 (45%) were positive in the MFP test while 37 (55%) were positive in the Buddemeyer assay (Data not shown).

The Buddemeyer assay proved to be more sensitive than MFP test in both these groups but the difference was not statistically significant. While it would be important to rule out false positivity in the case of the Buddemeyer assay, it is not unusual to find viable bacteria, albeit small in numbers, in BT cases as well as in treated cases of leprosy. Findings

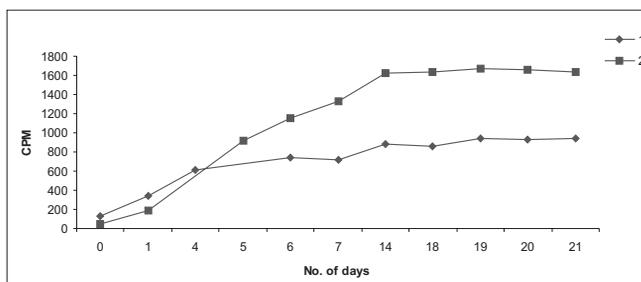


Figure 1: Two representative untreated BL-LL cases (1 and 2) showing peak cpm output at 14 days

indicate that the more sensitive the detection system, the better is the outcome.^[14-20]

Relationship of BI and MI with CO₂ evolution in the untreated BL-LL group of cases

The BI and MI are relatively simple to record, the latter being commonly used as an indicator of viability. Slit skin smear status was recorded from five different sites for all the patients. All the BT cases were smear-negative; while in BL-LL cases, the average BI varied between 1+ to 5+. The BI and MI determined from the homogenate for each biopsy specimen were correlated with cpm output to observe the relationship.

Although a trend of increase in CO₂ evolution with increase in BI was noted, it was not statistically significant ($r = 0.2159$, $P = 0.1067$) [Figure 2A]. The morphological indices (MI) of the specimens between 0–15% also did not exhibit absolute linear correlation ($r = 0.2424$, $P = 0.0517$) with CO₂ evolution [Figure 2B]. Similar discordance was observed by others using different *in vitro* methods.^[21-24]

Specificity and sensitivity of the Buddemeyer assay

The Buddemeyer assay results of two groups of cases were compared with MFP test results. Sensitivity and specificity of 75 and 74.2% respectively were observed for the MFP. Considering overall results, one to one concordance of 74.79% was observed between the two tests [Table 2]. A *Kappa* value of 0.459 also indicated a fairly good agreement between both the methods.

DISCUSSION

The Buddemeyer assay has shown good potential to be considered as a rapid index of viability in clinical samples. It is easy to perform with commercially available ingredients. Further, we also noted that there is no host tissue interference or modulation by the host immune system as is the case with the MFP test. Franzblau^[4] found the Buddemeyer assay to be more sensitive and cost-effective as compared to Bactec. In our study, the Buddemeyer assay was found to be more sensitive than the MFP test. However, it can be argued that the samples observed to be positive may not necessarily be

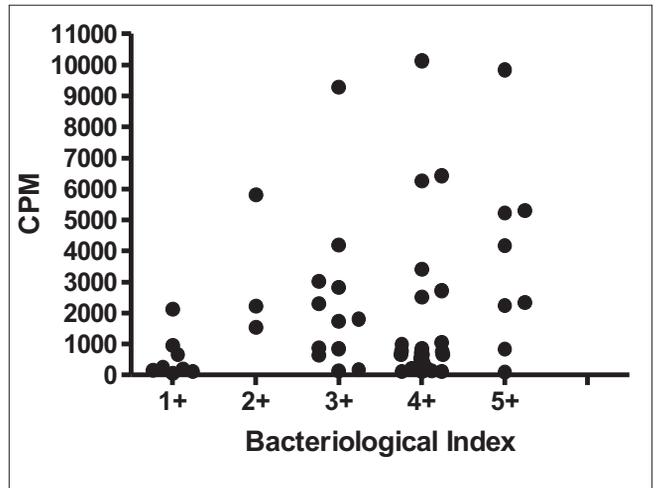


Figure 2A: Comparison between bacteriological index and cpm output of untreated BL-LL cases

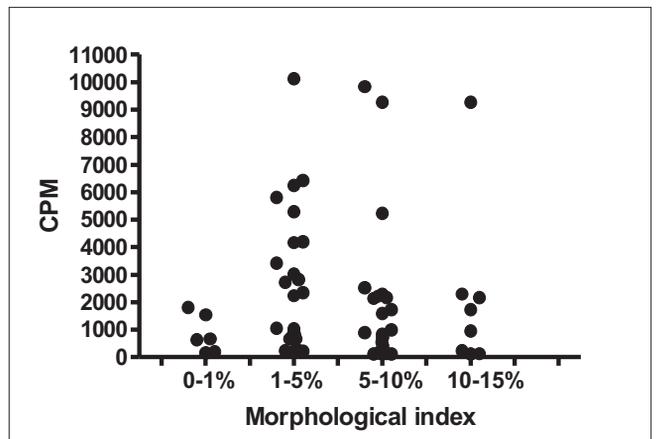


Figure 2B: Comparison between morphological index and cpm output of untreated BL-LL cases

Table 2: Concordance between Buddemeyer assay and mouse foot pad test results

Groups	Budd.+ MFP+	Budd.- MFP-	Budd.+ MFP-	Budd.- MFP+	Total
Untreated BL-LL	37 (74)	4 (8)	6 (12)	3 (6)	50
Untreated BT-BB	26 (35.6)	25 (34)	15 (20.5)	7 (9.5)	73

Figures in () indicate %, Budd., Buddemeyer assay positive; MFP+, Mouse foot pad test positive, Budd., Buddemeyer assay negative, MFP-, Mouse foot pad test negative. Figures in parentheses indicate percentages of test results from the total number of cases. *One to one concordance of 74.79% was observed between the results of both the tests when Budd + MFP + & Budd - & MFP - groups were compared. Results of eight cases (6%) were not considered due to contamination and other technical problems.

true positives, especially in BT cases. This assay system can be validated further using a more sensitive technique, e.g, targeting the 16S rRNA.

As cautioned by Truman *et al.*,^[10] the relative amount of ¹⁴CO₂ derived from the palmitate in the axenic cultures is influenced by the saturation of the label, any competing carbon source, temperature, pH of the medium and the atmosphere of the culture chamber. Thus, the use of palmitate oxidation as

Table 1: Comparison between Buddemeyer assay and mouse foot pad test results (%)

Groups	*Budd.+	*MFP+	P value
Untreated BL-LL	43/50 (86)	40/50 (80)	0.5944
Untreated BT-BB	41/73 (56)	33/73 (45)	0.2466

Figures in () indicate %, ($P < 0.05$ significant), *Buddemeyer assay positive *Mouse foot pad test positive. Results of eight cases (6%) were not considered due to contamination and other technical problems

an index of *M. leprae* viability requires standardization and validation of test results in any set-up. Moreover, utility; if any, in day-to-day patient care has not been investigated so far.

In clinical practice, it may not be of an additional benefit to perform this test with known positive specimens. On the other hand, the results clearly show that the RR assay will be more useful for detecting small numbers of viable cells, i.e., in smear-negative (tuberculoid) cases, in detecting persistent bacilli and in testing the efficacy of treatment.

Its inapplicability at the field level due to the requirement of a liquid scintillation counter and also usage and disposal of radioactive material are its limitations. Furthermore, it has to be borne in mind that any storage or temperature conditions that can affect the viability of *M. leprae* could hamper the test results, as is the case with MFP.

ACKNOWLEDGMENTS

We sincerely thank Dr. S. D. Ghate, Medical Officer, The Foundation for Medical Research (FMR), for performing the biopsies, Mrs. Anju Wakade, Research assistant, FMR, for helping with MFP test and Mr. K. Gandewar, Biostatistician, Department of Preventive Social Medicine, Sion Municipal Hospital, Mumbai for statistical help. We are also thankful to Dr. N. H. Antia for his valuable suggestions. This study was supported by a grant (5/8/3(6)/2001-ECD-1) from The Indian Council of Medical Research (ICMR).

REFERENCES

1. Shephard CC. The experimental disease that follows the infections of human leprosy bacilli into the foot pads of mice. *J Exp Med* 1960;112:445-54.
2. Camargo EE, Kertcher JA, Larson SM, Tepper BS, Wagner Jr HN. Radiometric measurement of differential metabolism of fatty acid by *Mycobacteria*. *Int J Lepr Other Mycobact Dis* 1982;50:200-4.
3. Franzblau SG. Oxidation of palmitic acid by *Mycobacterium leprae* in an axenic medium. *J Clin Microbiol* 1988;26:18-21.
4. Franzblau SG, Biswas AN, Jenner P, Colston MJ. Double-blind evaluation of BACTEC and Buddemeyer-type radiorespirometric assays for *in vitro* screening of antileprosy agents. *Lepr Rev* 1992;63:125-33.
5. Franzblau SG, Chan GP, Garcia-Ignacio BY, Chavez VE, Livelio JB, Jimenez CL, et al. Clinical trial of Fusidic acid for lepromatous leprosy. *Antimicrob Agents Chemother* 1994;38:1651-4.
6. Chan GP, Garcia-Ignacio BY, Chavez VE, Livelio JB, Jimenez CL, Parrilla, ML, et al. Clinical trial of sparfloxacin for lepromatous leprosy. *Antimicrob Agents Chemother* 1994;38:61-5.
7. Chan GP, Garcia-Ignacio BY, Chavez VE, Livelio JB, Jimenez CL, Parrilla ML, et al. Clinical trial of clarithromycin for lepromatous leprosy. *Antimicrob Agents Chemother* 1994;38:515-7.
8. Fajardo TT Jr, Villahermosa LG, dela Cruz EC, Abalos RM, Franzblau SG, Walsh GP. Minocycline in lepromatous leprosy. *Int J Lepr Other Mycobact Dis* 1995;63:8-17.
9. Lahiri R, Randhawa B, Krahenbuhl J. Application of a viability staining method for *Mycobacterium leprae* derived from the athymic (nu/nu) mouse footpad. *J Med Microbiol* 2005;54:235-42.
10. Truman RW, Krahenbuhl JL. Viable *M. leprae* as a research reagent. *Int J Lepr Other Mycobact Dis* 2001;69:1-12.
11. Ridley DS, Jopling WH. Classification of leprosy according to immunity: A five group system. *Int J Lepr Other Mycobact Dis* 1966;34:255-73.
12. World Health Organization (1987). Laboratory techniques for leprosy. WHO/CDS/LEP/86.4.
13. Buddemeyer EU, Hutchinson R, Cooper M. Automatic quantitative radiometric assay of bacterial metabolism. *Clin Chem* 1976;22:4159-64.
14. Shetty VP, Wakade A, Antia NH. A high incidence of viable *Mycobacterium leprae* in post-MDT recurrent lesions in tuberculoid leprosy patients. *Lepr Rev* 2001;72:334-7.
15. Kurabachew M, Wondimu A, Ryon JJ. Reverse transcription-PCR detection of *Mycobacterium leprae* in clinical specimens. *J Clin Microbiol* 1998;36:1352-6.
16. Haile Y, Ryon JJ. Colorimetric microtitre plate hybridisation assay for detection of *Mycobacterium leprae* 16S rRNA in clinical specimens. *Lepr Rev* 2004;75:40-9.
17. Katoch VM, Katoch K, Ramu G, Sharma VD, Datta AK, Shivannavar CT, et al. *In vitro* methods for determination of viability of mycobacteria: Comparison of ATP content, morphological index and FDA-EB fluorescent staining in *Mycobacteria leprae*. *Lepr Rev* 1988;59:137-43.
18. Gupta UD, Katoch K, Singh HB, Natrajan M, Sharma VD, Katoch VM. Detection of viable organisms in leprosy patients treated with MDT. *Acta Leprol* 1999;11:89-92.
19. Chae GT, Kim MJ, Kang TJ, Lee SB, Shin HK, Kim JP, et al. DNA-PCR and RNA-PCR for the 18-kDa gene of *Mycobacterium leprae* to assess the efficacy of multi-drug therapy for leprosy. *J Med Microbiol* 2002;51:417-22.
20. Ebenezer GJ, Daniel S, Norman G, Daniel E, Job CK. Are viable *Mycobacterium leprae* present in lepromatous patients after completion of 12 months' and 24 months' multi-drug therapy? *Indian J Lepr* 2004;76:199-206.
21. Satish M, Prasad HK, Mittal A, Nath I. Lack of correlation between Morphological Index and viability as assessed by the uptake of 3H-thymidine by macrophage resident *M. leprae*. *Lepr India* 1982;54:420-7.
22. Kvach JT, Munguia G, Strand SH. Staining tissue derived *Mycobacterium leprae* with fluorescein diacetate and ethidium bromide. *Int J Lepr Other Mycobact Dis* 1984;52:176-82.
23. Harshan KV, Prasad HK, Chopra NK, Mishra RS, Gogiya P, Nath I. Comparison of radiometric macrophage assay and fluorescein diacetate/Ethidium bromide staining for evaluation of *M. leprae* viability. *Int J Lepr Other Mycobact Dis* 1987;55:316-21.
24. Katoch VM, Katoch K, Ramanathan U, Sharma VD, Shivannavar CT, Datta AK, et al. Effect of chemotherapy on viability of *Mycobacterium leprae* as determined by ATP content, morphological index and FDA-EB fluorescent staining. *Int J Lepr Other Mycobact Dis* 1989;57:615-21.