

Bioluminescence assay of adenosine triphosphate in drug susceptibility testing of *Mycobacterium tuberculosis*

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Accepted January 5, 1998

Twenty three clinical isolates of *M. tuberculosis* and the reference strain, *M. tuberculosis* H37Rv were tested for their susceptibility to trifluoperazine (TFP) by the standard broth dilution method and the bioluminescence assay. The results showed that in 15 of the 23 isolates, the minimal inhibitory concentration (MIC) was identical in both the methods and in the remaining 8 isolates the difference in the MIC values between the methods, was less than two fold and was not significant. The findings suggest that the measurement of adenosine triphosphate (ATP) by bioluminescence assay can be employed as an alternative method for the rapid screening of clinical isolates for their susceptibility to anti-mycobacterial agents.

Key words ATP - bioluminescence assay - *Mycobacterium tuberculosis* - susceptibility test

In general, to determine the *in vitro* susceptibility of *Mycobacterium tuberculosis* to anti-mycobacterial agents, the broth dilution method or the agar dilution method are used as standard procedures¹. These methods involve sampling, plating and counting the colony forming units (cfu) to determine the bacterial growth and its inhibition. These methods are laborious, time consuming and the results are known only after 5 wk. There is hence, an urgent need to develop a rapid and reliable method for testing the antimicrobial sensitivity of *M. tuberculosis*. As there is a fairly good correlation between the ATP content and the viable counts of *M. tuberculosis*, the bioluminescence assay of ATP was suggested as an alternative, rapid and reliable method². However, the correlation of the bioluminescence assay with the broth dilution method in the determination of drug sensitivity of *M. tuberculosis* to antitubercular drugs, has not been reported in literature. The present study describes the determination of minimal inhibitory

concentration (MIC) of trifluoperazine (TFP), an antipsychotic drug with anti-mycobacterial activity³, against *M. tuberculosis* by the standard broth dilution method and the bioluminescence assay.

Material & Methods

Cultures : A total of 23 clinical isolates of *M. tuberculosis*, obtained from pulmonary tuberculosis patients attending Tuberculosis Research Centre, Chennai, was studied. The reference strain, *M. tuberculosis* H37Rv was included as control. All the cultures were coded before testing.

Broth dilution method : The broth dilution method, using Middlebrook 7H9 broth (BBL Microbiology Systems, Cockeysville, Md) supplemented with OADC (oleic acid, bovine albumin- factor - V, dextrose, catalase) and 0.05 per cent Tween 80, was set up as per the procedures described by Dickinson and Mitchison⁴. In brief, a 7 day old 7H9 broth

culture was adjusted, based on the Thoma bacteriological counter readings, to contain 10^6 bacilli/ml and distributed in bottles. TFP (gift from Dr P.S Murthy, University College of Medical Sciences, Delhi) was added to the culture bottles to attain final concentrations of 8, 16, 32 and 64 mg/l. The drug free culture was used as control. The culture bottles were incubated at 37°C for 7 days. The viable counts (VC) and the measurements of adenosine triphosphate (ATP) were determined for the cultures. The VC was determined, on day 0 for the control culture and on day 7 for all the cultures, by inoculating undiluted and 5 serial ten-fold dilutions of the cultures on Lowenstein-Jensen (LJ) medium slopes and counting the cfu at the end of 4 wk. MIC is defined as the lowest concentration of the drug which inhibited more than 99 per cent of the population in the control culture on day 7.

Bioluminescence assay of ATP : The extraction of ATP and its measurement by the bioluminescence assay were carried out by the procedures described by Prioli *et al*². In preliminary experiments the extraction of ATP was optimised. In brief, 100 μl of the culture was transferred to 200 μl of boiling 0.1 M Tris buffer (PH 7.75) containing 2 mM EDTA in a test tube. After boiling for 5 min in a water-bath the contents were cooled to room temperature. Then, 100 μl of the ATP monitoring reagent (Lumit, Lumac, Netherlands) was added and the light intensity was measured in a Biocounter (Lumac 3M, Biocounter 2010 A) as integral relative light units (RLU) in 10 sec.

Standard ATP curve : The Lumit ATP (10 $\mu\text{g/vial}$) was diluted in Lumit buffer to contain different concentrations ranging from 25,000 to 48 pg/ml and their RLU values were determined.

Estimation of ATP content of *M. tuberculosis* : The ATP content of the *M. tuberculosis* cultures was estimated from the equation ($\text{ATP} = 0.486 + 0.452 \text{ cfu}$) derived from the cfu and ATP values.

Determination of RLU index : As the ATP content of mycobacterial cultures was estimated from the RLU values the conversion of RLU values into ATP content was not done and RLU values were used for the analysis. The RLU index was calculated by dividing the RLU of the drug containing culture on

day 7 by the RLU of the drug free control culture.

The MIC is defined as the lowest concentration of the drug at which the RLU index is less than 0.1.

Statistical analysis : The difference in the agreement between the two methods was analysed by McNamar's test. The strength of the relationship between the variables was measured using Pearson's correlation coefficient and their linear relationship using regression lines.

Results & Discussion

The correlation coefficient between the standard ATP and RLU was 0.998. The correlation between cfu/RLU and ATP was 0.89 and they were significant. The average content of ATP/cfu of *M. tuberculosis* was estimated to be $5.36 \times 10^{-15}\text{g}$. Similar values were reported for mycobacterial cultures by Katoch *et al*⁵.

The distribution of MICs of TFP as determined by the broth dilution and bioluminescence assay is given in the Table. The MIC of 15 of the 23 strains was identical in both the methods. Of the remaining 8 isolates, none exhibited a two fold difference in the MIC value which is considered significant in bacterial susceptibility tests. The difference observed between the two methods was not statistically significant. In two earlier studies^{6,7} the bioluminescence assay was evaluated against the conventional indirect sensitivity test and the Bactec radiometric assay for the classification of *M. tuberculosis* cultures. Neisson *et al*⁶ reported that the results of the bioluminescence assay agreed with the conventional indirect sensitivity test in 99 per cent (103/104) of test results and with the radiometric assay in 98 per cent (102/104) of test results, using 25 strains. Beckers *et al*⁷ observed that

Table. Comparison of values of MIC obtained by the broth dilution and bioluminescence assay

	Broth dilution method				
	MIC				
	MIC	8	16	32	Total
Bioluminescence	8	0	5	0	
assay method	16	1	13	1	15
	32	0		2	3
	Total	1	19	3	23

the bioluminescence assay correlated with the indirect sensitivity test in 97 per cent (66/68) of the test results using 17 clinical isolates.

The other rapid methods that are being explored have their own limitations. The slide culture sensitivity test is prone to produce contagious aerosols and is difficult to perform in the routine set-up. In addition, it is unsuitable for the smear-negative but culture-positive samples since about 40 per cent of the samples fail to grow in the slide culture method⁸. The direct sensitivity test, based on inoculation of the sputum deposit on the drug containing LJ slopes, might give the susceptibility pattern of the isolates within 10 to 14 days but, it requires a reasonable number of bacilli in the deposit to get reliable results⁹. The radiometric assay is very well standardised and it is recommended for use in the routine mycobacteriology laboratories¹⁰. However, it also involves the use of radioactive isotopes and hence is a potential health hazard. Recent molecular methods such as polymerase chain reaction-single strand conformation polymorphism and the direct DNA sequencing techniques are very expensive¹¹⁻¹³ and their value needs to be evaluated in large clinical trials. The luciferase mycobacteriophage reporter assay is still in the experimental stage and much more needs to be done before it could be used in the laboratory¹⁴.

The results of the present study suggest that bioluminescence assay could be adopted as one of the methods for the determination of MIC of new anti-mycobacterial agents against *M. tuberculosis*. As the results of the bioluminescence assay are available in 14 days compared to 35 days in the broth dilution method, it can be employed for the rapid screening of clinical isolates/laboratory strains for their susceptibility to anti-mycobacterial agents.

Acknowledgment

The authors acknowledge the laboratory staff, in particular, Smt. N.S. Gomathi and Shri. R. Adimoolam for technical help.

References

1. Heifets I. Qualitative and quantitative drug susceptibility tests in mycobacteriology. *Am Rev Respir Dis* 1988; 137: 1217-22.
2. Prioli RP, Tannn A, Brown IN. Rapid methods for counting mycobacteria. Comparison of methods for extraction of mycobacterial adenosine triphosphate (ATP) determined by firefly luciferase assay. *Tubercle* 1985; 66 : 99-108.
3. Selvakumar N, Kumar Vanaja, Krishnamurthy PV, Prabhakar R, Murthy PS. *In vitro* susceptibility of *Mycobacterium tuberculosis* to trifluoperazine. *Curr Sci* 1997; 73 : 79-80.
4. Dickinson JM, Mitchison DA. *In vitro* properties of rifapentine (MDL 473) relevant to its use in intermittent chemotherapy of tuberculosis. *Tubercle* 1987; 68 : 113-8.
5. 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 *Mycobacterium leprae*. *Lepr Rev* 1988; 59 : 137-43.
6. Nelsson LE, Hoffner SE, Ansehn S. Rapid susceptibility testing of *M. tuberculosis* by bioluminescence assay of mycobacterial ATP. *Antimicrob Agents Chemother* 1988; 32 : 1208-12.
7. Beckers B, Lang HR, Schimke D, Lammers A. Evaluation of a bioluminescence assay for rapid antimicrobial susceptibility testing of mycobacteria. *Eur J Clin Microbiol* 1985; 4 : 556-61.
8. Dickinson JM, Allen BW, Mitchison DA. Slide culture sensitivity tests. *Tubercle* 1989; 70 : 115-21.
9. Mathew S, Paramasivan CN, Rehman F, Balambal R, Rajaram K, Prabhakar R. A direct rifampicin sensitivity test for tubercle bacilli. *Indian J Med Res* 1995; 102 : 99-103.
10. Siddiqui SH, Libonati JP, Middlebrook G. Evaluation of a rapid radiometric method for drug susceptibility testing of *M. tuberculosis*. *J Clin Microbiol* 1981; 13 : 908-12.
11. Telenti A, Imboden P, Marchesi F, Schmidheini T, Bodmer T. Direct, automated detection of rifampicin-resistant *M. tuberculosis* by polymerase chain reaction and single-strand conformation polymorphism analysis. *Antimicrob Agents Chemother* 1993; 37 : 2054-8.
12. Selvakumar N, Ding BC, Wilson SM. Separation of DNA strands facilitates detection of point mutations by PCR-SSCP. *Bio Techniques* 1997; 22 : 604-6.
13. Selvakumar N, Wilson SM, McNerney R, Narayanan PR. Single strand conformation polymorphism profiles with biotinylated PCR products to detect mutations in the *rpoB* gene of *Mycobacterium tuberculosis*. *Curr Sci* 1997; 73 : 774-7.
14. Jacobs WR Jr, Barletta RG, Udani R, Chan J, Kalkut G, Sosne G, *et al*. Rapid assessment of drug susceptibilities of *Mycobacterium tuberculosis* by means of luciferase reporter phages. *Science* 1993; 260 : 819-22.

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