RAPID SUSCEPTIBILITY TESTING OF MYCOBACTERIUM AVIUM COMPLEX AND MYCOBACTERIUM TUBERCULOSIS ISOLATED FROM AIDS PATIENTS

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In ominous projections issued by both U.S. Public Health Service and the World Health Organization, the epidemic of HIV infection will continue to rise more rapidly worldwide then predicted earlier. The AIDS patients are susceptible to diseases called opportunistic infections of which tuberculosis and M. avium Complex (MAC) infection are most common. This has created an urgent need to uncover new drugs for the treatment of these infections.

In the seventies, NASA scientists at Goddard Space Flight Center, Greenbelt, Maryland, had adopted a biochemical indicator, adenosine triphosphate (ATP), to detect presence of life in extraterrestrial space. Therefore, we proposed to develop ATP assay technique to determine sensitivity of antibacterial compounds against MAC and M. tuberculosis. The work was initiated in June 1992. In the last report, we described our efforts in developing ATP assay method using MAC. Studies were continued further, and during the period of this report, we established the relationship between colony forming units and ATP levels of these organisms during the growth cycle. Also, we evaluated the effects of standard antimycobacterial drugs using ATP assay technique and compared the results with those obtained with conventional tube dilution proportional method.

Relationship between bacterial counts and ATP levels: MAC was grown in 7H9 broth for five days at 37°C. Aliquots were taken for plating on 7H11 agar plates and also for ATP assays. The results in Figure 1 indicate that increasing volumes of bacterial suspension (0.05-1.4 ml) lead to higher ATP values (expressed as peak height or relative light units). The light emission after cell lysis and luciferase-luciferin reaction was proportional to the bacterial count, with coefficient of correlation of 0.983. With the aliquots of bacterial suspension, colony counts were made (as indication of viable cells), and these values were also proportional to ATP levels (Figure 2) with coefficient of correlation being 0.946.

In the next experiment, both MAC and M. tuberculosis were inoculated in 7H9 broth and incubated at 37°C. At periodic intervals (0-10 days for MAC and 0-34 days for M. tuberculosis) cultures were taken out. Aliquots were used for colony counts on 7H11 plates and also for ATP assays. The results (Tables 1 and 2) indicate that increase in ATP levels (biomass) was rapid during early phase of growth. In case of MAC, this increase was obvious within 12 hours indicating that, though bacteria did not initiate division, they were in the process of active metabolic activity. This resulted in higher ATP levels per 10^6 cells. When bacilli were in logarithmic phase, the increase in colony counts was parallel to that of ATP, thus giving steady value of ATP per 10^6 cells. During stationary phase, there was no increase in colony counts, but there was significant drop in the ATP levels, suggesting the occurrence of death phase. Similar results were also obtained during the growth cycle of M. tuberculosis. Thus, it seems from the ATP values, one can predict if there will be active growth or death of organism in a given environment much earlier than the colony counts.

Effect of antimycobacterial drugs: In these experiments, various drugs were added to 7H9 broth and inoculated with MAC and M. tuberculosis. Aliquots were removed after 5 days (MAC) and 20 days (M. tuberculosis) for colony counts and ATP assays. The results are presented in Tables 3 and 4. In both the organisms, ATP method was more sensitive than the colony counts. With each drug tested, one hundred percent inhibition was obtained with lower doses of the drug using ATP compared to doses of drug in colony count method.

In the next set of experiments, drugs were added to 7H9 broth, and cultures were inoculated with MAC and M. tuberculosis. During the incubation, cultures were removed for ATP assays, and colony counts are results are presented in Tables 5 and 6. For MAC, cells were exposed to 4 μg/ml rifampicin, and for M. tuberculosis, cells were exposed to 1 mg/ml streptomycin. Again, with both the organisms, ATP method was found to be more sensitive than colony count. In case of MAC, one hundred percent inhibition was

achieved within 24 hours by ATP method, while colony count method, same results were obtained in five days. In case of M. tuberculosis, when the cells were exposed to 1 μg/ml of streptomycin, there was a gradual decline of ATP levels from day 1 to day 3, indicating loss of metabolic capacity of the organism and hence, the loss of viability; by day 5, there was total loss of metabolic capacity. On the other hand, in the colony count method, total loss of viability was observed on day 10 with no further appearance of CFU up to day 34. This suggests the specificity and rapidity of the ATP assay method.

Data in Tables 7 and 8 represent results of drug susceptibility testing of 12 clinical isolates of M. tuberculosis. The results of ATP assay were available after five days of incubation in contrast to three weeks for standard colony count method. The results by both methods agree well, except that inhibition of M. tuberculosis by ATP method was observed with lower concentration of drugs compared to that observed by colony count method. Nevertheless, same strains were identified as drug-resistant by both the methods.

So, the results obtained so far prove that ATP assay by firefly bioluminescent method is feasible with MAC and M. tuberculosis. It is more sensitive and rapid than conventional colony count method. The next step is to compare the ATP assay method with the standard dilution proportional method (by adding drugs to agar plate) and the radiorespirometric method using BACTEC and evaluate the advantages of ATP method over the other two methods. This work will then be extended to clinical specimens and will be undertaken during year II of the project as described in the original proposal.

FIGURE 1. RELATIONSHIP BETWEEN BACTERIAL COUNTS (DENSITY) OF M. AVIUM COMPLEX AND ATP CONTENT AS MEASURED IN RELATIVE LIGHT UNITS (RLU).

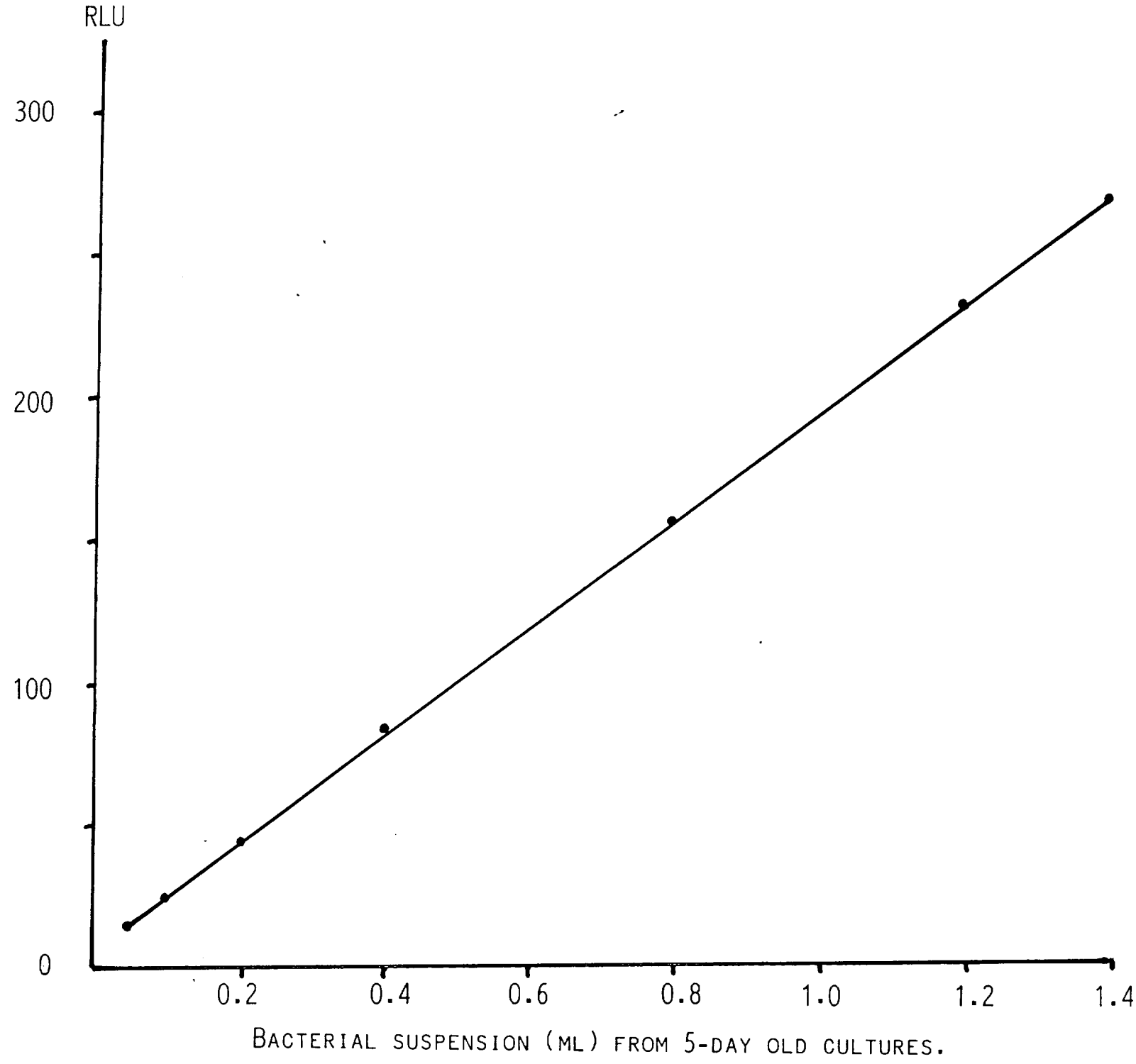

FIGURE 2. RELATIONSHIP BETWEEN THE CONCENTRATION (CFU/ML) OF M. AVIUM COMPLEX AND ATP CONTENT AS MEASURED IN RELATIVE LIGHT UNITS (RLU).

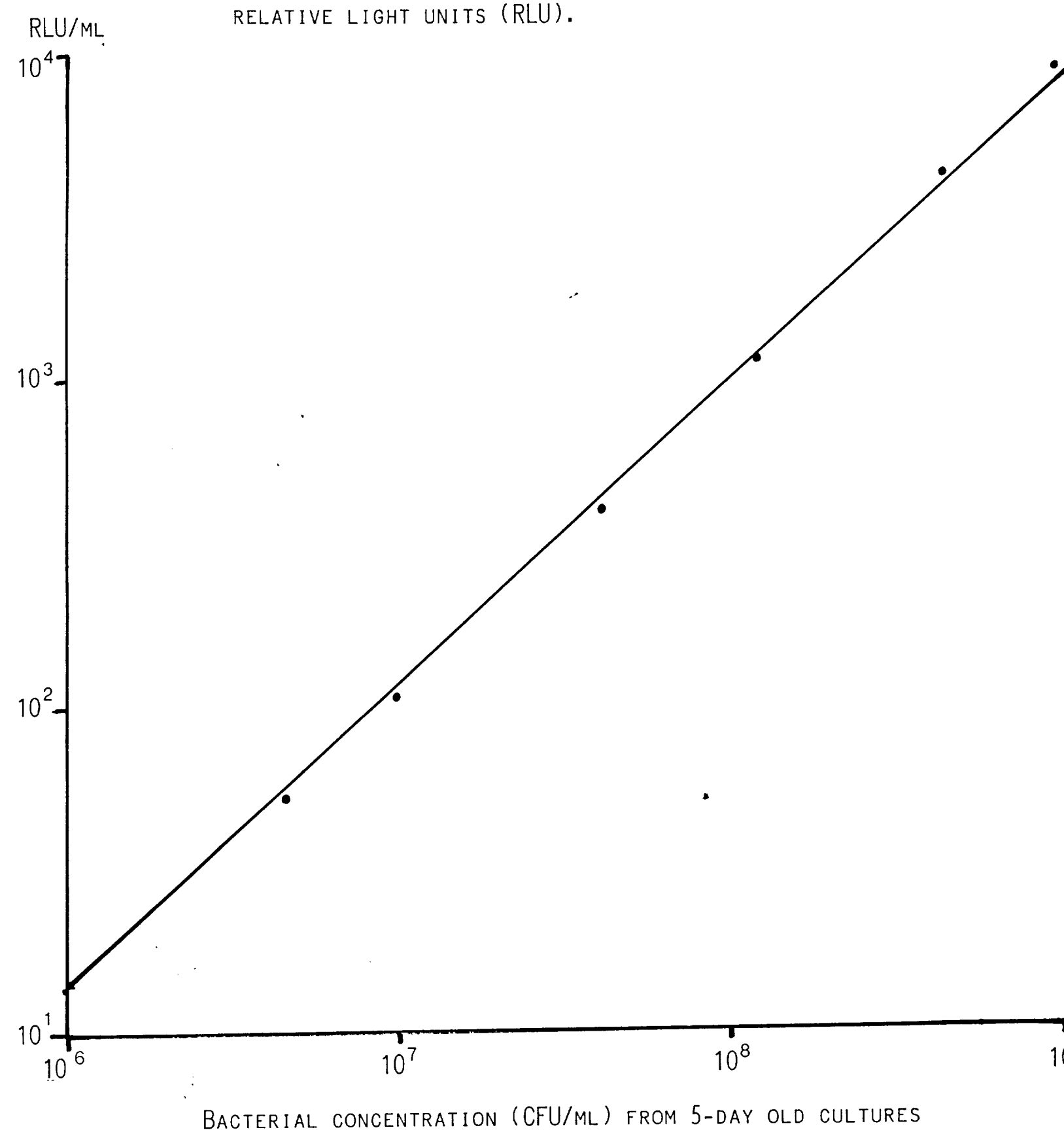

Table 1. Relationship between bacterial growth (CFU) and ATP of M. avium complex during the growth cycle.

Incubation (Days)	Growth (CFU/ml)	ATP levels (pg/ml)	ATP/10^6 CFU (pg)
0	0.25×10^5	1.41×10^1	564
0.5	0.41×10^5	4.52×10^1	1102
1	2.5×10^5	2.48×10^2	993
2	2.8×10^6	1.64×10^3	584
3	1.5×10^7	8.52×10^3	568
4	8.6×10^7	4.93×10^4	573
5	4.4×10^8	2.52×10^5	573
6	2.1×10^9	1.23×10^6	587
7	4.7×10^9	1.01×10^6	214
8	4.9×10^9	1.18×10^5	24

Table 2. Relationship between bacterial growth (CFU) and ATP of M. tuberculosis during the growth cycle.

Incubation (Days)	Growth (CFU/ml)	ATP levels (pg/ml)	ATP/10^6 CFU (pg)
0	0.31×10^5	1.59×10^1	513
1	0.42×10^5	2.65×10^1	631
3	0.98×10^5	7.72×10^1	788
5	2.27×10^5	2.34×10^2	1031
7	3.44×10^5	2.46×10^2	715
10	8.84×10^5	5.43×10^2	614
14	3.22×10^6	1.68×10^3	523
17	6.51×10^6	3.37×10^3	517
20	1.15×10^7	6.12×10^3	532
24	3.90×10^7	2.02×10^4	519
30	9.11×10^7	2.83×10^4	311
34	1.10×10^8	3.96×10^3	36

Table 3. Antimicrobial activities of various drugs against the growth of M. avium complex.[1]

Drug	Conc. µg/ml	CFU	% Inhibition	pg ATP/ml	% Inhibition
Streptomycin	0	4.73×10^8	0	2.47×10^5	0
	2	3.94×10^8	17	2.00×10^5	19
	4	2.39×10^8	50	1.31×10^5	47
	8	0.70×10^8	85	9.96×10^2	100
	16	0.14×10^8	97	0	100
	32	0	100	0	100
Ethambutol	0	4.91×10^8	0	3.11×10^5	0
	1	3.88×10^8	21	2.33×10^5	25
	2	3.44×10^8	30	2.02×10^5	35
	4	2.50×10^8	49	1.24×10^5	60
	8	0.54×10^8	89	1.31×10^3	100
	16	0	100	0	100
Rifampicin	0	4.81×10^8	0	2.64×10^5	0
	0.5	4.66×10^8	3	2.5×10^5	5
	1	4.28×10^8	11	2.24×10^5	15
	2	3.27×10^8	32	1.58×10^4	94
	4	0.14×10^8	97	0.71×10^3	100
	8	0	100	0	100
	16	0	100	0	100
Ciprofloxacin	0	4.49×10^8	0	2.37×10^5	0
	1	4.00×10^8	11	2.04×10^5	14
	2	2.96×10^8	34	1.40×10^5	41
	4	0.65×10^8	81	0	100
	8	0	100	0	100
	16	0	100	0	100

[1] All assays from cells from 5-day old cultures.

Table 4. Antimicrobial activities of various drugs against the growth of M. tuberculosis.[1]

Drug	Conc. (µg/ml)	CFU/ml	% Inhibition	pg ATP/ml	% Inhibition
Streptomycin	0	2.81×10^7	0	1.46×10^4	0
	0.01	2.93×10^7	0	8.17×10^3	44
	0.1	0.68×10^7	78	7.32×10^2	95
	1	0.17×10^7	94	0	100
	10	0	100	0	100
Isoniazid	0	2.74×10^7	0	1.41×10^4	0
	0.01	2.83×10^7	0	9.45×10^3	33
	0.1	0.31×10^7	89	4.23×10^2	97
	1	0	100	0	100
	10	0	100	0	100
Ethambutol	0	2.86×10^7	0	1.55×10^4	0
	0.01	2.94×10^7	0	1.55×10^4	0
	0.1	0.46×10^7	84	9.03×10^2	94
	1	0.20×10^7	93	0	100
	10	0	100	0	100
Rifampicin	0	2.68×10^7	0	1.39×10^4	0
	0.005	2.61×10^7	0	3.05×10^3	78
	0.05	2.51×10^7	6	0	100
	0.5	2.40×10^7	10	0	100
	5	1.96×10^7	27	0	100
	10	0.32×10^7	88	0	100
Streptomycin[2]	0	2.78×10^7	0	1.55×10^4	0
	0.01	3.00×10^7	0	1.52×10^4	0
	0.1	2.75×10^7	0	1.60×10^4	0
	1	2.84×10^7	0	1.50×10^4	0
	10	2.67×10^7	0	1.48×10^4	0

[1] All assays from cells from 20-day old cultures.
[2] Results with streptomycin-resistant M. tuberculosis

Table 5. Relationship between bacterial growth (CFU) and ATP of M. avium complex, exposed to rifampicin, during the growth cycle.

Incubation (Days)	Without Rifampicin		With Rifampicin[1]	
	Growth (CFU/ml)	ATP (pg/ml)	Growth (CFU/ml)	ATP (pg/ml)
0	0.33×10^5	1.76×10^1	0.36×10^5	1.93×10^1
1	4.10×10^5	3.67×10^2	0.31×10^5	0
2	3.81×10^6	2.17×10^3	0.26×10^5	0
3	2.10×10^7	1.14×10^4	0.12×10^5	0
4	1.32×10^8	6.98×10^4	0.03×10^5	0
5	6.46×10^8	3.44×10^5	0	0
6	2.83×10^9	1.51×10^6	0	0
7	5.72×10^9	1.34×10^6	0	0
10	5.8×10^9	1.68×10^5	0	0

[1] Rifampicin, 4 μg/ml, added to 7H9 broth.

Table 6. Relationship between bacterial growth (CFU) and ATP of M. tuberculosis, exposed to streptomycin, during the growth cycle.

Incubation (Days)	Without Streptomycin		With Streptomycin[1]	
	Growth (CFU/ml)	ATP (pg/ml)	Growth (CFU/ml)	ATP (pg/ml)
0	0.31×10^5	1.61×10^1	0.28×10^5	1.45×10^1
1	0.38×10^5	2.32×10^1	0.30×10^5	1.14×10^1
3	0.82×10^5	6.58×10^1	0.23×10^5	0.61×10^1
5	1.93×10^5	2.12×10^2	0.20×10^5	0
7	3.02×10^5	2.31×10^2	0.11×10^5	0
10	7.66×10^5	4.84×10^2	0.03×10^5	0
14	2.99×10^6	1.56×10^3	0	0
17	6.12×10^6	3.15×10^3	0	0
20	1.18×10^7	6.21×10^3	0	0
24	3.62×10^7	1.88×10^4	0	0
30	8.75×10^7	2.53×10^4	0	0
34	9.15×10^7	2.47×10^3	0	0

[1] Streptomycin, 1 µg/ml, added to 7H9 broth.

Table 7. Drug susceptibility testing 12 clinical isolates of M. tuberculosis using the standard dilution proportional method.

Strain	Streptomycin (µg/ml)				INH (µg/ml)				Rifampicin (µg/ml)					Ethambutol (µg/ml)			
	0.01	0.1	1	10	0.01	0.1	1	10	0.005	0.05	0.5	5	10	0.01	0.1	1	10
1	+	+	—	—	+	—	—	—	+	+	+	+	—	+	+	—	—
2	+	+	—	—	+	—	—	—	+	+	+	+	—	+	+	—	—
3	+	+	+	—	+	+	—	—	+	+	+	+	—	+	—	—	—
4	+	+	—	—	+	—	—	—	+	+	+	—	—	+	+	—	—
5	+	+	+	—	+	—	—	—	+	+	+	+	—	+	+	—	—
6	+	+	—	—	+	—	—	—	+	+	+	+	—	+	+	—	—
7	+	+	—	—	+	—	—	—	+	+	+	+	—	+	+	—	—
8	+	+	—	—	+	+	+	—	+	+	+	+	—	+	+	—	—
9	+	+	—	—	+	—	—	—	+	+	+	+	—	+	+	+	+
10	+	+	—	—	+	—	—	—	+	+	+	+	—	+	—	—	—
11	+	+	+	+	+	—	—	—	+	+	+	+	—	+	+	—	—
12	+	+	—	—	+	+	+	—	+	+	+	+	—	+	+	—	—

— = Inhibition
\+ = Growth

Table 8. Drug susceptibility testing 12 clinical isolates of M. tuberculosis using ATP assay.

Strain	Streptomycin (µg/ml)				INH (µg/ml)				Rifampicin (µg/ml)					Ethambutol (µg/ml)			
	0.01	0.1	1	10	0.01	0.1	1	10	0.005	0.05	0.5	5	10	0.01	0.1	1	10
1	+	—	—	—	+	—	—	—	+	—	—	—	—	+	—	—	—
2	+	—	—	—	+	—	—	—	+	—	—	—	—	+	—	—	—
3	+	+	—	—	+	—	—	—	—	—	—	—	—	+	—	—	—
4	+	—	—	—	+	—	—	—	+	—	—	—	—	+	—	—	—
5	+	—	—	—	+	—	—	—	+	—	—	—	—	+	—	—	—
6	+	—	—	—	+	—	—	—	+	—	—	—	—	+	—	—	—
7	+	—	—	—	+	—	—	—	—	—	—	—	—	+	—	—	—
8	+	—	—	—	+	+	—	—	+	—	—	—	—	+	—	—	—
9	+	—	—	—	+	—	—	—	+	—	—	—	—	+	+	+	+
10	+	—	—	—	+	—	—	—	+	—	—	—	—	+	—	—	—
11	+	+	+	+	+	—	—	—	+	—	—	—	—	+	—	—	—
12	+	—	—	—	+	+	—	—	+	—	—	—	—	+	—	—	—

— = Inhibition
\+ = Growth