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Improvements of ATP Assay as a Substitute for the CFU Method in Estimating Viable Cell Count for BCG/*r*BCG Vaccine Preparations

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Abstract

Bacillus Calmette–Guérin (BCG) and recombinant BCG (*r*BCG) vaccines can be genetically traced back to a live attenuated strain of *Mycobacterium bovis*. As organism viability is essential for the stimulation of a protective immune response, monitoring the count of viable organisms is an integral part of vaccine quality control. The colony-forming unit (CFU) test has been the conventional assay for determining BCG viability, and is a widely accepted surrogate for BCG potency. CFU analysis, however, is problematic and time consuming. The slowness and high variability of CFU test results are the main driving forces for manufacturers and control laboratories to look for a rapid, more reproducible alternative viable count assay. A modified adenosine triphosphate (ATP) luminescence assay was developed by Statens Serum Institut and was promoted by the WHO as an alternative viable count assay. However, certain conditions during the processes of sample preparation and ATP extraction have to be established before the ATP assay can meet the requirements of robustness and reproducibility. This study is focused on identifying the conditions necessary for a reliable process of ATP analysis for BCG/*r*BCG preparations. Using our improved ATP assay protocol, we demonstrated that the correlation coefficient between CFU count and ATP concentration of BCG/*r*BCG vaccines was high (R^2=0.83 for accelerated stability samples and R^2>0.97 for all other preparations). The ATP luminescence assay is a rapid, sensitive, reliable, strain-non-specific method in quantification of the viability of live attenuated mycobacterial vaccine preparations.

Keywords: BCG/*r*BCG; Vaccine; ATP assay

Introduction

The viability of Bacillus Calmette–Guérin (BCG) vaccine has conventionally been monitored using a colony-forming unit (CFU) assay. This method is widely accepted despite the high variability of results. The coefficient of variation percentage (CV%) of results from samples of the same culture can be anywhere between 5 and 50%, largely due to the characteristically substantial clumping of mycobacteria. Another disadvantage of the CFU assay is that the colony forming process is very slow and, depending on the growth medium, may require an incubation period of up to 4 to 6 weeks before the colonies can be counted. The difficulties associated with the CFU assay impact BCG manufacturing and qualification processes [1].

In recent years, the intracellular adenosine triphosphate (ATP) assay has been developed as an alternative more rapid procedure for quantifying BCG cultivatable particles [2-5]. In contrast to the multi-week CFU assay, it can be completed within 1 day. The ATP assay is based on the reaction of firefly luciferase with ATP which results in a bioluminescent product [6-9]. Since ATP is a major metabolite of living cells and is rapidly lost in dead cells, measuring ATP content can provide a reliable estimate of the number of living cells in a culture. The ATP method has been applied to BCG vaccine evaluation and the first studies were reported in the 1970s. It was introduced in the European Pharmacopoeia 5.5 as an alternative method for microbiological quality control. In the following years several studies measuring ATP content in BCG and an improved method for extraction of ATP from BCG have been described [4,10]. Although the usefulness of the ATP bioluminescence assay for assessing the viability of a few BCG strains has been evaluated, the requirements of robustness and reproducibility have not been eventually reached. The broad viability among different lots and strains, lack of process standardization of sample preparation and ATP extraction, hardly persuade BCG vaccine manufacturers and regulation agency to finalize the decision of method replacement from CFU to ATP. Using a modified ATP assay protocol, we proposed a procedure for reliable applying the ATP method in routine evaluation of BCG/*r*BCG vaccines and planned to confirm the following assumptions in this study: 1) ATP analysis is strain non-specific; 2) within each strain or among different strains the intracellular ATP content is highly correlated with the number of BCG bacilli, with no difference in fresh culture, frozen bulk or lyophilized final product; 3) ATP has a limitation in detecting vaccines with low bacterial counts (e.g., accelerated stability sample), for which a cut-off ATP reading needs to be determined.

Materials and Methods

Live, attenuated mycobacterial vaccine strains

Three live attenuated mycobacterial vaccine strains were evaluated. AERAS-422 (Ag85A, Ag85B, Rv3407, *Δure*C: pfoAG137Q, *Δpan*CD) is a live attenuated *r*BCG vaccine [11]. Lyophilized BCG Danish 1331 strain was purchased from the Statens Serum Institut (SSI) as a control. Pasteur BCG originated from Trudeau mycobacterial culture collection stock TMC#1011, lot#9A2 (CBER/FDA).

Preparations of fresh culture, frozen bulk, lyophilized (high and low dosages) and extra-low dosage (37°C accelerated) samples

Fresh cultures of AERAS-422, Danish 1331 and Pasteur BCG were initiated from their frozen working seeds (~4.5 mL) inoculated into Middlebrook 7H9 medium plus extra nutrients, with a volume ratio of 1:100 [12]. One set of fresh cultures was grown in a 37°C incubator at a shaking rate of 120 rpm for 3 to 4 days, until the OD_{600nm} reached 4.0 to 5.0. The grown bacterial cells were collected by centrifugation at 2400 × g for 20 min, followed by re-suspension in 1/10 of the original volume in a frozen formulation (12.5% lactose, 0.05% tyloxapol, pH 7.4), then aliquoted and stored at -80°C labeled as frozen bulk product. Another set of fresh cultures for CFU, ATP and OD_{600nm} comparison studies were grown for at 37°C 167 hr. A 1 mL sample was collected at 64, 88, 112, 119, 143 and 167 hr from cultures for each strain. A previously developed formulation and lyophilization cycle was used to prepare high dosage (HD, diluted 10 fold from prepared bulk) and low dosage (LD, diluted 40 fold) vials including all three strains [11]. Extra-low dosages (Extra-LD) were generated from the lyophilized LD vials incubated under accelerated conditions at 37°C for 4 weeks.

CFU measurement

Each BCG/*r*BCG specimen was properly reconstituted/diluted with saline-tyloxapol (ST) buffer (0.95% sodium chloride, 0.05% tyloxapol) [11]. 0.1 mL samples were withdrawn from each vial/tube and diluted in Middlebrook 7H9 broth (BBL, BD, Sparks, MD) using a series of 1:10 dilutions. Then 0.1 mL of each sample with the appropriate number of replicates was plated onto Middlebrook 7H11 plates (Hardy Diagnostics, CA). The number of serial dilutions varied depending upon the estimated CFU/mL range of the specimen. The plates were then put in Ziplock Bags (3 plates per bag) and incubated at 36 ± 2°C for two to four weeks until visible colonies had developed. For each dilution series, a dilution plate containing not less than 30 and not more than 300 colonies was counted. For CFU analysis, three vials were pooled from each lot, and measured in triplicate on different days.

Establishment of ATP Analysis

Standard curve

The first step for ATP analysis is to derive a standard curve. A standard 10 mM ATP bulk solution was purchased from VWR International, PA. A 100 nM ATP standard working solution was prepared by diluting bulk solution with deionized (DI) water and stored at -20°C. The working volumes for the standard curve were 0, 5, 10, 25, 50, 100 and 200 µL, adjusted to a final reaction volume of 500 µL with DI water. The corresponding amounts of ATP for creating the standard curve were 0, 1, 2, 5, 10, 20 and 40 nM, with relative luminescence units (RLU) reading between 0.0 and 2400.0.

Sample dilution factor (I)

Based on the ATP concentration range of the standard curve and CFU count for each strain, the dilution factor to be applied to each strain was determined before ATP analysis. 0.2 mL of thawed frozen bulk products were appropriately diluted and mixed well; all other lyophilized vials including HD, LD and temperature-treated Extra-LD samples were re-suspended in 0.5 mL ST buffer, vortexed, and 0.2 mL were used for ATP testing. The final RLU reading of each sample after applying the dilution factor should fall into the middle range of the standard curve if possible. Each sample was tested in duplicate for each analysis.

Determination of extraction volume of hot TAE buffer

In order to efficiently break down BCG/*r*BCG bacterial walls and therefore maximize ATP extraction, hot Tris-acetate-EDTA (TAE) buffer (VWR, 10× buffer) was used during ATP extraction [7]. The extraction volumes (Ve) (1, 2, 3, 5, 7.5, 10 and 12 mL for bulk, HD and LD samples; and 0.5, 1, 2, 3, 5, 7.5 and 10 mL for Extra-LD samples) of hot TAE buffer were pre-tested using 0.2 mL (V0.2) of each re-suspended/diluted sample (for further details refer to the following section of ATP analysis). The optimum volume of hot TAE buffer was selected based on the generated curve of ATP concentration and dilution factor (II). The calculation of the dilution factor (II) for each sample is:

Dilution factor (II) = $[(V_e + V_{0.2})/V_{0.2}] \div 0.1$ (after hot TAE treatment, 0.1 mL sample was used for following ATP analysis).

ATP Analysis

12.5 µL of 100 U/mL apyrase (Sigma, MO, 100 U/vial, reconstituted in 1 mL DI water, aliquoted at 50 µL/vial, stored at -20°C) was added to each appropriately diluted 0.5 mL sample, mixed well and left at room temperature (RT) for 30 min. 0.5 mL of ST buffer containing 12.5 µL apyrase was used as a control. 5 mL of 1 × TAE buffer was transferred to each 15 mL conical centrifuge tube. After the tubes were capped and kept in boiling water for 15 min, 0.2 mL apyrase-treated sample was pipetted directly into each tube. The tubes were capped and kept in boiling water for 6 min, cooled down to RT in ice-water, and then centrifuged at 4000 rpm for 15 min to avoid any analysis interference from cell debris. 100 µL of respective supernatant and diluted ATP standards were transferred in triplicate to Microtiter® 96-Well Fluorescence white microplates (Thermo Scientific, Rochester), followed by addition of 100 µL mixed BacTiter-Glo Microbial Cell Viability Assay reagent (Promega Corporation, WI) to each well by a multiple channel pipette. The BacTiter-Glo reagent was freshly prepared by mixing buffer and substrate included in the assay kit, and leaving it at RT for 2 min to release any bubbles generated. The plates were transferred onto a rotating plate shaker for 5 min at 350 rpm, and fluorescence intensity readings were then recorded by a Spectramax M2e plate reader (Molecular Devices, CA). ®

Establishment of linear relationship and conversion equation for CFU count and ATP concentration

To define the linear relationship between CFU count and ATP concentration for each BCG/*r*BCG strain, log values of corresponding CFU count and ATP concentration were plotted using Excel (scatter chart format), and corresponding conversion equations and correlation coefficient (R) values were determined.

Results

Handling of BCG/*r*BCG samples

To identify conditions that would reduce both size and number of cell clumps, we re-suspended BCG/*r*BCG samples in DI water, saline or ST buffer, respectively. Representative clumping appearances are

shown in Figure 1. The least amount of clumping was seen using ST buffer, followed by saline and then DI water which had the most clumpings. Therefore, ST buffer was selected for re-suspension or dilution of the BCG/*r*BCG samples tested in this study.

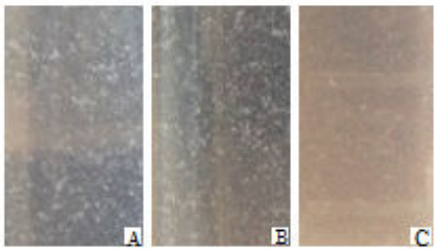

Figure 1: Clumping appearances of a BCG sample re-suspended in different solutions. A) DI water; B) Saline; C) ST buffer.

Establishment of a standard curve

Using the prepared standard ATP (no BCG/*r*BCG being involved), there was a strong correlation between concentration of ATP and RLU, with an R2 value typically more than 0.99 (Figure 2).

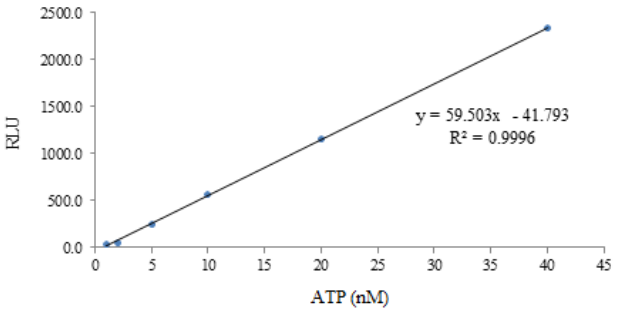

Figure 2: A representative standard curve in ATP analysis.

Dilution factor (I) of BCG/*r*BCG preparations

The dilution factor (I) to be applied to each of the bulk, HD, LD and Extra-LD preparations was determined (Table 1). After bulk materials of AERAS-422 and Danish were diluted 100-fold, and Pasteur was diluted 200-fold, the average RLU reading of each tested sample was in the linear range of the standard curve (data not shown).

Dosage range (CFU/mL)	Danish	AERAS-422	Pasteur
E+8 to 9 (Bulk)	100	100	200
E+7 to 8 (High dosage)	1	1	10
E+6 to 7 (Low dosage)	1	1	1
<E+6 (Extra low dosage)	1	1	1

Table 1: Dilution factor (I) of BCG/*r*BCG preparations.

Samples from lyophilized vials with different dosages were directly analyzed without any dilution (dilution factor is regarded as one). High dosage Pasteur was diluted 10-fold prior to analysis.

Extraction volume of hot TAE buffer

The minimum extraction volume of hot TAE buffer was determined for different BGC/*r*BCG samples. Example plot generated with dilution factor (II) vs ATP concentration for the LD product is shown in Figure 3. Plot of bulk, HD or Extra-LD preparation also had the similar trend (data not shown) as the LD product. There was an increasing amount of ATP was detected with increasing volume of extraction buffer. However, a plateau was reached when 10 to 20-fold volume (5-10 mL) of hot TAE buffer to treat 0.2 mL sample. It showed that 5 mL of TAE buffer was sufficient to extract ATP from 0.2 mL tested samples. 5 mL of hot TAE buffer was therefore used for the rest of this study.

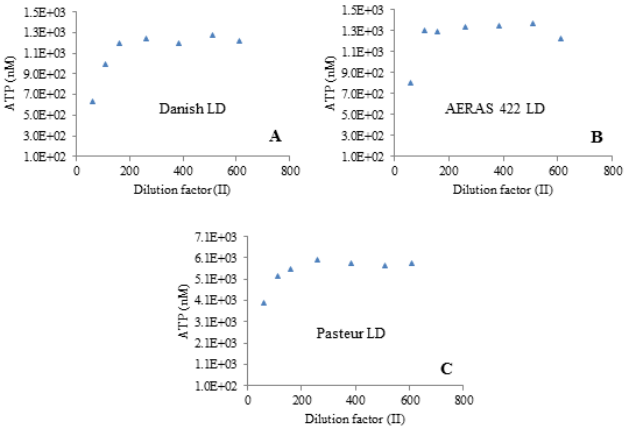

Figure 3: Determination of extraction volume (hot TAE buffer) for each strain. The x-axis of dilution factor (II) was calculated from the samples after adding different volumes of hot TAE buffer.

The relationship among ATP, CFU and OD_{600nm} readings

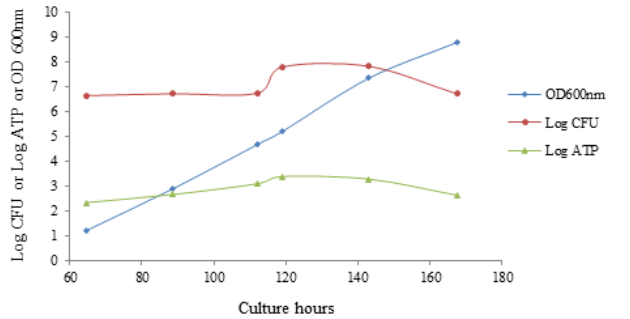

Figure 4: Relationship among the ATP, CFU and OD_{600nm} readings

To evaluate the relationship of ATP, CFU and OD_{600nm} readings of fresh culture, a time course comparison study up to 167 hr was executed using the Danish BCG strain (Figure 4). A good linear

relationship was seen between ATP and CFU at each time point, but not for corresponding OD_{600nm} readings. The ATP and CFU results were relatively stable between 64 and 112 hr and between 119 and 143 hr, with an increase from 112 to 119 hr and a decrease after 143 hr. As expected, during the studied culture period, the OD_{600nm} readings of the tested culture continually increased, presumably due to the decline of cultural viability and the accumulation of dead cells.

The relationship between ATP concentration and CFU count of different BCG/*r*BCG strains

The relationship between CFU count and ATP concentration of BCG/*r*BCG strains (Danish, AERAS-422 and Pasteur) is shown in Figure 5. Overall, a good linear relationship was shown between ATP concentration and CFU count for each strain. The linear relationship between ATP concentration and CFU count of the bulk, HD and LD products in Danish and AERAS-422 strains was most robust (R^2>0.99, Figure 5A and 5C), while the Pasteur strain also showed a good linear relationship (R^2=0.9773, Figure 5E). If the Extra-LD product was included, the Danish and Pasteur strains again displayed a good linear relationship (R^2>0.97, Figure 5B and 5F), the AERAS-422 strain showed a less strong but still acceptable linear relationship (R^2= 0.8635, Figure 5D).

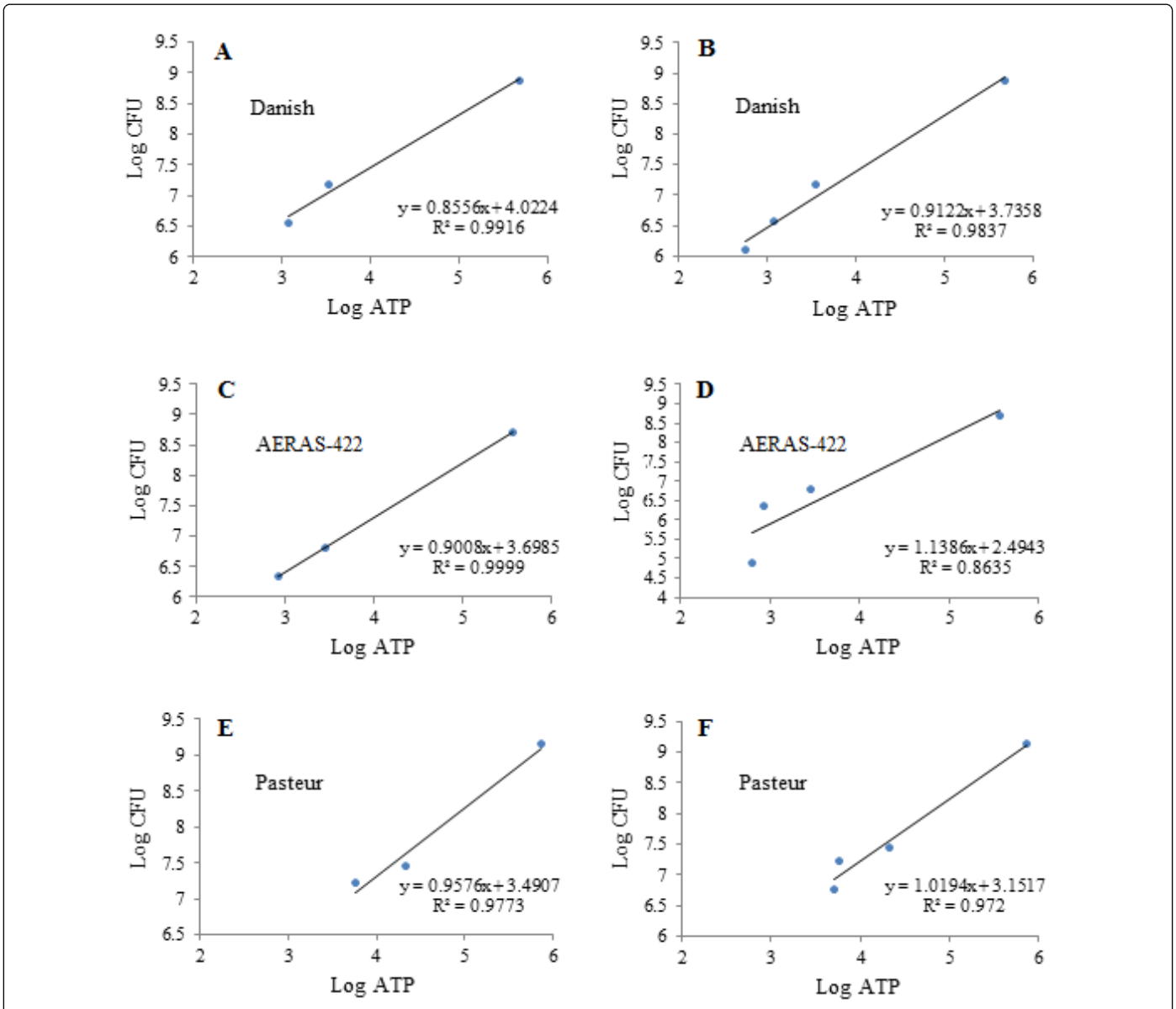

Figure 5: Linear relationship between the CFU count and ATP concentration of the same BCG/*r*BCG strain. A, C and E were generated from the average results of 3 different dosage forms (bulk, HD and LD) for each strain; B, D and F were generated from addition of Extra-LD data for each strain (for original values refer to Table 2).

To evaluate the relationship between ATP concentration and CFU count among different strains with different dosages (bulk, HD, LD and Extra-LD), cross-comparison plots were drawn in Figure 6 (Refer Table 2 for the average values of ATP and CFU calculated from multiple samples).

Except a low CFU count and maybe limitation of ATP measurement generated from AERAS-422 Extra-LD samples, all curves with the wide dosage ranges among different BCG/*r*BCG strains showed the similar tendency between ATP concentrations (E+2 to E+5 nM) and CFU counts (E+6 to E+9/mL).

	ATP*			CFU*		
	nM	CV%	No. of tested samples	CFU/mL	CV%	No. of tested samples
Danish						
Bulk	4.75E+05	10.2	28	7.49E+08	42.7	27
High dosage	3.50E+03	21.1	24	1.48E+07	28.3	27
Low dosage	1.20E+03	30.5	24	3.67E+06	26.7	27
Extra low dosage	5.79E+02	24.6	24	1.22E+06	20	27
AERAS-422						
Bulk	3.66E+05	12	28	5.16E+08	24.1	27
High dosage	2.87E+03	24.6	24	6.37E+06	37.1	27
Low dosage	8.50E+02	25.2	24	2.24E+06	19.9	27
Extra low dosage	6.11E+02	17.2	24	4.64E+04	15.4	8
Pasteur						
Bulk	7.17E+05	8.8	28	1.40E+09	49.2	18
High dosage	2.13E+04	34.4	24	2.88E+07	30.7	18
Low dosage	5.81E+03	20	24	1.65E+07	18.7	18
Extra low dosage	5.05E+03	10.1	10	5.84E+06	52.6	18

Table 2: Comparison between ATP concentrations and CFU counts. *The results of ATP and CFU for each dosage form of BCG/*r*BCG were an average value of the tested samples.

Discussion

There are many reasons for extracting and measuring ATP from cells, but they usually can be placed into one of two main categories: 1) use the level of endogenous ATP as an index of energy status in metabolic and physiological studies; and 2) estimate cell numbers in microbial and tissue cultures, assuming that the ATP per cell remains a fairly constant and known value under defined conditions. Thus by measuring total intracellular ATP in a sample of culture, cell numbers may be rapidly determined. This is the basis of rapid enumeration using the ATP-firefly luminescence technique. In this study, we re-evaluated critical conditions for the ATP luminescence assay, and established a linear relationship between ATP concentration and CFU count for all stage preparations (live culture, frozen bulk, lyophilized high and low dosages, and stability samples) of three different BCG/*r*BCG vaccines.

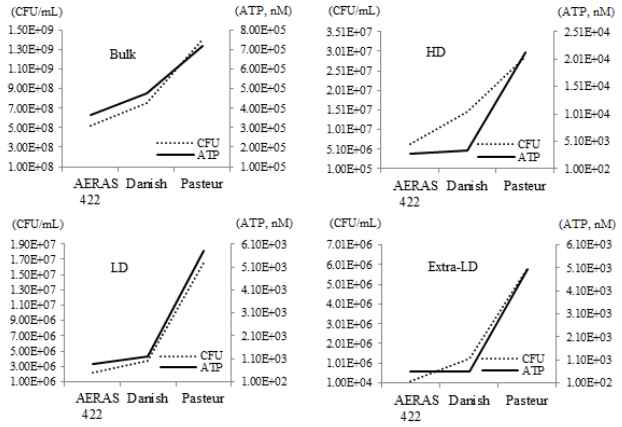

Figure 6: ATP concentration and CFU count cross-comparison plots for the same range of dosage among different strains.

Mycobacteria have a tendency to clump together in all different stages of BCG/*r*BCG production, mostly because of the lipid content of the cell wall [12]. Highly branched structures heavily covered by lipid cell walls with few free bacilli are common observations under microscopy using the acid fast staining procedure. Without proper handling of BCG/*r*BCG samples, unreliable results with a high percentage of variation are typically generated in both ATP and CFU assays. Another problematic issue resulting from clumping in relation to the CFU assay is that lot-to-lot and vendor-to-vendor differences of agar media (such as Middlebrook 7H10 and Middlebrook 7H11) can generate 1- to 2-log CFU variance for samples from the same culture (data not shown). To decrease the variation caused by clumping in BCG/*r*BCG samples, we incorporated tyloxapol, a non-ionic detergent, into the ATP and CFU analytical processes to reduce clumping propensity and provide a more even suspension of branched bacilli samples. In addition, we propose that pre-selection with different agar media/vendors should be completed to determine optimal growth of each strain before CFU result is to be used for further comparison ATP analysis. A known standard control needs to be included and a qualification test needs to be performed on each lot of agar plate to verify its suitability for CFU evaluation.

The next critical step is to completely extract intracellular ATP from clumping mycobacterial cells. The ideal extraction agent should:

Penetrate the cell wall and membrane more or less instantaneously.

Extract ATP more or less instantaneously.

Extract the target intracellular ATP pool completely.

Stabilize the ATP pool by instantaneously and irreversibly inactivating all enzymes that use ATP as a substrate or produce ATP from other substrates.

Have no inhibitory (quenching) effect on firefly luciferase during the ATP assay.

Not cause breakdown of ATP (e.g., hydrolysis) either in the short term (at the extraction time) or long term (during storage).

Not have an effect on the kinetics of the firefly reaction.

Which would cause signal distortion and consequently affect internal standardization. It is essential that extractant is rapidly and intimately associated with all cells. If the cells clump together, the extractant may take longer to penetrate to the inner cells by which time considerable changes in ATP content may have taken place. The use of boiling buffer or cold acid (the latter requires immediate neutralization following extraction) fulfills these needs but also introduces sample dilution (and thus loss in sensitivity for low ATP concentrations) or extra manipulation. However, this is not a concern with respect to BCG/*r*BCG preparations which have relatively more available cells for ATP extraction. We emphasize that addition of BCG/*r*BCG sample directly into hot TAE buffer (pre-heated in boiling water) is critical. It is important not to overload the extractant with too many cells. Usually a few milligrams dry weight of sample per milliliter of extractant can fit the purpose. If boiling buffer is applied, we recommend that the ratio of sample volume to boiling buffer is 1:10 to 1:20 for routine BCG/*r*BCG sample treatment. The ATP extraction process selected hot TAE buffer as recommended extractant was based on its operational simplicity and reliable result.

Before ATP extraction is performed it is necessary to be aware of there are still many factors that could impact the reproducibility of a ATP analysis:

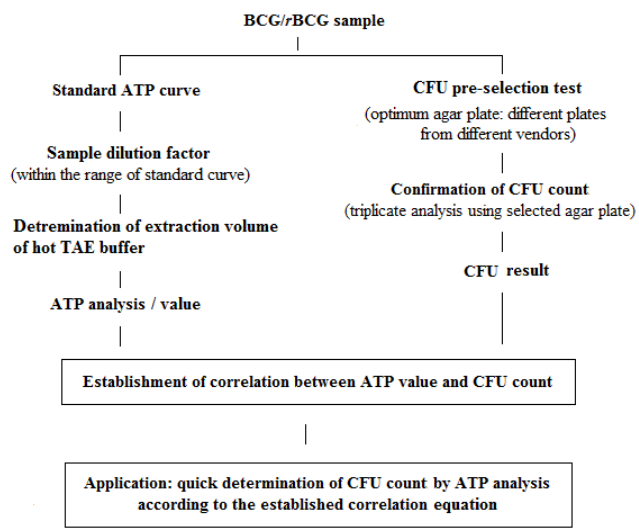

Figure 7: Proposed procedure: ATP method application in quality evaluation of a BCG/*r*BCG vaccine.

- Age of cells or stage of growth.
- Stage of cell division.
- Density of cells.
- Change of growth rate.
- Change of pH.
- Environmental change of light flux (for light sensitive organisms, such as BCG/*r*BCG).
- Microbial contamination [7,13,14].

As a result, qualified procedures to determine both CFU count and ATP concentration are necessary before the CFU assay is to be replaced by ATP method, especially for the final quality control application of ATP method in routine BCG/*r*BCG vaccine preparations.

In summary, we propose a procedure on applying the ATP method in quality evaluation of BCG/*r*BCG vaccine (Figure 7). Our results showed that the luciferin-luciferase bioluminescence reaction provides a sensitive assay for ATP, with light output directly proportional to the amount of ATP present, demonstrating a highly significant correlation between the results of ATP analysis and CFU test. The ATP analysis was shown to be non-specific, within samples from the same or different strain(s), the intracellular ATP content was highly correlated with the number of BCG bacilli for both in-process and final lyophilized products. Moreover, we demonstrated that the ATP method may have a limitation in detecting low bacterial count sample, such as stability sample in accelerated study. Although it was still in the acceptable range tested with our high temperature treated samples, a cut off value or acceptable range may need to be evaluated and determined. The relative lower correlation between the ATP and CFU methods for accelerated samples may be caused by interference from dead clumping cells blocking release of ATP, or undetectable but still live bacilli in the CFU assay which results in a low CFU count but a high ATP reading.

It is difficult to determine whether ATP concentration or CFU count represents a "close to real" measurement of viability in BCG/*r*BCG samples, since the "gold-standard" CFU method is highly variable, and no other approach has been identified as a control for the qualification of ATP method. The reality of impaired culturability, as well as resuscitation from a state of dormancy for high temperature treated BCG/*r*BCG samples, further complicate the qualification process. The implementation of pre-testing of optimum agar plate for maximal bacterial growth to individual strain is a critical step in the replacement of CFU method with fast and convenient ATP analysis.

Using the improved and more reliable ATP assay protocol, we showed that the intracellular ATP content correlated with the number of bacilli in different BCG/*r*BCG strains. Overall, these data indicate that the modified ATP luminescence assay is a rapid, sensitive, reliable, strain-non-specific method in quantification of the viability of live attenuated mycobacterial vaccine preparations.

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