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Suitability of alternative adenosine triphosphate potency assay for lot release of Tokyo bacilli Calmette-Guerin - 172-1 Vaccines in Thailand

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ABSTRACT

Objectives: The objective of this study is to establish the suitability of alternative adenosine triphosphate potency assay for lot release of Tokyo bacilli Calmette-Guerin-172-1 Vaccines in Thailand. **Materials and Methods:** Each vial of the BCG vaccine was reconstituted in 1 ml of Dubos medium supplemented with 0.045% of Tween 80 and 0.5% of bovine albumin. For the IRP, the BCG Tokyo172 substrain ampoule was diluted at 1:4 in the same medium used for the vaccine. After incubation, the BCG culture was extracted with buffers and then ATP reagent was added in to each well and mixed. The luminescence of the sample and standard were measured in an Orion L Microplate Luminometer at 25 °C. **Results:** The ATP assay kits included extraction reagents from three commercial kits that showed different ATP activity of the BCG vaccine; the cell viability kit and extraction reagent from the BioThema found a higher amount of ATP activity than the other two commercial kits. To reduce the cost of the assay, three lots of in-house extraction reagents were separately prepared and assessed along with the cell viability kit for the detection of ATP activity of the BCG vaccines. Using a commercial kit that was a combination of the BioThema and in-house extraction reagent, it was proven effective for determining the viability of mycobacterium from the BCG vaccines. No difference in ATP activity was found when the in-house extraction reagent was used instead of commercial reagents. **Conclusion:** The suitability of an alternative ATP potency assay was routinely used for the lot release of the BCG vaccine in Thailand by using an in-house extraction reagent since it was easy to utilise and took only 2 days to produce the estimate of the viable cell content compared with 4 weeks for the conventional method.

Keywords: Adenosine triphosphate assay, bacilli Calmette-Guerin vaccine, potency and activity

INTRODUCTION

The bacilli Calmette-Guerin (BCG) vaccine has existed for 80 years and is widely used as a vaccine against *Mycobacterium tuberculosis*, including meningitis and disseminated tuberculosis bacilline in children.[1,2] BCG vaccine is a live attenuated strain of *Mycobacterium bovis*. Its viability is essential for the stimulation of protective immune responses, and therefore monitoring the viable count is an integral part of quality control. Three different substrains of BCG (Danish 1331, Russian BCG-1, and Tokyo 172-1) were identified by multiplex polymerase chain reaction and qualified using both a cultural viable count and modified adenosine triphosphate (ATP) assay.[3] They represented the dominant substrains used for BCG vaccine production and distribution for worldwide use. On the basis of the results from these studies, the National Institute of Biological Standards and Control (NIBSC) propose, all three substrains of the BCG vaccine preparations (NIBSC code: 07/270: Danish BCG 1331-2, NIBSC code: 07/272: Tokyo BCG TY 1002, and NIBSC code: 07/274: Moscow BCG 254-2) are established as reference reagents with an assigned colony forming unit (CFU) and ATP content per ampoule.[4] Freeze-dried BCG vaccine is a preparation of live mycobacteria produced by the culture of a substrain of the BCG. The potency of this vaccine is dependent on its content of cultural viable cells known as a CFU test. Assay is widely used for live vaccine as a standard method used worldwide. *M. bovis* BCG is a slow-growing organism; cultivation is time-consuming, and the

estimation of a viable count takes 1 month. Recently, the World Health Organization (WHO) proposed recommendations for BCG vaccine potency testing; alternatively, a bioluminescence or other biochemical method could be used.[1,2,4] The modified ATP method was introduced and developed by the Staten Serum Institute as an alternative rapid assay for the viable count of the BCG Danish 1331 vaccine.[5] However, no other countries or laboratories have used an alternative ATP assay for routine potency testing of the Tokyo BCG-172-1 vaccine. To compare the commercial ATP assay kits and set the protocol for the alternative ATP potency assay for the release of the BCG vaccines in Thailand, the ATP assay was conducted for the suitability of an alternative ATP assay for further use as a routine potency testing of the BCG vaccine in Thailand.

MATERIALS AND METHODS

International Reference Preparation (IRP)

An international reference standard (IRP) was prepared by the Japan BCG Laboratory, Tokyo, Japan in 2006. It was established as the first WHO reference reagent of the BCG vaccine of the Tokyo 172 substrain in 2009. The intended uses of this material were as a comparator or reference for the validity and consistency monitoring in viability assays; such as the cultural viable count and modified ATP assays. The IRP of the Tokyo 172 substrain (NIBSC code: 07/272) consisted of 49.4×10^6 CFU/ml of culture particles with a standard deviation (SD) of 5.9 and 217.6 ng ATP with a SD of 27.5 per ampoule. This preparation was stored at −20°C for long-term storage to preserve the viability and protect it from direct sunlight.

Working Reference Preparation (WRP)

A single batch of freeze-dried WRP lot no. BCG030313 obtained from the Thai Red Cross Society was used in the ATP assays and stored at −70°C for use as an in-house reference standard. It contained 12.26×10^6 CFU/ml of culture particle with a SD of 1.96.

BCG Vaccine Samples

Seventeen batches of freeze-dried BCG vaccine of the Tokyo 172-1 strain were obtained from the Thai Red Cross Society and kept at 2–8°C.

Culture Medium

Dubos medium with 0.045% of Tween 80 and 0.5% of bovine albumin (Sigma, U.S.A.) and Middlebrook 7H9 (Cat. No. 271310, BD Difco, USA) supplemented with 10% of Oleic Albumin Dextrose Complex (OADC), and 0.2% of glycerol was used for a 1-day culture of BCG vaccines.

Reagents for the ATP Assay

The intracellular ATP was measured using a cell viability kit SL (BioThema, Sweden) that was intended for the cell proliferation by determining the total ATP. Extractant B/S was included in the kit releases of the ATP from most types of cells and inactivated ATP degrading enzymes. ATP was assayed using the ATP Reagent. The intensity of the light was proportional to the amount of ATP and was measured in an Orion L Microplate Luminometer (Titertek-Berthold technologies, Sweden). For maximum accuracy, the light emission was measured before and after the addition of a known amount of the ATP standard.

Determination of the ATP in the BCG Vaccine

Each vial of the BCG vaccine was reconstituted in 1 ml of Dubos medium supplemented with 0.045% of Tween 80 and 0.5% of bovine albumin, and the reconstituted vaccine was transferred into a 2.0 ml sterile screw-capped tube. For the IRP, the BCG Tokyo172 substrain ampoule was diluted at 1:4 in the same medium used for the vaccine. The reconstituted BCG suspensions were incubated at 37°C for 22–26 h. The following day, the BCG culture was then prepared in a pre-warmed Dubos medium as an undiluted, 1:2 and 1:4 dilutions. 100 µl of each dilution of the BCG suspension was added directly to 0.9 ml of the pre-heated extraction buffer after boiling in a water bath for 2 min. The BCG-buffer mixture was returned to the boiling water bath and kept for 1 min to complete the extraction.[6] Each extract was allowed to cool down at room temperature, and 20 µl were then transferred into three individual wells of the white OptiPlate-96 microplate (Cat. No. 3912, costar, USA). 80 µl of the ATP reagent SL was added to all wells and mixed. Furthermore, 10 μl of the 10 µmol/L ATP standard was added to all wells and mixed again. The luminescence (I_{smp} and $I_{smp+std}$) of the sample and standard from all wells were measured in an Orion L Microplate Luminometer (Titertek-Berthold technologies, Sweden) at 25°C.

Preparation of an In-house Extraction Reagent

The extraction buffer was prepared in the laboratory to compare with the commercial buffers. 2 g of dodecyl trimethyl ammonium bromide were dissolved in 100 ml of Tris-Ethylenediaminetetraacetic acid (EDTA) buffer, which contained 0.1 mol/L of Tris(hydroxymethyl) aminomethane and 2 mmol/L of EDTA and then adjusted to pH 7.75 with acetic acid.

Measurement of the ATP

The calculation of the ATP content of an ampoule was completed on an Excel sheet (Microsoft® Office Excel 2010). The ATP content was calculated and expressed as ng ATP per ampoule or vial.

The amount of ATP was calculated using the following formula:

ATP_{smp} (ng/ampoule) $= 100 \times I_{smp}/(I_{smp+std} - I_{smp}) \times 100 \times 573.1 \times$ dilution factor

ATP_{smp} = ATP in the sample

I_{smp} = The luminescence of the sample

$I_{smp+std}$ = The luminescence of the sample and the ATP standard

The factor 100 is the amount of the ATP Standard added (100 pmol).

573.1 is the molecular weight of ATP.

Determination of the Culture Particle Count in the BCG Vaccine

The conventional method for BCG potency testing is routinely performed by culture particle counts in a selective medium.

In this study, the culture particle counts were assessed and compared the testing results with the alternative ATP assay using WRP. Briefly, 10 vials of BCG vaccines were reconstituted with 1 ml/vial of Sauton solution. The BCG suspensions from the 10 vials were pooled, and the suspension was diluted in 100-fold serial dilutions at the first and second dilutions. Then, the second dilution made two-fold serial dilutions for inoculation onto a Lowenstein-Jensen medium. After inoculation, 10 tubes of each dilution were incubated at 37°C for 4 weeks before counting and calculating the number of the CFU.[7]

Comparison between the Culture Particle Count and ATP Methods

The BCG samples were assayed with both the conventional particle count and the ATP method. The viability of the samples included a stability test by keeping the samples at 37°C for 28 days, whereas the potency test was refrigerated for a short period.

RESULTS

Estimation of the Intracellular ATP from the Assay Kits and Extraction Reagents

At least three kits (cell viability kit, BioThema; Bactiter glow, Promega, and ATP lite, ABI) and extraction reagents (BioThema, Promega, and ABI) were compared to select the most appropriate kit and reagent for the assay. The amount of ATP activity of WRP was measured and shown in Table 1.

It was found that using a cell viability kit and extraction reagent from BioThema showed a high amount of ATP (23.60 ng/dose), whereas the other companies (Bactiter glow and ATP lite) had a low yield of ATP (2.31 and 9.11 ng/dose). From these results, the cell viability kit and reagent from BioThema were the most appropriate for the ATP assay.

Comparison of the Extraction Reagents with Three Different Assay Kits

To seek a good combination of the ATP kit and extraction reagent, the BioThema extraction reagent and another two reagents were compared with each assay kit from each company. As shown in Table 2, the assay kit and reagent from BioThema had 15.22 ng/dose that was almost similar to the amount of ATP when using the Promega kit and BioThema reagent, whereas other combinations between the kits and reagents showed a lower amount of ATP or an undetectable ATP.

The results revealed that both the BioThema and Promega kit were able to use the extraction reagent from BioThema.

Comparison of a Suitable Extraction Buffer with an In-house Extraction Reagent

To ensure which combination was the most appropriate for the ATP assay, an in-house extraction reagent was prepared and tested for the replacement of the BioThema extraction reagent. In the following experiment, the ATP yield from a commercial extraction buffer was compared with an in-house extraction reagent. Three independent assays using BCG working reference standards were 23.17, 19.52, and 19.68 ng/dose (geometric mean [GM] = 20.79 ng/dose) for the commercial extraction reagent, BioThema, and were 26.57, 32.77, and 26.91 (GM = 28.75 ng/dose) for the in-house extraction reagent. It was found that the ATP yield of the in-house extraction reagent was comparable with BioThema when used as an extraction reagent [Table 3].

Evaluation of In-house Extraction Reagent

To ensure that the in-house extraction reagent could be used instead of a commercial extraction reagent, three extraction reagents prepared by three analysts were compared at the same time. The results showed that there were no different ATP activities among the three preparations with 6.83% of the mean CV [Table 4].

Comparison between the Culture Particle Count and ATP Assay

To conduct a comparison between the two methods for a culture viable count and ATP assay, the BCG samples from real storage at 4°C and heat-treated at 37°C for 28 days were performed. It was found that the ATP content showed comparable results to the particle counts [Figure 1].

The BCG vaccine samples with different viabilities were assayed with both the ATP method and culture viable count. The high viability samples were newly prepared, lyophilized BCG refrigerated for potency testing, and the low viability samples were heat-treated for 28 days at 37°C for stability testing. A WRP was also included. The correlation of the

Table 1: Estimation of the intracellular ATP assay from the assay kits and extraction reagents

Assay kit	Extraction reagent	ATP (ng/ampoule)*
Cell viability kit (BioThema)	BioThema	23.60
Bactiter glow (Promega)	Promega	2.31
ATP lite (ABI)	ABI	9.11

*WRP, lot BCG 030313 was used in the assay to compare the ATP activity among the three companies. Three independent assays were performed, and a GM was expressed in ng/ampoule of the ATP activity. ATP: Adenosine triphosphate, WRP: Working reference preparation, GM: Geometric mean, BCG: Bacilli Calmette-Guerin

Table 2: Comparison of the extraction reagents with three different assay kits

Assay kit	Extraction reagent	ATP (ng/dose)*
Cell viability kit (BioThema)	BioThema	15.22
Bactiter glow (Promega)	Promega	1.34
	BioThema	14.66
ATP lite (ABI)	ABI	2.75
	BioThema	Undetermined
	Promega	5.46

*WRP, lot BCG 030313 was used in the assay to compare the ATP activity among the six combinations. Three independent assays were performed, and a GM was expressed in ng/ampoule of each combination. ATP: Adenosine triphosphate, WRP: Working reference preparation, GM: Geometric mean, BCG: Bacilli Calmette-Guerin

ATP content and CFU is shown in Figure 1. Both methods demonstrated the effect of the temperature on the viability and a smaller loss of viability obtained by the ATP than by a CFU measurement.

Determination of the Optimal Culture Medium for Viable Mycobacteria

From Table 4, the ATP content of WRP BCG (lot FB01915) using an in-house extraction buffer decreased that was different from the ATP content found in Table 3. It was possible that the culture medium may influence the viable mycobacteria. From the further study, the BCG vaccine was cultured in different kinds of media, and the results are shown in Table 5.

The results showed that the ATP content of the BCG cultured by the Dubos medium (supplemented with 0.5% of albumin and 0.045% of Tween 80 and sterilized by Millipore filtration) produced the highest amount of ATP followed by culturing the BCG with Middlebrook 7H9 broth (supplemented with 10% of OADC and 0.2% of glycerol), whereas the BCG vaccine cultured with the Dubos medium (supplemented with 0.5% of albumin and 0.045% of Tween 80 and sterilized by autoclave) gave the lowest amount of ATP.

DISCUSSION

The quality control of BCG vaccine includes a necessary counting of living organisms. The classical method involving colony counting is not optimal because *M. bovis* BCG is a slow-growing bacterium and cultivation is time-consuming. In addition, because of their hydrophobicity and tendency to clump, enumeration using colony forming units does not provide an exact or reproducible estimate of the number of viable mycobacteria in a vaccine.[8] A more rapid and accurate method for quantitation would be a considerable improvement in the quality control of the BCG vaccine.[9] In recent years, there has been increasing interest in the development of the intracellular ATP assay for BCG vaccines to rapidly quantitate the number of viable organisms within live attenuated mycobacterial vaccine preparations.[10] However, the alternative ATP method has not yet widely implemented as a routine form of testing for the lot release of BCG vaccines. According to European Pharmacopoeia and WHO for the assay of the viable cell content of BCG vaccines, determination of ATP by a bioluminescence reaction could be used as an alternative method.[11] In the researchers' previous study, alternative methods were validated and compared to the conventional method (in press). Thus, the aim of this study was to compare the commercial ATP assay kits and set the protocol for the alternative ATP potency assay to examine the suitability of an alternative ATP potency assay for the lot release of Tokyo BCG 172-1 vaccines in Thailand. These experiments showed that the bacterial number determined by the ATP assay using cell viability and an extraction reagent from the BioThema gave higher amounts of ATP than the other two companies; namely Bactiter glow, Promega, and ATP lite from ABI. When comparing the suitable extraction reagent from the BioThema with different assay kits, it was found that both the BioThema and Promega kit could be used with the same extraction buffer of the BioThema, resulting in the BioThema reagent being the most appropriate reagent for the extraction of ATP from the viable cells. To reduce the cost of the assay, an in-house extraction reagent was prepared and used for the ATP assay by comparing it with the BioThema extraction reagent. In all cases, in-house extraction reagents provided comparable results and showed good reproducibility in each lot prepared. During the development stage, the

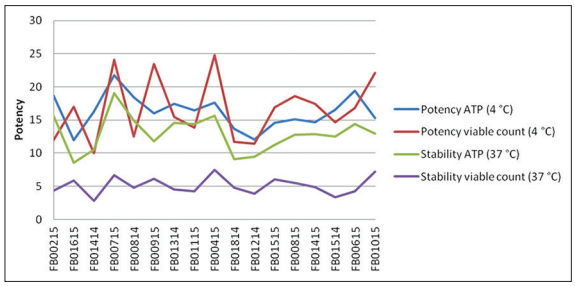

Figure 1: Comparison between the culture particle count and adenosine triphosphate assay*. *Working reference preparation, lot bacilli Calmette-Guerin (BCG) FB01915 was used in the assay to compare the adenosine triphosphate activity and cultural particle count. Independent assays in each lot of BCG vaccine were performed using the BioTherm assay kit and in-house extraction buffer

Table 3: Comparison of a suitable extraction reagent with in-house extraction reagent

WRP no.	ATP (ng/ampoule)*	
	Commercial extraction reagent (BioThema)	In-house extraction reagent
1	23.17	26.57
2	19.52	32.77
3	19.68	26.91
Mean ± SD	20.79 ± 2.06	28.75 ± 3.49
%CV	9.92	12.12

*WRP, lot BCG FB01915 was used in the assay to compare the ATP activity between the BioThema and in-house extraction reagent. Three independent assays were performed, and a GM was expressed in ng/ampoule of each extraction reagent. ATP: Adenosine triphosphate, WRP: Working reference preparation, GM: Geometric mean, BCG: Bacilli Calmette-Guerin, SD: Standard deviation

Table 4: Evaluation of in-house extraction reagent

Analyst	ATP (ng/ampoule)*		
	Lot no. 1	Lot no. 2	Lot no. 3
1	13.93	13.21	12.73
2	14.34	14.69	15.39
3	12.83	13.87	14.18
Mean	13.70	13.92	14.10
SD	0.78	0.74	1.33
%CV	5.70	5.33	9.45
Mean±SD (total)		13.91±0.87	
%CV (total)		6.28	

*WRP, lot BCG FB01915 was used in the assay to compare the ATP activity among three lots of in-house extraction reagents. Three independent assays were performed, and a GM was expressed in ng/ampoule of each lot. ATP: Adenosine triphosphate, WRP: Working reference preparation, GM: Geometric mean, BCG: Bacilli Calmette-Guerin, SD: Standard deviation

Table 5: Determination of the optimal culture medium for the viable mycobacteria

Type of medium	ATP (ng/ ampoule)*
Dubos medium (supplemented with 0.5% of albumin and 0.045% of Tween 80 and sterilized by autoclave)	11.40
Dubos medium (supplemented with 0.5% of albumin and 0.045% of Tween 80 and filtrated by Millipore filtration)	119.88
Middlebrook 7H9 broth (supplemented with OADC and 2% of glycerol)	86.0

*In- house reference standard, lot BCG FB01915 was used in the assay to compare the ATP activity among the different media for the viable mycobacteria. OADC: Oleic Albumin Dextrose Complex, BCG: Bacilli Calmette-Guerin, ATP: Adenosine triphosphate

ATP assay was compared with a conventional CFU method to count the number of viable units. A good comparable testing between the ATP content and CFU was found when the samples were kept at 4°C for potency testing and 37°C for stability testing. Furthermore, the researchers demonstrated that the optimal culture medium for viable mycobacteria was the Dubos medium (supplemented with 0.5% of albumin and 0.045% of Tween 80 and filtrated by Millipore filtration). This was quite a new finding for the culture medium since most studies used only the Dubos medium or other suitable culture media,[12] but none were specified for the supplementary medium. Albumin added between the filtration and autoclave showed a completely different ATP content. More importantly, the researchers demonstrated that BCG potency testing could be evaluated using the ATP bioluminescence assay. In general, the luciferase enzyme used in the method was specific for ATP,[13] but not for BCG. This was because the BCG samples were assayed, and negative control without the BCG vaccine was also performed in parallel to determine the ATP content from other microorganisms. Although ATP activity can be detected in all living organisms that are not specific for Mycobacterium, in this experiment no microorganism in the negative control culture was detected (data not shown). The current CFU method for assessing the live vaccine viability is time-consuming, labor-intensive, and often not reproducible. The 4-week incubation period required to generate colonies substantially delays the production process. The necessity for using complex media to cultivate mycobacteria can increase the variability of the ATP assay. The researchers investigated this variable factor and showed that the ATP activity of a filtrated medium (Dubos medium) was extensively higher than an autoclaved medium, whereas when using another medium like 7H9 broth, this also showed nearly the same amount of ATP activity as using a filtrated Dubos medium. From other studies, the ATP assay showed high variability in the results[12] because there were some critical parameters such as culture medium and assay test kits; in contrast, the researchers found that the CV in each assay ranged from 5.33% to 12.12% and was >10% when using three lots of each in-house extraction buffer preparation. Overall, the researchers have shown that the alternative ATP assay is suitable for assessing the potency values of the Tokyo BCG- 172 vaccine in Thailand and can be used routinely for lot release of the products.

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