ORIGINAL ARTICLE

Development of a New Approach to Determine the Potency of Bacille Calmette–Guérin Vaccines Using Flow Cytometry

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Objectives: To circumvent the limitations of the current golden standard method, colony-forming unit (CFU) assay, for viability of Bacille Calmette–Guérin (BCG) vaccines, we developed a new method to rapidly and accurately determine the potency of BCG vaccines.
Methods: Based on flow cytometry (FACS) and fluorescein diacetate (FDA) as the most appropriate fluorescent staining reagent, 17 lots of BCG vaccines for percutaneous administration and 5 lots of BCG vaccines for intradermal administration were analyzed in this study. The percentage of viable cells measured by flow cytometry along with the total number of organisms in BCG vaccines, as determined on a cell counter, was used to quantify the number of viable cells.
Results: Pearson correlation coefficients of FACS and CFU assays for percutaneous and intradermal BCG vaccines were 0.6962 and 0.7428, respectively, indicating a high correlation. The coefficient of variation value of the FACS assay was less than 7%, which was 11 times lower than that of the CFU assay.
Conclusion: This study contributes to the evaluation of new potency test method for FACS-based determination of viable cells in BCG vaccines. Accordingly, quality control of BCG vaccines can be significantly improved.

Key Words: BCG vaccine, vaccine potency, flow cytometry

INTRODUCTION

Bacille Calmette–Guérin (BCG) vaccine is a live attenuated strain of *Mycobacterium bovis* used for the prevention of tuberculosis caused by *Mycobacterium tuberculosis* [1]. BCG vaccines can be administrated percutaneously or intradermally. At present, the golden method recommended by the World Health Organization (WHO) for potency testing of BCG vaccine is the colony-forming unit (CFU) assay. This direct method determines the number of micro-organisms by serially diluting the test sample and cultivating on the Lowenstein-Jensen (LJ) medium [2]. However, analyst variation of the CFU assay is high (5%–50%) owing to dilution error, modification of medium during cultivation, and counting error arising from mycobacterial clumping. In addition, 4 to 5 weeks are needed for colony formation because of a very slow growth rate, resulting in long lead-time during production of vaccines [3,4]. Accordingly, it is necessary to develop a new potency testing method to rapidly determine the viable cells in BCG

vaccine. Various methods, such as adenosine triphosphate (ATP) and tetrazolium salt (XTT) assays, have been developed, but they have not been used in the commercial manufacturing of BCG vaccines owing to a low correlation with the CFU assay [5–7].

Flow cytometry (FACS) is known to be highly sensitive and accurate in the counting of cells or bacteria and able to measure the bacterial size and content at the rate of approximately 1,000 cells/s [8]. This automated FACS system can minimize inter-analyst variation. In addition, it allows a rapid determination as it is not necessary to cultivate the cells. Furthermore, the viability of various bacteria can be determined with fluorescent staining reagents, such as propidium iodide (PI), fluorescein diacetate (FDA), and SYTO 9. Accordingly, the aim of this study was to develop a FACS-based test method to detect viable and non-viable cells in the BCG vaccine, which was evaluated with several percutaneous or intradermal BCG vaccines against the CFU assay.

MATERIALS AND METHODS

1. Preparation of BCG vaccines

BCG vaccines distributed in the Korean market, freeze-dried BCG vaccine for percutaneous administration that was manufactured from the Tokyo 172 strain (Japan BCG Laboratory, Tokyo, Japan), and freeze-dried BCG vaccine for intradermal administration that was manufactured from the Danish 1331 strain (Statens Serum Institut, Copenhagen, Denmark), were used in this study. In total, 17 lots of percutaneous BCG vaccines and 5 lots of intradermal BCG vaccines were used as samples. For FACS measurement, freeze-dried BCG vaccine samples (0.5 mg/mL for percutaneous administration and 0.75 mg/mL for intradermal administration) were diluted in Middlebrook 7H9 medium (10:1 and 100:1, respectively; Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C for 24 hours.

2. Determination of total cell count in BCG vaccines using a Coulter counter

After dilution of BCG vaccines, total cell count was determined using Beckman Coulter Multisizer 4 (Beckman Coulter, Carlsbad, CA, USA). This equipment measures microorganisms of 0.4 μm or larger in size, including very small *M. bovis* (1–3 μm). Isoton II diluent buffer (100 mL; Beckman Coulter) and diluted sample solution (0.5 mL) were mixed in a glass beaker, and cells in the solution were counted.

3. Determination of viable cell counts in BCG vaccines using a FACS analyzer

Cells (> 1 mL) previously cultured in Middlebrook 7H9 medium were heat-treated in a glass tube at 121°C for 20 minutes. FDA (25 μg/mL; Sigma-Aldrich) was then added to 500 mL of live (non-heat-killed) and heat-killed sample solutions at 37°C for 30 minutes. Separately, 500 mL of unstained sample was prepared. Green fluorescence of the individual sample solutions was measured using FACSVerse (BD Biosciences, San Jose, CA, USA). PMT voltage conditions were set to 100 V for FSC-H, 100 V for SSC-H, 250 V for FITC-H, and 400 V for PE-H. First, the cell population of the unstained sample solution shown in the SSC-H/FSC-H window was set as P1 gate. Subsequently, heat-killed and live sample solutions were analyzed under the same conditions. Fluorescence-staining results were checked in the SSC-H/FITC-H window. The percentage of live cells measured by flow cytometry was checked and multiplied by the total count measured using the Coulter counter to determine the viable cell count. The equation for determination of the viable cell count in BCG vaccines is as follows; Coulter counter's count (number) × FACS viable rate (%). FACS assay was performed three times for accuracy.

4. Determination of viable cell counts in BCG vaccines using the CFU assay

After serial dilution of percutaneous and intradermal BCG vaccines, each sample preparation was inoculated onto the LJ medium (BD Biosciences). After cultivation, colonies were counted to determine the number of viable cells. Specifically, one vial of percutaneous BCG vaccine (12 mg) was dissolved in 150 μL of accompanying diluents, and 450 μL of sterilized water was added to prepare a sample solution (20 mg/mL). This sample solution was serially diluted to the degree of 0.1×10^4 mg/mL, and 100 μL of the last-step sample solution (0.1×10^4 mg/mL) was used to inoculate 10 LJ mediums. One vial of intradermal BCG vaccine (0.75 mg) was dissolved in 1 mL of accompanying diluent to prepare a sample solution (0.75 mg/mL). This sample solution was serially diluted in 1/4 Sauton medium (10,000×, 20,000×, and 40,000×), and 100 μL of each diluted solution (10,000×, 20,000×, and 40,000×) was added to LJ medium (5, 5, and 10 samples, respectively). After inoculation, LJ media were slant-incubated at 37°C for 1 to 2 days. They were then put upright in the media box and incubated for a further 4 to 5 weeks. Colonies were counted at week 3, week 4, and week 4 + day 3 to determine the number of viable cells.
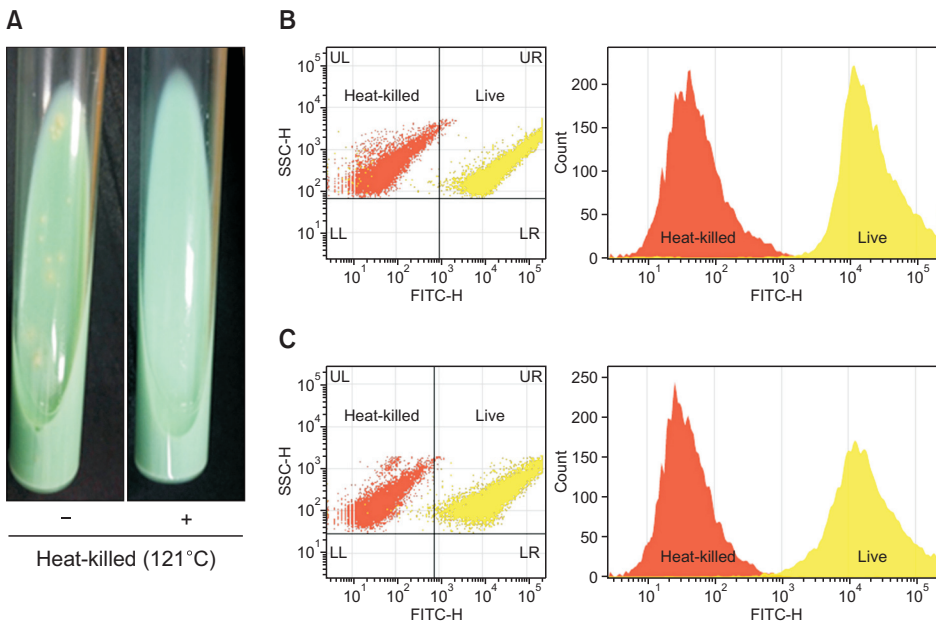
Figure 1. Selection of fluorescent staining reagent for flow cytometry (FACS) assay. Heat-killed samples (121°C, 20 minutes) and live samples were used. After serial dilutions, 100 μL of each sample was inoculated onto Lowenstein-Jensen medium, and cultivation was performed at 37°C for 4 to 5 weeks (A). After staining with fluorescent staining reagents, the percentages of viable cells were measured by flow cytometry. Test result of percutaneous Bacille Calmette–Guérin (BCG) vaccine after staining with fluorescein diacetate (FDA) (B). Test result of intradermal BCG vaccine after staining with FDA (C).
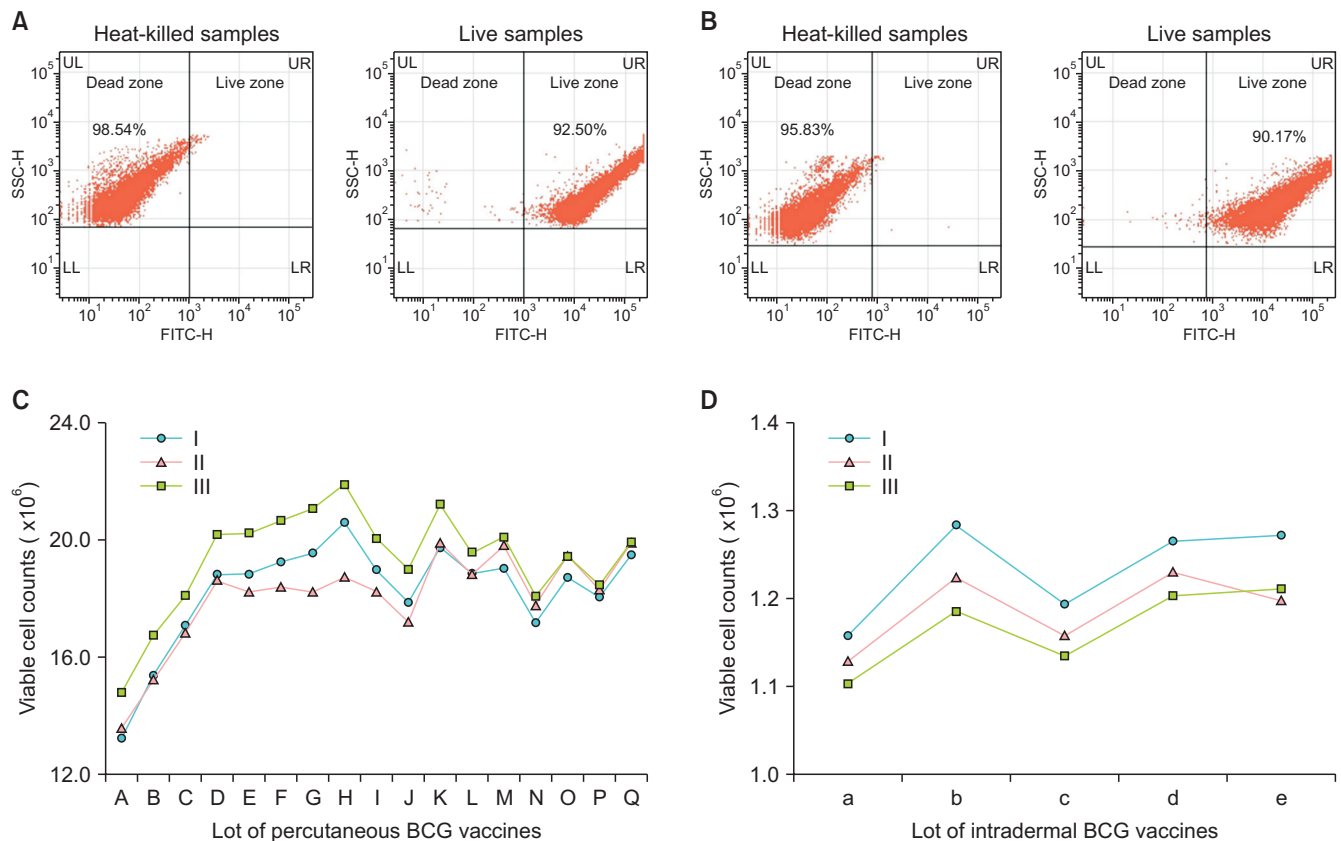UL, upper left; UR, upper right; LL, lower left; LR, lower right.

Figure 2. Determination of viable cell counts in Bacille Calmette–Guérin (BCG) vaccines by flow cytometry (FACS) assay. Seventeen lots of BCG vaccines for percutaneous administration and 5 lots of BCG vaccines for intradermal administration were tested. The percentage of viable cells in BCG vaccines for percutaneous administration (lot Q) (A) and intradermal administration (lot e) (B) was determined by flow cytometry. The percentage of viable cells in each lot was multiplied by the total count quantified on a Coulter counter to determine the viable cell count. Viable cell counts of 17 lots of percutaneous BCG vaccines measured by three laboratories (C), and viable cell counts of 5 lots of intradermal BCG vaccines measured by three laboratories (D).
UL, upper left; UR, upper right; LL, lower left; LR, lower right.

5. Collaborative studies

In total, 17 lots of percutaneous BCG vaccines and 5 lots of intradermal BCG vaccines were tested for viable cell count by FACS and CFU assays, as described in Materials and Methods sections 1–4 in three independent laboratories (I, II, and III).

6. Statistical analysis

To assess the correlation between FACS and CFU assays, vi-able cell counts measured by individual test methods (unit: $\times 10^6$) were transformed into $\log_{10}$ values, and Pearson correlation coefficients (r) were calculated. Values between $0.7 < r < 1$ indicate a strong positive linear relationship. Values between $0.3 < r < 0.7$ indicate an obvious positive linear relationship. All statistical analyses were based on parametric test results. SAS® (version 9.4; SAS Institute, Cary, NC, USA) was used for statistical analysis.

Table 1. Determination of viable cell counts in BCG vaccines by FACS and CFU assays

Lot	FACS assay					CFU assay				
	I[a]	II[a]	III[a]	SD	CV(%)	I[a]	II[a]	III[a]	SD	CV (%)
A. BCG vaccines for percutaneous administration										
A	13.22	13.57	14.78	0.82	5.91	7.43	45.90	12.40	20.92	95.49
B	15.37	15.24	16.75	0.83	5.28	8.35	30.65	17.60	11.20	59.38
C	17.08	16.84	18.10	0.67	3.88	8.47	31.35	18.80	11.46	58.65
D	18.83	18.64	20.18	0.84	4.39	7.72	28.45	25.60	11.24	54.58
E	18.85	18.24	20.22	1.01	5.31	8.15	32.75	20.00	12.30	60.60
F	19.27	18.42	20.67	1.14	5.85	7.65	22.55	27.60	10.37	53.84
G	19.56	18.24	21.07	1.42	7.22	9.07	27.10	25.60	10.01	48.60
H	20.61	18.74	21.92	1.59	7.81	9.78	37.11	26.80	13.80	56.18
I	18.99	18.25	20.04	0.90	4.72	10.12	36.10	23.60	12.99	55.84
J	17.86	17.20	18.99	0.91	5.03	10.65	34.90	16.40	12.67	61.35
K	19.75	19.91	21.22	0.81	3.98	12.18	11.70	23.20	6.51	41.46
L	18.87	18.81	19.58	0.43	2.23	11.53	26.83	28.40	9.32	41.87
N	19.04	19.82	20.11	0.55	2.81	11.16	46.20	22.80	17.84	66.78
M	17.18	17.73	18.07	0.45	2.56	11.79	41.72	27.20	14.97	55.64
O	18.73	19.50	19.46	0.43	2.25	11.22	44.06	33.60	16.78	56.63
P	18.05	18.31	18.46	0.21	1.13	12.14	32.55	11.60	11.94	63.64
Q	19.51	19.92	19.94	0.24	1.20	12.23	24.80	20.00	6.34	33.36
B. BCG vaccines for intradermal administration										
f	0.88					0.23				
g	1.24					0.38				
a	1.16	1.13	1.10	0.03	2.44	1.22	0.64	1.32	0.37	34.67
b	1.28	1.22	1.19	0.05	4.01	1.13	1.30	2.11	0.52	34.50
c	1.19	1.16	1.13	0.03	2.56	1.23	0.94	1.08	0.15	13.39
d	1.26	1.23	1.20	0.03	2.54	1.17	0.90	1.34	0.22	19.52
e	1.27	1.20	1.21	0.04	3.25	1.32	1.56	1.58	0.14	9.60
h	1.29					1.47				
i	1.29					1.53				

BCG, Bacille Calmette–Guérin; FACS, flow cytometry; CFU, colony-forming unit; SD, standard deviation; CV, coefficient of variation.
[a]$\times 10^6$.

RESULTS

1. Selection of a fluorescent staining reagent for FACS assay

To select the fluorescent staining reagent for FACS assay of the BCG vaccine, BCG vaccine samples were stained with various reagents widely used in flow cytometry, such as thiazole orange, SYTO 13, SYTO 9, PI, calcein-AM, c-FDA, and FDA. BCG vaccines for percutaneous or intradermal administration were resuspended and heat-treated at 121°C for 20 minutes. In addition to the heat-killed samples, live samples were also prepared. Prior to FACS analysis, each sample preparation was serially diluted, inoculated onto LJ media, and incubated at 37°C for 4 to 5 weeks to determine formation of colonies. For heat-killed samples, no colony was found (Figure 1A). Heat-killed samples and live samples were stained with fluorescent staining reagents, and analysis was performed by flow cytometry. When stained with FDA, dead and live zones were observed for heat-killed and live samples (Figure 1B, C). For the remaining six fluorescent staining reagents, an overlapping distribution was found (data not shown). In conclusion, only FDA was suitable for specific detection of viable cells in BCG vaccines.

2. Determination of viable cell counts in BCG vaccines by FACS assay

The percentage of viable cells in BCG vaccines determined by FACS was 92.50 ± 2.16% for percutaneous lot Q and 90.17 ± 3.50% for intradermal lot e (Figure 2A, B). Other lots also showed similar percentages (data not shown). The percentage of viable cells in each BCG vaccine lot determined by FACS was multiplied by the total count quantified on the Coulter counter to determine viable cell counts. The calculated number of viable cells in percutaneous BCG vaccines was 13.22–19.75 × 10^6 cells (laboratory I), 13.57–19.92 × 10^6 cells (II), and 14.78–21.92 × 10^6 cells (III), and coefficient of variation (CV) was 1.13%–7.81% (Figure 2C and A of Table 1). The number of viable cells in intradermal BCG vaccine was 0.88–1.29 × 10^6 cells (I), 1.13–1.23 × 10^6 cells (II), and 1.10–1.21 × 10^6 cells (III), and CV was 2.44%–4.01% (Figure 2D and B of Table 1). In conclusion, three laboratories reported similar levels of viable cell count in all lots of BCG vaccines for percutaneous and intradermal administration. When FACS results were analyzed for correlation between the three laboratories, Pearson correlation coefficient was 0.8729–0.9810, indicating a highly positive correlation (Table 2).

Table 2. Analysis of correlation between laboratories for FACS assay of viable cell counts in BCG vaccines

BCG vaccine	Laboratory	Pearson correlation coefficient (r)
Percutaneous	I–II	0.9313
	I–III	0.9810
	II–III	0.8729
Intradermal	I–II	0.9470
	I–III	0.9565
	II–III	0.9118

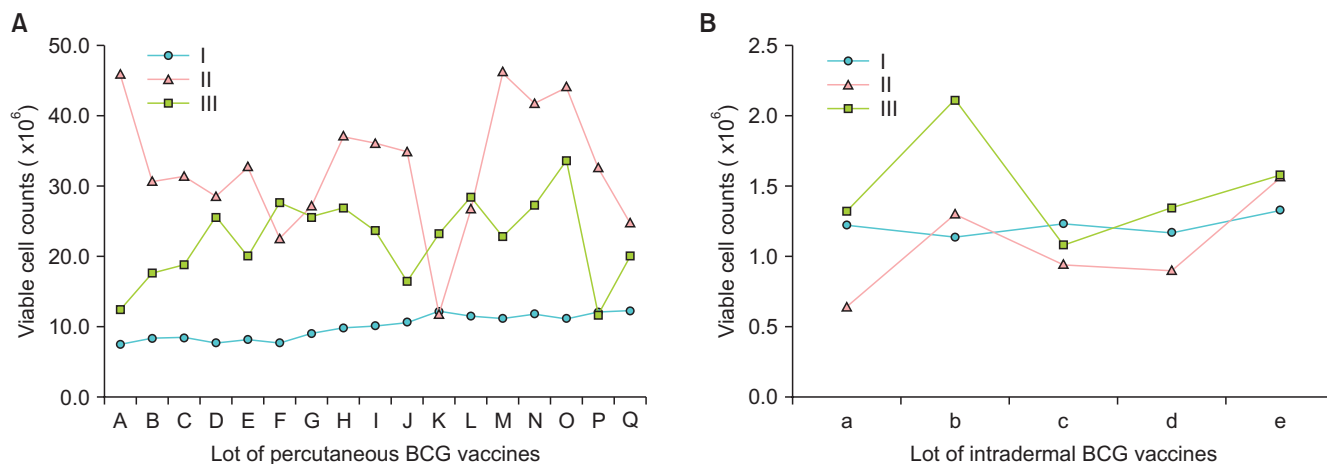FACS, flow cytometry; BCG, Bacille Calmette–Guérin.

Figure 3. Determination of viable cell counts in Bacille Calmette–Guérin (BCG) vaccines by colony-forming unit (CFU) assay. As with the flow cytometry (FACS) assay, 17 lots of percutaneous BCG vaccines and 5 lots of intradermal BCG vaccines were tested. After serial dilutions, samples were inoculated onto Lowenstein-Jensen media, and colonies were counted after cultivation at 37°C for 4 to 5 weeks. Viable cell counts of 17 lots of percutaneous BCG vaccines measured by three laboratories (A), and viable cell counts of 5 lots of intradermal BCG vaccines measured by three laboratories (B).
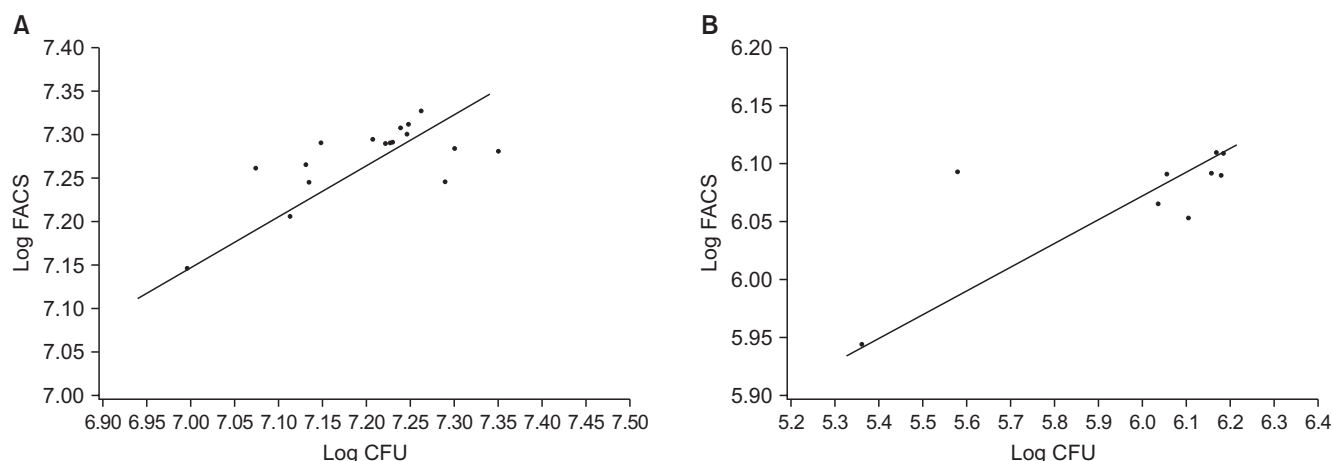
A

B

Figure 4. Analysis of correlation between flow cytometry (FACS) and colony-forming unit (CFU) assays. Based on results from FACS and CFU assays of 17 lots of percutaneous Bacille Calmette–Guérin (BCG) vaccines and 5 lots of intradermal BCG vaccines at three laboratories, Pearson correlation coefficient (r) was calculated to assess correlation between the two methods. Analysis of correlation between two test methods for percutaneous BCG vaccines (A), and for intradermal BCG vaccines (B).

3. Determination of viable cell counts in BCG vaccines by CFU assay

Viable cell counts in percutaneous BCG vaccines determined by CFU assay were $7.43–12.23 \times 10^6$ cells (I), $11.70–46.20 \times 10^6$ cells (II), and $11.60–33.60 \times 10^6$ cells (III) (Figure 3A and A of Table 1). Viable cell counts in intradermal BCG vaccines determined by CFU assay were $0.23–1.53 \times 10^6$ cells (I), $0.64–1.56 \times 10^6$ cells (II), and $1.08–2.11 \times 10^6$ cells (III) (Figure 3B and B of Table 1). CV values were 33.36% to 95.49% for percutaneous BCG vaccines and 9.60% to 34.67% for intradermal BCG vaccines, indicating high variation between laboratories.

4. Analysis of correlation between FACS and CFU assays

To assess the correlation between FACS and CFU assay, test results of BCG vaccines for percutaneous or intradermal administration were statistically analyzed to calculate Pearson correlation coefficient. For percutaneous BCG vaccines, test results from laboratories I and III were used in correlation analysis. Test results from laboratory II were excluded because of high intra-laboratory variation. When test results of the total 17 lots obtained at laboratories I and III were analyzed, mean viable cell count was 7.273 ± 0.044 $\log_{10}$ FACS and 7.201 ± 0.090 $\log_{10}$ CFU, and Pearson correlation coefficient was 0.6962, indicating an obvious positive linear relationship. In other words, as CFU assay value increased, FACS assay value also increased (Figure 4A and A of Table 3). For intradermal BCG vaccines, test results of a total of 9 lots (4 lots additionally tested) from laboratory I and test results of 5 lots from laboratories II and III were used in correlation analysis. Mean viable cell count was 6.072 ± 0.051 $\log_{10}$ FACS and 5.981 ± 0.299 $\log_{10}$ CFU, and Pearson correlation coef-

Table 3. Analysis of correlation between FACS and CFU assay of viable cell counts in BCG vaccines

BCG vaccine	Log₁₀ FACS	Log₁₀ CFU	Pearson correlation coefficient (r)
A. Percutaneous	7.273 ± 0.044	7.201 ± 0.090	0.6962
B. Intradermal	6.072 ± 0.051	5.981 ± 0.299	0.7428

Values are presented as mean ± standard deviation.
FACS, flow cytometry; CFU, colony-forming unit; BCG, Bacille Calmette–Guérin.
Pearson correlation coefficient (r) is 0.6962 for A and 0.7428 for B.

ficient was 0.7428, indicating a strong positive linear relationship. In other words, as CFU assay value increased, FACS assay value also increased (Figure 4B and B of Table 3).

DISCUSSION

Tuberculosis mainly occurs in the lungs, but it can be also found in most tissues, including the kidneys, nervous system, and bone. Tuberculosis causes various symptoms such as cough, hemoptysis, fever, dyspnea, feeling of helplessness, anorexia, and weight loss. With improved public health, medical system, and socioeconomic development, the number of patients with tuberculosis has significantly decreased in Korea. However, the incidence of tuberculosis and death rate owing to tuberculosis in Korea are still high, when compared to those in other Organization for Economic Co-operation and Development (OECD) member countries [9]. An epidemiological study revealed that, although the number of patients with tuberculosis has consistently de-

creased since 2013, the incidence in schools, work places, and other collective facilities has increased by 122% in 3 years from 2013 to 2015. This data indicates an increase in transmission of tuberculosis, as well as in the number of patients with latent tuberculosis [10].

In this study, in order to develop a FACS-based assay to determine the potency of BCG vaccine prescribed for prevention of tuberculosis, FDA was selected as the fluorescent staining reagent for the FACS assay, and percentages of viable cells were determined in 17 lots of BCG vaccines for percutaneous administration and 5 lots of BCG vaccines for intradermal administration by staining with FDA and measurement on a FACS analyzer. The percentages of viable cells were multiplied by the total cell counts quantified on a Coulter counter to determine the number of viable cell in BCG vaccines. Furthermore, FACS and CFU assay results were comparatively assessed to determine their correlation. When compared to the CFU assay, the FACS assay showed very low CV values. In correlation analysis of the two methods, Pearson correlation coefficients of BCG vaccines for percutaneous and intradermal administration were 0.6962 and 0.7428, respectively, indicating a strong positive linear relationship. In conclusion, when a total of 22 lots of BCG vaccines for percutaneous and intradermal administration were tested and comparatively assessed, CFU assay values increased as FACS assay values increased, revealing a correlation between the two test methods.

In Korea, a percutaneous BCG vaccine manufactured from the Tokyo 172 strain, an intradermal BCG vaccine manufactured from the Tokyo 172 strain, and an intradermal BCG vaccine manufactured from the Danish 1331 strain are authorized and distributed. In addition, BCG vaccine for intradermal administration from the Pasteur strain is under development. At present, the CFU assay is the only potency test method to determine viable cell count in BCG vaccine, the gold standard method recommended by the WHO. However, because this method has

limitations, development of alternative test methods is needed. Although various alternative methods, such as ATP and XTT assays, have been developed, they cannot be used in place of the CFU assay, because enzyme activity level does not necessarily mean cell viability. Indeed, it was reported that an enzymatic activity could be still detected in dead cells or esterase activity was increased in dead cells, which was due to the remaining enzymatic activity facilitating the transport of fluorescent staining reagents [11]. The FACS assay investigated in this study also rely on an enzymatic activity, that of FDA. Therefore, in addition to the viable cells, false-positive dead cells might contribute to the FACS assay results. Although it is difficult to use this new method as a potency test of the BCG vaccine on the basis of the results obtained in this study, it may be used in development and manufacturing of BCG vaccines to quickly assess potency, as a correlation between FACS and CFU assays was confirmed. As a result, it may significantly contribute to improve quality control. To accumulate data and improve reliability of the FACS assay for determination of viable cell count in BCG vaccines, further studies are needed.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGMENTS

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