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Comparison of TEMPO BC and MYP Plate Methods for the Enumeration of *Bacillus cereus* in Various Foods

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ABSTRACT - This study aimed to compare the automated most-probable-number (MPN) TEMPO BC and the quantitative mannitol-egg yolk-polymyxin (MYP) plate methods for enumeration of *Bacillus cereus* in food samples known to be frequently contaminated. Food products that were naturally or artificially contaminated with *B. cereus* were analyzed by both methods. A difference of less than 1 log (CFU/g) between the two methods was noted in 95.3% samples. There were no significant differences in artificially contaminated products between the two methods in terms of R^2 values for sauce products, jorim products, fish products, etc. However, a significant difference was noted for sunsik, fermented soybean products, and products. The linear equation of naturally versus artificially contaminated food was $\log_{(\text{TEMPO BC})} = 0.8453 \times \log_{(\text{MYP plate agar})} + 0.1642$. Statistical analysis of the results showed good agreement between the two methods. Due to growing interest in food safety, the use of the TEMPO BC method may increase. In response to this trend, the results from this study will offer valuable comparative data on the feasibility of existing methods and help develop new approaches for food safety testing.

Key words : *Bacillus cereus*, TEMPO BC Method, MYP Plate Method, Food Safety

Bacillus cereus is a well-known food pathogen that causes emesis and diarrhea. Though both forms of food poisoning are normally self-limiting, occasional fatal outcomes have been reported[1)]. The *Bacillus cereus* group consists of six different closely related species: *B. anthracis*, *B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, and *B. weihenstephanensis*. Of these, *B. cereus* is most commonly associated with foodborne illness. *B. cereus* is a facultative anaerobic, spore-forming, motile microorganism[2)].

Traditional methods for enumeration of quality indicators such as *B. cereus* in food are laborious and material-intensive. In addition, quality assurance in the food industry requires rapid testing methods. Moreover, since existing tests consider a particular growth phase of the candidate microorganisms, factors such as dilution factor need to be considered as well, making the process time-consuming and requiring technical skill. This has given rise to several alternative and rapid methods[3)]. The most-probable-number (MPN) method requires inoculation of multiple tubes with serially diluted sample, and the statistical basis for this test allows quantification of even a low number of target microorganisms[4)]. However, this method is particularly complicated; therefore, a computer operated automated system (TEMPO; Biomériux, Marcy L'Etoile, France) was based on 16 MPN technique and reduce the effort required to estimate bacterial populations[5)]. The instrument employs a unique micro-channeled card into which the sample is introduced under vacuum, automatically creating the serial dilutions necessary for MPN estimation. Samples are then added to vials of prepared dehydrated medium and introduced onto cards in an automated vacuum chamber. The cards are then removed from the vacuum chamber and incubated as instructed. After incubation, the cards are placed in a reading chamber equipped with a detector capable of determining fluorescence associated with growth of organisms in specific channels on the cards. Subsequently, MPN is automatically calculated using computer software[6,7)]. The most important parameters for feasibility of quantifying quality-related microorganisms in various foods are short turn-around time and ease of use, particularly due to large numbers of samples to be collected and processed each day. This has driven further studies on the TEMPO system to monitor and control the hygienic quality of food products.

Therefore, the aim of this study was to compare and analyze results of quantitative estimation of *B. cereus* in various food samples currently available using the TEMPO

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B. cereus (BC) system and the mannitol-egg yolk-polymyxin (MYP) plate method accoding to the Korea Food Code[8].

Materials and Methods

Samples

In this study, both naturally contaminated samples (samples without inoculation) and artificially contaminated food samples were used. A total of 616 samples were collected from different retail outlets in Korea and were immediately transported to the laboratory in insulated cooler boxes. Samples were stored at 4ºC until analysis. All samples were analyzed by both the TEMPO BC and ISO 7932 MYP plate methods. Naturally contaminated samples consisted of fish products (n = 96), meat products (n = 15), vegetable products (n = 33), sunsik (a powdered mixture of roasted grains and other foods; n = 24), fermented soy products (n = 4), red pepper products (e.g., red pepper, red pepper paste, etc.; n = 109), products containing oil (n = 18), bakery and rice cakes (n = 45), ready-to-eat foods (n = 21), and prepared foods (n = 19), as listed in Table 1. Artificially contaminated samples (n = 232) consisted of special purpose products (infant formula), sauce products, red pepper products, bean paste products, fermented soybean products, fish products, pickled products, sunsik, kimchi products, and jorim products. Descriptions of individual types of food used in this study are given below.

Artificial contamination of samples

B. cereus (ATCC 21772) was used for artificial contamination of samples. Stock cultures of *B. cereus* were grown on nutrient agar (Merck Darmstadt, Germany) for 24 h, and a suspension was prepared in Butterfield's phosphate buffered dilution water (BPD; Difco, Detroit, MI, USA). The dilutions included blank (0 CFU/g), low level (1-10 CFU/g), medium level I (10-100 CFU/g), medium level II (100-1000 CFU/g), and high level (1000-10000 CFU/g). Food samples were prepared by homogenizing 25 g food in 225 mL phosphate-buffered saline in a stomacher (Seward, London, UK) at 260 rpm for 1 min, followed by quantitative analysis by both TEMPO BC and MYP plate methods[9]. The sample of 25 g for the test using MYP plates was diluted in 225 mL BPD. For the MYP plating method, homogenized samples were serially diluted, and 0.2 mL was spread on MYP growth medium (Merck Darmstadt) for a total of five times to achieve a total inoculum of 1 mL. Plates were incubated for 24 h at 30ºC, and pink colonies with a hazy zone surrounding them due to lecithinase production were counted. From these plates, more than five typical colonies were selected and transferred to nutrient agar medium (Merck Darmstadt) and incubated at 30ºC for 18-24 h. Colonies were identified using VITEK II (bioMerieux). The enumeration of *B. cereus* in the food sample was based on the percentage of colonies that were morphologically consistent with *B. cereus*. For example, in addition to an average count of 65, obtained with 10^{-4} dilution of the sample, if four out of five colonies tested were confirmed as *B. cereus*, the *B. cereus* CFU/g of food was calculated as = 65 × 4/5 × 10000 × dilution factor (10) = 5,200,000[10].

Table 1. Category and number of samples

Sample category	Number
Fish products	96
Meat products	15
Vegetable products	33
Sunsik	24
Fermented soybean products	4
Red pepper products	109
Products containing oil	18
Bakery and rice cakes	45
Ready to eat foods	21
Prepared foods	19
Total	384

TEMPO BC method

The samples were processed for the TEMPO BC method by mixing 1 mL serially diluted sample with 3 mL buffer in a TEMPO BC medium vial. The mixture was then injected onto a BC card using the TEMPO preparation system and incubated at 30ºC for 24 h. Results were obtained from the TEMPO read system.

Statistical analysis

Bacterial counts were converted to logarithmic form. Values obtained within the enumeration ranges for both methods were subjected to Pearson correlation coefficient and linear regression analysis. All statistical analyses were performed using Microsoft Excel software. The significance level was defined as $p < 0.05$. If the absolute value of the

Table 2. Agreement between TEMPO BC and MYP plate methods

TEMPO BC and MYP results	Number of samples	Agreement[1)]	Discrepancy
Out of range [2)]	344	327 (95.1%)	17 (5.0%)
Within range [3)]	40	37 (92.5%)	3 (7.5%)
Total	384	364 (95.3%)	20 (5.2%)

[1)] Difference of less than 1 log between the methods.
[2)] Data for which at least one of the two methods gave results of "less than or more than".
[3)] Numerical data that could be transformed in $\log_{10}$ for both methods.

correlation coefficient, R^2 was > 0.9, the values measured by the two methods were considered as correlated. Concordance analysis was performed using the method described in the Campden and Chorleywood Food Research Association (CCFRA) Guideline number 29[11] and agreement indicated that the difference between the two methods was lower than 1 log base 10.

Results and Discussion

Quantitative test results of samples

Contamination by *B. cereus* in samples (Table 1) of various commercially available food products was quantitatively compared using the TEMPO BC and ISO 7932, MYP plate methods. Of the 384 samples tested, 364 samples (95.3%) showed a difference of less than 1 log CFU/g, while 20 samples (5.2%) showed a difference of greater than 1 log CFU/g (Table 2). The latter consisted of five rice cake samples (23.8%) out of a total of 21 rice cakes samples, as shown in Table 3. The difference in quantitative results from TEMPO BC and MYP plate methods was between log 2.1 CFU/g and log 3.6 CFU/g. Additionally, the MYP plate method showed a higher count of *B. cereus*. Red pepper products (food products containing red pepper paste or red pepper powder) also showed discrepancies (Table 3). The principle underlying the TEMPO BC testing method is measuring fluorescence generated by enzymes produced due to proliferation of bacteria on a TEMPO card. It is thought that the matrix contained in rice cakes may have influenced the results from the TEMPO BC method. According to the manufacturer instructions[7,12], the recommended dilution ratio for food products containing buckwheat flour, bean flour, or dietary fiber is 1:200. Additionally, it is not recommended to use TEMPO BC for enumeration of the *B. cereus* group in buckwheat flour, soy flour, and external pea fiber. Therefore, quantitative analysis using these products should be performed with consideraion of the dilution ratio.

Quantitative test results of artificially contaminated samples

Quantitative tests were performed on food products (10 types) artificially contaminated with *B. cereus* using the MYP plate and TEMPO BC methods, and the results were analyzed using paired t-tests. The results showed that statistically no significant ($p > 0.05$) for sauce products ($R^2 = 0.98$), jorim products ($R^2 = 0.98$), fish products ($R^2 = 0.96$), special purpose products ($R^2 = 0.95$), pepper paste products ($R^2 = 0.95$), kimchi products ($R^2 = 0.92$), and pickled products ($R^2 = 0.90$). In contrast, the results for sunsik ($R^2 = 0.94$), fermented soybean products ($R^2 = 0.86$), and bean paste products ($R^2 = 0.71$) showed significant differences between the two test methods ($p < 0.05$). A comparison of the two test results for each food type could yield more precise results by further correction with linear regression equation (Table 4).

Sunsik, fermented soybean products, and bean paste products may provide false-positive results in the fluorescence reaction. Fermented soybean products often harbor diverse *Bacilli* accumulated after fermentation and aging[13], which makes it difficult to morphologically select and enumerate *B. cereus* using the MYP plate method. The TEMPO BC method quantitatively analyzes *B. cereus* groups, and in cases where the sample is heavily contaminated, only *B. cereus* would be identified. This may have contributed to discrepancies between the results from these two methods. The default dilution used in this study was 1:4 (detection limit < 10 CFU), and all food types were compared and analyzed according to the type. The reason we diluted to 1:4 in our study was to minimize detection limit. However, the results showed a lot of difference from the MYP method. Therefore, in order to apply the automated TEMPO testing method on sunsik, fermented soybean products, and bean paste products, it is necessary to perform an additional test with a dilution ratio of 1:200 (detection limit < 50 CFU), as described in the manufacturer's instructions.

Statistical analysis of various food products using TEMPO BC and MYP plate methods

Linear regression analysis was performed on the comparison of the two testing results applied on various natural food product samples and artificially contaminated samples (n = 616). The linear equation was $\log_{(\text{TEMPO BC})} = 0.8453 \times$

Table 3. Discrepancies between TEMPO BC and the MYP plate methods

Sample category	Number of samples	Discrepancy	%	MYP>TEMPO	MYP<TEMPO	Lower	Upper
Fish products	96	3	3.1	1	2	−1.3	1.9
Vegetable products	33	2	6.1	0	2	2.1	4.0
Sunsik	24	1	4.2	0	1	1.0	1.3
Red pepper products	109	7	6.4	0	7	1.3	2.6
Bakery and rice cakes	21	5	23.8	0	5	2.1	3.6
Prepared foods	13	2	15.4	1	1	−1.1	1.5

Table 4. Statistical results of samples artificially injected with *Bacillus cereus* per food product type

Food product type	Correlation coefficient (R^2)	*p* value	Linear regression analysis yielded equation
Special purpose products	0.95	0.03	$\log_{(TEMPO\ BC)} = 0.8070 \times \log_{(MYP\ plate\ agar)} + 0.7331$
Sauce products	0.98	0.02	$\log_{(TEMPO\ BC)} = 0.9252 \times \log_{(MYP\ plate\ agar)} + 0.1385$
Red pepper products	0.95	0.00	$\log_{(TEMPO\ BC)} = 0.5935 \times \log_{(MYP\ plate\ agar)} + 0.6206$
Bean paste products	0.71	0.06	$\log_{(TEMPO\ BC)} = 0.4445 \times \log_{(MYP\ plate\ agar)} + 1.6302$
Fermented soybean products	0.86	0.09	$\log_{(TEMPO\ BC)} = 1.1290 \times \log_{(MYP\ plate\ agar)} - 0.5308$
Fish products	0.96	0.00	$\log_{(TEMPO\ BC)} = 0.9297 \times \log_{(MYP\ plate\ agar)} - 0.0551$
Pickled products	0.90	0.00	$\log_{(TEMPO\ BC)} = 1.0231 \times \log_{(MYP\ plate\ agar)} - 0.4861$
Sunsik	0.94	0.17	$\log_{(TEMPO\ BC)} = 0.8817 \times \log_{(MYP\ plate\ agar)} + 0.2220$
Jorim products	0.98	0.03	$\log_{(TEMPO\ BC)} = 0.9327 \times \log_{(MYP\ plate\ agar)} + 0.1101$
Kimchi products	0.92	0.00	$\log_{(TEMPO\ BC)} = 1.1184 \times \log_{(MYP\ plate\ agar)} - 0.9497$

$\log_{(MYP\ plate\ method)} + 0.1642$, and the R^2 value was 0.803. The results from the paired t-test showed no significant difference (correlation coefficient $R^2 > 0.9$, and $p > 0.05$). A high correlation was obtained between the two methods except for a few food products.

Due to the recent surge in the number of food samples received for testing for contamination with *B. cereus* to determine food safety levels, prompt test results are required. However, current standard methods, such as the MYP plate method, require a minimum of 4 days for the results to be available. Based on the results from this study, using the automated TEMPO BC apparatus will not only shorten the turn-around time required to just 1 day but also reduce the requirement for trained personnel. This method also offers the benefit of increased reliability of results due to automation and subsequent reduction in human error. However, additional analysis and testing are needed based on the characteristic of testing products because the two quantitative test methods showed a low level of agreement for Korean traditional food products with high levels of contamination by *B. cereus*.

With growing consumer interest in food safety, the Korean Food Code and international standards are gaining importance. Consequently, the use of TEMPO BC testing is expected to increase as well. The results from this study will offer valuable comparative data on the feasibility of existing methods and help develop new approaches for food safety testing.

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Conflicts of Interest Statement

The authors declare no conflicts of interest.

국문요약

본 연구는 자동화 MPN 분석 장비인 TEMPO (TEMPO BC)와 MYP 배지법을 이용한 바실러스 정량 시험 결과에 대한 상관관계와 유의성을 연구하였다. 자연적으로 오염되어 있는 *B. cereus* 다빈도 검출 식품을 선별하여(N = 384) 두 시험법으로 정량 시험한 결과값의 차가 1 log CFU/g 미만이 95.3%였으며, *B. cereus*가 검출된 시료에서는 92.5%였다. 통계학적으로 TEMPO BC 시험법과 MYP 배지법은 유의적 차이가 없었다($p < 0.05$). 국내 정량 규격이 있는 식품에 인위적으로 *B. cereus*를 접종하고 두 시험법을 비교하였다. R^2값은 소스류(0.98), 조림식품류(0.98), 젓갈류(0.96)에서 유의적인 차이가 없었으나, 생식류(0.94), 청국장류(0.86), 된장류(0.71)는 시험법 간의 유의적인 차이가 있었다. 콩, 메밀 등의 곡류 제품은 TEMPO BC 결과값의 오류가 날 수 있는 재료로 명시되어 있으므로 선식류와 청국장류, 된장류는 자동화 시험법인 TEMPO BC로 사용하기 위해서는 시험법을 추가적으로 검증 할 필요성이 있다고 생각된다. 자연적으로 오염되어 있는 식품 시료와 인위적으로 접종한 시료에 대한 선형 방정식은 log(TEMPO BC) = 0.8453 × log (MYP배지) + 0.1642이며 paired t-test를 이용하여 검증한 결과 유의적인 차이가 없었다. 최근 식품의 안전성에 대한 소비자의 관심이 증가하여 식품공전 및 국제 기준에 정량기준이 강화되고 있는 추세이므로 TEMPO BC의 수요증가가 예상된다. 따라서 본 연구는 이에 대비하여 식품의 안전성 평가에 사용되는 기존의 실험법과의 비교 및 타당성 연구 기초 자료로 쓰일 것이라 생각된다.

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