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Note

Tryptic soy medium is feasible for the in situ preparation of standards containing small defined numbers of microbial cells

Hideaki Matsuoka [a,*], Tomoya Shigetomi [a], Hisakage Funabashi [a,1], Mikako Saito [a], Shizunobu Igimi [b]

[a] Tokyo University of Agriculture and Technology, Department of Biotechnology and Life Science, 2-24-16 Naka-cho, Koganei, Tokyo 184-8588, Japan
[b] National Insititute of Health Science, Division of Biomedical Food Research, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

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ABSTRACT

A standard material comprising a few viable cells of a microorganism is essential for rational validation of microbiological methods. We propose a method of a flow cytometric sorting of cells stained with 6-carboxyfluorescein diacetate. The feasibility of tryptic soy medium in this method is demonstrated with 5 strains.

The detection of food-borne toxic bacteria is one of the critical issues in food safety. Regulatory standards require that high-risk bacteria, such as *Salmonella* spp. and *Escherichia coli* O157, should not be detected in food. In order to guarantee the absence of these bacteria, it is necessary to verify that the detection methods used can actually detect only a single cell in food, even if those methods have been well validated (AOAC, 2002.10; AOAC, 2005.05). Therefore, standard food samples that contain a few viable cells are essential.

From this viewpoint, a flow cytometric cell sorter is a promising apparatus for the single-cell sorting of microbial cells (Bunthof et al., 2001; van Dijk et al., 2010). Moreover, the sorted single-cells can be frozen without loss of colony-forming potentiality (Morgan et al., 2004). Thus, cells prepared in this way have been commercialized and used as standard materials of living cells (Wohlsen et al., 2006). However the number of available strains was only 8. It was suspected that most of the other strains tested might have been inactivated or killed by unintended stresses during freezing and then thawing before use.

As a result, we aimed to develop an in situ preparation method applicable to many other strains. Once such a preparation protocol has been established, food samples containing small defined numbers of bacteria may be prepared at much higher throughput by this method than by repeated dilutions by experts using the utmost care. We planned to attain our goal by adopting the following criteria. The range of cell numbers to be sorted in one sample is 0–100. The sorting site of each single cell is addressable. The criterion for successful sorting is a 95% or higher colony formation rate (CFR). Culture media for general use are preferable.

We have studied intensively pre-sorting treatment, viable staining, sort gate setting, and post-sorting culture for the following 5 strains. Initially, we used standard method agar (STA) for culture as a medium in general use. STA is composed of pancreatic digest of casein 0.5%, yeast extract 0.25%, dextrose 0.1%, and agar 1.5%, and adjusted at pH7.0 ± 0.2.

Bacillus subtilis (NBRC 3009), *E. coli* K-12 (NBRC 3301), *Pseudomonas aeruginosa* (NBRC 12689), and *Staphylococcus aureus* (NBRC 102135) were obtained from the National Institute of Technology and Evaluation — Biological Resource Center (NBRC) (Kisarazu, Chiba, Japan). *E. coli* (ATCC 8739) was obtained from American Type Culture Collection (ATCC). Each strain was plated on STA plates and cultured at 37 °C for 18 h. The colonies were re-plated on STA plates and cultured again under the same condition. The resulting colonies were used as the test sample.

The test sample was suspended in a phosphate buffer solution (PBS) (0.1 M, pH 7.0) at 10^8 cells/ml, and a 1-ml aliquot was transferred to a microtube. In order to stain viable cells, 6-carboxyfluorescein diacetate (CFDA) (Sigma) was added to the microtube at 150 ppm. CFDA permeates the cell membrane. Then CFDA is hydrolyzed to generate a fluorescent derivative, 6-carboxyfluorescein (CF) by intracellular esterase only in living cells. CFDA, as well as CF, is known to be non-toxic to the cell (Bunthof et al., 2001). Therefore, living cells may be stained without loss of cell viability. After incubation with CFDA for 30 min, the cells were collected by centrifugation and washed with PBS. The resulting cell suspension was applied to a fluorescence-activated cell sorter (FACSAria II, Becton Dickinson and Company).

Although non-staining cells emitted slight fluorescence (Fig. 1Aa–Ea), the stained cells of *B. subtilis* (Fig. 1Ab), *E. coli* K-12 (Fig. 1Bb), and *S. aureus*

* Corresponding author. Tel.: +81 423887029; fax: +81 423871503.
E-mail address: mhide@cc.tuat.ac.jp (H. Matsuoka).
[1] Present address: Hiroshima University, Institute for Sustainable Science and Development, 2-313 Kagamiyama, Higashihiroshima, Hiroshima 739-8527, Japan.

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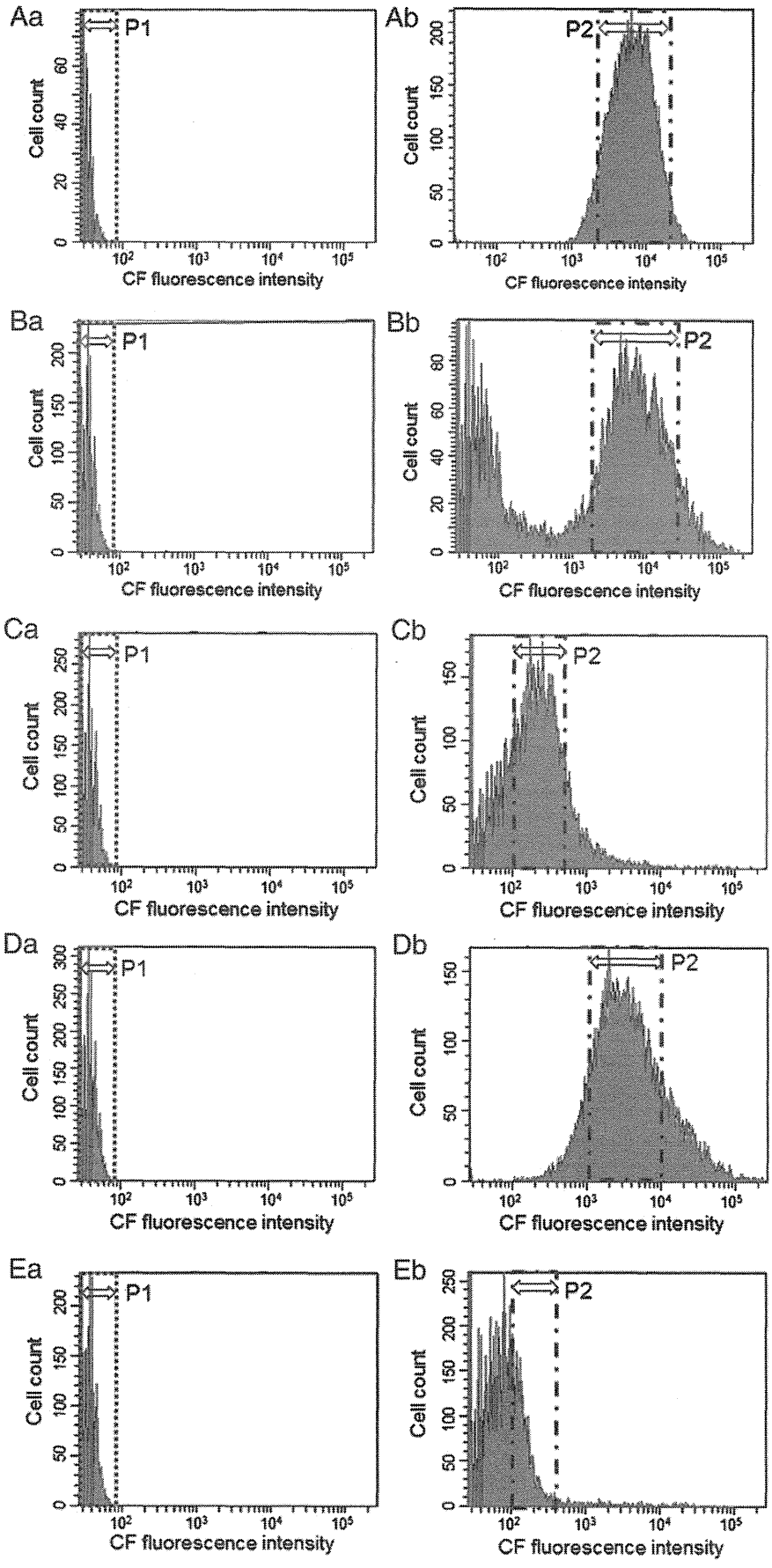

Fig. 1. Results of the flow cytometry ofmicrobial cells after or without staining with CFDA. (A) B. subtilis, (B) E. coli K-12, (C) P. aeruginosa, (D) S. aureus, (E) E. coli ATCC8739. (a) Without staining, (b) after staining with CFDA. P1: sort gate for non-staining cells, P2: sort gate for stained cells.

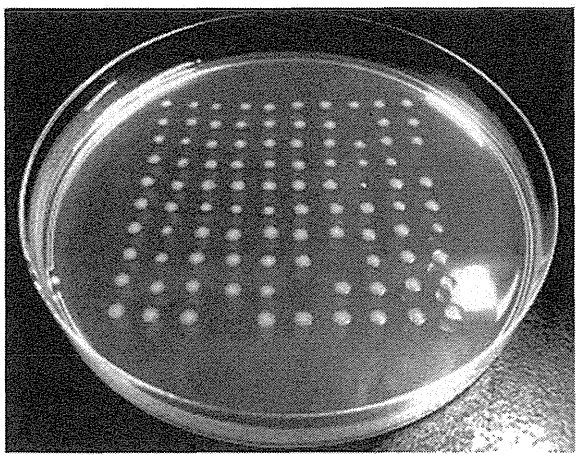

Fig. 2. Colony formation pattern of *E. coli* K-12 sorted on an STA plate.

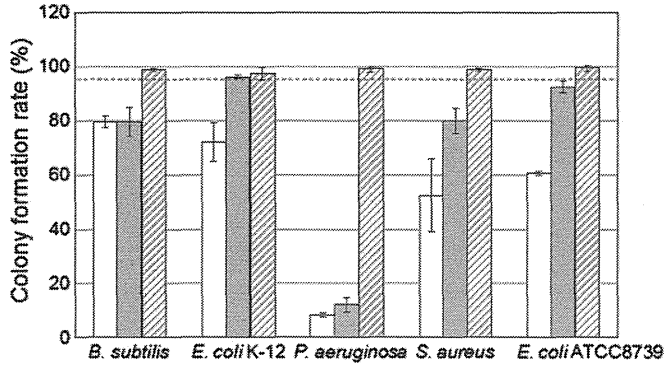

Fig. 3. CFRs of tested strains. ▭: non-staining cells sorted on STA plates, ▩: CFDA-stained cells sorted on STA plates, ▨: CFDA-stained cells sorted on TSA plates. Error bar: mean ± SD ($n=3$). Broken line: 95% CFR.

(Fig. 1Db) emitted 100-fold higher intensity fluorescence as compared with non-staining cells. As a result, the sort gate for stained cells (P2) could be set distinctively to exclude non-stainable cells. Although the fluorescence of *P. aeruginosa* and *E. coli* ATCC 8739 was less intense, it was thought that the sort gate P2 would also be effective at excluding non-stainable cells (Fig. 1Cb, Eb). As a control, non-staining cells were also sorted by setting the gate P1.

CFDA is relatively hydrophobic and the outer membranes of many wild type Gram-negative organisms have relatively low permeability to hydrophobic compounds. The Gram-negative strains tested in this study, *E. coli* ATCC8739 in particular, showed lower fluorescence than the Gram-positive strains. Because of such differences of stainability, it was necessary to find appropriate sort gates for respective strains. Therefore more useful dyes with general stainability to a wide spectrum of strains should be important in future.

The P1- or P2-gated fraction was sorted onto an STA plate of 86 mm in diameter. The STA plate was set on an automatic stage installed in the cell sorter. The automatic stage was driven so that 100 cells could be sorted onto 10×10 grid-like spots at 1 cell/spot. After the sorting, the STA plate was incubated at 37 °C for 18 h. The colony-formation pattern of *E. coli* K-12 is depicted in Fig. 2. In this case, 95 colonies were observed and therefore the CFR was 95%. However, the CFRs of the other strains were lower than 95% (Fig. 3); in particular, that of *P. aeruginosa* was 12%.

Next, we investigated various factors to improve the CFR. Ultimately, we found that the use of tryptic soy broth (TSB) for the pre-sorting culture and tryptic soy agar (TSA) for the post-sorting culture was effective. TSB is composed of pancreatic digest of casein 1.7%, papaic digest of soybean 0.3%, dextrose 0.25%, NaCl 0.5%, and agar 1.5%, K_2HPO_4 0.25%, and adjusted at pH7.3 ± 0.2. TSA is composed of pancreatic digest of casein 1.5%, papaic digest of soybean 0.5%, NaCl 0.5%, and agar 1.5%, and adjusted at pH7.3 ± 0.2. As demonstrated in Fig. 3, all of the 5 strains tested satisfied the 95% criteria. In comparison with STA, TSA and TSB contain soy protein hydrolysates. Therefore, we suspect that those hydrolysates might contain effective components. In conclusion, the tryptic soy medium is feasible in the present method for the in situ preparation of standard materials containing a few defined viable cells.

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FOOD BIOLOGICAL CONTAMINANTS

Evaluation of the Culture Method NIHSJ-02 Alternative to ISO 10272-1:2006 for the Detection of *Campylobacter jejuni* and *Campylobacter coli* in Chicken: Collaborative Study

YOSHIKA MOMOSE, YUMIKO OKADA, HIROSHI ASAKURA, TOMOYA EKAWA, and KAZUYA MASUDA
National Institute of Health Sciences, Tokyo 158-8501, Japan
HIDEAKI MATSUOKA
Tokyo University of Agriculture and Technology, Tokyo 184-8588, Japan
KEIKO YOKOYAMA and AKEMI KAI
Tokyo Metropolitan Institute of Public Health, Tokyo 169-0073, Japan
SHIOKO SAITO
Akita Prefectural Research Center for Public Health and Environment, Akita, 010-0874, Japan
REIJI HIRAMATSU
Aichi Prefectural Institute of Public Health, Aichi 462-8576, Japan
MASUMI TAGUCHI
Osaka Prefectural Institute of Public Health, Osaka 537-0025, Japan
KATSUYUKI ISHIMURA
Hiroshima City Institute of Public Health, Hiroshima 733-8650, Japan
KIYOSHI TOMINAGA
Yamaguchi Prefectural Institute of Public Health and Environment, Yamaguchi 753-0821, Japan
SHUNSUKE YAHIRO
Kumamoto Prefectural Institute of Public Health and Environmental Science, Kumamoto 869-0425, Japan
MASAHIRO FUJITA
Gunma Meat Inspection Center, Gunma 370-1103, Japan
SHIZUNOBU IGIMI[1]
National Institute of Health Sciences, Tokyo 158-8501, Japan

Collaborators: K. Aikawa; N. Asai; M. Furukawa; S. Harada, Y. Inoguchi; F. Itoh; T. Konno; M. Matsumoto; R. Saito; M. Sato; T. Wada; K. Yamaguchi; A. Yoshida

For the surveillance of the prevalence of *Campylobacter jejuni* and *Campylobacter coli* in raw chicken products in Japan, a qualitative method, National Institute of Health Sciences Japan (NIHSJ)-02, was developed as an alternative to International Organization for Standardization (ISO) 10272-1:2006. In the NIHSJ-02 culture method, the enrichment step is carried out in a reduced volume of Preston broth at 42 ± 1°C to reduce cost and space, and to prevent the overgrowth of background bacteria. To evaluate the performance of NIHSJ-02, a collaborative study was conducted, and the results obtained by NIHSJ-02 were compared with those obtained using the reference method, ISO 10272-1:2006. Fifteen laboratories participated; each examined 48 minced chicken samples consisting of test samples uninoculated, inoculated with *C. jejuni* at a low or high level, and inoculated with *C. coli* at a low level. The average probabilities of detection by NIHSJ-02 across laboratories were 0.033, 0.222, 0.678, and 0.267 in samples uninoculated, inoculated with *C. jejuni* at a low and high level, and with *C. coli* at a low level, respectively. Those by ISO 10272-1:2006 were 0.051, 0.128, 0.551, and 0.090. Significantly higher probabilities of detection were determined by NIHSJ-02 compared to ISO 10272-1:2006, except for uninoculated samples. On the other hand, significantly lower frequency of occurrence of background bacteria was observed by NIHSJ-02 (43.1%) compared with ISO 10272-1:2006 (92.6%). NIHSJ-02 showed better performance than ISO 10272-1:2006 with regard to the selective detection of *C. jejuni* and *C. coli* in chicken.

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[1] Corresponding author's e-mail: igimi@nihs.go.jp
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Campylobacter is one of the leading causes of human enteritis worldwide (1,2). *Campylobacter jejuni* is the most common causative species, followed by *C. coli* (1,2), and chicken meat is the most common source of campylobacteriosis (1, 3–5). In Japan, the incidence of *Campylobacter* enteritis has not decreased markedly over the last 10 years (6), and careful monitoring is required to take effective measures to reduce the number of illnesses. The prevalence of *C. jejuni* and *C. coli* in raw chicken products has been monitored by prefectural and municipal public health institutes and reported annually to the Ministry of Health, Labour, and Welfare in Japan. The detection rate of *Campylobacter* spp.

differs depending on the method used; therefore, a unified and validated method is needed for their surveillance.

The International Organization for Standardization (ISO) standard method, ISO 10272-1:2006, recommended Bolton broth for enrichment, focusing on the recovery of damaged cells (7). The use of Bolton broth is also described in the U.S. Food and Drug Administration *Bacteriological Analytical Manual* (8). However, the selectivity of Bolton broth is mainly achieved by cephem antibiotic, and the first selective medium, modified charcoal cefoperazone deoxycholate agar (mCCDA), also contains the common cephem antibiotic, cefoperazone, as a selective reagent. In recent years, an increased prevalence of extended-spectrum β-lactamase-producing (ESBL) bacteria in broilers and raw chicken meat has been reported in Japan and other countries (9–12), suggesting that these bacteria hydrolyze cefoperazone, thereby hindering the detection of campylobacters.

Considering the high prevalence of ESBL bacteria in raw chicken meat, Preston broth was chosen as an alternative to Bolton broth in our culture method, National Institute of Health Sciences Japan (NIHSJ)-02. Other modifications were introduced with regard to the test portion/enrichment medium ratio and the incubation temperature to increase the practicability. Applying a reduced volume of enrichment broth (adding four volumes of a test portion to make 5-fold homogenate) was intended to reduce cost and space, and to increase the initial concentration of *Campylobacter* in the homogenate. In a preliminary study in which naturally contaminated *Campylobacter* were isolated from 60 out of 79 retail chicken samples enriched in 5-fold homogenate, 59 out of 79 retail chicken samples were positive for *Campylobacter* by enrichment in 10-fold homogenates, showing no significant difference between enrichment in 5-fold and 10-fold homogenates (unpublished data). The incubation temperature of 42 ± 1°C is commonly used for the detection of both *Salmonella* spp. and thermotolerant *Campylobacter* in Japan, and is introduced in the NIHSJ-02 method. In the ISO 10272-1:2006 method, nine volumes of Bolton broth are used to make a 10-fold homogenate, which is incubated at 37 ± 1°C from 4 to 6 h, and then at 41.5±1°C.

To evaluate the performance of NIHSJ-02, a collaborative study was conducted according to ISO 16140:2003 validation guidelines (13). The results obtained by NIHSJ-02 were then compared with those obtained using the reference method ISO 10272-1:2006.

Methods

NIHSJ-02 for the Detection of C. jejuni and C. coli in Chicken

A 25 g amount of the test portion is inoculated into four volumes (100 mL) of Preston broth and homogenized by stomaching for 30 s. This homogenate is incubated in a microaerophilic atmosphere generated by commercially available gas-generation kits or in an apparatus suitable for achieving a microaerophilic atmosphere at 42±1°C. After 26±2 h and 44±4 h enrichment, the cultures are inoculated onto two selective solid media; the first medium is mCCDA and the second any other agar plate using selective ingredients different from mCCDA. The media are incubated at 42±1°C in a microaerophilic atmosphere for 44±4 h, and five presumptive colonies are picked for further confirmation. These colonies are subcultured on *Brucella* agar, checked for purity, and confirmed by microscopic examination, oxidase reaction, and catalase reaction. Results are expressed as "positive/25 g" when *Campylobacter* has been isolated after 26±2 h or 44±4 h enrichment. Species identification is optional.

Study Design

A collaborative study was conducted according to ISO 16140:2003 (13), with some modifications. ISO 16140 uses eight replicates at each level in 10 laboratories. In this collaborative study, six replicates in 13 laboratories were used as required conditions for the validation, considering the capacity of laboratory equipment and research workers. A total of four inoculation levels were used, and six blind replicates at each level were analyzed. Three replicates at each level were analyzed at a time, and the same procedure was duplicated to achieve six replicates. Of the 15 participating laboratories, 13 achieved results without outliers. The media and reagents used for enrichment, isolation, and confirmation were provided from the same lot and sent to the participating laboratories within 1 week before the examination started. All participants started examination within the day they received the test samples. All results were reported to and analyzed by the organizing laboratory, NIHS (Tokyo, Japan).

Media and Reagents

Preston broth (CM0067 supplemented with SR0232 and SR0117; Oxoid, Basingstoke, UK) and Bolton broth (100068 supplemented with 100079; Merck, Darmstadt, Germany) were used for enrichment in the NIHSJ-02 and ISO 10272-1:2006 methods, respectively. Both methods used mCCDA (CM0739 supplemented with SR0155; Oxoid) and Butzler agar (CM0331 supplemented with SR0085; Oxoid) as first and second selective isolation media. Preston broth, Bolton broth, and Butzler agar were supplemented with 5% (v/v) lysed horse blood. Cytochrome Oxidase Test Strip (Nissui, Japan) was provided for confirmation.

Bacterial Strains

C. jejuni NCTC 11168 and *C. coli* JCM 2529 were used as inoculation strains. They were grown at 37°C on Mueller-Hinton agar plates (Becton, Dickinson and Co., Sparks, MD) under microaerophilic conditions and suspended in Maximum Recovery Diluent (Oxoid) with OD_{595} values of 0.7–0.8. The suspensions were serially diluted to meet the target inoculation levels of 1–9 CFU/25 g for the low-level group and 10–99 CFU/25 g for the high-level group. Each inoculum was spread onto 10 Mueller-Hinton agar plates, incubated under microaerophilic conditions, and the concentrations of the inocula were determined.

Preparation and Distribution of Samples

Retail chilled minced chicken was used as the test matrix. A freeze-thaw procedure was repeated twice before inoculation of test strains to reduce the level of naturally contaminated *Campylobacter* but retain the level of background bacteria. The

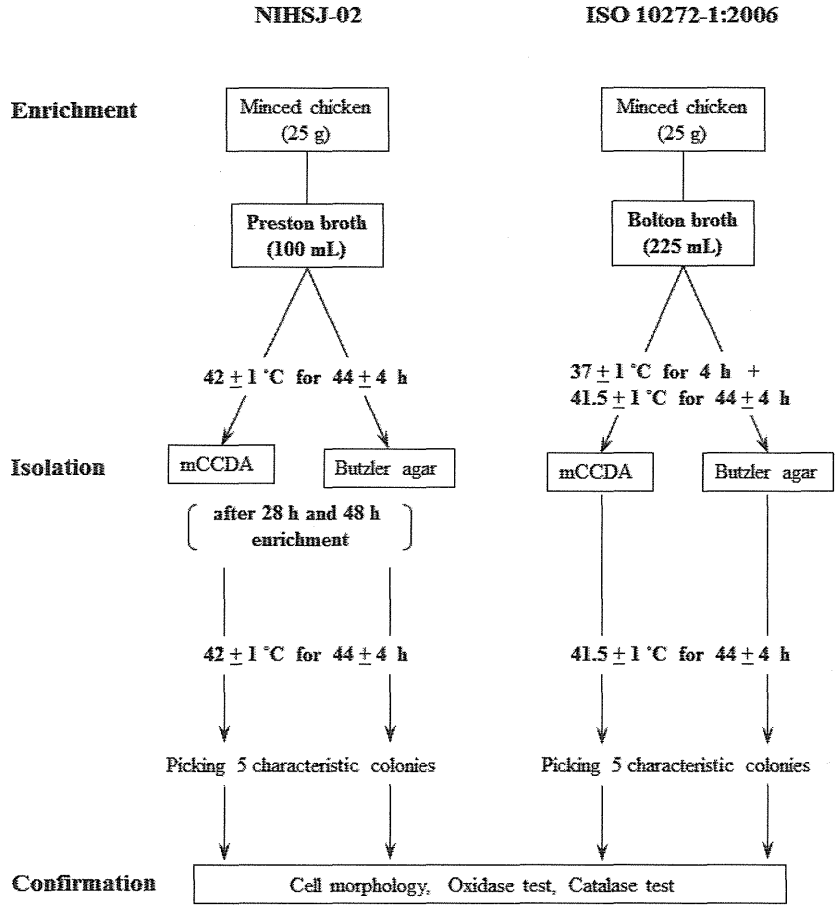

Figure 1. Schematic summary illustrating steps involved in the comparison of NIHSJ-02 and ISO 10272-1:2006; 60 × 88 mm (300 × 300 DPI).

minced chicken was frozen at –80°C overnight and thawed at room temperature twice, then divided into 25 g portions and inoculated with *C. jejuni* or *C. coli*, or left uninoculated. Samples were randomly numbered, packaged in biosafety containers, and sent on dry ice to 15 collaborating laboratories within 3 days.

Microbiological Analysis

Each collaborating laboratory received 24 test samples consisting of six uninoculated portions, six inoculated with *C. jejuni* at a low level, six inoculated with *C. jejuni* at a high level, and six inoculated with *C. coli* at a low level. Three samples out of each level were tested by NIHSJ-02, and the other three samples by ISO 10272-1:2006 according to the procedure shown in Figure 1. This procedure was duplicated, and 48 samples in total were examined in each laboratory.

Data Expression

Results obtained by duplicate examinations were combined, and the probability of detection (POD) was calculated for each laboratory at each inoculation level (14). LPOD (the average POD across laboratories) was further estimated, and the performances of the two methods were compared by the difference in LPOD values (dLPOD). POD is defined as the probability of the method giving a positive result; LPOD is a composite POD pooled across laboratories and includes between-laboratory variation, in addition to variation inherent in the binomial nature of the binary probabilities (14). The statistical significance between the candidate and reference methods is determined when the confidence interval (CI) of dLPOD does not include zero.

Results

Inoculation Levels

The inocula of *C. jejuni* for the first samples were 7.0±2.1 CFU/25 g for the low-level group and 72.2±13.8 CFU/25 g for the high-level group. Those for the second samples were 4.3±2.3 CFU/25 g for the low-level group and 52.1±15.7 CFU/25 g for the high-level group. The inocula of *C. coli* for the first and second samples were 5.2±2.2 CFU/25 g and 6.2±3.5 CFU/25 g, respectively.

LPOD of Campylobacter Results

The test samples were sent to 15 laboratories across Japan, which conducted the two methods to detect the inoculum.

Table 1. Detection of *Campylobacter* and background bacteria in chicken by NIHSJ–02[a]

		NIHSJ–02																					
		Uninoculated						*C. jejuni* (low level)						*C. jejuni* (high level)						*C. coli* (low level)					
Lab No.		1	2	3	7	8	9	1	2	3	7	8	9	1	2	3	7	8	9	1	2	3	7	8	9
1	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	–/–	–/–	–/–	+/+	+/+	+/+	–/–	–/–	–/–	–/–	+/+	+/+	–/–	–/–	+/+
	Background	–/–	–/–	+/–	–/–	–/–	+/+	–/–	–/+	+/+	–/–	+/–	–/+	–/–	–/–	+/–	–/–	+/+	+/+	–/–	+/–	+/+	–/–	–/–	–/–
2	*Campylobacter*	–/–	–/–	+/+	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	+/+
	Background	–/–	–/–	–/–	–/–	–/–	–/+	–/–	–/+	+/+	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	–/–	–/–	+/+	–/–	–/+	+/+
3	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–
	Background	–/–	–/–	+/+	–/–	+/+	+/+	–/–	+/–	–/+	–/–	–/–	–/–	–/–	+ / –	–/+	–/–	–/–	–/–	–/–	–/+	+/+	–/–	–/–	+/+
4	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	+/+	+/+
	Background	–/–	–/–	+/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	– / +	– / +	–/–	–/+
5	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	–/–	+/+	+/+	+/+	–/–	–/–	+/+	–/–	–/–	+/+	–/–	–/–	–/–
	Background	–/–	–/–	+/+	–/–	–/–	+/+	–/–	–/+	–/–	–/–	–/+	–/+	+/–	+/+	+/+	–/–	+/+	+/+	–/–	–/–	+/+	–/–	–/+	–/+
6	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/–	–/–	–/–	+/+	+/+	+/+	+/+	–/–	–/–	+/+	–/–	–/–	–/–	–/–	+/+	+/+
	Background	–/–	–/–	+/+	–/–	–/–	+/+	–/–	–/–	–/+	–/–	–/+	–/–	–/–	–/–	–/–	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+
7	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	–/–	+/+	+/+	+/+	–/–	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+
	Background	–/+	+/+	+/+	–/–	–/+	+/+	–/–	+/+	–/–	–/–	–/+	+/+	–/–	+/+	+/+	–/–	–/–	–/–	–/–	+/+	+/+	–/–	+/+	–/–
8	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	–/–	+/+	+/+	+/+	–/–	–/–	+/+	–/–	+/+	+/+	–/–	+/+	+/+
	Background	–/–	–/–	–/+	–/–	–/+	+/+	–/+	+/+	–/–	–/–	–/–	+/+	–/–	–/–	–/–	–/–	–/+	–/–	–/–	–/–	–/–	–/–	–/–	–/–
9	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	–/–	+/+	+/+	+/+	–/–	–/–	+/+	–/–	–/–	–/–	–/–	–/–	–/–
	Background	–/–	+/–	–/+	–/–	–/–	+/–	–/–	+/–	+/–	–/–	–/–	–/+	–/–	–/–	+/+	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	–/–
10	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	+/+	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+
	Background	–/–	+/–	+/–	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/+	–/+	+/+	–/+	+/+	–/–	+ / –	+/+	–/–	–/–	+/+	+/–	+ / –	+/+
11	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	–/–	–/–	–/–	–/–	+/+	+/+	–/–	–/–	+/+	–/–	–/–	–/–	–/–	–/–	–/–
	Background	–/–	–/–	–/–	–/–	–/–	+/+	–/+	–/+	+/+	–/–	–/–	+/+	–/+	–/–	+/+	–/–	+/+	+/+	–/–	+/+	+/+	+/+	+/+	+/+
12	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	+/+	–/–	–/–	–/–	–/–	–/–	+/+
	Background	–/–	–/–	–/–	+/+	+/+	+/+	–/–	–/–	+/+	–/–	+/–	+/+	–/–	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+
13	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	–/–	–/–	+/+	+/+	–/–	–/–	+/+	–/–	–/–	–/–	–/–	–/–	–/–
	Background	–/–	–/–	–/–	–/–	–/–	–/–	+/–	+/+	+/+	–/–	–/–	–/–	+/+	–/–	+/+	–/–	–/+	–/–	+/–	–/+	+/+	+/+	+/+	+/+
14	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	+/+	–/–	+/+	+/+	–/–	+/+	+/+	–/–	+/+	+/+	–/–	–/–	+/+
	Background	–/–	–/–	+/–	–/–	+/+	+/+	–/–	–/–	–/–	–/–	+/+	–/–	+/+	+/+	+/+	–/–	–/–	+/+	–/–	–/–	+/+	–/–	+/+	–/–
15	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	+/+	–/–	–/–	–/–	–/–	+/+	+/+
	Background	–/–	–/–	+/+	–/–	–/–	–/–	+/+	+/+	+ / –	–/–	–/–	–/–	–/–	–/–	–/–	–/–	– / +	–/–	–/–	–/–	+/+	–/–	–/–	–/+

[a] Portions (25 g) of minced chicken were inoculated with *C. jejuni* or *C. coli*, or left uninoculated. Each collaborating laboratory received 24 test samples consisting of six portions uninoculated, six portions inoculated with *C. jejuni* at a low level, six portions inoculated with *C. jejuni* at a high level, and six portions inoculated with *C. coli* at a low level. Three samples out of each level were tested by NIHSJ–02 and the other three samples by ISO 10272–1:2006. This procedure was duplicated to achieve six replicates (1–3, first samples; 7–9, second samples tested by NIHSJ–02) at each level. Results are expressed as + (detected) or – (not detected) on mCCDA/Butzler agar.

The reported results are shown in Table 1 (NIHSJ-02) and Table 2 (ISO 10272-1:2006). The results by NIHSJ-02 from all 15 laboratories were accepted. The results obtained by ISO 10272-1:2006 from one laboratory for each examination were excluded because of deviation from the protocol; the results reported from the remaining 13 laboratories (Laboratory No. 2–14) were used for the data analysis. Frequency of *Campylobacter* did not differ regardless of the selective isolation agars used in NIHSJ-02. On the other hand, *Campylobacter* was isolated only from Butzler agar in 24 out of 64 samples with positive results by ISO 10272-1:2006 (Table 2), and the selectivity of mCCDA for the isolation of *Campylobacter* was found to be inferior to that from Butzler agar, the second selective isolation medium, in ISO 10272-1:2006.

Positive rates of *Campylobacter* are expressed as LPOD with 95% CI and are summarized in Table 3. LPOD values by

Table 2. Detection of *Campylobacter* and background bacteria in chicken by ISO 10272–1:2006[a]

		ISO 10272–1:2006																							
		Uninoculated						*C. jejuni* (low level)						*C. jejuni* (high level)						*C. coli* (low level)					
Lab No.		4	5	6	10	11	12	4	5	6	10	11	12	4	5	6	10	11	12	4	5	6	10	11	12
1	*Campylobacter*	–/–	–/–	–/–	ND	ND	ND	–/–	–/+	+/+	ND	ND	ND	–/+	+/+	+/+	ND	ND	ND	–/–	–/–	–/–	ND	ND	ND
	Background	–/–	+/+	+/+	ND	ND	ND	+/+	+/+	–/–	ND	ND	ND	+/+	–/–	+/+	ND	ND	ND	+/+	+/+	+/+	ND	ND	ND
2	*Campylobacter*	–/–	+/–	–/+	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	–/–	–/+	+/+	+/+	–/+	–/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–
	Background	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	+/+	+/+	+/+	+/+	+/+	+/+
3	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/+	–/–	–/–	–/–	–/–	–/–	+/+	–/+	–/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–
	Background	+/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
4	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/+	–/–	–/–	+/+	–/–	–/–	–/–	–/–	–/–	–/–
	Background	+/+	+/+	+/+	–/–	+/+	+/+	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
5	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	–/–	–/+	+/+	+/+	–/–	–/+	+/+	–/–	–/–	–/–	–/–	–/–	+/+
	Background	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–
6	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/+	+/+	–/–	–/–	–/–	–/–	–/–
	Background	+/+	+/+	+/+	+/–	+/+	+/+	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	+/+	–/–	+/+	+/+	+/+	–/–	+/+	+/+
7	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/+	–/–	+/+	+/+	–/–	–/–	+/+	–/–	–/–	+/+	–/–	–/–	+/+
	Background	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
8	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	–/–	–/–	–/–	–/+	–/–	+/+	+/+	–/–	–/–	–/+	–/–	–/–	–/–
	Background	+/+	+/+	+/+	+/–	+/+	+/+	+/–	+/+	+/+	+/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
9	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/+	+/+	–/–	–/–	+/+	–/–	–/–	+/–	–/–	–/–	–/–
	Background	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	+/+	–/–	+/+	+/+	+/+	–/–	+/+	+/+
10	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/+	–/+	–/–	–/–	+/+	–/+	+/+	+/+	–/+	+/+	+/+	–/–	–/–	–/+	–/–	–/–	+/+
	Background	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	+/+	–/–	+/+	+/–	+/+	+/+	–/–	+/+	+/+	+/+	+/+	–/–	+/+	+/+
11	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–
	Background	+/+	+/+	+/+	+/–	+/+	–/–	+/+	+/+	+/+	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	+/+	+/+	+/+	+/–	+/+	+/+
12	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	–/+	–/–	–/–	–/+	–/–	–/–	–/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–
	Background	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	+/+	+/+
13	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
	Background	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
14	*Campylobacter*	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
	Background	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
15	*Campylobacter*	ND	ND	ND	–/–	–/–	–/–	ND	ND	ND	–/–	–/–	–/–	ND	ND	ND	–/–	–/–	+/+	ND	ND	ND	–/–	–/–	–/–
	Background	ND	ND	ND	+/+	+/+	+/+	ND	ND	ND	–/–	+/+	+/+	ND	ND	ND	+/+	+/+	–/–	ND	ND	ND	+/+	+/+	+/+

[a] Portions (25 g) of minced chicken were inoculated with *C. jejuni* or *C. coli*, or left uninoculated. Each collaborating laboratory received 24 test samples consisting of six portions uninoculated, six portions inoculated with *C. jejuni* at a low level, six portions inoculated with *C. jejuni* at a high level, and six portions inoculated with *C. coli* at a low level. Three samples out of each level were tested by NIHSJ–02 and the other three samples by ISO 10272–1:2006. This procedure was duplicated to achieve six replicates (4–6, first samples; 10–12, second samples tested by ISO 10272–1:2006) at each level. Results are expressed as + (detected) or – (not detected) on mCCDA/Butzler agar, or ND (not determined) because of the deviation from the protocol.

NIHSJ-02 were 0.033, 0.222, 0.678, and 0.267 in test samples uninoculated, inoculated with *C. jejuni* at a low level and a high level, and inoculated with *C. coli* at a low level, respectively. Those by ISO 10272-1:2006 were 0.051, 0.128, 0.551, and 0.090, respectively. No significant difference was observed in uninoculated samples. On the other hand, the CI of the dLPOD did not straddle zero in *C. jejuni*- (both low and high) and *C. coli*-inoculated samples, meaning that significant differences in LPOD were identified between the two methods (Table 4). Compared to ISO 10272-1:2006, NIHSJ-02 showed significantly higher detection rates of *C. jejuni* and *C. coli* in chicken.

Frequency of Occurrences of Background Bacteria

The frequency of occurrences of background bacteria was calculated in the same manner as LPOD of *Campylobacter*, and

Table 3. Probability of detection of *Campylobacter* and background bacteria[a]

	NIHSJ–02				
		C. jejuni		*C. coli*	
	Uninoculated	Low level	High level	Low level	Background bacteria
No. of total replicates	90	90	90	90	360
LPOD	0.033	0.222	0.678	0.267	0.431
LPOD 95% CI	(0.011, 0.093)	(0.212, 0.233)	(0.659, 0.700)	(0.246, 0.287)	(0.420, 0.441)
S_r	0.183	0.437	0.455	0.416	0.490
S_L	0	0	0.123	0.161	0.077
S_R	0.183	0.437	0.471	0.446	0.496
	ISO 10272–1:2006				
No. of total replicates	78	78	78	78	312
LPOD	0.051	0.128	0.551	0.090	0.926
LPOD 95% CI	(0.020, 0.125)	(0.071, 0.220)	(0.526, 0.576)	(0.044, 0.174)	(0.892, 0.950)
S_r	0.215	0.328	0.467	0.282	0.256
S_L	0.058	0.077	0.187	0.059	0.056
S_R	0.222	0.337	0.503	0.288	0.262

[a] LPOD = The POD value obtained by combining all valid collaborator data sets for a method; 95% CI = 95% confidence intervals; S_r = repeatability SD; S_L = SD of laboratory effect; and S_R = reproducibility SD.

as shown in Table 3, was 0.431 in the total samples tested by NIHSJ-02 and 0.926 by ISO 10272-1:2006. The frequency of occurrences of background bacteria was significantly lower by NIHSJ-02 than by ISO 10272-1:2006 because the dLPOD was found to be negative and the CI did not straddle zero (Table 4).

Discussion

In 2000, Baylis et al. (15) compared three enrichment broths and reported that Bolton broth and *Campylobacter* enrichment broth showed better performance than Preston broth for the isolation of *Campylobacter* from naturally contaminated foods, including chicken meat and livers. In recent years, however, the lower sensitivity of ISO 10272-1:2006 was suggested by several authors (16, 17). Jasson et al. (18) showed that Bolton broth allowed the growth of ESBL *Escherichia coli* present in poultry meat, and these bacteria could mask the growth of *Campylobacter*. *Pseudomonas* spp. were also frequently recovered from Bolton broth (15) due to the absence of polymixin and rifampicin. The effect of background bacteria could be problematic for the isolation of *Campylobacter* in foods with a high background level of non-*Campylobacter* bacteria. For this reason, Preston broth, which contains polymixin and rifampicin as selective reagents (19), was chosen for enrichment in NIHSJ-02, and because the previous version of ISO 10272 used incubation in Preston broth at 42°C as one of the enrichment procedures (20), the enrichment conditions used in NIHSJ-02 should be considered reasonable.

Three out of 90 and four out of 78 uninoculated samples were positive for *Campylobacter* by NIHSJ-02 and ISO 10272-1:2006, respectively. This is probably due to the survival of some naturally contaminated *Campylobacter* after the freeze-thaw procedure during sample preparation. Because the positive rates were low and did not differ significantly in uninoculated samples between both methods, the surviving *Campylobacter* probably did not affect the test results.

In a qualitative method, three criteria (relative accuracy, relative sensitivity, and relative specificity) are expressed for an interlaboratory study according to the guidelines of ISO 16140:2003 using paired samples. However, unpaired samples were used in this study, and it was difficult to calculate all of these parameters. A new statistical approach has been

Table 4. Comparison of LPOD of NIHSJ–02 with ISO 10272–1:2006

	Comparison of LPOD				
		C. jejuni		*C. coli*	
	Uninoculated	Low level	High level	Low level	Background bacteria
dLPOD[a]	–0.018	0.094	0.126	0.177	–0.500
dLPOD 95% CI[b]	(–0.094, 0.050)	(0.001, 0.152)	(0.095, 0.158)	(0.090, 0.227)	(–0.522, –0.460)

[a] dLPOD = the difference between LPOD values of NIHSJ–02 and ISO 10272–1:2006.

[b] 95% CI = 95% confidence intervals.

proposed for such validation to combine sensitivity, specificity, false-positive and false-negative parameters into a single parameter, POD (14). The LPOD for two methods could be successfully compared by the difference, the dLPOD, at a given analyte concentration. These parameters were adopted in the AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Foods and Environmental Surfaces (21) and applied to data analysis in this study.

In conclusion, a significantly higher detection rate of *Campylobacter* and a lower frequency of occurrences of background bacteria were observed by NIHSJ-02 than by ISO 10272-1:2006. NIHSJ-02 would work well with regard to the selective detection of *C. jejuni* and *C. coli* in chicken, which is usually contaminated with a high background level of non-campylobacters.

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The participation of the following collaborators is greatly appreciated:

Takayuki Konno, Akita Prefectural Research Center for Public Health and Environment, Akita, Japan

Masakado Matsumoto, Aichi Prefectural Institute of Public Health, Aichi, Japan

Maho Sato and Fumiaki Itoh, Hiroshima City Institute of Public Health, Hiroshima, Japan

Masato Furukawa and Seiya Harada, Kumamoto Prefectural Institute of Public Health and Environmental Science, Kumamoto, Japan

Keiji Yamaguchi, Hokkaido Institute of Public Health, Hokkaido, Japan

Katsuhiro Aikawa, Kanagawa Prefectural Institute of Public Health, Kanagawa, Japan

Norio Asai, Kyoto Prefectural Institute of Public Health and Environment, Kyoto, Japan

Yumi Inoguchi, Association of Meat Science and Technology Institute, Tokyo, Japan

Rie Saito, Japan Frozen Foods Inspection Corp., Hyogo, Japan

Atsushi Yoshida, Food Analysis Technology Center SUNATEC, Mie, Japan

Takaomi Wada, Hiroshima Environment and Health Association, Hiroshima, Japan

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FOOD BIOLOGICAL CONTAMINANTS

Flow Cytometric Method for in situ Preparation of Standard Materials of a Small Defined Number of Microbial Cells with Colony-Forming Potentiality

HIDEAKI MATSUOKA, KOICHIRO NAKANO, NORIMASA TAKATANI, and TOMONORI YOSHIDA
Tokyo University of Agriculture and Technology, Department of Biotechnology and Life Science, 2-24-16 Naka-cho, Koganei, Tokyo 184-8588, Japan
SHIZUNOBU IGIMI
National Institute of Health Sciences, Division of Biomedical Food Research, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
MIKAKO SAITO
Tokyo University of Agriculture and Technology, Department of Biotechnology and Life Science, 2-24-16 Naka-cho, Koganei, Tokyo 184-8588, Japan

Standard materials of a small defined number of cells with colony-forming potentiality are essential for the rational validation of food microbiological methods. An in situ flow cytometric method using viable staining with 6-carboxyfluorescein diacetate (CFDA) and tryptic soy agar (TSA) was previously proposed and its feasibility was demonstrated with five strains. In this study, this method was applied to 16 strains to support its broad applicability. The cell sorting gate was previously determined based on the CFDA stainability alone. Now the structural properties of cells designated by forward and side-scattering intensities have been introduced as the second gating criteria. Under the optimum gate condition, 100 cells have been selected and sorted on TSA. Consequently, a 95% or higher colony-forming rate has been attained for every strain. A successful application to microaerophilic *Campylobacter* spp. is especially of great importance because it suggests further broader applicability.

All standard and reference methods for determining microbial cells in food are based on colony counting methods using appropriate agar media. The number of colonies that appear on agar media is usually expressed in terms of CFUs. A CFU is usually a single cell but may represent a pair, a cluster, or a chain of cells that can be formed by a cell population. However, another concept concerning a single cell is necessary, and for this concept we have introduced the term colony-forming potentiality (CFP), which is a single cell that can grow to form a colony with a visible size. Thus, a CFP is not just a viable cell but is one that can replicate to form a visible colony. Injured cells are typical of viable cells that may not necessarily maintain CFP (1, 2), and thus might cause a reduction in viable cell count. Count loss is also caused by the presence of microcolonies; the formation of colonies provides evidence of viable cells, but it is well-known that microcolonies can hardly be observed by manual colony counting (3). The reduction in cell count due to these causes is a critical issue even in the absence of food matrixes.

On the other hand, in common test samples containing food matrixes, the problem of false signals is more serious because the distinction between microbial colonies and nonbiological particles is difficult. To date, various methods for the continuous or time-lapse measurement of the colony-forming process have been developed (4, 5) and may effectively reduce the number of false signals. However, it still remains difficult to approve the results as accurate values unless the methods have been well validated separately.

In this context, the development of standard materials of cells maintaining CFP is an urgent requirement. Certified reference materials (CRMs) for microbiological analysis provided by the Institute for Reference Materials and Measurements (IRMM) in Belgium are thought to be feasible tools for this purpose. The CRMs comprise gelatin capsules containing milk powder contaminated with a spray containing bacterial cells of a target strain. For instance, BCR-528 is a CRM for *Bacillus cereus* American Type Culture Collection (ATCC) (Manassas, VA) 9139. According to its certificate of analysis, the number of colony-forming particles is 53.4 ± 0.9 per capsule (mean ± SD, $n = 11$ (6). To date, however, the number of CRMs available is only six strains.

BioBall® developed in 2003 is a useful standard material containing a few cells (7, 8). This standard material enables the preparation of an exact number of 30 ± 2 colonies simply by plating the small cotton-like ball on an agar plate. On the basis of this technology, IRMM developed two BioBall strains: *Escherichia coli* O157 (NTCT 12900) (9) and *Salmonella enteritidis* (NCTC 12694) (10). However, the number of BioBalls developed so far is only 10 strains.

These standard materials can be preserved, and their performance after storage has been validated. Nevertheless, the number of standard strains available is very small, and the development of new ones is still difficult because of high costs, long preparation times, and variations in strain-specific properties. Therefore, an alternative approach to standard materials is urgently required. An in situ preparation method using a flow cytometric cell sorter (FCCS) was considered. Originally, FCCS was applied to animal cells greater than 10–20 μm, but recently it has been applied successfully to

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Corresponding author's e-mail: mhide@cc.tuat.ac.jp
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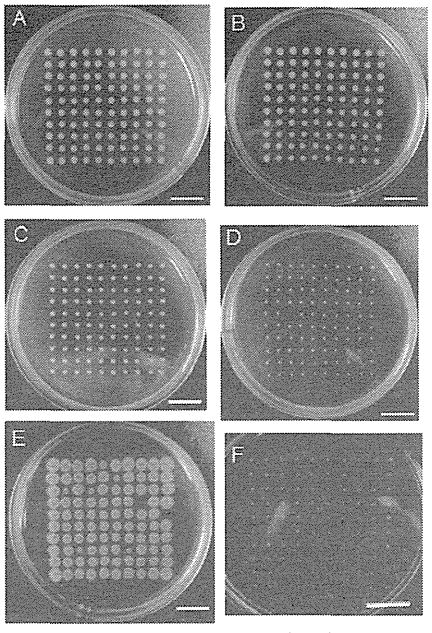

Figure 1. Colony formation patterns (A) *E. coli* ATCC 8739; (B) *C. freundii*; (C) *K. pneumoniae*; (D) *M. morganii*; (E) *M. luteus*; and (F) *C. coli*. A bar indicates 15 mm.

smaller bacterial cells (11, 12) and is used for the production of BioBalls.

In a previous study (13), we found that staining target cells with 6-carboxyfluorescein diacetate (CFDA) and sorting of the CFDA stainable cells on tryptic soy agar (TSA) plates was effective for two strains of *E. coli*, and one strain each of *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. CFDA is well-known as a probe for viable cells (14, 15), and the CFDA stainability of each cell is displayed as a histogram of fluorescent intensity versus number of cells. Therefore, a sorting gate can be set on this histogram as a definite range of fluorescence intensity. Sorting based on the CFDA stainability alone, however, was not sufficient for broader application. Therefore, we have introduced the second sorting gate based on structural properties of cells. The structural properties of each cell are plotted on a panel of forward scatter intensity (FSC) versus side scatter intensity (SSC). FSC is an indicator of cell size while SSC indicates the complexity of intracellular structure. This second sorting gate can be set on the FSC-SCC panel as a definite area. In this study, we intended to find an optimum gate condition in the FSC-SSC panel by trial and error for each of 16 strains including the microaerophilic strains of *Campylobacter*.

Experimental

Microorganisms

E. coli K-12 (NBRC 3301), *Klebsiella pneumoniae* (NBRC 3318), *Enterobacter aerogenes* (NBRC 12010), *Pseudomonas aeruginosa* (NBRC 12689), *B. subtilis* (NBRC 3009), *S. aureus* (NBRC 102135), and *Micrococcus luteus* (NBRC 12708) were obtained from the National Institute of Technology and Evaluation-Biological Resource Center (NBRC; Kisarazu, Chiba, Japan). *Escherichia coli* ATCC 8739

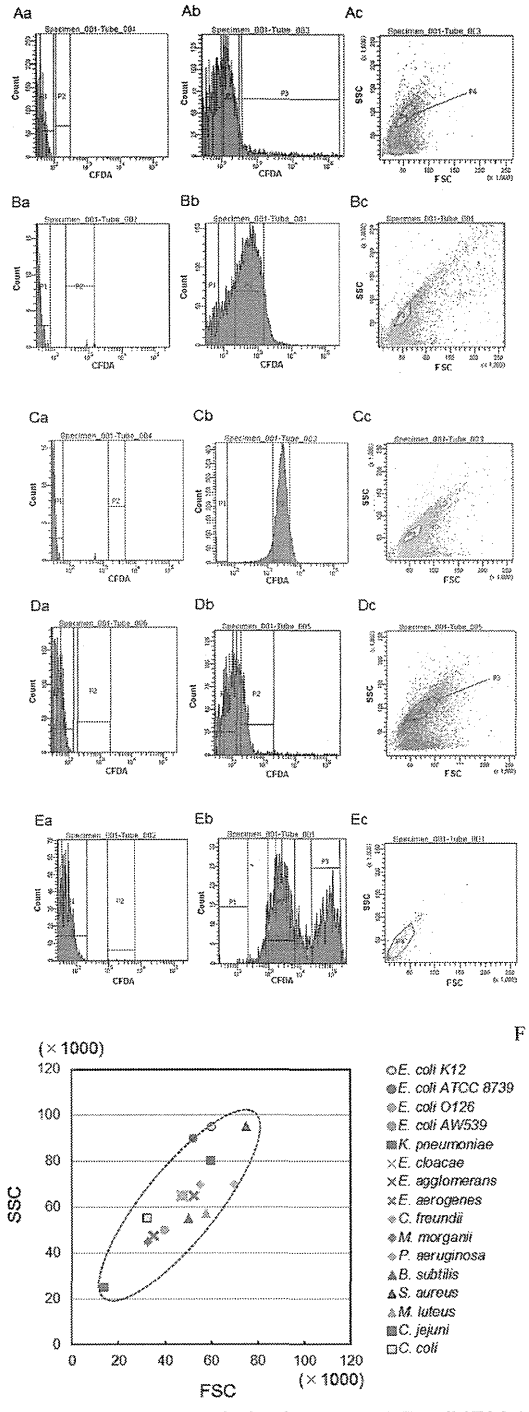

Figure 2. Flow cytograms of microbial cells (A) *E. coli* ATCC 8739; (B) *C. freundii*; (C) *M. luteus*; (D) *S. aureus*; (E) *E. agglomerans*: (a) Fluorescence intensity profile before staining with CFDA; (b) fluorescence intensity profile after staining with CFDA; (c) particle distribution pattern. Sorting gates are P2&P4 (A, E) or P2&P3 (B, C, D). Particles in these gates are indicated as yellow points; (F) center points of the optimum sorting gate.

was obtained from ATCC. *E. coli* O126, *Enterobacter cloacae*, *Enterobacter agglomerans*, and *Citrobacter freundii* were isolated from food. *Morganella morganii* is a histamine-producing bacteria and was kindly provided by T. Fujii (Tokyo University of Marine Science and Technology, Tokyo, Japan). *E. coli* AW539 is an aspartate taxis mutant and was obtained

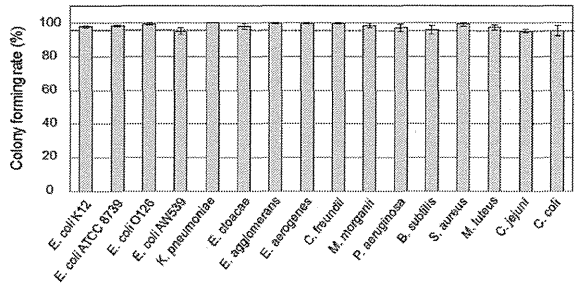

Figure 3. CFRs of the strains tested. Data are mean ± SD (n = 3). A broken line indicates the 95% criteria.

from Nagoya University (Nagoya, Japan). *Campylobacter jejuni* (NCTC 11168) and *C. coli* (JCM 2529) were obtained from National Institute of Health Sciences (Tokyo, Japan).

Culture and Fluorescent Staining

Every strain except *C. jejuni* and *C. coli* was precultured in tryptic soy broth, and its cell suspension was plated on TSA. After culture at 37°C for 18 h, the resulting colonies were picked up and suspended in phosphate buffer solution (PBS), 0.1 M, pH 7.0. The cell concentration was adjusted at 10^8 cells/mL. A 1 mL aliquot of this cell suspension was transferred to a microtube; then CFDA (Sigma, St. Louis, MO) was added to the microtube at 150 ppm. After the reaction with CFDA for 30 min, the cell suspension was diluted 100-fold with PBS and then transferred into a 5 mL polystyrene round-bottom tube (BD Falcon, 352235; Becton Dickinson Co., Cockeysville, MD) through a strainer cap attached to the tube. The mesh size of the strainer was 35 µm.

C. jejuni and *C. coli* stored at –80°C were placed on Müller-Hinton agar containing vancomycin (1 µg/mL) and incubated at 37°C for 48 h under a microaerophilic atmosphere (O_2 6–12%, CO_2 5–8%) maintained with AnaeroPack in a 2.5 L rectangular jar (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan). The surface of resulting cells was swabbed with a cotton chip and the cells were suspended in Müller-Hinton broth. After incubation at 37°C for 13 h, the cell concentration was adjusted to 10^8 cells/mL by diluting with PBS. A 1 mL aliquot of this suspension was transferred to a microtube; then CFDA was added to the microtube at 150 ppm. Successive procedure for CFDA staining was performed as described above under aerobic condition because air exposure for no longer than 1 h caused no inhibition effect on their CFP.

Cell Sorting

The CFDA-stained cells were applied to a fluorescence-activated cell sorter (FACSAria II; BD Co.). The CFDA-stained cells were excited by a blue laser at 488 nm. The cell sorting gate was searched for by repeating trial and error based on the two criteria: structural properties and CFDA stainability. The structural properties of each cell were automatically plotted on an FSC-SSC flow cytogram, while CFDA stainability of each cell was displayed as a histogram of fluorescent intensity versus number of cells. A cell population within a region indicated on these two flow cytograms was selected and sorted on a TSA plate of 86 mm in diameter. The TSA plate was set on an automatic stage installed in the cell sorter. The automatic stage was driven so that 100 cells could be sorted into a 10 × 10 grid pattern of spots at 1 cell/spot. After sorting, the TSA plate of every strain except *Campylobacter* spp. was incubated at 37°C in an incubator (SLI 600ND, Tokyo Rika Kikai Co. Ltd, Tokyo, Japan). *C. jejuni* and *C. coli* were incubated at 37°C under a microaerophilic atmosphere, described above. After incubation, the number of colonies was counted. The colony-forming rate (CFR) was determined by the following equation:

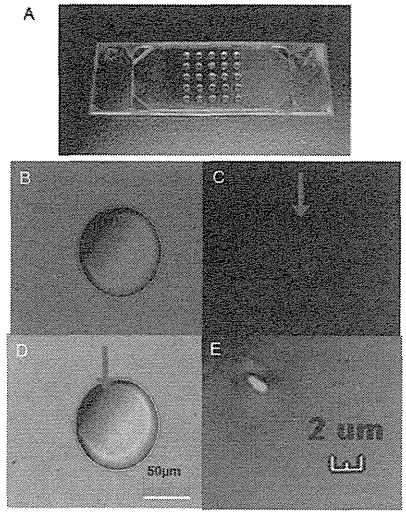

Figure 4. Detection of a single cell immediately after sorting. (A) 5 × 5-well plate devised on a slide glass (2.5 × 7.5 cm^2); (B) microscopic view of a droplet; (C) fluorescent microscopic view of the droplet; (D) merge of B and C; and (E) magnified image of a single cell. A fluorescent point was found at the arrow in (C) and (D).

$$CFR = 100 \times N/N_0\ (\%)$$

where N_0 was the number of sorted cells and N was the number of colonies. In this study, N_0 was 100 and, therefore, CFR = N (%). Experiments were repeated three times for respective conditions.

Results

Colony-Forming Pattern

Single-cell sorting was performed on a grid of 10 × 10 spots on a TSA plate for each strain. In many cases, the colony size within each plate was uniform, which might indicate that single-cells with uniform properties were sorted at every spot. The culture time was 18 h for every strain except the following three strains: culture times for *B. subtilis* and two strains of *Campylobacter* spp. were 12 h and 48 h, respectively. Typical examples of colony-forming pattern are depicted in Figure 1.

Optimum Sorting Gates for Respective Strains

E. coli ATCC 8739 cells emitted slight fluorescence without staining (Figure 2Aa) that was thought to be intrinsic fluorescence; this fraction was designated as P1. After CFDA staining, the cells emitted much more intense fluorescence (Figure 2Ab). The fluorescence intensity range was divided

into P1, P2, and P3 fractions. The cells in the P2 fraction were regarded as properly stained cells, and the first gate for cell sorting was set as P2. A narrow area in the FSC-SSC flow cytogram was defined by a closed circle and set as the second gate, designated as P4. The P2 and P4 combined gate condition was optimized so that CFR could be 95% or higher. Under the optimized condition (yellow points in P4 in Figure 2Ac), CFR exceeded 95%. The relative number of yellow points in this P2 and P4 combined gate was only 4.9% among all sorted cells. However, this small population was thought to be a uniform cell group with uniform CFP. *E. coli* K-12, *K. pneumoniae*, *E. aerogenes*, and *C. coli* showed similar flow cytograms to that of *E. coli* ATCC 8739. The optimum combined gates for these strains could be determined in the same way.

Figure 2B–E depict the results of *C. freundii*, *M. luteus*, *S. aureus*, and *Enterobacter agglomerans*, respectively. Their flow cytograms of CFDA stainability (Figure 2Bb, Cb, Db, and Eb) were different from that of *E. coli* ATCC 8739. However, the optimum condition of the second gate designated as P3 (in Figure 2Bb, Cb, and Db) or P4 (in Figure 2Eb) could be determined so that CFR was 95% or higher.

In summary, the center points of the optimum sorting gate for respective strains are plotted in Figure 2F. These center points are localized inside an oval leaning about 40 degrees versus the FSC intensity axis. This result suggests that the center points of the optimum sorting gate for any strains might also be located inside this oval. Therefore, it is recommended to start a search for the optimum sorting gate for any other strains from this oval area.

Colony-Forming Rates

The CFR of every strain attained under the optimum gate condition is shown in Figure 3. Every CFR value became 95% or higher levels. In the case of *E. coli* ATCC 8739, for instance, the colony count data were 99, 98, and 98 colonies/100 spots. Therefore, the CFR was 98.3 ± 0.6%. In the case of *K. pneumoniae*, the colony count data were 100, 100, and 100 colonies/100 spots. Therefore, the CFR was 100 ± 0%.

Discussion

The number of cells sorted at a single spot was assumed to be one or zero. This performance level of the cell sorter is thought to be a matter of course; however, the sorted matter is actually a single particle and not two or more particles by accident. Therefore, we checked the sorted particle(s) by microscopy immediately after sorting.

A 5 × 5-well plate was devised for this purpose (Figure 4A), and a single droplet was sorted into each well. Shortly after sorting, each droplet was observed with a fluorescence microscope. The droplet size was about 100 μm in diameter (Figure 4B). In each droplet, a single fluorescent point could be detected (Figure 4C and D); this point was recognized as a rod-like cell (Figure 4E). Among all the droplets checked in this way, every one contained a single fluorescent particle of a similar size and shape. Therefore, single-cell sorting was performed as expected.

In this study, CFDA was used as a probe for viable cells. The cell membrane is permeable to CFDA. Only in viable cells, CFDA is hydrolyzed to generate a fluorescent derivative, 6-carboxyfluorescein (CF), by intracellular esterase. Both CFDA and CF are nontoxic to the cell (11); therefore, viable cells may be stained without loss of cell viability. However, the intensity of CFDA stainability was different among strains (Figure 2). There was also an occasion that a single strain such as *Enterobacter agglomerance* showed two peaks of fluorescent intensity (Figure 2E). We investigated the dependence of CFDA stainability intensity on CFR, but no clear relationship was found. Consequently, the optimum gate for CFDA stainability was determined empirically, although there was no limitation of CFDA application in the strains used here.

We expect that CFDA can be applied to many other strains. However, to develop a general platform for whatever strains it should be necessary to find other fluorescent probes. From this viewpoint, a fluorescent glucose such as 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2NBDG) is promising. It can be taken up only by viable cells of microorganisms (16, 17) and animals (18), supposedly via glucose transporters. The use of 2NBDG alone or in combination with its antipode, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-L-glucose (2NBDLG; 19) or its derivative of different fluorescent color would be feasible for the viable or dead distinction by a different principle from that of CFDA.

From the viewpoint of broad applicability, the successful application to *Campylobacter* spp. was exciting. This supports the usefulness of TSA, which is a nonselective medium and can therefore support the repair processes of injured cells. *Campylobacter* cells partially injured by air exposure stress and hydrodynamic sheer stress during cell sorting might be repaired by TSA. These suggest a possibility of further extension to other bacteria more susceptible to aerobic conditions. Moreover, it may possibly be extended to anaerobic bacteria by facilitating the CFDA attaining and cell sorting procedures. This should be a future challenge.

Finally, we emphasize that the present flow cytometric method may provide an experimental clue to study about one of the most substantial subjects in microbiology: the homogeneity and heterogeneity of colonies.

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Hideaki Matsuoka and Shizunobu Igimi acknowledge support from Grant-in-aid for Scientific Research for the Promotion of Safety and Security of Foods on the subject, "Microbiological Methods in Foods and Their Validation" (H23-Food-General-010), The Ministry of Health, Labour, and Welfare, Tokyo, Japan.

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JASISコンファレンス

～国際化に対応する分析値の質の向上と
AOACの新しい分析法妥当性確認～

微生物試験法の妥当性確認の新ガイドライン

2012年9月7日

松 岡 英 明
東京農工大学　大学院工学研究院　生命機能工学部門

1

準拠する規格等

- ISO 16140:2003: Microbiology of food and animal feeding stuffs — Protocol for the validation of alternative methods.
- AOAC INTERNATIONAL, Official Methods of Analysis 18th Edition, 2005,
  Appendix D: Guidelines for Collaborative Study Procedures to Characteristics of a Method of Analysis.
  Appendix E: Laboratory Quality Assureance.
- JIS Z 8402-1～5; 1999～2002 (ISO 5725-1～5; 1994～1998): 測定法及び測定結果の精確さ(真度及び精度)Accuracy (trueness and precision) of measurement methods and results
- AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces. (2012. 2. 24)

2

AOAC INTERNATIONAL

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AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces

This document provides guidelines for the AOAC validation of microbiological methods for food and environmental surfaces. It includes terms and their definitions associated with the *Official Methods of Analysis*℠ and *Performance Tested Methods*℠ programs and validation requirements for qualitative, quantitative, and confirmatory identification methods.

The guideline working group consisted of Sharon Brunelle, Robert LaBudde, Maria Nelson, and Paul Wehling. Consensus was reached on the guidelines by the AOAC Methods Committee for Microbiology and approved by the AOAC Official Methods Board.

3

AOAC INTERNATIONAL JAPAN SECTION

AOAC2012版の項目

1	視点・範囲				
2	適用範囲				
3	用語と定義				
4	定性試験				
	4.1	開発者の自主バリデーション、または単一試験所でのバリデーション(またはプレコラボスタディ)[注1]			
		4.1.1	視点・範囲		
		4.1.2	包含性・排他性試験(pure cultureでの試験、参照法は不要)[注2]		マトリクスが無い場合
			4.1.2.1	菌種・菌株の選定 (Species/Strain Se	菌株選定の考え方は記載してあっても、具体的表記はない。
			4.1.2.2	試験のデザイン (Study Design)	
			4.1.2.3	結果報告 (Data Reporting)	
		4.1.3	マトリクス試験(食品だけでなく表面汚染を入れる、参照法との比較試験)		マトリクスがある場合
			4.1.3.1	参照法	
			4.1.3.2	食品カテゴリー[注3]	
			4.1.3.3	環境表面	
			4.1.3.4	汚染レベル[注4]	PODの概念。一方、ISO16140での「精確さ」「特異性」「感度」は記載なし。それらと「検出レベル」を合わせてPODか？
			4.1.3.5	繰り返し数(試験画分数、Number of test portion)[注5]	

4

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	4.1.3.6	試験画分サイズ、混合と貯留		具体的で詳しく例示
	4.1.3.7	汚染源		菌株選定の考え方は記載してあっても、具体的表記はない。基本的に一つのマトリクスには1菌株。ただし、複数菌株を同時に添加するのはmulti-analyte mehodsの場合のみ、とあるが、それがどのような試験かについては記載なし
	4.1.3.8	人工的な汚染試料の調製		
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4.1.4	堅牢性試験（PTMの場合のみ）			
	4.1.4.1	菌株の選定		包含性・排他性試験では「菌種・菌株」
	4.1.4.2	試験のデザイン		
	4.1.4.3	結果の解析と報告		「結果の解析と報告」になっている

5

AOAC2012版の項目

4.2	外部委託バリデーション			記述が簡略。「4.1 開発者の自主バリデーション」を引用
	4.2.1	視点・範囲		
	4.2.2	参照法		
	4.2.3	マトリクス		
	4.2.4	試験のデザイン		
4.3	コラボスタディ			
	4.3.1	視点・範囲		
	4.3.2	試験所の数[注6]		
	4.3.3	参照法		
	4.3.4	マトリクスの選択[注7]		
	4.3.5	分析種濃度の推定		
	4.3.6	繰り返し数（試験画分数、Number of test portion）[注5]		
	4.3.7	試験画分サイズ、混合と貯留		
	4.3.8	汚染源		
	4.3.9	人工的な汚染試料の調製		
	4.3.10	自然汚染試料の調製		
	4.3.11	試験画分の確認		
	4.3.12	結果の解析と報告		「結果の解析と報告」になっている
		4.3.12.1	生データの表	
		4.3.12.2	併行精度の評価	
		4.3.12.3	室間再現精度の評価	
		4.3.12.4	全試験所平均検出確率	
		4.3.12.5	参照法と代替法の全試験所平均検出率の差	
		4.3.12.6	結果の要約表	
		4.3.12.7	結果の図	
		4.3.12.8	参照法が無い場合の結果の解析と報告	「参照法が無い場合が意外に多い」という背景か？

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AOAC2012版の項目

5	定量試験			
	5.1	開発者の自主バリデーション、または単一試験所でのバリデーション(またはプレコラボスタディ)		
		5.1.1	視点・範囲	
		5.1.2	包含性・排他性試験(pure cultureでの試験、参照法は不要)[注7]	適用対象は選択的あるいは差分定量法の場合
			5.1.2.1 菌株の選定 (Strain Selection)	定性では「菌種・菌株」
			5.1.2.2 試験のデザイン (Study Design)	
			5.1.2.3 結果報告 (Data Reporting)	
		5.1.3	マトリクス試験(表面汚染は含まない?、参照法との比較試験)	
			5.1.3.1 参照法	
			5.1.3.2 食品カテゴリー[注13]	
			5.1.3.3 汚染レベル[注11]	
			5.1.3.4 繰り返し数(試験画分数、Number of test portion)[注11]	
			5.1.3.5 汚染源	
			5.1.3.6 人工的な汚染試料の調製	
			5.1.3.7 人工的汚染試料と自然汚染試料の使用	
			5.1.3.8 競合菌叢の必要性	
			5.1.3.9 試験画分の確認	
			5.1.3.10 結果の解析と報告	
			5.1.3.10.1 一般的事項	
			5.1.3.10.2 結果の初期レビュー	
			5.1.3.10.3 外れ値	
			5.1.3.10.4 併行再現精度	
			5.1.3.10.5 適用できる場合の参照法と代替法の平均の差	
		5.1.4	堅牢性試験(PTMの場合のみ)	
			5.1.4.1 菌株の選定	
			5.1.4.2 試験のデザイン	
			5.1.4.3 結果の解析と報告	

AOAC2012版の項目

	5.2	外部委託バリデーション		
		5.2.1	視点・範囲	
		5.2.2	参照法	
		5.2.3	マトリクス	
		5.2.4	試験のデザイン	
	5.3	コラボスタディ		
		5.3.1	視点・範囲	
		5.3.2	試験所の数[注12]	
		5.3.3	参照法	
		5.3.4	マトリクスの選択[注13]	
		5.3.5	汚染のレベル	定性では「分析種濃度」
		5.3.6	繰り返し数(試験画分数、Number of test portion)[注14]	
		5.3.7	特定菌の計数	
		5.3.8	汚染源	
		5.3.9	人工的な汚染試料の調製	
		5.3.10	人工的汚染試料と自然汚染試料の使用	
		5.3.11	試験画分の確認	
		5.3.12	結果の解析と報告	
			5.3.12.1 一般的事項	
			5.3.12.2 結果の初期レビュー	
			5.3.12.3 外れ値	
			5.3.12.4 性能指標	
			5.3.12.4.1 併行再現精度	
			5.3.12.4.2 室間再現精度	
			5.3.12.5 適用できる場合の参照法と代替法の平均の差	
			5.3.12.6 計算	

AOAC2012版の項目

6	同一性確認試験				
	6.1	開発者の自主バリデーション、または単一試験所でのバリデーション(またはプレコラボスタディ)			
		6.1.1	視点・範囲		
		6.1.2	包含性・排他性試験		
			6.1.2.1	菌種・菌株の選定	
			6.1.2.2	試験のデザイン	
			6.1.2.3	結果の解析と報告	
		6.1.3	堅牢性試験(PTMの場合のみ)		
			6.1.3.1	菌株の選定	
			6.1.3.2	試験のデザイン	
			6.1.3.3	結果の解析と報告	
	6.2	外部委託バリデーション			
		6.2.1	視点・範囲		
		6.2.2	試験のデザイン		
		6.2.3	結果の解析と報告		
	6.3	コラボスタディ			
		6.3.1	視点・範囲		
		6.3.2	試験所の数		
		6.3.3	試験の数(Number of tests)		
		6.3.4	結果の解析と報告		
7	安全性				
8	文献				

付録	X-A	
付録	X-B	
付録	X-C	
付録	X-D	
付録	X-E	
付録	X-F	
付録	X-G	
付録	X-H	9

注1: OMAの前段としてのPTM

AOACプログラム	必要とされる試験	AOAC2012版での章節番号		
		定性試験	定量試験	同一性の確認試験 *)
PTM	開発者の自主バリデーション	4.1	5.1	6.1
	外部委託バリデーション	4.2	5.2	6.2
OMA	単一試験所でのバリデーション、あるいはプレコラボ	4.1	5.1	6.1
	外部委託バリデーション	4.2	5.2	6.2
	コラボスタディ	4.3	5.3	6.3
PTM→OMA	開発者の自主バリデーション	4.1	5.1	6.1
	外部委託バリデーション	4.2	5.2	6.2
	コラボスタディ	4.3	5.3	6.3

*)標的菌の同一性を確認することだけが目的の試験法。バイオテロ関連が具体的標的。

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