Evaluation of the Quantitative Dry Culture Method (Sanita-kun™ SA) for the Enumeration of Staphylococcus aureus in Artificially Contaminated Food Samples

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Sanita-kun™ SA for Staphylococcus aureus (SkSA), a novel dry sheet quantitative culture system, was evaluated. When the inclusivity and exclusivity of SkSA were assessed using 121 microorganisms including 47 S. aureus strains, the tested S. aureus strains formed blue-colored colonies on the SkSA and all the other microbes failed to grow.

The SkSA was then compared with Baird-Parker agar (BP) according to ISO 6888-1, Mannitol salt agar with egg yolk (MSEY), and 3M Petrifilm™ STX (3M-STX) in 100 artificially contaminated food samples. The correlation coefficients between SkSA and BP, SkSA and MSEY, and SkSA and 3M-STX were 0.971, 0.989 and 0.996, respectively.

Our results demonstrated that SkSA is a suitable alternative for the enumeration of S. aureus in foods.

Key words : Staphylococcus aureus / Sanita-kun™ / Dry sheet culture / Chromogenic medium.

Staphylococcus aureus has been shown to have a broad habitat including human skin. Hence, S. aureus can easily contaminate food through inappropriate handling (Baird, 1995). Since S. aureus produces several kinds of heat-stable enterotoxins, consumption of S. aureus-contaminated food can cause vomiting, diarrhea and abdominal pain (Balaban and Rasooly, 2000; Jablonski and Bohach, 1997; Nema et al., 2007). Hence, the control of S. aureus is critical in food processing and distribution facilities for prevention of food poisoning.

ISO 6888-1 (International Organization for Standardization, 1999) as well as the U.S. Food and Drug Administration Bacteriological Analytical Manual (2014) have recommended the use of Baird-Parker agar (PB; Baird-Parker, 1962) for the enumeration of S. aureus in food. Additionally, Mannitol salt agar with egg yolk (MSEY; Gunn et al., 1972) has also been used historically in Japan. However, these agar plate methods require preparation of the agar plates, cumbersome spreading techniques and comparatively long incubation times for the formation of the specific egg yolk reaction (Baird, 1995; De Buyser et al., 2003).

To make the operation easier, the 3M Petrifilm™ STX (3M-STX; 3M Microbiology Products, St. Paul, MN, USA) and Compact Dry X-SA™ (CD-XSA; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) have been accepted as suitable ready-to-use alternatives (Fedio et al., 2008; Ingham et al., 2003; Teramura et al., 2010). However, 3M-STX requires a spreader device and confirmation disk for the differentiation of S. aureus, and CD-XSA requires the morphological discrimination of colonies since microorganisms other than S. aureus also form blue-colored colonies.

In consideration of these factors, the Sanita-kun™ SA method (SkSA; JNC Corporation, Tokyo, Japan), a novel, ready-to-use dry sheet quantitative culture system, was developed. The SkSA is based on the unique Sanita-kun™ system which consists of a transparent cover film, non-woven fabric and water absorption polymer incorporated with growth medium compo-
FIG. 1. The colony appearance of S. aureus on SkSA. S. aureus NBRC 100910 grew as blue-colored colonies on SkSA after 24h of incubation at 37°C.

The medium composition of SkSA consists of soy peptone, meat peptone, yeast extract, meat extract, mannitol, lithium chloride, polysorbate 80, 3 kinds of antibiotics and a chromogenic substrate for acid-phosphatase. After inoculation of a 1 ml aliquot of a sample onto SkSA, the sample is automatically diffused to the whole plate through the capillary action of the non-woven fabric. S. aureus grows to form blue colored colonies after 24 h of incubation at 37°C (Fig.1). It is not necessary to prepare the medium or manually spread the sample since SkSA is a pre-sterilized and self-diffusible culture system. Additionally, it is not necessary to use a confirmation disk or to differentiate colonies by their appearance since only S. aureus forms blue colonies on SkSA. The aim of this study was to evaluate the performance of SkSA as a new alternative for the enumeration of S. aureus.

The inclusivity of SkSA was assessed using 47 strains of S. aureus including 33 isolates from food samples. Twenty-two Staphylococcus sp. strains other than S. aureus, 11 gram-positive strains other than Staphylococcus sp., 38 gram-negative strains and 3 yeasts were tested in the exclusivity study. After bacterial and yeast strains were cultured on Tryptic Soy Agar (Difco, Becton Dickinson, Detroit, MI, USA) at 37°C for 24 h, each culture was suspended in sterile saline (0.85 % NaCl) at a turbidity equivalent to No.1 McFarland standard (3.0 x 10^3 CFU/ml; McFarland, 1907). Each inoculum strain was then subjected to 10-fold serial dilution in sterile saline. One milliliter of each suspension was inoculated onto SkSA and 3M-STX, whereas 0.1 ml was plated onto BP (SYSMEX-bioMérieux Co., Ltd., Tokyo, Japan) and MSEP (Nissui Pharmaceutical Co., Ltd.) with a plastic spreader (Nissui Pharmaceutical Co., Ltd.). The colonies on SkSA and 3M-STX were observed and counted after 24 h of incubation at 37°C whereas those on BP and MSEP were enumerated after 48 h of incubation at 37°C.

The results from the inclusivity and exclusivity studies are shown in Table 1. A total of 47 S. aureus strains grew and formed blue-colored colonies on SkSA. Similarly, these 47 S. aureus strains grew and formed colonies of a typical appearance on BP, MSEP and 3M-STX. With respect to SkSA, none of the microorganisms tested other than S. aureus grew. In contrast, of the 33 gram-positive bacteria other than S. aureus and the 3 yeasts, 29, 22 and 9 strains grew with atypical appearance on BP, MSEP and 3M-STX, respectively. Additionally, one S. intermedius strain formed typical colonies on 3M-STX (mauve) and BP (black with egg yolk reaction). All of the 38 gram-negative bacteria failed to grow on 3M-STX and MSEP. There were 4 strains (1 strain of Enterobacter gergoviae, 2 strains of Pseudomonas aeruginosa and 1 strain of Salmonella enterica) which grew on BP. Unexpectedly no microorganisms other than S. aureus grew on SkSA in the present studies, but Bacillus species strains were not inhibited on the other 3 selective media.

Teramura et al. (2010) reported that CD-XSA required the morphological discrimination of colonies since Bacillus species strains formed blue-colored colonies with a scabrous surface. SkSA needed neither a confirmation disk nor discrimination of other colonies since SkSA had excellent selectivity for S. aureus. SkSA is based on a chromogenic agar medium which Teramura et al. (2014) reported for the detection of the methicillin-resistant S. aureus. However, this agar medium could not inhibit Bacillus sp. strains. Moreover, SkSA differed from this chromogenic agar medium in the use of bacitracin as one of selective agents. Hence, it was suggested that bacitracin contributed to the selectivity for Bacillus sp. strains. According to ISO 16140 "Protocol for the validation of alternative methods" (International Organization for Standardization, 2003), at least 30 reaction-positive strains and 20 negative strains should be used for the inclusivity and exclusivity studies, respectively. It is therefore suggested that the performance of SkSA met the criteria for both inclusivity and exclusivity as described in ISO 16140.

The SkSA method was compared with the BP, MSEP and the 3M-STX methods using artificially contaminated food samples. One hundred samples (20 meats, 20 seafoods, 12 vegetables, 8 fruits, 11 confectioneries, 9 dairy products, 9 dough products and 11 delicatessen items) were purchased from retail stores in Yokohama city, Japan. Testing to establish that these samples were negative for S. aureus was conducted according to NIHJSJ-05 (National Institute of Health Sciences...
### TABLE 1. Growth and colony color of microbes tested on various media

| Name of organism | No. of tested strains | No. of strains grown<sup>a</sup> | SkSA | Baird-Parker | MSEY | 3M-STX |
|------------------|-----------------------|----------------------------------|------|--------------|------|--------|
| **Gram positive bacteria** | | | | | | |
| *Bacillus cereus* | 1N 0 1 1 0 | 1 (b) 1 (pEY) 0 | | | | |
| *B. licheniformis* | 1N 0 1 0 1 | 1 (b) 1 (pEY) 0 | | | | |
| *B. subtilis* | 1N 0 1 0 1 | 1 (b) 1 (pEY) 0 | | | | |
| *Corynebacterium ammoniagenes* | 1N 0 1 1 0 | 1 (b) 1 (pEY) 0 | | | | |
| *Enterococcus faecalis* | 1J 0 1 0 1 | 1 (b) 1 (pEY) 0 | | | | |
| *E. faecium* | 1J 0 1 0 1 | 1 (b) 1 (pEY) 0 | | | | |
| *E. hirae* | 1I 0 1 0 1 | 1 (b) 0 0 | | | | |
| *Lactobacillus lactis* | 1I 0 1 0 1 | 0 0 0 | | | | |
| *L. plantarum* | 1I 0 1 0 1 | 0 0 0 | | | | |
| *Leuconostoc mesenteroides* | 1I 0 1 0 1 | 1 (b) 0 0 | | | | |
| *Micrococcus luteus* | 1N 0 1 0 1 | 0 1 (b) 0 0 | | | | |
| *Staphylococcus arlettae* | 1D 0 1 1 0 | 0 1 (b) 1 (pEY) 0 | | | | |
| *S. aureus* | 5J 9N 33 47 | 47 (B) 47 (pEY) 47 (yEY) 47 (M) | | | | |
| *S. auricularis* | 1A 0 1 0 1 | 1 (b) 1 (pEY) 0 | | | | |
| *S. capitis* | 1J 0 1 0 1 | 1 (b) 0 0 | | | | |
| *S. caprae* | 1J 0 1 0 1 | 0 1 (b) 1 (pEY) 0 | | | | |
| *S. chromogenes* | 1N 0 1 0 1 | 0 1 (b) 0 0 | | | | |
| *S. cohnii* | 1J 0 1 1 0 | 1 (b) 1 (yEY) 0 | | | | |
| *S. delphini* | 1D 0 1 0 1 | 1 (b) 1 (yEY) 0 | | | | |
| *S. epidermidis* | 2N 0 2 0 2 | 2 (b) 2 (pEY) 0 | | | | |
| *S. equorum* | 1A 0 1 0 1 | 0 1 (b) 0 0 | | | | |
| *S. gallinarum* | 1A 0 1 0 1 | 1 (b) 0 0 | | | | |
| *S. haemolyticus* | 1J 0 1 0 1 | 0 0 0 0 | | | | |
| *S. hominis* | 1J 0 1 0 1 | 0 0 0 0 | | | | |
| *S. hyicus* | 1J 0 1 0 1 | 0 1 (pEY) 0 | | | | |
| *S. intermedius* | 1A 0 1 0 1 | 0 1 (pEY) 0 | | | | |
| *S. kentus* | 1A 0 1 0 1 | 0 0 0 0 | | | | |
| *S. saprophyticus* | 1J 0 1 0 1 | 0 1 (pEY) 0 | | | | |
| *S. schleiferi* | 1A 0 1 0 1 | 0 1 (pEY) 0 | | | | |
| *S. sciuri* | 1A 0 1 0 1 | 1 (b) 1 (yEY) 0 | | | | |
| *S. simulans* | 1J 0 1 0 1 | 0 1 (b) 1 (pEY) 0 | | | | |
| *S. warneri* | 1J 0 1 0 1 | 0 0 0 0 | | | | |
| *S. xylosus* | 1N 0 1 0 1 | 1 (b) 1 (pEY) 0 | | | | |
| **Subtotal** | | 47 33 80 47 76 68 56 | | | | |
| **Gram negative bacteria** | | | | | | |
| *Aeromonas hydrophila* | 1J 0 1 0 0 | 0 0 0 0 | | | | |
| *Cedeces lapagei* | 1J 0 1 0 0 | 0 0 0 0 | | | | |
| *Citrobacter amalonaticus* | 1I 0 1 0 0 | 0 0 0 0 | | | | |
| *C. freundii* | 1J 0 1 0 0 | 0 0 0 0 | | | | |
| *C. koseri* | 1I 0 1 0 0 | 0 0 0 0 | | | | |
| *Cronobacter sakazakii* | 1A 0 1 0 0 | 0 0 0 0 | | | | |
| *Edwardsiella tarda* | 1J 0 1 0 0 | 0 0 0 0 | | | | |
| *Enterobacter aerogenes* | 1I 0 1 0 0 | 0 0 0 0 | | | | |
| *E. cloacae* | 2I 0 2 0 2 | 0 0 0 0 | | | | |
| *E. gergoviae* | 1J 0 1 0 1 | 0 0 0 0 | | | | |
| *Enterirchiria coli* | 4N 0 1 0 0 | 0 0 0 0 | | | | |
| *E. fergusonii* | 1N 0 1 0 0 | 0 0 0 0 | | | | |
| *E. vulneris* | 1N 0 1 0 0 | 0 0 0 0 | | | | |
| *Hafnia alvei* | 1J 0 1 0 0 | 0 0 0 0 | | | | |
| *Klebsiella oxytoca* | 1J 0 1 0 0 | 0 0 0 0 | | | | |
| *K. pneumoniae* | 1J 0 1 0 0 | 0 0 0 0 | | | | |
| *Kluyvera ascorbata* | 1J 0 1 0 0 | 0 0 0 0 | | | | |
| *K. intermedius* | 1J 0 1 0 0 | 0 0 0 0 | | | | |
| *Leclercia adecarboxylata* | 1N 0 1 0 0 | 0 0 0 0 | | | | |
| *Morganella morgani* | 1I 0 1 0 0 | 0 0 0 0 | | | | |
| *Proteus mirabilis* | 1N 0 1 0 0 | 0 0 0 0 | | | | |
| *P. vulgaris* | 1N 0 1 0 0 | 0 0 0 0 | | | | |
| *Providencia alcalifaciens* | 1I 0 1 0 0 | 0 0 0 0 | | | | |
| *Pseudomonas aeruginosa* | 3N 0 3 0 2 | 0 0 0 0 | | | | |
| *P. fluorescens* | 1N 0 1 0 0 | 0 0 0 0 | | | | |
| *P. stutzeri* | 1N 0 1 0 0 | 0 0 0 0 | | | | |
| *Rhizobium aquatilis* | 1I 0 1 0 0 | 0 0 0 0 | | | | |
| *Salmonella enterica* | 3N 0 3 0 1 | 0 0 0 0 | | | | |
| *Serratia marcescens* | 1J 0 1 0 0 | 0 0 0 0 | | | | |
| *S. rubidiae* | 1N 0 1 0 0 | 0 0 0 0 | | | | |
| **Subtotal** | 3B 0 3B 0 4 | 0 0 0 0 | | | | |
| **Yeasts** | | | | | | |
| *Candida albicans* | 1N 0 1 0 0 | 0 0 0 0 | | | | |
| *C. tropicalis* | 1N 0 1 0 1 | 0 0 0 0 | | | | |
| *Saccharomyces cerevisiae* | 1J 0 1 0 1 | 0 0 0 0 | | | | |
| **Subtotal** | 3 0 3 0 1 | 1 1 1 1 | | | | |

<sup>a</sup> Standard strains were from A; ATCC (American Type Culture Collection); D; DSM (Leibniz Institute DSMZ-German Collection of microorganisms and Cell Cultures, Germany); J; JCM (Japan Collection of Microorganisms) and N; NBRC (NITE Biological Resource Center, Japan).

<sup>b</sup> Isolated strains were derived from food.

<sup>c</sup> Parentheses indicate colony color: B, blue; M, mauve; b, black; y, yellow; p, pink; EY, egg yolk reaction.
In brief, each 10 g of sample was homogenized and cultured with Tryptic Soy Broth (Difco) with 7.5% NaCl and 1% Sodium pyruvate for 24 h at 37°C. Pre-cultured samples were then streaked onto two plates of BP and MSEY, and incubated for 48 h at 37°C.

For the preparation of artificially contaminated foods, three strains of *S. aureus* (NBRC 14462, NBRC 15035, NBRC 100910, NITE Biological Resource Center, Japan) were used for spiking the samples. Each 10 g of sample was inoculated at the following levels: low (2-3 log CFU/g), medium (3-4 log CFU/g) and high (4-5 log CFU/g). After being kept for 3 days at 4°C, each artificially contaminated food sample was added to a 9-fold volume of Butterfield’s phosphate buffer and homogenized for 90 sec by a homogenizer (MASTICATOR 400S, IUL, S. A., Barcelona, Spain). Each homogenized sample was subjected to 10-fold serial dilution in Butterfield’s phosphate buffer. Comparative analyses were then carried out according to the following procedures. For the reference method analysis, each 0.1 ml of sample was spread onto BP and MSEY using a sterile plastic spreader. Each 1 ml of sample was inoculated onto SkSA and 3M-STX. After 24 h of incubation at 37°C, blue colonies on SkSA and mauve colonies on 3M-STX were determined as *S. aureus*, respectively.

For the BP and MSEY methods, typical colonies were counted after 48 h of incubation at 37°C. At least 1 typical colony on each medium was confirmed to be *S. aureus* by latex agglutination test for coagulase (PS-latex; EIKEN CHEMICAL CO., LTD, Tokyo, Japan)

The result obtained with each method was converted into log CFU of *S. aureus* per gram of the tested food. All statistical analyses were carried out using Microsoft Excel 2003 at the significance level of *P* = 0.05. The linear correlation coefficients (r), slopes, and intercepts between SkSA and each other method were calculated. A one-way analysis of variance (ANOVA) was performed to determine differences among the compared methods.

Of the 100 artificially contaminated food samples, the SkSA, BP and 3M-STX recovered *S. aureus* from all tested samples whereas MSEY failed to recover *S. aureus* from 2 samples. The correlation coefficients, slopes, intercepts and 95% confidence limits between SkSA and BP, SkSA and MSEY, and SkSA and 3M-STX are shown in Table 2. The correlation coefficients of SkSA with BP, MSEY and 3M-STX, were 0.971, 0.989 and 0.996, respectively. The slopes and intercepts of the regression lines for all comparisons were close to 1 and 0, respectively. The means log CFU ± standard deviation (SD) of SkSA, BP, MSEY and 3M-STX were 3.82 ± 0.92, 3.89 ± 0.90, 3.79 ± 1.05, and 3.80 ± 0.90, respectively. There were no significant differences (*p* > 0.05) in counts between SkSA and BP, MSEY, or 3M-STX by one-way analysis of variance (ANOVA) as shown in Table 2.

With the SkSA, there was no need for either the use of a confirmation disk or morphological examination to discriminate different colonies for the determination of *S. aureus*, since only *S. aureus* colonies grew on SkSA even if the sample produced high levels of other microorganisms on the other 3 media. This result indicated that SkSA had not only excellent selectivity but also high correlation with the 3 conventional selective media. Additionally, SkSA could detect *S. aureus* with only a sample inoculation step since SkSA is a pre-sterilized and self-diffusible chromogenic medium. In contrast, even though molecular methods such as PCR-based methods (Chiang et al., 2007; Sowmya et al., 2012) have been developed for rapid and definite detection, these methods require specific devices, skilled users and higher operating costs. Hence, it is suggested that SkSA would provide not only the ease of test operations but also the ease of reading results in the daily monitoring for *S. aureus*. In conclusion, Sanita-kun™ SA (SkSA), a novel quantitative dry culture method, was evaluated for the enumeration of *S. aureus* in food.

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**TABLE 2. Statistical relationship of SkSA with conventional methods for enumerating of *S. aureus* in artificially contaminated food samples**

| Parameter              | SkSA vs. Baird-Parker | SkSA vs. MSEY | SkSA vs. 3M-STX |
|------------------------|-----------------------|---------------|-----------------|
| No. of samples         | 100                   | 100           | 100             |
| Correlation coefficient| 0.971                 | 0.989         | 0.996           |
| Slope                  | 0.982                 | 1.000         | 1.010           |
| Intercept              | 0.003                 | -0.011        | -0.016          |
| 95% confidence limits  | ±0.178                | ±0.192        | ±0.178          |
| p value (ANOVA)        | 0.597                 | 0.786         | 0.868           |
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