FOOD BIOLOGICAL CONTAMINANTS

The Validation of the Dai Nippon Medi·Ca SA Method for AOAC Research Institute *Performance Tested Methods*[SM] Certification

AOAC *Performance Tested Method*[SM] 111703

Abstract

A ready-made dry medium method for *Staphylococcus aureus* count, the Medi·Ca SA method incubated at 35 or 37°C, was compared with the Baird-Parker method (AOAC *Official Method*[SM] **975.55**) for 11 food matrices: raw beef, raw ground beef, raw lamb, cooked ham, raw salmon, frozen prawn, fresh chilled pasta, pasteurized milk, natural cheese, cream puff, and potato salad. The mean difference between the two methods at each contamination level for each matrix was <0.5 $\log_{10}$, and the 95% confidence intervals on the mean differences fell within the range of −0.50 to 0.50. Standard deviation of repeatability and RSD_r values of the Medi·Ca SA method were generally the same level as those of the Baird-Parker method, and r^2 ranged from 0.98 to 1.00. Product consistency and stability studies showed little variability between productions lots and a shelf-life of 16 months. Incubation time within the range of 22–26 h and variations to the sample volume did not adversely affect the results. These results showed that the Medi·Ca SA method is a reasonable alternative to the reference method for selected food matrices and makes it possible to simultaneously detect and enumerate *S. aureus* in only 24 h.

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The method was independently tested, evaluated, and certified by the AOAC Research Institute as a *Performance Tested Method*[SM]. *See* http://www.aoac.org/testkits/steps.html for information on certification.

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Scope of Method

(**a**) *Target organisms.—Staphylococcus aureus*.

(**b**) *Matrices*.—Raw beef, raw ground beef (73% lean), raw lamb, cooked ham, raw salmon filet, frozen prawn (whole), fresh chilled pasta (not boiled), pasteurized milk (3.6% fat), natural cheese (Camembert), cream puff, and potato salad (components; potato, carrot, onion).

(**c**) *Summary of validated performance claims*.—Performance equivalent to that of the AOAC *Official Method*[SM] **975.55**, *Staphylococcus aureus* in Foods (1) for raw beef, raw ground beef, raw lamb, cooked ham, raw salmon, frozen prawn, fresh chilled pasta, pasteurized milk, natural cheese, cream puff, and potato salad.

Principle of the Method

Medi·Ca SA is a ready-made dry medium for *S. aureus* count made up of four components: a waterproof sheet, a dry medium containing a gelling agent and the chromogenic enzyme substrates, a hydrophobic resin ring surrounding the medium, and a transparent cover over the medium (Figure 1). Figure 2 shows the principle of the Medi·Ca SA Method. A sample suspension is dispensed on the center of the medium while the cover is lifted. After that, the cover is gently dropped to evenly spread the suspension on the medium. The suspension rapidly soaks into the medium, which turns into a gel in 3 min. The incubation of the sheet at 35 ± 1 or 37 ± 1°C for 24 ± 1 h develops blue colonies for *S. aureus* because of the enzymatic reaction involving the substrate.

General Information

S. aureus are facultative anaerobic, nonmotile Gram-positive cocci, 0.5–1.0 μm in diameter, which can be found in the environment, foods, humans, and animals. Properties are catalase positive, coagulase positive, and oxidase negative. Growth is best under aerobic conditions. Temperature range for growth is 10–45°C, optimum 30–37°C (2). *S. aureus* has been confirmed to be a major causative agent of food poisoning because of its enterotoxins. The presence of a large number of *S. aureus* organisms in a food may indicate poor handling or sanitation (3).

Materials and Methods

Test Kit Information

(**a**) *Kit name*.—Medi·Ca SA.

(**b**) *Cat. No*.—SA-01.

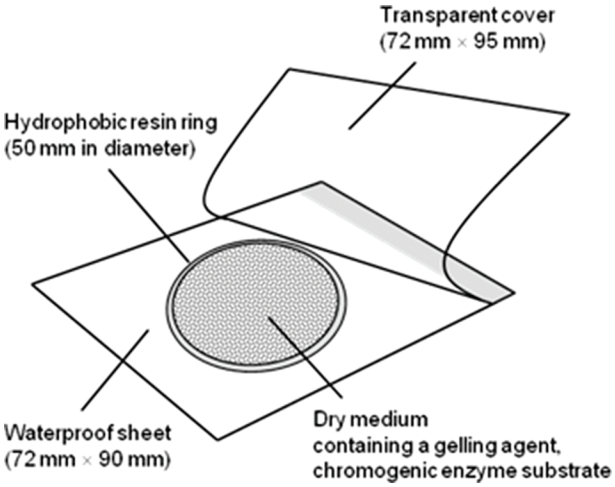

Figure 1. Structure of a Medi·Ca SA.

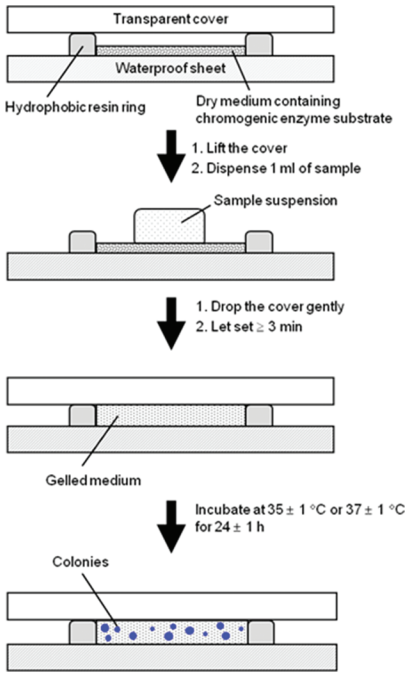

Figure 2. Principle of the Medi·Ca SA method.

(**c**) *Ordering information*.—Available from Dai Nippon Printing Co., Ltd. (Tokyo, Japan).

Additional Media and Reagents

Media and reagents were prepared according to the U.S. Food and Drug Administration Bacteriological Analytical Manual (BAM; 3).

(**a**) *Baird-Parker medium*.—Dissolve 10 g tryptone, 5 g beef extract, 1 g yeast extract, 10 g sodium pyruvate, 12 g glycine, 5 g lithium chloride·6H_2O, and 20 g agar. Autoclave 15 min at 121°C. Final pH, 7.0 ± 0.2. Aseptically add 5 mL prewarmed (45–50°C) Bacto EY tellurite enrichment to 95 mL melted base. Mix well (avoiding bubbles) and pour 15–18 mL portions into 15 × 100 mm Petri dishes.

(**b**) *Tripticase (Triptic) soy broth (TSB)*.—Dissolve 17 g trypticase peptone, 3 g phytone peptone, 5 g NaCl, 2.5 g K_2HPO_4, and 2.5 g glucose in 1 L distilled water. Heat with gentle agitation to dissolve. Dispense 225 mL into 500 mL Erlenmeyer flasks. Autoclave 15 min at 121°C. Final pH, 7.3 ± 0.2.

(**c**) *Tripticase (Triptic) soy agar (TSA)*.—Dissolve 15 g trypticase peptone, 5 g phytone peptone, 5 g NaCl, and 15 g agar in 1 L distilled water. Heat with gentle agitation to dissolve agar. Boil 1 min. Dispense into suitable tubes or flasks. Autoclave 15 min at 121°C. Final pH, 7.3 ± 0.2.

(**d**) *Brain heart infusion (BHI) broth*.—Dissolve 6 g brain heart infusion, 6 g peptic digest of animal tissue, 5 g NaCl, 3 g dextrose, 14.5 g pancreatic digest of gelatin, and 2.5 g Na_2PO_4 in 1 L distilled water. Boil for 1 min to completely dissolve. Dispense 225 mL into 500 mL Erlenmeyer flasks. Autoclave 15 min at 121°C. Final pH, 7.4 ± 0.2.

(**e**) *Butterfield's phosphate-buffered diluent (BPD)*.—Dissolve 34 g KH_2PO_4 in 500 mL distilled water. Adjust pH to 7.2 with 1 N NaOH. Bring volume to 1 L with distilled water. Sterilize 15 min at 121°C. Store in refrigerator. Take 1.25 mL of above stock solution and bring volume to 1 L with distilled water. Dispense into bottles to 90 mL. Sterilize 15 min at 121°C.

(**f**) *Mannitol salt agar*.—Dissolve 1 g beef extract, 10 g polypeptone, 75 g NaCl, 10g mannitol, 0.025 g phenol red, and 15 g agar in 1 L distilled water. Heat with gentle agitation to dissolve. Dispense 20 mL portions into 15 × 100 mm Petri dishes. Autoclave 15 min at 121°C. Final pH, 7.4 ± 0.2.

(**g**) *Coagulase plasma (rabbit) with EDTA*.

Apparatus

(**a**) *Blender*.—High-speed blender with a sterile jar.

(**b**) *Balance*.—2000 ± 0.1 g capacity.

(**c**) *Pipettes*.—Calibrated 1.0 mL micropipette and 10.0 mL serological pipette with 0.1 mL graduations.

(**d**) *Incubator*.—Maintaining at 35 ± 1, 37 ± 1°C.

Reference Cultures

Reference cultures used in this study were obtained from the American Type Culture Collection (ATCC; Manassas, VA), Biological Resource Center, National Institute of Technology and Evaluation (NBRC; Chiba, Japan), and National Collection of Type Cultures, a Culture Collection of Public Health England (Salisbury, United Kingdom).

Safety Precautions

If medium or reagent gets into eyes or mouth, rinse immediately with plenty of water and consult a doctor. Analysis must be performed under a laboratory analyst with microbiological training and supervision. All waste must be handled as a biohazard and disposed by autoclaving.

General Preparation

Remove each Medi·Ca SA sheet from an aluminum bag under aseptic conditions. Fold end of the bag over twice and seal with tape. Store the bag under refrigerated conditions. The shelf life under refrigerated conditions is 3 months after opening.

Sample Preparation

Perform sample preparation according to BAM Chapter 12. Weigh each 50 g test portion into a sterile blender jar, add 450 mL BPD, and blend for up to 2 min. Prepare all decimal dilutions with 90 mL BPD plus 10 mL previous dilution. Shake all dilutions 25 times in a 30 cm arc.

Analysis

Place each Medi·Ca SA sheet on a flat surface and allow it to reach room temperature (15–25°C). Lift the cover, place a 1 mL sample suspension on the center of the medium, and drop the cover onto the sample. Leave the sheet on a horizontal surface for 3 min or more until solidification of the suspension is completed. Hold both ends of the sheet and place it in an incubator. Incubate the sheets at 35 ± 1 or 37 ± 1°C for 24 ± 1 h. It is possible to stack up to 25 sheets.

Interpretation and Test Result Report

Count blue colonies. The use of *S. aureus* ATCC 25923 as the control for blue colony is recommended. The suitable colony counting range is 1–250. See the following for troubleshooting regarding interpretation and reporting of test results:

(**a**) When the number of colonies per sheet exceeds 250 for all dilutions, record the count as too numerous to count (TNTC). If an estimated count is required, count colonies within 1–3 squares (1 cm × 1 cm) printed on the cover and calculate an average. Multiplying the average number by 20 provides the estimated count because the circular growth area is approximately 20 cm^2.

(**b**) When the entire growth area becomes blue colored, record the count as TNTC.

(**c**) When a bubble disrupts a colony so that the colony outlines the bubble, count it as one colony.

(**d**) When a colony spreads, count it as one colony.

(**e**) When two or more spreading colonies appear to originate from separate sources, count each source as one colony.

(**f**) When the sample is not clear (i.e., cloudy or dark), prepare a higher dilution.

(**g**) When the entire growth area becomes colored due to food components involving the chromogenic reaction, prepare a higher dilution.

Validation Study

This validation study was conducted under the AOAC Research Institute *Performance Tested Method*SM program and the AOAC INTERNATIONAL Method Committee Guidelines for the Validation of Microbiological Methods for Food and Environmental Surfaces (4). Method developer studies included the inclusivity/exclusivity study, matrix studies for all claimed matrices, product consistency and stability studies, and robustness testing. The independent laboratory study was conducted by Q Laboratories, Inc., and included a matrix study for raw beef, raw ground beef, and natural cheese of the claimed food matrices.

Method Developer Validation Studies

Inclusivity and exclusivity study.—The inclusivity and exclusivity study examined the ability of the Medi·Ca SA method to detect a variety of *S. aureus* strains and to distinguish those from closely related non-*S. aureus* strains and species. For inclusivity, 52 different isolates of *S. aureus* were selected (Table 1). Each strain was cultured in BHI at 35 ± 1°C for 24 ± 1 h, and decimal dilutions of each strain were prepared using BPD. For exclusivity, 55 isolates of closely related non-*S. aureus* species and strains were selected (Table 2). Each exclusivity strain was cultured in TSB at 35 ± 1°C for 24 ± 1 h, and decimal dilutions of each strain were prepared using BPD. The inclusivity and exclusivity isolates were tested in a randomized blind coded fashion so that the analyst did not know the identity of the test samples. Each sample from appropriate dilutions was cultured in Medi·Ca SA medium at 35 ± 1°C for 24 ± 1 h.

Matrix study.—The Medi·Ca SA method was compared with AOAC **975.55** for 11 different food matrices: raw beef, raw ground beef, raw lamb, cooked ham, raw salmon, frozen prawn, fresh chilled pasta, pasteurized milk, natural cheese, cream puff, and potato salad. The study included five replicate test portions at each contamination level for each matrix, including an uncontaminated level. Because the contamination levels of *S. aureus* were quite low, the strains listed in Tables 3 and 4 were used to artificially contaminate matrices. A volume of 0.1 mL of diluted 24 h TSB cultures of the appropriate strains were spread over the surface of a bulk sample or spike into a sample of matrices and shaken in sterile plastic containers. It was allowed to equilibrate with the food environment at 4 ± 1°C for 72 ± 1 h. For heat-processed matrices, cooked ham and pasteurized milk, 24 h TSB cultures were heated at 52°C for 10 min before being added to a sample. The degree of injury of the culture was estimated using the following formula:

$$(1-n_{select}/n_{nonselect}) \times 100$$

where n_{select} = number of colonies on selective agar; and $n_{nonselect}$ = number of colonies on nonselective agar. Mannitol salt agar and TSA were used for selective and nonselective agar, respectively.

For AOAC **975.55**, 50 g test portions were diluted with 450 mL BPD in a blender jar and blended for 2 min. Serial dilutions were prepared in BPD. A volume of 1 mL of the appropriate dilution was aseptically transferred to triplicate plates of Baird-Parker medium, and 1 mL inoculum was equitably distributed over the triplicate plates (0.4, 0.3, 0.3 mL). Inoculum was spread over the surface of agar using sterile bent glass streaking rods. Agar plates were inverted and incubated 45–48 h at 35–37°C. Following incubation, only typical *S. aureus* colonies were enumerated: colonies presenting a black center with a surrounding halo. To confirm, at least 10 representative *S. aureus* colonies across the triplicate plates were picked, and a coagulase test was conducted.

For data analysis, a logarithmic transformation was performed on the reported CFU/g:

$$\text{Log}_{10}[\text{CFU/g} + (0.1)\text{f}]$$

where f = the reported CFU/unit corresponding to the smallest reportable result. The standard deviation of repeatability (s_r) and the relative standard deviation were calculated after the Cochran and Grubbs outlier test. The candidate method result

Table 1. Inclusivity study

No.	Strain Name	Source	Origin	Medi Ca SA[a]
1	*Staphylococcus aureus*	ATCC 6538[b]	Human lesion	Blue
2	*Staphylococcus aureus*	ATCC 8095	Cream pie	Blue
3	*Staphylococcus aureus*	ATCC 9144	Unknown	Blue
4	*Staphylococcus aureus*	ATCC 13565	Ham	Blue
5	*Staphylococcus aureus*	ATCC 25904	Unknown	Blue
6	*Staphylococcus aureus*	ATCC 25923	Clinical isolate	Blue
7	*Staphylococcus aureus*	ATCC 27664	Chicken tetrazzini	Blue
8	*Staphylococcus aureus*	ATCC 33862	Unknown	Blue
9	*Staphylococcus aureus*	NBRC 12732[c]	Unknown	Blue
10	*Staphylococcus aureus*	NBRC 13276	Human lesion	Blue
11	*Staphylococcus aureus*	NBRC 15035	Wound	Blue
12	*Staphylococcus aureus*	NBRC 100910	Human pleural fluid	Blue
13	*Staphylococcus aureus*	NCTC 10788[d]	Human lesion	Blue
14	*Staphylococcus aureus*	#D0072[e]	Ground beef and pork	Blue
15	*Staphylococcus aureus*	#D0075	Chicken	Blue
16	*Staphylococcus aureus*	#D0076	Chicken	Blue
17	*Staphylococcus aureus*	#D0088	Ground pork	Blue
18	*Staphylococcus aureus*	#D0106	Human skin	Blue
19	*Staphylococcus aureus*	#D0107	Human skin	Blue
20	*Staphylococcus aureus*	#D0108	Human skin	Blue
21	*Staphylococcus aureus*	#D0109	Human skin	Blue
22	*Staphylococcus aureus*	#D0112	Food poisoning	Blue
23	*Staphylococcus aureus*	#D0113	Food poisoning	Blue
24	*Staphylococcus aureus*	#D0116	Food poisoning	Blue
25	*Staphylococcus aureus*	#D0117	Food poisoning	Blue
26	*Staphylococcus aureus*	#D0118	Food poisoning	Blue
27	*Staphylococcus aureus*	#D0120	Food poisoning	Blue
28	*Staphylococcus aureus*	#D0121	Food poisoning	Blue
29	*Staphylococcus aureus*	#D0124	Food poisoning	Blue
30	*Staphylococcus aureus*	#D0125	Food poisoning	Blue
31	*Staphylococcus aureus*	#D0130	Food poisoning	Blue
32	*Staphylococcus aureus*	#D0131	Food poisoning	Blue
33	*Staphylococcus aureus*	#D0133	Food poisoning	Blue
34	*Staphylococcus aureus*	#D0134	Food poisoning	Blue
35	*Staphylococcus aureus*	#D0135	Food poisoning	Blue
36	*Staphylococcus aureus*	#D0138	Food poisoning	Blue
37	*Staphylococcus aureus*	#D0151	Milk	Blue
38	*Staphylococcus aureus*	#D0152	Milk	Blue
39	*Staphylococcus aureus*	#D0153	Milk	Blue
40	*Staphylococcus aureus*	#D0154	Milk	Blue
41	*Staphylococcus aureus*	#D0156	Milk	Blue
42	*Staphylococcus aureus*	#D0182	Ground pork	Blue
43	*Staphylococcus aureus*	#D0183	Ground pork	Blue
44	*Staphylococcus aureus*	#D0185	Chicken	Blue
45	*Staphylococcus aureus*	#D0206	Unknown	Blue
46	*Staphylococcus aureus*	#D0207	Unknown	Blue
47	*Staphylococcus aureus*	#D0208	Pork	Blue
48	*Staphylococcus aureus*	#D0209	Pork	Blue
49	*Staphylococcus aureus*	#D0210	Pork	Blue
50	*Staphylococcus aureus*	#D0211	Pork	Blue
51	*Staphylococcus aureus*	#D0216	Food	Blue
52	*Staphylococcus aureus*	#D0217	Food	Blue

[a] Colony color.

[b] ATCC = American Type Culture Collection, Manassas, VA.

[c] NBRC = Biological Resource Center, National Institute of Technology and Evaluation, Chiba, Japan.

[d] NCTC = National Collection of Type Cultures, a Culture Collection of Public Health England, Salisbury, United Kingdom.

[e] Numbers starting with #D indicates strains that were isolated by Dai Nippon Printing Co., Ltd.

Table 2. Exclusivity study

No.	Strain name	Source	Origin	Medi Ca SA[a]
1	*Staphylococcus auricularis*	ATCC 33753[b]	External auditory meatus	—[c]
2	*Staphylococcus capitis*	ATCC 27840	Human skin	—
3	*Staphylococcus caprae*	ATCC 35538	Goat milk	—
4	*Staphylococcus carnosus*	NBRC 109622[d]	Dry sausage	—
5	*Staphylococcus carnosus*	#D0086[e]	Roast beef	—
6	*Staphylococcus cohnii*	NBRC 109713	Human skin	—
7	*Staphylococcus epidermidis*	NBRC 12993	Unknown	—
8	*Staphylococcus epidermidis*	NBRC 100911	Nose	—
9	*Staphylococcus gallinarum*	NBRC 109767	Chicken nares	Pink
10	*Staphylococcus* sp.	#D0058	Ground beef and pork	—
11	*Staphylococcus haemolyticus*	NBRC 109768	Human skin	—
12	*Staphylococcus hominis*	ATCC 700586	Blood	—
13	*Staphylococcus hyicus*	ATCC 11249	Pig with exudative epidermitis	—
14	*Staphylococcus intermedius*	ATCC 29663	Pigeon nares	—
15	*Staphylococcus lentus*	ATCC 29070	Goat udder	—
16	*Staphylococcus saprophyticus*	NBRC 102446	Urine	—
17	*Staphylococcus schleiferi*	ATCC 43808	Jugular catheter	Blue
18	*Staphylococcus sciuri*	ATCC 29062	Eastern gray squirrel skin	—
19	*Staphylococcus simulans*	NBRC 109714	Human skin	—
20	*Staphylococcus warneri*	NBRC 109769	Human skin	—
21	*Staphylococcus xylosus*	NBRC 109770	Human skin	—
22	*Bacillus circulans*	NBRC 13626	Soil	—
23	*Bacillus cereus*	NBRC 3836	Unknown	—
24	*Bacillus cereus*	NBRC 15305	Unknown	—
25	*Bacillus cereus*	NBRC 13494	Unknown	Pink
26	*Bacillus cereus*	#D0068	Food powder	Pink
27	*Bacillus licheniformis*	NBRC 12200	Unknown	Pink
28	*Bacillus subtilis*	NBRC 3134	Unknown	—
29	*Bacillus thuringiensis*	NBRC 3951	Unknown	—
30	*Bacillus pumilus*	NBRC 12092	Unknown	—
31	*Enterococcus faecalis*	NBRC 100481	Unknown	—
32	*Enterococcus faecalis*	ATCC 29212	Urine	—
33	*Enterococcus faecium*	NBRC 100486	Unknown	—
34	*Leuconostoc mesenteroides*	NBRC 3426	Unknown	—
35	*Macrococcus caseolyticus*	ATCC 13548	Dairy products	—
36	*Macrococcus caseolyticus*	#D0073	Ground beef	—
37	*Micrococcus luteus*	NBRC 3333	Unknown	—
38	*Aeromonas hydrophila*	NBRC 12658	Unknown	—
39	*Citrobacter freundii*	ATCC 8090	Unknown	—
40	*Enterobacter aerogenes*	NBRC 13534	Sputum	—
41	*Enterobacter cloacae*	NBRC 13535	Spinal fluid	—
42	*Escherichia coli*	NBRC 3972	Feces	—
43	*Escherichia coli*	NBRC 102203	Urine	—
44	*Escherichia coli*	ATCC 25922	Clinical isolate	—
45	*Klebsiella oxytoca*	NBRC 105695	Pharyngeal tonsil	—
46	*Klebsiella pneumoniae*	ATCC 13883	Unknown	—
47	*Kluyvera cryocrescens*	NBRC 102467	Kitchen food	—
48	*Proteus mirabilis*	NBRC 105697	Unknown	—
49	*Pseudomonas aeruginosa*	NBRC 3899	Well water	—
50	*Pseudomonas aeruginosa*	ATCC 9027	Outer ear infection	—
51	*Salmonella enterica*	NBRC 105726	Human feces	—
52	*Serratia marcescens*	NBRC 102204	Pond water	—
53	*Aspergillus niger*	NBRC 33023	Tannin gallic acid fermentation	—
54	*Candida albicans*	NBRC 1594	Clinical bronchomycosis	—
55	*Saccharomyces cerevisiae*	NBRC 10217	Brewer's top yeast	—

[a] Colony color.

[b] ATCC = American Type Culture Collection, Manassas, VA.

[c] — = Not detected.

[d] NBRC = Biological Resource Center, National Institute of Technology and Evaluation.

[e] Numbers starting with #D indicates strains that were isolated by Dai Nippon Printing Co., Ltd.

Table 3. Matrix study: Medi·Ca SA versus AOAC 975.55–35°C

			35°C									
			Medi·Ca SA			Baird-Parker			Mean difference	95% CI		
Matrix	Inoculation microorganism	Contamination level	Mean[a]	s_r	RSD_r	Mean	s_r	RSD_r		LCL[b]	UCL[c]	r^2
Cooked ham	*Staphylococcus aureus* D0109	Uninoculated	<1.00			<1.00						
		Low	2.56	0.05	1.90	2.70	0.07	2.42	0.15	0.06	0.23	
		Medium	3.53	0.05	1.35	3.65	0.07	1.95	0.13	0.04	0.22	0.99
		High	4.48	0.06	1.24	4.70	0.04	0.90	0.22	0.13	0.32	
Cream puff	*Staphylococcus aureus* ATCC 8095	Uninoculated	<1.00			<1.00						
		Low	2.05	0.07	3.29	2.11	0.13	6.21	0.06	−0.12	0.24	
		Medium	2.95	0.04	1.30	3.07	0.07	2.13	0.12	0.01	0.23	0.99
		High	3.96	0.05	1.33	4.10	0.02	0.59	0.14	0.08	0.21	
Fresh chilled pasta	*Staphylococcus aureus* NBRC 100910	Uninoculated	<1.00			<1.00						
		Low	3.16	0.04	1.36	3.16	0.02	0.75	0.00	−0.04	0.04	
		Medium	3.88	0.06	1.41	4.01	0.05	1.36	0.13	0.02	0.23	0.99
		High	4.86	0.07	1.33	4.96	0.01	0.17	0.11	0.03	0.18	
Frozen prawn	*Staphylococcus aureus* NBRC 13276	Uninoculated	<1.00			<1.00						
		Low	2.93	0.04	1.45	3.10	0.06	1.93	0.17	0.10	0.25	
		Medium	4.14	0.09	2.25	4.21	0.05	1.17	0.07	−0.06	0.20	0.99
		High	4.95	0.03	0.50	5.05	0.08	1.58	0.10	−0.03	0.23	
Natural cheese	*Staphylococcus aureus* ATCC 25923	Uninoculated	<1.00			<1.00						
		Low	2.45	0.07	3.01	2.61	0.14	5.37	0.16	−0.03	0.34	
		Medium	3.36	0.08	2.42	3.46	0.05	1.41	0.10	0.01	0.20	0.99
		High	5.07	0.04	0.79	5.27	0.04	0.69	0.19	0.15	0.23	
Natural cheese[d]	*Staphylococcus aureus* ATCC 25923	Uninoculated	<1.00			<1.00						
		Low	1.60	0.17	10.63	1.74	0.07	3.86	0.25	−0.50	0.00	
		Medium	2.53	0.03	1.14	2.48	0.06	2.41	0.06	−0.15	0.03	0.98
		High	3.56	0.04	1.12	3.50	0.03	0.91	0.05	−0.12	0.03	
Pasteurized milk	*Staphylococcus aureus* D0152	Uninoculated	<1.00			<1.00						
		Low	2.87	0.03	1.01	3.08	0.05	1.74	0.21	0.12	0.29	
		Medium	3.82	0.04	0.96	4.08	0.07	1.61	0.26	0.18	0.34	1.00
		High	5.14	0.02	0.45	5.38	0.03	0.58	0.24	0.19	0.29	

Table 3. (*continued*)

			35°C									
			Medi·Ca SA			Baird-Parker			Mean difference	95% CI		
Matrix	Inoculation microorganism	Contamination level	Mean[a]	s_r	RSD_r	Mean	s_r	RSD_r		LCL[b]	UCL[c]	r^2
Potato salad	*Staphylococcus aureus* D0138	Uninoculated	<1.00			<1.00						
		Low	2.65	0.04	1.65	2.81	0.04	1.52	0.17	0.09	0.24	
		Medium	4.27	0.02	0.53	4.38	0.04	0.93	0.11	0.05	0.18	1.00
		High	5.07	0.04	0.79	5.27	0.04	0.69	0.19	0.15	0.23	
Raw beef	*Staphylococcus aureus* NBRC 15035	Uninoculated	<1.00			<1.00						
		Low	2.03	0.09	4.21	1.99	0.20	10.1	0.04	−0.30	0.21	
		Medium	3.05	0.06	2.07	3.04	0.03	0.95	0.01	−0.10	0.08	0.98
		High	3.87	0.04	1.03	3.91	0.04	0.89	0.09	0.00	0.17	
Raw beef[d]	*Staphylococcus aureus* ATCC 12600	Uninoculated	<1.00			<1.00						
		Low	2.76	0.04	1.50	2.68	0.07	2.57	0.08	−0.13	−0.04	
		Medium	3.66	0.09	2.32	3.57	0.06	1.80	0.09	−0.20	0.02	0.99
		High	4.56	0.05	0.99	4.52	0.08	1.71	0.05	−0.12	0.03	
Raw ground beef[d]	*Staphylococcus aureus* ATCC 29213	Uninoculated	<1.00			<1.00						
		Low	2.15	0.12	5.79	2.05	0.12	5.70	0.11	−0.01	0.23	
		Medium	3.30	0.04	1.20	3.23	0.14	4.42	0.07	−0.12	0.27	0.99
		High	4.23	0.08	1.93	4.20	0.08	1.89	0.04	−0.81	0.21	
Raw lamb	*Staphylococcus aureus* ATCC 12600	Uninoculated	<1.00			<1.00						
		Low	2.85	0.04	1.49	2.89	0.05	1.69	0.04	−0.06	0.15	
		Medium	3.79	0.04	0.93	3.86	0.04	1.08	0.07	0.04	0.10	1.00
		High	4.69	0.02	0.47	4.82	0.05	1.12	0.14	0.07	0.21	
Raw salmon	*Staphylococcus aureus* D0076	Uninoculated	<1.00			<1.00						
		Low	2.34	0.05	1.98	2.36	0.11	4.72	0.02	−0.08	0.12	
		Medium	3.48	0.06	1.76	3.43	0.05	1.43	0.05	−0.10	0.01	0.99
		High	4.73	0.04	0.90	4.80	0.07	1.48	0.08	0.00	0.15	

[a] Mean of 5 replicates after the logarithmic transformation: $Log_{10}[CFU/g + (0.1)f]$.

[b] LCL = Lower confidence limit.

[c] UCL = Upper confidence limit.

[d] Matrix study conducted by the independent laboratory.

Table 4. Matrix study: Medi·Ca SA versus AOAC 975.55–37°C

			37°C									
			Medi·Ca SA			Baird-Parker			Mean difference	95% CI		
Matrix	Inoculation microorganism	Contamination level	Mean[a]	s_r	RSD_r	Mean	s_r	RSD_r		LCL[b]	UCL[c]	r^2
Cooked ham	*Staphylococcus aureus* D0109	Uninoculated	<1.00			<1.00						
		Low	2.49	0.08	3.25	2.74	0.08	2.96	0.25	0.09	0.40	
		Medium	3.57	0.07	1.90	3.66	0.03	0.81	0.09	−0.02	0.19	0.98
		High	4.48	0.03	0.57	4.69	0.07	1.40	0.21	0.12	0.30	
Cream puff	*Staphylococcus aureus* ATCC 8095	Uninoculated	<1.00			<1.00						
		Low	2.02	0.07	3.58	2.15	0.13	6.12	0.13	−0.05	0.31	
		Medium	2.97	0.05	1.62	3.10	0.04	1.14	0.14	0.04	0.23	0.99
		High	3.97	0.05	1.21	4.12	0.03	0.78	0.15	0.10	0.20	
Fresh chilled pasta	*Staphylococcus aureus* NBRC 100910	Uninoculated	<1.00			<1.00						
		Low	3.18	0.04	1.21	3.12	0.04	1.31	0.06	−0.12	0.01	
		Medium	3.93	0.04	1.08	4.05	0.06	1.54	0.12	0.01	0.24	0.99
		High	4.76	0.10	2.01	4.93	0.03	0.67	0.17	0.06	0.29	
Frozen prawn	*Staphylococcus aureus* NBRC 13276	Uninoculated	<1.00			<1.00						
		Low	3.06	0.05	1.65	3.07	0.05	1.55	0.00	−0.08	0.09	
		Medium	4.19	0.03	0.70	4.22	0.05	1.18	0.02	−0.03	0.07	0.99
		High	4.96	0.08	1.53	5.06	0.05	0.97	0.10	−0.04	0.23	
Natural cheese	*Staphylococcus aureus* ATCC 25923	Uninoculated	<1.00			<1.00						
		Low	2.47	0.07	3.02	2.57	0.09	3.56	0.10	−0.02	0.22	
		Medium	3.32	0.12	3.47	3.36	0.10	2.97	0.04	−0.20	0.28	0.98
		High	4.60	0.04	0.77	4.81	0.06	1.14	0.21	0.16	0.27	
Natural cheese[d]	*Staphylococcus aureus* ATCC 25923	Uninoculated	<1.00			<1.00						
		Low	1.61	0.12	7.55	1.74	0.07	3.86	0.23	−0.43	−0.02	
		Medium	2.59	0.03	1.05	2.49	0.06	2.40	0.02	−0.05	0.12	0.98
		High	3.59	0.04	1.02	3.51	0.03	0.87	0.00	−0.08	0.08	
Pasteurized milk	*Staphylococcus aureus* D0152	Uninoculated	<1.00			<1.00						
		Low	2.84	0.07	2.60	3.06	0.04	1.45	0.22	0.11	0.33	
		Medium	3.84	0.06	1.44	4.06	0.03	0.64	0.23	0.17	0.29	0.99
		High	5.06	0.06	1.23	5.39	0.04	0.65	0.33	0.22	0.44	

Table 4. (*continued*)

Matrix	Inoculation microorganism	Contamination level	37°C									
			Medi·Ca SA			Baird-Parker			Mean difference	95% CI		r^2
			Mean[a]	s_r	RSD_r	Mean	s_r	RSD_r		LCL[b]	UCL[c]	
Potato salad	*Staphylococcus aureus* D0138	Uninoculated	<1.00			<1.00						
		Low	2.66	0.07	2.50	2.86	0.06	2.02	0.20	0.13	0.27	
		Medium	4.29	0.01	0.33	4.33	0.05	1.21	0.04	−0.02	0.09	0.99
		High	5.09	0.04	0.80	5.30	0.04	0.83	0.21	0.13	0.30	
Raw beef	*Staphylococcus aureus* NBRC 15035	Uninoculated	<1.00			<1.00						
		Low	1.91	0.16	8.12	2.03	0.07	3.55	0.13	−0.07	0.32	
		Medium	3.06	0.04	1.30	3.05	0.02	0.66	0.01	−0.08	0.06	0.98
		High	3.85	0.06	1.62	3.87	0.03	0.78	0.02	−0.08	0.12	
Raw beef[d]	*Staphylococcus aureus* ATCC 12600	Uninoculated	<1.00			<1.00						
		Low	2.73	0.04	1.48	2.68	0.07	2.57	0.05	−0.17	0.07	
		Medium	3.68	0.09	2.39	3.57	0.06	1.80	0.11	−0.20	−0.03	0.99
		High	4.63	0.03	0.59	4.52	0.08	1.71	0.12	−0.22	−0.01	
Raw ground beef[d]	*Staphylococcus aureus* ATCC 29213	Uninoculated	<1.00			<1.00						
		Low	2.20	0.15	6.73	2.09	0.11	5.07	0.16	0.00	0.32	
		Medium	3.29	0.05	1.51	3.26	0.13	4.07	0.06	−0.11	0.23	0.99
		High	4.26	0.04	0.97	4.21	0.08	1.82	0.06	−0.08	0.21	
Raw lamb	*Staphylococcus aureus* ATCC 12600	Uninoculated	<1.00			<1.00						
		Low	2.87	0.02	0.72	2.91	0.03	1.10	0.04	0.01	0.07	
		Medium	3.81	0.05	1.34	3.86	0.06	1.54	0.04	−0.08	0.16	0.99
		High	4.68	0.09	1.91	4.73	0.04	0.90	0.05	−0.11	0.21	
Raw salmon	*Staphylococcus aureus* D0076	Uninoculated	<1.00			<1.00						
		Low	2.35	0.05	2.07	2.36	0.06	2.48	0.01	−0.07	0.09	
		Medium	3.42	0.06	1.68	3.38	0.06	1.72	0.05	−0.12	0.03	1.00
		High	4.73	0.05	1.03	4.80	0.01	0.19	0.07	0.01	0.13	

[a] Mean of 5 replicates after the logarithmic transformation: Log_{10}[CFU/g + (0.1)f].

[b] LCL = Lower confidence limit.

[c] UCL = Upper confidence limit.

[d] Matrix study conducted by the independent laboratory.

(y-axis) versus the reference method result (x-axis) was plotted to calculate the slope and square of the correlation coefficient (r^2). The mean difference between the candidate and reference method transformed results with 95% confidence interval (CI) at each contamination level for each matrix was analyzed using an Excel worksheet developed by AOAC Statistical Advisor Robert Labudde, Paired Method Analysis for Micro Testing, Version 1.0 (5).

Product consistency and stability study.—Three different production lots of Medi·Ca SA sheets were examined for lot-to-lot variability and product stability. Production lots that were near the expiration date (October 26, 2015; 16 months), near the middle of the expiration period (June 3, 2016; 7 months), and recently manufactured (March 14, 2017; 0 months) were selected. Natural cheese samples were inoculated with *S. aureus* (ATCC 25923). A volume of 0.1mL of diluted 24-h TSB cultures was added to a bulk sample of natural cheese and allowed to equilibrate with the food environment at 4 ± 1°C for 48 ± 1 h. Each production lot of Medi·Ca SA sheets with five replicates of target at the high level, five replicates of target at the low level, and five replicates of uninoculated level was tested.

Robustness study.—Sample volume and incubation time were varied using a factorial design to evaluate the ability of the Medi·Ca SA method to remain unaffected by small variations. Natural cheese samples were inoculated with *S. aureus* (ATCC 25923). A volume of 0.1 mL of diluted 24-h TSB cultures was added to a bulk sample of natural cheese and allowed to equilibrate with the food environment at 4 ± 1°C for 48 ± 1 h. Each production lot of Medi·Ca SA sheets with five replicates of target at the high level, five replicates of target at the low level, and five replicates of uninoculated level was tested. The incubation temperature was set at 35 ± 1°C for all combinations. Test combinations were described below:

Combination 1: 0.9 mL sample; 22 h
Combination 2: 0.9 mL sample; 26 h
Combination 3: 1.1 mL sample; 22 h
Combination 4: 1.1 mL sample; 26 h
Combination 5: 1.0 mL sample; 24 h (nominal combination)

Independent Laboratory Validation Study

Matrix study.—The methodology for this study was followed as outlined in the AOAC Research Institute's Independent Laboratory Validation Protocol: *Independent Laboratory Study for Dai Nippon Printing Co., Ltd. for the Medi·Ca SA Medium for Enumeration of Staphylococcus aureus* (6). The Medi·Ca SA method was compared with the AOAC **975.55** reference method for three matrices: raw beef, raw ground beef, and natural cheese, at two temperatures: 35 or 37 ± 1°C. The study included five replicate test portions at each contamination level for each matrix. Each matrix was artificially contaminated with a different strain of *S. aureus* described in Table 3 and 4. The inocula were prepared by transferring a single bacterial colony from trypticase soy agar with 5% sheep blood into BHI and incubating at 35 ± 1°C for 18–24 h. Following incubation, the culture was diluted to a target level using BHI broth as the diluent. Separate bulk portions of the matrices were contaminated to create three different contamination levels. The reference method and Medi·Ca SA method were performed as described above.

Table 5. Inoculum heat stress result

Matrix	Inoculation microorganism	Percent injury
Cooked ham	*S. aureus* #D0109	73.2%
Pasteurized milk	*S. aureus* #D0152	62.0%

Results

Method Developer Validation Studies

Inclusivity and exclusivity study.—The 52 *S. aureus* strains, including 39 foodborne strains, were tested for the inclusivity study. All the *S. aureus* strains formed blue colonies (Table 1). Fifty-five non-*S. aureus* strains, including 21 *Staphylococcus*, 9 *Bacillus*, 7 Gram-positive strains, 15 Gram-negative strains, and 3 fungi, were tested for the exclusivity study (Table 2). Fifty strains were not detected, and four strains formed pink colonies. Only one strain formed a blue colony. The strains that formed pink colonies were *S. gallinarum* (NBRC 109767), *Bacillus cereus* (NBRC 13494, #D0068), and *B. licheniformis* (NBRC 12200). The strain that formed a blue colony was *S. schleiferi* (ATCC 43808).

Matrix study.—Nine raw foods and two heat-processed foods were analyzed by the two methods. Tables 3 and 4 show mean values, s_r, mean differences, and 95% CIs on mean differences for the matrix study. Table 5 presents the results of the heat-stressed cultures for heat-processed meat products. According to the results, the mean differences between the two methods at each contamination level for each matrix were less than 0.33 $\log_{10}$, and much smaller in most cases. The 95% CIs for the mean differences between the two methods at each contamination level for each matrix fell within the range of −0.50 to 0.50. The s_r and RSD_r values of the Medi·Ca SA method were almost the same as those of the reference method. The r^2 obtained for each matrix was over 0.98. The r^2 values across all matrices were 0.99 at 35°C and 0.98 at 37°C, respectively (Figures 3 and 4). There was no statistical difference between two incubation temperatures.

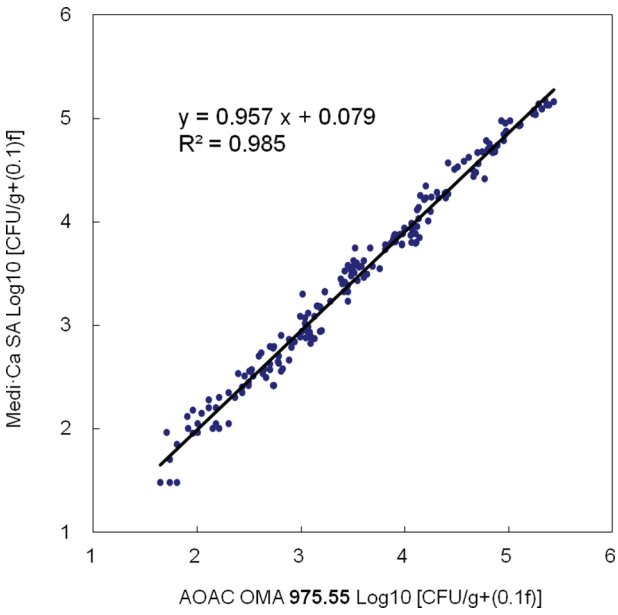

Figure 3. Linear regression analysis for all raw data of the matrix study (35°C).

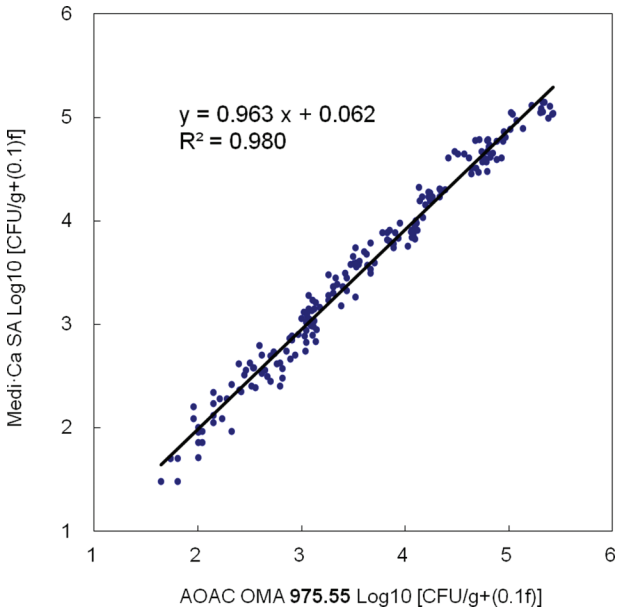

Figure 4. Linear regression analysis for all raw data of the matrix study (37°C).

Product consistency and stability study.—No significant difference in *S. aureus* counts between production lots and no significant time slope were observed (Table 6). These results indicated that the lot-to-lot variability of the Medi·Ca SA medium is very low, and the shelf-life of the medium is at least 16 months.

Robustness study.— Table 7 shows the result of the robustness study. The difference was less than 0.11 $\log_{10}$, and no significant difference was observed among all combinations.

Independent Laboratory Validation Study

Matrix study.—Artificially contaminated raw beef, raw ground beef, and natural cheese were evaluated by the two methods (Tables 3 and 4). As in the method developer study, the mean differences between the two methods at each contamination level for each matrix were less than 0.33 $\log_{10}$, and much smaller in most cases. The 95% CI for the mean difference between the two methods at each contamination level for each matrix fell within the range of −0.50 to 0.50, and no statistical difference was observed at all three contamination levels and incubation temperatures. For the raw beef and raw ground beef, both r^2 values were 0.99, and the value for natural cheese was 0.98.

Discussion

In the exclusivity study, *S. gallinarum* (NBRC 109767), *B. cereus* (NBRC 13494, #D0068) and *B. licheniformis* (NBRC 12200) formed pink colonies. The reason for this was that the type of enzyme produced from these strains was different from *S. aureus*. Those strains were easily distinguished from *S. aureus* by colony color. However, *S. schleiferi* (ATCC 43808) formed a blue colony, which was the same as *S. aureus*. In this case, a coagulase test is necessary to identify *S. aureus* or not. *S. schleiferi* showed negative coagulase reaction.

Some of the *S. aureus*-related bacteria such as *Staphylococci* and *Bacillus* species grow in Baird-Parker medium. When testing the raw food matrices, these colonies sometimes make enumeration of *S. aureus* difficult. In the case of Medi·Ca SA method, the growth of these bacteria is inhibited, and *S. aureus* colony is stained clearly. Medi·Ca SA method does not need to confirm halo of colony.

Table 6. Product consistency and stability study

Matrix	Inoculation microorganism	Contamination level	October 26, 2015		June 3, 2016		March 14, 2017	
			Mean[a]	s_r	Mean	s_r	Mean	s_r
Natural cheese	*S. aureus* ATCC 25923	Uninoculated	<1.00		<1.00		<1.00	
		Low	3.05	0.03	3.08	0.02	3.09	0.03
		High	5.19	0.03	5.11	0.02	5.22	0.02

[a] Mean of 5 replicates after the logarithmic transformation: Log_{10}[CFU/g + (0.1)f].

Table 7. Robustness study

Matrix	Inoculation microorganism	Contamination level	Combination 1		Combination 2		Combination 3		Combination 4		Combination 5[a]	
			0.9 mL sample; 22 h[b]		0.9 mL sample; 26 h		1.1 mL sample; 22 h		1.1 mL sample; 26 h		1.0 mL sample; 24 h	
			Mean[c]	s_r	Mean	s_r	Mean	s_r	Mean	s_r	Mean	s_r
Natural cheese	*S. aureus* ATCC 25923	Uninoculated	<1.00		<1.00		<1.00		<1.00		<1.00	
		Low	3.04	0.05	3.08	0.05	3.12	0.04	3.12	0.03	3.15	0.03
		High	5.13	0.03	5.19	0.06	5.14	0.04	5.22	0.01	5.20	0.02

[a] Combination 5 is the nomination combination.

[b] Incubation time.

[c] Mean of 5 replicates after the logarithmic transformation: Log_{10}[CFU/g + (0.1)f].

In the matrix study, all matrices were incubated at two temperatures, 35 or 37 ± 1°C. It was observed that the colony intensity was stronger at 37 than 35°C for some of the matrices. However, the number of colonies was almost the same in these two temperatures, and there were no significant differences.

Overall, it was generally observed that the Medi·Ca SA method produced statistically similar results when compared with the reference method. This rapid method makes it possible to simultaneously detect and enumerate *S. aureus* in only 24 h, whereas the reference method requires 48 h.

Conclusions

It can be concluded that the Medi·Ca SA method is a reasonable alternative to the AOAC **975.55** reference method for 11 food matrices: raw beef, raw ground beef, raw lamb, cooked ham, raw salmon, frozen prawn, fresh chilled pasta, pasteurized milk, natural cheese, cream puff, and potato salad.

Acknowledgments

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