FOOD BIOLOGICAL CONTAMINANTS

Dai Nippon Printing Co., Ltd Medi·Ca CC for Enumeration of Coliform Bacteria

Performance Tested Method[SM] 021401

Abstract

A ready-made dry medium method for coliform count, the Medi·Ca CC method, was compared to the Violet Red Bile Agar method (*Bacteriological Analytical Manual*, Chapter 4, Enumeration of *Escherichia coli* and the Coliform Bacteria, Section G) for nine raw foods from four food categories: raw ground pork, raw lamb, raw ground chicken, raw tuna fillet, raw salmon fillet, raw shrimp, fresh peeled banana, fresh cut pineapple, and fresh cut apple. The 95% confidence interval for the mean difference between the two methods at each contamination level for seven matrixes from all four categories fell within the range of –0.50 to 0.50, and no statistical difference was observed at all three contamination levels for four matrixes from three categories. These results demonstrated that the Medi·Ca CC method is a reasonable alternative to the reference method for raw meat, raw poultry, raw fish, and fresh fruits.

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Submitted for publication April 4, 2014.

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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DOI: 10.5740/jaoacint.14-076

Scope of Method

(**a**) *Target organisms.*—Coliform bacteria.

(**b**) *Matrixes.*—Raw ground pork, raw lamb, raw ground chicken, raw tuna fillet, raw salmon fillet, raw shrimp, fresh peeled banana, fresh cut pineapple, and fresh cut apple.

(**c**) *Summary of validated performance claims.*—The Medi·Ca CC method is a reasonable alternative to the Violet Red Bile Agar (VRBA) method [*Bacteriological Analytical Manual* (BAM), Enumeration of *Escherichia coli* and the Coliform Bacteria, Chapter 4, Section G] (1) for raw meat, raw poultry, raw fish, and fresh fruits.

Principle of the Method

Medi·Ca CC is a ready-made dry medium for coliform count made up of four components: a waterproof sheet; a dry medium containing a gelling agent and the chromogenic enzyme substrate, 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal); 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 CC method. Sample suspension is dispensed on the center of the medium while the cover is lifted. After that, the cover is dropped gently to spread the suspension on the medium evenly. The suspension rapidly soaks into the medium, which turns into a gel in 3 min. The incubation of the sheet at 35 ± 1°C for 24 ± 1 h develops blue colonies because of the enzymatic reaction involving the substrate; the β-galactosidase produced by bacteria catalyzes the hydrolysis of the X-gal to yield an insoluble blue product.

Materials and Methods

Test Kit Information

Medi·Ca CC.—Available from Dai Nippon Printing Co., Ltd (Tokyo, Japan). Cat. No. CC-01.

Media and Reagents

Media and reagents were prepared according to the BAM (2).

(**a**) *VRBA.*—Dissolve 3 g yeast extract, 7 g peptone, 5 g sodium chloride (NaCl), 1.5 g bile salts, 10 g lactose, 0.03 g neutral red, 0.002 g crystal violet, and 15 g agar in 1 L distilled water. Mix thoroughly and adjust to pH 7.4 ± 0.2. Heat with agitation and boil for 2 min. Do not autoclave.

(**b**) *Lauryl Sulfate Tryptose (LST) Broth.*—Dissolve 20 g tryptose or trypticase, 5 g lactose, 2.75 g K_2HPO_4, 2.75 g

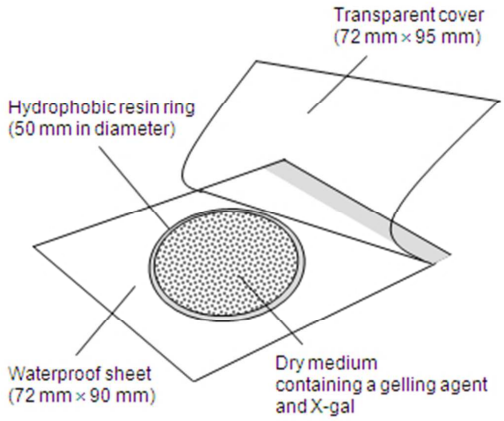

Figure 1. Structure of a Medi·Ca CC sheet.

KH_2PO_4, 5 g NaCl, 0.1 g sodium lauryl sulfate in 1 L distilled water. Dispense 10 mL portions into 20 × 150 mm tubes containing inverted 10 × 75 mm fermentation tubes. Autoclave 15 min at 121°C. Final pH, 6.8 ± 0.2.

(**c**) *Brilliant Green Lactose Bile (BGLB) Broth*.—Dissolve 10 g peptone and 10 g lactose in 500 mL distilled water. Add 20 g dehydrated oxgall dissolved in 200 mL distilled water. The pH of this solution should be 7.0–7.5. Mix and add water to make 975 mL. Adjust pH to 7.4. Add 13.3 mL 0.1% aqueous brilliant green in distilled water. Add distilled water to make 1 L. Dispense into fermentation tubes, making certain that fluid level covers inverted vials. Autoclave 15 min at 121°C. Final pH, 7.2 ± 0.1.

(**d**) *Tryptic 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.

(**e**) *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_2HPO_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.

(**f**) *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.

Apparatus

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

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

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

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

Reference Materials

(**a**) *Escherichia coli*.—(Migula 1895) Castellani and Chalmers 1919 (NBRC 15034) obtained from NITE Biological Resource Center (Chiba, Japan).

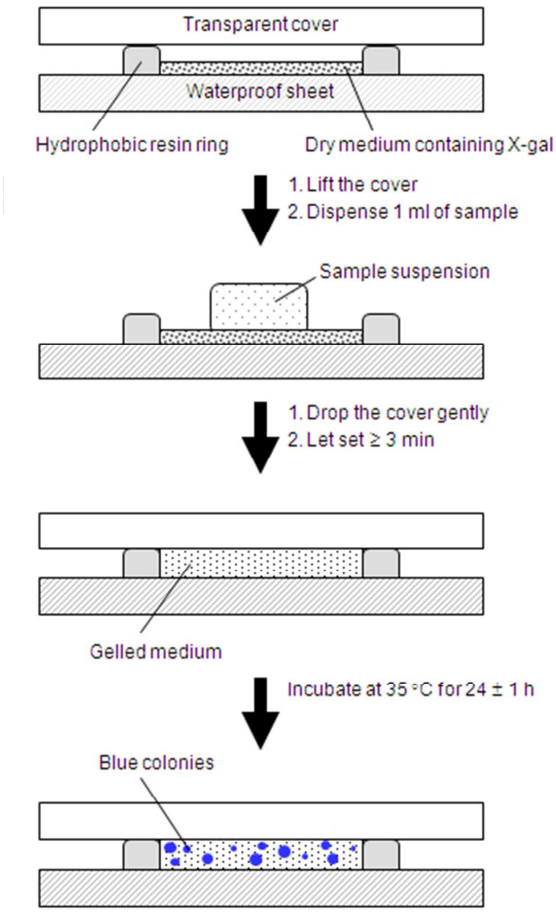

Figure 2. Principle of the Medi·Ca CC method.

(**b**) *E. coli*.—(Migula) Castellani and Chalmers (ATCC 25922) obtained from American Type Culture Collection (Manassas, VA).

(**c**) *Enterobacter aerogenes*.—Hormaeche and Edwards 1960 (NBRC 13534) obtained from NITE Biological Resource Center.

Safety Precautions

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

Sample Preparation

Sample preparation is carried out according to the BAM Chapter 4. Weigh each 50 g test portion into a blender jar, add 450 mL BPD, and blend for up to 2 min. Prepare all decimal

Table 1. Inclusivity/exclusivity panel results

Coliforms				
			Result[b]	
Strain name	Source[a]	Origin	Medi·Ca CC	BGLB[c]
Citrobacter koseri	NBRC[d] 105690	Unknown	+	+
Citrobacter amalonaticus	NBRC 13547	Unknown	+	+
Citrobacter freundii	ATCC[e] 8090	Unknown	+	+
Citrobacter freundii	NBRC 12681	Unknown	+	+
Citrobacter koseri	Natural isolate #20	White radish sprouts	+	+
Cronobacter sakazakii	NBRC 102416T	Child's throat	+	+
Cronobacter sakazakii	NBRC 105698	Child's throat	+	+
Cronobacter sakazakii	Natural isolate #6	Green soybeans	+	+
Enterobacter aerogenes	NBRC 13534T	Sputum	+	+
Enterobacter aminigenus	NBRC 105700T	Soil	+	+
Enterobacter cloacae	NBRC 13535T	Spinal fluid	+	+
Enterobacter cloacae	NBRC 13536	Unknown	+	+
Enterobacter cloacae	NBRC 12935	Unknown	+	+
Enterobacter cloacae	NBRC 12937	Diseased silk-worm	+	+
Enterobacter cloacae	ATCC 222	Unknown	+	+
Enterobacter cloacae	Natural isolate #21	White radish sprouts	+	+
Enterobacter gergoviae	NBRC 105706T	Urine	+	+
Escherichia blattae	NBRC 105725T	Hindgut of cockroach	–	+
Escherichia coli	NBRC 15034	Clinical specimen	+	+
Escherichia coli	NBRC 102203T	Urine	+	+
Escherichia coli	NBRC 13500	Unknown	+	+
Escherichia coli	NBRC 15034	Clinical specimen	+	+
Escherichia coli	ATCC 25922	Unknown	+	+
Escherichia coli	NBRC 13966	Unknown	+	+
Escherichia coli	NBRC 13898	Unknown	+	+
Escherichia coli	NBRC 3301	Unknown	+	+
Escherichia coli	NBRC 3302	Unknown	+	+
Escherichia coli	NBRC 13540	Unknown	+	+
Escherichia coli	NBRC 3366	Unknown	+	–
Escherichia coli	NBRC 3543	Unknown	+	+
Escherichia coli	NBRC 3544	Unknown	+	+
Escherichia coli	NBRC 14129	Unknown	+	+
Escherichia coli	NBRC 15484	Unknown	+	+
Escherichia coli	NBRC 12062	Unknown	+	+
Escherichia coli	NBRC 12433	Unknown	+	+
Escherichia coli	NBRC 12734	Unknown	+	+
Escherichia coli	NBRC 3972	Feces	+	+
Escherichia coli	NBRC 3991	Unknown	+	+
Escherichia coli	NBRC 13891	Unknown	+	–
Escherichia coli	NBRC 13892	Unknown	+	–
Escherichia coli	NBRC 3545	Unknown	+	+
Escherichia coli	NBRC 3546	Unknown	+	+
Escherichia coli	NBRC 3806	Unknown	+	+
Escherichia coli	NBRC 3993	Unknown	+	–
Escherichia fergusonii	NBRC 102419	Feces of human (1-year-old boy)	+	+
Escherichia hermanii	NBRC 105704T	Toe of 17-year-old female	+	+
Escherichia vulneris	NBRC 102420	Human wound	+	+

Table 1. (*continued*)

Strain name	Source[a]	Origin	Result[b]	
			Medi·Ca CC	BGLB[c]
Klebsiella oxytota	NBRC 105695	Pharyngeal tonsil	+	+
Klebsiella pneumoniae	NBRC 14940T	Unknown	+	+
Klebsiella pneumoniae	ATCC 13883	Unknown	+	+
Klebsiella pneumoniae	Natural isolate #31	Raw yellowtail	+	+
Kluyvera cryocrescens	Natural isolate #2	Food	+	+
Kluyvera intermedia	NBRC 102594T	Surface water	+	+
Leclercia adecarboxylata	NBRC 102595	Drinking water	+	+
Pantoea agglomerans	Natural isolate #3	Cake	+	+
Rahnella aquatilis	Natural isolate #10	Raw ground pork	+	+
Raoultella planticola	NBRC 14939	Radish root	+	+
Raoultella terrigena	Natural isolate #33	Raw salmon	+	+
Raoultella terrigena	NBRC 14941T	Drinking water	+	+
		Non-coliforms		
Achromobacter denitrificans	NBRC 15125T	Soil	–	–
Achromobacter xylosoxidans	NBRC 15126	Ear discharge	–	–
Aeromonas hydrophila	NBRC 12658	Unknown	–	–
Alcaligenes faecalis	NBRC 13111T	Unknown	–	–
Bacillus amylolichefaciens	Natural isolate #8	Powdered paprika	–	–
Bacillus cereus	NBRC 15305T	Unknown	–	–
Bacillus cereus	NBRC 3836	Unknown	–	–
Bacillus cereus	NBRC 13494	Unknown	–	–
Bacillus licheniformis	Natural isolate #4	Cheese cake	–	–
Bacillus subtilis	Natural isolate #14	Chinese barbecued pork	–	–
Bacillus subtilis	NBRC 3134	Unknown	–	–
Corynebacterium variabile	NBRC 15286	Food	–	–
Edwardsiella tarda	NBRC 105688T	Human feces	–	–
Kocuria kristinae	Natural isolate #5	Cheese cake	–	–
Lactobacillus delbrueckii	NBRC 3202	Sour grain mash	–	–
Lactococcus lactis	Natural isolate #40	Yogurt	–	–
Lactbacillus casei	Natural isolate #42	Lactic acid drink	–	–
Micrococcus luteus	NBRC 3333T	Unknown	–	–
Micrococcus luteus	NBRC 13867	Air	–	–
Micrococcus lylae	NBRC 15355T	Human skin	–	–
Proteus hauseri	NBRC 3851	Unknown	–	–
Proteus hauseri	NBRC 105696	Unknown	–	–
Proteus mirabilis	NBRC 105697T	Unknown	–	–
Providencia alcalifaciens	NBRC 105687T	Feces	–	–
Pseudomonas mendocina	NBRC 14162	Soil enrichment with ethanol as carbon source	–	–
Pseudomonas aeruginosa	NBRC 3453	Unknown	–	–
Pseudomonas aeruginosa	NBRC 12689	Unknown	–	–
Pseudomonas aeruginosa	ATCC 9027	Unknown	–	–
Pseudomonas aeruginosa	NBRC 3446	Urine	–	–
Pseudomonas aeruginosa	NBRC 3449	Urine	–	–
Pseudomonas fluorescens	Natural isolate #16	Raw lamb	–	–
Pseudomonas fluorescens	Natural isolate #22	White radish sprouts	–	–
Pseudomonas pseudoaligenes	NBRC 14167	Sinus drainage	–	–
Pseudomonas stutzeri	NBRC 14165	Human spinal fluid	–	–

Table 1. (*continued*)

Strain name	Source[a]	Origin	Result[b]	
			Medi·Ca CC	BGLB[c]
Serratia liquefaciens	Natural Isolate #12	Raw ground chicken	–	–
Serratia marcescens	NBRC 102204	Pond water	+	–
Staphylococcus aureus	ATCC 33862	Unknown	–	–
Staphylococcus aureus	NBRC 14462	Clinical isolate	–	–
Staphylococcus aureus	NBRC 100910T	Human pleural fluid	–	–
Staphylococcus aureus	NBRC 12732	Human lesion	–	–
Streptococcus equinus	NBRC 12553T	Unknown	–	–
Streptococcus thermophilus	Natural Isolate #41	Yogurt	–	–

[a] The natural isolate strains were isolated and numbered in our laboratory, and then identified by molecular and biochemical analyses.

[b] + = Detected, – = not detected.

[c] Brilliant green lactose bile broth.

[d] NITE Biological Resource Center, Chiba, Japan.

[e] American Type Culture Collection, Manassas, VA.

dilutions with 90 mL BPD plus 10 mL of previous dilution and shake 25 times in a 30 cm arc.

Analysis

Place each Medi·Ca CC sheet on a flat surface and allow it to reach room temperature (15–25°C). Lift the cover, place 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 into an incubator. Incubate the sheets at 35 ± 1°C for 24 ± 1 h. It is possible to stack up to 25 sheets.

Interpretation and Test Result Report

Count all blue colonies regardless of size or intensity. The suitable colony counting range is 1–250. See the following troubleshooting for the interpretation and test result report:

(**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 since the circular growth area is approximately 20 cm^2.

(**b**) When the entire growth area becomes blue, 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 spreading colony appears to be caused by diffusion, 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 blue due to food components involving the chromogenic reaction or containing many lactic acid bacteria, prepare a higher dilution.

(**h**) When colonies are extremely small or light, incubate continuously for a few hours to make the colony size larger or the colony color intensity darker.

Validation Study

The *Performance Tested Methods*[SM] validation study was performed according to the *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces* (3).

Method Developer Validation Studies

Inclusivity and exclusivity study.—The inclusivity and exclusivity study examined the ability of the Medi·Ca CC method to detect a variety of coliform strains and to distinguish those from closely related non-coliform strains and species. For inclusivity, 59 different isolates of coliform strains were selected (Table 1). Each strain was cultured in LST broth at 35 ± 1°C for 24 ± 1 h, and decimal dilutions of each strain were prepared using BPD. For exclusivity, 42 isolates of closely related non-coliform species and strains were selected (Table 1). 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 CC medium at 35 ± 1°C for 24 ± 1 h.

Matrix study.—The Medi·Ca CC method was compared to the VRBA method for nine raw foods from four food categories: raw ground pork, raw lamb, raw ground chicken, raw tuna fillet, raw salmon fillet, raw shrimp, fresh peeled banana, fresh cut pineapple, and fresh cut apple. The study included five replicate test portions at each contamination level for each matrix, including a dilution blank control and an uninoculated level. For natural contamination, raw ground pork, raw lamb, raw ground chicken, raw tuna fillet, raw salmon fillet, and raw shrimp samples were stored at 3–30°C for up to 24 ± 1 h to achieve

Table 2. Matrix study results (method developer)

			Medi·Ca CC			VRBA					95% CI[d]		
Matrix	Inoculation micoorganism	Contamination level	Mean[a]	s_r[b]	RSD_r[c]	Mean	s_r	RSD_r	*p*-value	Mean diff.	LCL[e]	UCL[f]	r^2[g]
Raw ground pork	NA[h]	Low	4.19	0.03	0.71	4.18	0.06	1.36	0.69	–0.01	–0.09	0.06	1.00
		Medium	5.06	0.04	0.80	5.09	0.02	0.48	0.11	0.03	–0.01	0.07	
		High	8.42	0.06	0.69	8.47	0.11	1.30	0.53	0.23	–0.15	0.25	
Raw lamb	NA	Low	2.31[i]	0.09	3.83	2.48	0.05	1.95	0.01	0.18	0.08	0.27	1.00
		Medium	7.79	0.04	0.48	7.82	0.03	0.33	0.25	0.02	–0.02	0.07	
		High	8.59[i]	0.04	0.46	8.69	0.07	0.86	0.04	0.09	0.01	0.18	
Raw ground chicken	NA	Low	2.11	0.07	3.47	2.22	0.09	4.20	0.08	0.11	–0.02	0.23	0.99
		Medium	3.62[i]	0.08	2.08	3.75	0.09	2.35	0.05	0.13	0.00	0.26	
		High	5.02	0.02	0.43	4.93	0.12	2.43	0.14	–0.09	–0.22	0.04	
Raw tuna fillet	NA	Low	2.53	0.06	2.23	2.54	0.05	1.85	0.76	0.01	–0.10	0.13	1.00
		Medium	3.56	0.10	2.89	3.42	0.10	2.98	0.06	–0.13	–0.27	0.01	
		High	6.41	0.03	0.52	6.27	0.13	2.12	0.09	–0.13	–0.30	0.03	
Raw salmon fillet	NA	Low	2.16	0.19	8.99	2.02	0.08	4.06	0.25	–0.15	–0.44	0.15	0.97
		Medium	3.04	0.02	0.79	3.06	0.06	1.84	0.65	0.01	–0.06	0.08	
		High	3.97	0.03	0.71	3.96	0.06	1.45	0.61	–0.01	–0.06	0.00	
Raw shrimp	NA	Low	2.42[i]	0.08	3.41	2.16	0.17	7.66	0.01	–0.26	–0.43	–0.10	1.00
		Medium	3.53	0.06	1.81	3.47	0.06	1.70	0.24	–0.06	–0.18	0.06	
		High	8.41[i]	0.03	0.34	8.23	0.03	0.42	0.00	–0.18	–0.25	–0.11	
Fresh peeled banana	*E. coli* NBRC 15034	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	—	—
		Low	3.51	0.11	3.28	3.49	0.06	1.74	0.73	–0.02	–0.16	0.12	0.99
		Medium	4.75	0.09	1.86	4.76	0.09	1.84	0.88	0.01	–0.17	0.19	
		High	5.65	0.03	0.58	5.68	0.07	1.24	0.40	0.03	–0.06	0.13	
Fresh cut pineapple	*E. coli* ATCC 25922	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	—	—
		Low	3.28[i]	0.02	0.68	3.34	0.03	0.93	0.01	0.06	0.02	0.10	1.00
		Medium	4.41	0.04	0.95	4.41	0.04	0.94	0.94	0.00	–0.06	0.06	
		High	5.42	0.03	0.57	5.40	0.03	0.49	0.33	–0.02	–0.06	0.03	
Fresh cut apple	*E. aerogenes* NBRC 13534	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	—	
		Low	3.60	0.06	1.54	3.58	0.05	1.46	0.34	–0.02	–0.08	0.03	1.00
		Medium	4.67	0.08	1.71	4.64	0.11	2.38	0.70	–0.03	–0.25	0.19	
		High	5.74	0.04	0.63	5.65	0.08	1.46	0.11	–0.09	–0.21	0.03	

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

[b] s_r = Standard deviation.

[c] RSD_r = Relative standard deviation.

[d] CI = Confidence interval.

[e] LCL = Lower confidence limit.

[f] UCL = Upper confidence limit.

[g] r^2 = Square of the correlation coefficient.

[h] NA = Not applicable. Samples are naturally contaminated.

[i] Significantly different ($P < 0.05$).

Table 3. Product consistency and stability study results

Matrix	Inoculation micoorganism	Contamination level	Mar. 9, 2012		Jan. 31, 2013		Oct. 15, 2013	
			Mean[a]	s_r[b]	Mean	s_r	Mean	s_r
Fresh peeled banana	*E. coli*	Uninoculated	<1.00	—	<1.00	—	<1.00	—
	NBRC 15034	Low	3.69	0.03	3.72	0.05	3.75	0.04
		High	5.74	0.06	5.73	0.06	5.81	0.06

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

[b] s_r = SD.

three different contamination levels so that each level was approximately one log higher than the previous. For artificial contamination, fresh peeled banana samples, fresh cut pineapple samples, and fresh cut apple samples were inoculated with *E. coli* (NBRC 15034), *E. coli* (ATCC 25922) and *Enterobacter aerogenes* (NBRC 13534), respectively. A 24 h TSB culture was added to a bulk sample of each fresh fruit product, and allowed to equilibrate with the food environment at 4 ± 1°C for 72 ± 1 h.

For the VRBA method, 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 and aliquots of each dilution were plated in duplicate. The plates were then poured with approximately 10 mL of tempered (48°C) VRBA. After the plates solidified, an overlay with 5 mL of VRBA was added to the plates to prevent surface growth and spreading of colonies. Solidified plates were inverted and incubated for 18–24 h at 35 ± 1°C. Total coliform counts were determined by enumeration of purple-red colonies that were 0.5 mm or larger in diameter and surrounded by zone of precipitated bile acids. Colonies were confirmed as coliforms by transferring a minimum of 10 colonies each to a tube of BGLB broth and incubating at 35 ± 1°C. Tubes were examined at 24 and 48 h for gas production. Final results were reported by averaging counts between duplicate plates. Final results were presented as coliform plate count/g.

For data analysis, a logarithmic transformation was performed on the reported CFU/g: Log_{10}[CFU/g + (0.1)f], where f is the reported CFU/unit corresponding to the smallest reportable result. The SD (s_r) and the RSD (RSD_r) were calculated after the Cochran and Grubs outlier test. The candidate method result (*x*-axis) vs the reference method result (*y*-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. A paired *t*-test with equal variance was also performed to calculate the *P*-value.

Product consistency and stability study.—Three different production lots of Medi·Ca CC sheets were examined for lot-to-lot variability and product stability. Production lots that were near the expiration date (March 9, 2012), near the middle of the expiration period (January 31, 2013), and recently manufactured (October 15, 2013) were selected. Fresh peeled banana samples were inoculated with *E. coli* (NBRC 15034). A 24 h TSB culture was added to a bulk sample of fresh peeled banana and allowed to equilibrate with the food environment at 4 ± 1°C for 72 ± 1 h. Each production lot of Medi·Ca CC 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 in a randomized blind-coded fashion.

Robustness study.—Volume of sample and incubation time were varied to evaluate the ability of the Medi·Ca CC method to remain unaffected by small variations. The factorial design was as follows:

Combination 1.—0.9 mL sample; 22 ± 0.5 h.
Combination 2.—0.9 mL sample; 26 ± 0.5 h.
Combination 3.—1.1 mL sample; 22 ± 0.5 h.
Combination 4.—1.1 mL sample; 26 ± 0.5 h.
Combination 5.—1.0 mL sample; 24 ± 0.5 h.

Fresh peeled banana samples were inoculated with *E. coli* (NBRC 15034). A 24 h TSB culture was added to a bulk sample of fresh peeled banana and allowed to equilibrate with the food environment at 4 ± 1°C for 72 ± 1 h. Medi·Ca CC sheets with five replicates of target at the high level, five replicates of target at the low level, and five replicates of uninoculated level were tested in a randomized blind-coded fashion. The incubation temperature was set at 35 ± 1°C for all combinations.

Independent Laboratory Study

Matrix study.—The methodology for this study was followed as outlined in the AOAC Research Institute's Independent Laboratory Validation Protocol. The Medi·Ca CC method was compared to the VRBA method for two matrixes; raw salmon fillet and fresh peeled banana. The study included five replicate test portions at each contamination level for each matrix. Raw salmon fillet was analyzed for natural contamination of coliform bacteria. Raw salmon fillet samples were stored at 2–5°C for up to 5 days to achieve three different contamination levels so that each level was approximately one log higher than the previous. Fresh peeled banana samples were inoculated with *E. coli* (NBRC 15034). The inoculum was prepared by transferring a pure isolated colony of the specified organism from trypticase soy agar with 5% sheep's blood into BHI and incubating the BHI at 35 ± 2°C for 24 ± 2 h. Post incubation, the BHI was diluted and the diluted culture was used to inoculate a bulk sample of fresh peeled bananas. The inoculated test portion was mixed thoroughly and held under refrigeration conditions (2–5°C) for 48–72 h to allow time for the organism to equilibrate within the matrix. The VRBA and Medi·Ca CC methods were performed as described above.

Results

Method Developer Validation Studies

Inclusivity and exclusivity studies.—Of the 59 coliform inclusivity strains tested, 58 were detected and one was not detected (Table 1). The strain not detected was *Escherichia blattae* (NBRC 105725T). Of the 42 exclusivity strains tested, 41 were

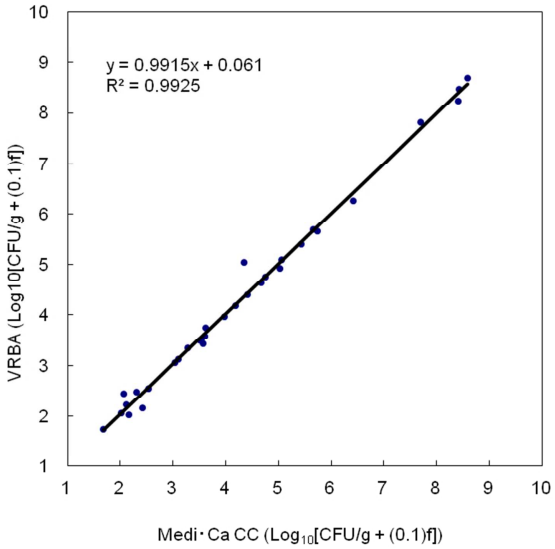

Figure 3. Linear regression analysis for all data of the two matrix studies.

not detected and one was detected (Table 1). The strain detected was *Serratia marcescens* (NBRC 102204).

Matrix study.—Nine raw foods from four food categories were analyzed by the two methods (Table 2). 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 for raw ground pork, raw tuna fillet, raw salmon fillet, fresh peeled banana, and fresh cut apple. However, the mean $\log_{10}$ counts of the Medi·Ca CC method at the low and high levels for raw lamb, at the medium level for raw ground chicken, and at the low level for fresh cut pineapple were significantly lower than those of the VRBA method. In addition, the mean $\log_{10}$ counts of the Medi·Ca CC method at low and high levels for raw shrimp were significantly higher than those of the VRBA method. The s_r and RSD_r values of the Medi·Ca CC method were equal to or lower than those of the VRBA method for 16 out of 27 samples. A square of the correlation coefficient (r^2) obtained for each matrix was 0.97 or more.

Product consistency and stability study.—No significant difference in coliform counts between production lots and no significant time slope were observed (Table 3). These results indicated that the lot-to-lot variability of the Medi·Ca CC medium is very low and the shelf-life of the medium is at least 19 months.

Robustness study.—No significant difference between combination 1 and 2 or between combination 3 and 4 was observed, indicating that incubation time within the range of 22 to 26 h does not adversely affect coliform count results (Table 4). However, the variation in volume of sample within the range of 0.9 to 1.1 mL slightly affected the coliform counts.

Independent Laboratory Study

Matrix study.—Naturally contaminated raw salmon fillet and artificially contaminated fresh peeled banana were evaluated by the two methods (Table 5). No statistical difference between the two methods was observed for one of the three levels analyzed for the raw salmon fillet and each contamination level for the fresh peeled banana. However, the mean $\log_{10}$ counts of the Medi·Ca CC method at the high level for raw salmon fillet was significantly lower than that of the VRBA method. A second high level for the raw salmon fillet was tested to verify the initial result. The s_r and RSD_r values of the Medi·Ca CC method were lower than those of the VRBA method for the low and medium levels for the raw salmon fillet and low level for the fresh peeled banana. The linear regression analysis for all raw data of the two matrix studies presented a square of the correlation coefficient of 0.99 (Figure 3).

Discussion

The Medi·Ca CC method was compared to the VRBA method (BAM, Chapter 4, Section G) for the nine raw foods from the four food categories. The 95% CI for the mean difference between the two methods at each contamination level for seven matrixes from all four categories fell within the range of –0.50 to 0.50, and no statistical difference was observed at all three contamination levels for four matrixes from three categories (Tables 2 and 5). In addition, the repeatability of the Medi·Ca CC method was similar overall to that of the VRBA method. These results demonstrated that the Medi·Ca CC method is a reasonable alternative to the VRBA method for raw meat, raw poultry, raw fish, and fresh fruits.

In contrast to the internal laboratory study, the Medi·Ca CC method produced a remarkably lower coliform count than the VRBA method at the high level for raw salmon fillet in the independent laboratory study (Table 5). The explanation for this is that non-coliform bacteria in large numbers competed with total coliform and made it difficult for coliforms to be detected;

Table 4. Robustness study results

			0.9 mL sample				1.1 mL sample				1.0 mL sample	
			22 h		26 h		22 h		26 h		24 h	
Matrix	Inoculation microorganism	Contamination level	Mean[a]	s_r[b]	Mean	s_r	Mean	s_r	Mean	s_r	Mean	s_r
Fresh	*E.coli*	Uninoculated	<1.00	—	<1.00	—	<1.00	—	<1.00	—	<1.00	—
peeled	NBRC 15034	Low	3.56	0.04	3.65	0.04	3.79	0.04	3.77	0.04	3.73	0.09
banana		High	5.65	0.05	5.72	0.05	5.82	0.04	5.77	0.06	5.74	0.05

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

[b] s_r = Standard deviation.

Table 5. Matrix study results (independent laboratory)

			Medi·Ca CC			VRBA					95% CI[d]		
Matrix	Inoculation microorganism	Contamination level	Mean[a]	s_r[b]	RSD_r[c]	Mean	s_r	RSD_r	Mean difference	*p*-value	LCL[e]	UCL[f]	r^{2g}
Raw	NA[h]	Low	2.02	0.26	12.7	2.06	0.27	13.1	0.67	0.04	–0.18	0.25	0.97
salmon		Medium	3.01[i]	0.18	5.9	3.12	0.24	7.9	0.02	0.11	0.02	0.20	
fillet		High	4.34[i]	0.29	6.6	5.04	0.22	4.3	0.00	0.70	0.54	0.86	
		High (retest)	3.33[i]	0.18	5.5	4.09	0.22	5.5	0.01	0.76	0.54	0.98	
Fresh	*E.coli*	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	—	0.95
peeled	NBRC 15034	Low	1.68	0.08	4.4	1.74	0.19	10.9	0.55	0.07	–0.22	0.35	
banana		Medium	2.06	0.76	33.5	2.45	0.42	17.1	0.45	0.38	–0.89	1.66	
		High	3.53	0.09	2.5	3.50	0.08	2.4	0.66	0.03	–0.15	0.24	

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

[b] s_r = SD.

[c] RSD_r = RSD.

[d] CI = Confidence interval.

[e] LCL = Lower confidence limit.

[f] UCL = Upper confidence limit.

[g] r^2 = Square of the correlation coefficient.

[h] NA = Not applicable. Samples are naturally contaminated.

[i] Significantly different ($P < 0.05$).

approximately 80% of the total coliform colonies could not be recognized due to their tiny sizes or slight intensities. The Medi·Ca CC method is subject to that kind of growth inhibition because a gel volume of a Medi·Ca CC medium is roughly 10 times smaller than an agar volume of VRBA. In fact, a total viable count of the sample stored in a storage condition in the independent lab study (at 2–5°C for 5 days) was approximately 10^7 CFU/g, being 100 times higher than that of the one stored in a storage condition in the internal laboratory study (at 10±1°C for 24±1 h). In addition, the third party pointed out that the low level had distinct dark blue colonies and the high level had small light blue colonies. Probably, psychrophiles such as *Pseudomonas* selectively grew to inhibit the growth of coliforms in the independent laboratory study. In that case, an additional incubation for a few hours to make the colony size larger or the colony color intensity darker is recommended to obtain the colony count results equivalent to the VRBA method.

Conclusions

We conclude that the Medi·Ca CC method is a reasonable alternative to the VRBA method for raw meat, raw poultry, raw fish, and fresh fruits.

Acknowledgments

We thank Patrick Bird, Megan Boyle, and Erin Crowley (Q Laboratories, Inc.) for their professional work in the independent laboratory study.

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