FOOD BIOLOGICAL CONTAMINANTS

Validation of Medi·Ca EC Method for AOAC Research Institute *Performance Tested Methods*[SM] Certification

***Performance Tested Method*[SM] 031601**

Abstract

A ready-made dry medium method for *Escherichia coli* and coliform count, the Medi·Ca EC method, was compared with the most probable number (MPN) method using Brilliant Green Lactose Bile broth and *E. coli* broth (AOAC INTERNATIONAL *Official Method*[SM] **966.24**) for seven food matrixes: raw beef, raw pork, raw frozen pork, raw lamb, raw salmon, frankfurter sausage, and cooked ham. The mean difference between the two methods at each contamination level for each matrix was <0.5 $\log_{10}$, and the 95% confidence intervals for the mean differences fell within the range of –0.5 to 0.5, with the exception of a few cases in the independent laboratory study. s_r and RSD_r values of the Medi·Ca EC method were generally lower than those of the MPN method, and r^2 ranged from 0.91 to 0.99. Product consistency and stability studies showed little variability between production lots and the shelf-life of 20 months. An incubation time within the range of 22–26 h did not adversely affect the results; however, variations in sample volume did affect final counts. These results showed that the Medi·Ca EC method is a reasonable alternative to the reference method for the selected food matrixes and makes it possible to simultaneously detect and enumerate *E. coli* and coliform in only 24 h.

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The method was independently tested, evaluated, and certified by the AOAC Research Institute (RI) 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.—Escherichia coli* and coliform bacteria.

(b) *Matrixes.*—Raw beef, raw pork, raw frozen pork, raw lamb, raw salmon, frankfurter sausage, and cooked ham.

(c) *Summary of validated performance claims.*—Performance equivalent to that of AOAC INTERNATIONAL *Official Method*[SM] **966.24**, *Coliform Group and Escherichia coli in Tree Nut Meats, Microbiological (MPN) Method* (1) for raw beef, raw pork, raw frozen pork, raw lamb, raw salmon, frankfurter sausage, and cooked ham.

Principle

Medi·Ca EC is a ready-made dry medium to count *E. coli* and coliform. It has four components: a waterproof sheet; dry medium containing a gelling agent and chromogenic enzyme substrates 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) and 6-bromo-5-chloro3-indolyl-β-D-galactopyranoside (Magenta-Gal); a hydrophobic resin ring that surrounds the medium; and a transparent cover over the medium (Figure 1). Figure 2 shows the principle of the Medi·Ca EC method. A sample suspension was dispensed in the center of the medium while the cover was lifted. The cover was then gently dropped back into place to evenly spread the suspension over the medium. The suspension was rapidly soaked into the medium, which turned into a gel in 3 min. Incubation of the sheet at 35 ± 1°C for 24 ± 1 h resulted in the development of navy blue/blue–purple and pink/red–purple colonies because of enzymatic reaction involving the substrate: β-Glucuronidase produced by bacteria catalyzed the hydrolysis of the X-Gluc to yield an insoluble blue product, whereas β-galactosidase produced by bacteria catalyzed the hydrolysis of Magenta-Gal to yield an insoluble red–purple product. Navy blue/blue–purple colonies indicated *E. coli* and pink/red–purple colonies indicated non-*E. coli* coliform. Ninety-eight percent of

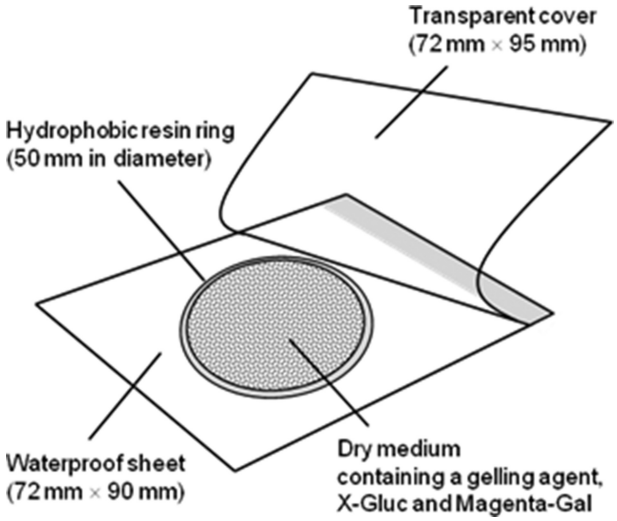

Figure 1. Structure of Medi·Ca EC.

E. coli produce both β-glucuronidase and β-galactosidase and non-*E. coli* coliform only produces β-galactosidase (2).

General Information

E. coli are highly motile Gram-negative facultative anaerobic rod bacteria that can be found in the environment, foods, and intestines of human and animals. Most *E. coli* are harmless and are actually an important part of a healthy human intestinal tract. However, some *E. coli* are pathogenic, meaning they can cause illness either within the intestinal tract (i.e., diarrhea) or outside of it. The types of *E. coli* that can cause diarrhea can be transmitted through contaminated water or food, or through contact with animals or humans (3). Coliform is not a taxonomic classification, but rather a working definition used to describe a group of Gram-negative facultative anaerobic rod-shaped bacteria that ferment lactose to produce acid and gas within 48 h at 35°C. Coliform is known as a convenient standard of sanitary significance (4).

Materials and Methods

Test Kit Information

(a) *Kit name*.—Medi·Ca EC.

(b) *Catalog No*.—EC-01.

(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; 4).

(a) *Plate count agar (PCA)*.—Dissolve 5 g tryptone, 2.5 g yeast extract, 1 g dextrose, and 15 g agar in 1 L distilled water. Heat to dissolve the ingredients and then dispense into 500 mL Erlenmeyer flasks. Autoclave for 15 min at 121°C. Final pH: 7.0 ± 0.2.

(b) *Violet Red Bile Agar (VRBA).*—Dissolve 3 g yeast extract, 7 g peptone, 5 g NaCl, 1.5g 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.

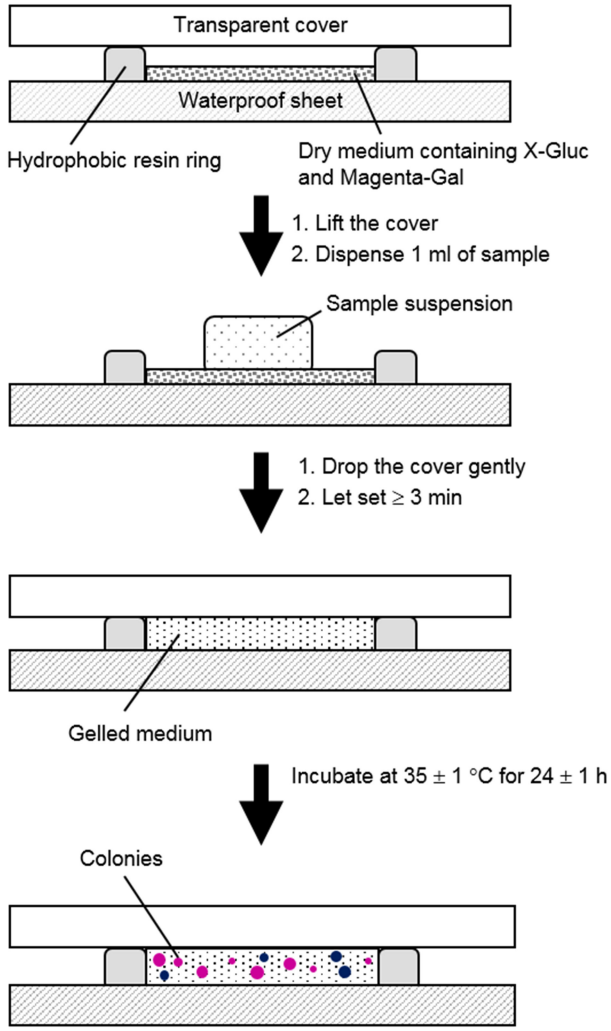

Figure 2. Principle of the Medi·Ca EC method.

(c) *Lauryl tryptose (LST) broth.*—Dissolve 20 g tryptose or trypticase, 5 g lactose, 2.75 g K_2HPO_4, 2.75 g KH_2PO_4, 5 g NaCl, and 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 for 15 min at 121°C. Final pH: 6.8 ± 0.2.

(d) *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 for a volume of 975 mL. Adjust the pH to 7.4. Add 13.3 mL 0.1% aqueous brilliant green to the distilled water. Add distilled water to dilute to a volume of 1 L. Dispense into fermentation tubes, ensuring that the fluid level covers the inverted vials. Autoclave for 15 min at 121°C. Final pH: 7.2 ± 0.1.

(e) *Escherichia coli* (*EC) broth.*—Dissolve 20 g tryptose or trypticase, 5 g lactose, 4 g K_2HPO_4, 1.5 g KH_2PO_4, 5 g NaCl, and 1.5 g bile salt in 1 L distilled water. Dispense 8 mL portions into 16 × 150 mm test tubes containing inverted 10 × 75 mm

fermentation tubes. Autoclave for 15 min at 121°C. Final pH: 6.9 ± 0.2.

(f) *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 for 15 min at 121°C. Final pH: 7.3 ± 0.2.

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

(h) *Levine's eosin-methylene blue (L-EMB) agar.*—Dissolve 10 g peptone, 10 g lactose, 2 g K_2HPO_4, 15 g agar, 0.4 g eosin Y, and 0.065 g methylene blue in 1 L distilled water. Boil to dissolve the peptone, phosphate, and agar in 1 L water. Add water to dilute to the original volume. Dispense in 100 or 200 mL portions and autoclave for 15 min at a maximum of 121°C. Final pH: 7.1 ± 0.2. Before use melt each potion, and to each 100 mL portion add 5 mL sterile 20% lactose solution, 2 mL aqueous 2% eosin Y solution, and 4.3 mL 0.15% aqueous methylene blue solution.

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 pipet with 0.1 mL graduations.

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

(e) *Water bath.*—Maintained at 45.5 ± 0.05°C.

Reference Cultures

Reference cultures used in this study were obtained from the American Type Culture Collection (Manassas, VA); Biological Resource Center, National Institute of Technology and Evaluation (Chiba, Japan); and the Research Institute of Microbial Diseases (Osaka University, Japan).

Safety Precautions

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

General Preparation

Remove each Medi·Ca EC sheet from the aluminum bag under aseptic conditions. Fold the end of the bag over twice and seal with tape. Store the bag under refrigerated conditions.

Sample Preparation

Perform the sample preparation according to BAM Chapter 4. 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 and 10 mL of the previous dilution. Shake all dilutions 25 times in a 30 cm arc.

Analysis

Place each Medi·Ca EC sheet on a flat surface and allow to reach room temperature (15–25°C). Lift the cover, place 1 mL sample suspension in the center of the medium, and gently drop the cover onto the sample. Leave the sheet on a horizontal surface for 3 min or more until solidification of the suspension is complete. Holding both ends of the sheet, place the sheet in an incubator. Incubate the sheets at 35 ± 1°C for 24 ± 1 h. Up to 25 sheets can be stacked.

Interpretation and Test Result Report

Count the navy blue/blue–purple colonies for *E. coli* and pink/red–purple colonies for the non-*E. coli* coliform. The use of *E. coli* NBRC 15034 as the control for the blue colony and *E. cloacae* NBRC 13536 as the control for the red–purple colony is recommended. The suitable colony counting range is 1–250. See below for troubleshooting the 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 × 1 cm) printed on the cover and calculate an average. Multiplying the average number by 20 provides an estimated count because the circular growth area is approximately 20 cm^2.

(b) When the entire growth area becomes 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 chromogenic reaction, prepare a higher dilution.

Validation Study

This validation study was conducted under the *Performance Tested Method*[SM] program of the AOAC Research Institute (RI) and *AOAC INTERNATIONAL Method Committee Guidelines for the Validation of Microbiological Methods for Food and Environmental Surfaces* (5). Method developer studies included an inclusivity and exclusivity study, matrix studies for all claimed matrixes, product consistency and stability studies, and robustness testing. The independent laboratory study was conducted by Q Laboratories, Inc., and included a matrix study for the raw beef and cooked ham in the claimed food matrixes.

Method Developer Validation Studies

Inclusivity and exclusivity study.—The inclusivity and exclusivity study examined the ability of the Medi·Ca EC method to detect a variety of *E. coli* and non-*E. coli* coliform

strains and to distinguish these from closely related noncoliform strains and species. For inclusivity, 51 different isolates of coliform strains, including 25 *E. coli* 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, 41 isolates of closely related noncoliform 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 the appropriate dilutions was cultured in Medi·Ca EC medium at 35 ± 1°C for 24 ± 1 h.

Matrix study.—The Medi·Ca EC method was compared with AOAC *Official Method* **966.24** for seven different food matrixes: raw beef, raw pork, raw frozen pork, raw lamb, raw salmon, frankfurter sausage, and cooked ham. For the meat products, steak meat was used. The study included five replicate test potions at each contamination level for each matrix, including an uncontaminated level. Raw pork, raw frozen pork, raw lamb, and raw salmon were analyzed for natural contamination from coliform bacteria. Because the contamination levels of *E. coli* and non-*E. coli* coliform bacteria on some matrixes were very low, the strains listed in Tables 3 and 4 were used to artificially contaminate matrixes. Twenty-four hour TSB cultures of the appropriate strains were spread over the surface of a bulk sample of matrixes and blended in sterile plastic containers, which allowed for equilibration with the food environment at 4 ± 1°C for 72 ± 1 h. For heat-processed meat matrixes, frankfurter sausage and cooked ham, 24 h TSB cultures were heated at 50°C for 10 min before being added to samples. The degree of injury of the culture was estimated using the following formula:

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

where n_{select} = number of colonies on the selective agar; and $n_{\text{nonselect}}$ = number of colonies on the nonselective agar.

VRBA and PCA were used as the selective and nonselective agar, respectively. After incubation, at least 10 representative non-*E. coli* coliform colonies were chosen and each transferred to a tube of BGLB broth for confirmation. These tubes were incubated at 35 ± 1°C for 48 ± 2 h and examined for gas formation at 24 and 48 h. In addition, at least 10 representative *E. coli* colonies were chosen and each transferred to a tube of EC broth. These tubes were incubated at 45.5 ± 0.05°C for 48 ± 2 h in a water bath and examined for gas formation at 24 and 48 h.

For AOAC *Official Method* **966.24**, 50 g test potions were diluted with 450 mL BPD in a blender jar and blended for 2 min. Each 1 mL sample suspension at dilutions of 1:10, 1:100, and 1:1000 was inoculated into a three-tube most probable number (MPN) series with triplicate tubes of LST broth. These tubes were incubated at 35 ± 1°C for 48 ± 2 h and examined for gas formation at 24 and 48 h. Evidence of gas formation is indicated by the displacement of liquid in a Durham tube. A loopful of gassing tubes was transferred to BGLB and EC broths. BGLB broth tubes were incubated 35°C for 48 ± 2 h. Table **966.24A** containing MPNs was used to compute the MPN on the basis of the number of tubes of BGLB broth that produced gas. EC broth tubes were incubated at 45.5 ± 0.05°C for 48 ± 2 h in a water bath and examined for gas formation at 24 and 48 h intervals. When the tubes were incubated, the water level rose above the highest level of medium. Gas-positive tubes were streaked on L-EMB agar plates. These plates were incubated at 35 ± 1°C for 24 ± 2 h. Typical *E. coli* colonies from the L-EMB agar were transferred to PCA slants for further testing. The slants were incubated at 35 ± 1°C for 18–24 h. The cultures were identified by IMViC tests.

For data analysis, a logarithmic transformation was performed on the reported CFU/g and MPN/g: $\log_{10}[\text{CFU or MPN/g} + (0.1)f]$, where f = reported CFU or MPN/unit corresponding to the smallest reportable result. The s_r and RSD were calculated according to the Cochran and Grubbs outlier test. The candidate method result (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 a 95% confidence interval (CI) at each contamination level for each matrix was analyzed using an Excel Worksheet, Paired Method Analysis for Micro Testing, Version 1.0 (6), which was developed by AOAC Statistical Advisor Robert Labudde.

Product consistency and stability study.—Three different production lots of Medi·Ca EC sheets were examined for lot-to-lot variability and product stability. Production lots that were near the expiration date (December 20, 2013), near the middle of the expiration period (January 6, 2015), and recently manufactured (September 4, 2015) were selected. Cooked ham samples were inoculated with *E. coli* (NBRC 13500) and *Enterobacter cloacae* (NBRC 13536). Twenty-four hour TSB cultures were added to a bulk sample of cooked ham and allowed to equilibrate with the food environment at 4 ± 1°C for 48 ± 1 h. Each production lot of Medi·Ca EC sheets with five replicates of the target at the high level of inoculation, five replicates of the target at the low level of inoculation, and five replicates at the uninoculated level was tested.

Robustness study.—The sample volume and the incubation time were varied using a factorial design to evaluate the ability of the Medi·Ca EC method to remain unaffected by small variations.

Cooked ham samples were inoculated with *E. coli* (NBRC 13500) and *E. cloacae* (NBRC 13536). Twenty-four hour TSB cultures were added to a bulk sample of cooked ham and allowed to equilibrate with the food environment at 4 ± 1°C for 48 ± 1 h. Each production lot of Medi·Ca EC sheets with five replicates of the target at the high level of inoculation, five replicates of the target at the low level of inoculation, and five replicates at the uninoculated level was tested. The incubation temperature was set at 35 ± 1°C for all combinations.

Independent Laboratory Validation Study

Matrix study.—The methodology for this study was followed as outlined in the AOAC-RI's independent laboratory validation protocol, *Independent Laboratory Study for Dai Nippon Printing Co., Ltd for the Medi·Ca EC Medium for Enumeration of Escherichia coli and Coliform Bacteria* (7). The Medi·Ca EC method was compared with AOAC reference method **966.24** for two matrixes: raw beef and cooked ham. The study included five replicate test portions at each contamination level for each matrix. The raw beef (*E. coli* and *Klebsiella oxytoca*) and cooked ham (*E. coli* and *E. cloacae*) were artificially

Table 1. Inclusivity study

Isolate No.	Strain name	Source	Origin	Medi·Ca EC[a,b,c]
1	*Buttiauxella noackiae*	D0077[d]	Chicken	+ (Pink)
2	*Citrobacter amalonaticus*	NBRC 13547[e]	Unknown	+ (Red–purple)
3	*Citrobacter freundii*	NBRC 12681	Unknown	+ (Red–purple)
4	*Citrobacter freundii*	ATCC 8090[f]	Unknown	+ (Red–purple)
5	*Citrobacter koseri*	NBRC 105690	Unknown	+ (Red–purple)
6	*Cronobacter sakazakii*	D0003	Soybean	+ (Red–purple)
7	*Enterobacter aerogenes*	NBRC 13534	Sputum	+ (Red–purple)
8	*Enterobacter amnigenus*	D0037	Cabbage	+ (Red–purple)
9	*Enterobacter asburiae*	D0029	Radish sprout	+ (Red–purple)
10	*Enterobacter cloacae*	D0030	Radish sprout	+ (Red–purple)
11	*Enterobacter cloacae*	ATCC222	Unknown	+ (Red–purple)
12	*Enterobacter cloacae*	D0033	Bean sprout	+ (Pink)
13	*Escherichia blattae (Shimwellia blattae)*	NBRC 105725	Hindgut of cockroach	ND
14	*Escherichia coli*	NBRC 102203	Urine	+ (Navy blue)
15	*Escherichia coli*	NBRC 12062	Unknown	+ (Navy blue)
16	*Escherichia coli*	NBRC 12433	Unknown	+ (Navy blue)
17	*Escherichia coli*	NBRC 12734	Unknown	+ (Navy blue)
18	*Escherichia coli*	NBRC 13500	Unknown	+ (Navy blue)
19	*Escherichia coli*	NBRC 15034	Clinical specimen	+ (Navy blue)
20	*Escherichia coli*	NBRC 3972	Feces	+ (Blue–purple)
21	*Escherichia coli*	ATCC 25922	Unknown	+ (Navy blue)
22	*Escherichia coli*	NBRC 3301	Unknown	+ (Blue–purple)
23	*Escherichia coli*	D0100	Ground beef and pork	+ (Navy blue)
24	*Escherichia coli*	D0099	Ground chicken	+ (Navy blue)
25	*Escherichia coli*	D0101	Chicken	+ (Navy blue)
26	*Escherichia coli*	D0102	Chicken	+ (Navy blue)
27	*Escherichia coli*	NBRC 13540	Unknown	+ (Navy blue)
28	*Escherichia coli*	NBRC 3543	Unknown	+ (Blue–purple)
29	*Escherichia coli*	NBRC 3806	Unknown	+ (Navy blue)
30	*Escherichia coli*	NBRC 3991	Unknown	+ (Navy blue)
31	*Escherichia coli*	NBRC 13898	Unknown	+ (Navy blue)
32	*Escherichia coli*	D0104	Coconut water	+ (Navy blue)
33	*Escherichia coli*	NBRC 14195	Unknown	+ (Navy blue)
34	*Escherichia coli*	NBRC 3302	Unknown	+ (Blue–purple)
35	*Escherichia coli*	NBRC 3544	Unknown	+ (Navy blue)
36	*Escherichia coli*	NBRC 14129	Unknown	+ (Blue–purple)
37	*Escherichia coli* O157	ATCC 43895	Raw hamburger meat	+ (Red–purple)
38	*Escherichia coli* O26	RIMD 05091876[g]	Patient	+ (Blue–purple)
39	*Escherichia fergusonii*	NBRC 102419	Human feces	+ (Red–purple)
40	*Escherichia hermanii*	NBRC 105704	Toe of 17 year old female	+ (Red–purple)
41	*Escherichia vulneris*	NBRC 102420	Human wound	+ (Red–purple)
42	*Klebsiella oxytoca*	D0032	Yellowtail	+ (Pink)
43	*Klebsiella oxytoca*	NBRC 105695	Pharyngeal tonsil	+ (Red–purple)
44	*Klebsiella pneumoniae*	ATCC 13883	Unknown	+ (Red–purple)
45	*Kluyvera cryocrescens*	NBRC 102467	Food	+ (Red–purple)
46	*Leclercia adecarboxylata*	NBRC 102595	Drinking water	+ (Red–purple)
47	*Pantoea agglomerans*	D0004	Cake	+ (Pink)
48	*Rahnella aquatilis*	D0038	Pork	+ (Red–purple)
49	*Rahnella aquatilis*	D0053	Salmon	+ (Red–purple)
50	*Raoultella terrigena*	D0022	Salmon	+ (Red–purple)
51	*Raoultella planticola*	NBRC 14939	Radish root	+ (Red–purple)

[a] + = Detected.

[b] ND = Not detected.

[c] Text in parentheses indicates the color of the colony detected.

[d] Numbers starting with "D" indicate strains that were isolated by Dai Nippon Printing Co., Ltd.

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

[f] ATCC = American Type Culture Collection.

[g] RIMD = Research Institute of Microbial Diseases, Osaka University.

Table 2. Exclusivity study

Isolate No.	Strain name	Source	Origin	Medi·Ca EC[a,b,c]
1	*Achromobacter xylosoxidans*	NBRC 15126[d]	Ear discharge	ND
2	*Aeromonas hydrophila*	NBRC 12658	Unknown	+ (Pink)
3	*Bacillus amylolychefaciens*	D0015[e]	Paprika powder	ND
4	*Bacillus cereus*	NBRC 3836	Unknown	ND
5	*Bacillus circulans*	NBRC 13626	Soil	ND
6	*Bacillus coagulans*	NBRC 12583	Evaporated milk	ND
7	*Bacillus licheniformis*	D0010	Cheese cake	ND
8	*Bacillus megaterium*	NBRC 15308	Unknown	ND
9	*Bacillus subtilis*	D0021	Chinese barbecued pork	ND
10	*Bacillus thuringiensis*	NBRC 3951	Unknown	ND
11	*Corynebacterium variabile*	NBRC 15286	Food	ND
12	*Edwardsiella tarda*	NBRC 105688	Human feces	ND
13	*Enterococcus faecalis*	ATCC 29212[f]	Urine	ND
14	*Enterococcus faecium*	NBRC 100486	Unknown	ND
15	*Kocuria rhizophila*	D0008	Raw pork	ND
16	*Lactobacillus casei*	D0025	Lactic acid drink	ND
17	*Lactobacillus delbrueckii*	NBRC 3202	Sour grain mash	ND
18	*Lactococcus lactis*	D0026	Yogurt	ND
19	*Leuconostoc mesenteroides*	D0057	Korean pickle	ND
20	*Micrococcus luteus*	NBRC 3333	Unknown	ND
21	*Proteus hauseri*	NBRC 3851	Unknown	ND
22	*Proteus mirabilis*	NBRC 105697	Unknown	ND
23	*Pseudomonas aeruginosa*	NBRC 3899	Well water	ND
24	*Pseudomonas aeruginosa*	ATCC 9027	Unknown	ND
25	*Pseudomonas mendocina*	NBRC 14162	Soil enrichment with ethanol as carbon source	ND
26	*Pseudomonas* sp.	D0054	Salmon	ND
27	*Salmonella enterica*	NBRC 105726	Human feces (food poisoning in a male)	ND
28	*Serratia liquefaciens*	D0027	Chicken	ND
29	*Serratia marcescens*	NBRC 102204	Pond water	+ (Pink)
30	*Serratia rubidaea*	NBRC 12973	Seawater	+ (Red–purple)
31	*Staphylococcu epidermidis*	NBRC 100911	Nose	ND
32	*Staphylococcus aureus*	D0072	Ground beef and pork	ND
33	*Staphylococcus aureus*	ATCC 25923	Clinical isolate	ND
34	*Staphylococcus carnosus*	D0086	Roast beef	ND
35	*Staphylococcus gallinarum*	D0061	Japanese tea leaf	ND
36	*Staphylococcus intermedius*	ATCC 29663	Pigeon nares	ND
37	*Staphylococcus saprophyticus*	D0009	Pork	ND
38	*Staphylococcus simulans*	NBRC 109714	Human skin	ND
39	*Staphylococcus* sp.	D0058	Ground beef and pork	ND
40	*Staphylococcus xylosus*	NBRC 109770	Human skin	ND
41	*Yersinia frederiksenii*	D0052	Salmon	ND

[a] + = Detected.

[b] ND = Not detected.

[c] Text in parentheses indicates the color of the colony detected.

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

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

[f] ATCC = American Type Culture Collection.

Table 3. Matrix study: Total coliforms for the Medi·Ca EC method versus AOAC Official Method 966.24

			Total coliforms									
			Medi·Ca EC			BGLB				95% CI[b]		
Matrix	Inoculation microorganism	Contamination level	Mean[a]	s_r	RSD_r	Mean	s_r	RSD_r	Mean difference	LCL[c]	UCL[d]	r^2
Raw pork	*E. coli* ATCC 9637	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	—
		Low	2.40	0.08	3.42	2.49	0.15	6.20	–0.10	–0.24	0.05	0.92
		Medium	3.90	0.12	2.98	3.67	0.19	5.12	0.24	–0.01	0.48	
		High	4.39	0.09	2.01	4.61	0.13	2.74	–0.22	–0.45	0.02	
Raw frozen pork	*E. coli* D0099	Uninoculated	2.14	0.09	4.27	1.93	0.20	10.16	0.21	0.04	0.38	—
		Low	2.36	0.05	2.20	2.30	0.19	8.26	0.06	–0.14	0.26	0.94
		Medium	3.06	0.07	2.30	2.77	0.18	6.59	0.29	0.09	0.49	
		High	4.12	0.02	0.58	4.16	0.19	4.62	–0.04	–0.30	0.22	
Raw beef	*E. coli* ATCC 25922 and *K. oxytoca* NBRC 105695	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	—
		Low	2.52	0.05	1.88	2.53	0.26	10.39	–0.02	–0.34	0.31	0.95
		Medium	3.16	0.10	3.10	3.12	0.25	7.86	0.04	–0.25	0.34	
		High	4.86	0.03	0.53	4.70	0.21	4.48	0.16	–0.09	0.41	
Raw beef[e]	*E. coli* ATCC 25922 and *K. oxytoca* NBRC 105695	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	—
		Low	2.44	0.10	4.07	2.30	0.19	8.39	0.13	–0.01	0.34	0.95
		Medium	3.50	0.09	2.47	3.22	0.17	5.35	0.28	0.01	0.55	
		High	4.23	0.13	2.95	4.04	0.00	0.00	0.18	0.03	0.34	
Raw lamb	*E. coli* D0101	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	—
		Low	2.94	0.05	1.81	3.05	0.11	3.73	–0.11	–0.31	0.09	0.99
		Medium	4.01	0.04	0.96	4.01	0.09	2.31	0.00	–0.10	0.10	
		High	5.85	0.06	0.99	6.18	0.15	2.36	–0.32	–0.46	–0.19	
Raw salmon	*E. coli* NBRC 3806	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	—
		Low	1.67	0.08	4.69	1.64	0.22	13.12	0.03	–0.27	0.32	0.93
		Medium	3.03	0.04	1.30	3.24	0.14	4.17	–0.21	–0.36	–0.05	
		High	4.23	0.11	2.52	4.29	0.10	2.42	–0.05	–0.31	0.20	
Frankfurter sausage	*E. coli* NBRC 12433 and *E. cloacae* ATCC 222	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	—
		Low	3.12	0.04	1.36	2.99	0.11	3.70	0.13	–0.02	0.28	0.97
		Medium	4.05	0.05	1.20	4.01	0.09	2.31	0.04	–0.13	0.21	
		High	4.89	0.07	1.42	4.73	0.13	2.78	0.16	–0.03	0.35	
Cooked ham	*E. coli* NBRC 13500 and *E. cloacae* NBRC 13536	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	—
		Low	1.67	0.26	15.61	1.88	0.14	7.66	–0.22	–0.47	0.03	0.95
		Medium	2.69	0.05	1.71	2.80	0.15	5.48	–0.10	–0.34	0.13	
		High	3.36	0.03	0.92	3.33	0.09	2.65	0.03	–0.10	0.16	
Cooked ham[e]	*E. coli* NBRC 13500 and *E. cloacae* NBRC 13536	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	—
		Low	1.92	0.11	5.68	1.73	0.37	21.52	0.19	–0.27	0.66	0.93
		Medium	3.12	0.18	5.74	2.89	0.21	7.18	0.23	–0.06	0.52	
		High	4.15	0.07	1.68	3.93	0.17	4.25	0.22	0.05	0.38	

[a] Mean of five replicates after the logarithmic transformation, $\log_{10}[CFU/g + (0.1)f]$.

[b] CI = Confidence interval.

[c] LCL = Lower confidence limit.

[d] UCL = Upper confidence limit.

[e] Matrix study conducted by the independent laboratory.

Table 4. Matrix study: *E. coli* in the Medi·Ca EC method versus AOAC *Official Method* 966.24

			E. coli									
			Medi·Ca EC			EC			Mean difference	95% CI[b]		
Matrix	Inoculation microorganism	Contamination level	Mean[a]	s_r	RSD_r	Mean	s_r	RSD_r		LCL[c]	UCL[d]	r^2
Raw pork	*E. coli* ATCC 9637	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	—
		Low	1.53	0.33	21.79	1.53	0.15	9.64	0.00	–0.46	0.46	
		Medium	2.47	0.16	6.44	2.33	0.08	3.50	0.14	–0.09	0.37	0.93
		High	3.80	0.04	1.12	3.81	0.25	6.57	–0.01	–0.29	0.27	
Raw frozen pork	*E. coli* D0099	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	—
		Low	1.80	0.11	5.90	1.99	0.22	11.1	–0.18	–0.44	0.07	
		Medium	2.84	0.04	1.44	3.03	0.09	3.08	–0.19	–0.31	0.22	0.97
		High	3.95	0.05	1.21	4.12	0.21	5.10	–0.17	–0.44	0.10	
Raw beef	*E. coli* ATCC 25922 and *K. oxytoca* NBRC 105695	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	—
		Low	1.91	0.09	4.73	1.77	0.18	10.32	0.14	–0.15	0.43	
		Medium	2.35	0.08	3.21	2.32	0.08	3.41	0.02	–0.09	0.14	0.91
		High	3.15	0.08	2.48	3.18	0.21	6.47	–0.03	–0.31	0.25	
Raw beef[e]	*E. coli* ATCC 25922 and *K. oxytoca* NBRC 105695	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	—
		Low	2.12	0.14	6.41	2.20	0.15	6.91	–0.08	–0.22	0.06	
		Medium	3.22	0.06	1.89	3.14	0.16	5.00	0.07	–0.06	0.21	0.93
		High	3.81	0.14	3.72	3.61	0.31	8.46	0.20	–0.14	0.54	
Raw lamb	*E. coli* D0101	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	—
		Low	2.83	0.04	1.58	3.01	0.20	6.74	–0.19	–0.46	0.09	
		Medium	3.75	0.08	2.01	3.77	0.18	4.84	–0.02	–0.23	0.19	0.95
		High	4.79	0.06	1.18	4.84	0.18	3.78	–0.05	–0.24	0.15	
Raw salmon	*E. coli* NBRC 3806	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	—
		Low	1.68	0.11	6.59	1.69	0.15	9.14	–0.01	–0.24	0.21	
		Medium	2.42	0.01	0.49	2.46	0.16	6.60	–0.04	–0.25	0.17	0.96
		High	3.67	0.07	2.01	3.70	0.24	6.38	–0.02	–0.25	0.20	
Frankfurter sausage	*E. coli* NBRC 12433 and *E. cloacae* ATCC 222	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	—
		Low	2.72	0.06	2.28	2.52	0.17	6.56	0.20	–0.07	0.47	
		Medium	3.92	0.04		3.84	0.18	4.76	0.08	–0.16	0.32	0.98
		High	5.69	0.04	0.62	0.90	0.21	3.57	–0.12	–0.41	0.17	
Cooked ham	*E. coli* NBRC 13500 and *E. cloacae* NBRC 13536	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	—
		Low	1.58	0.20	12.95	1.70	0.15	8.75	–0.13	–0.34	0.08	
		Medium	2.52	0.09	3.75	2.49	0.22	8.80	0.03	–0.17	0.23	0.93
		High	3.23	0.09	2.80	3.33	0.09	2.65	–0.10	–0.25	0.05	
Cooked ham[e]	*E. coli* NBRC 13500 and *E. cloacae* NBRC 13536	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	—
		Low	1.52	0.21	13.77	1.46	0.22	15.25	0.06	–0.31	0.43	
		Medium	2.80	0.16	5.58	2.76	0.27	9.82	0.04	–0.17	0.25	0.93
		High	3.83	0.09	2.29	3.51	0.25	7.24	0.32	–0.04	0.68	

[a] Mean of five replicates after the logarithmic transformation, $\log_{10}[CFU/g + (0.1)f]$.

[b] CI = Confidence interval.

[c] LCL = Lower confidence limit.

[d] UCL = Upper confidence limit.

[e] Matrix study conducted by the independent laboratory.

contaminated with a different strain of *E. coli* and non-*E. coli* coliform. Each inoculum was prepared by transferring a single colony from TSA with 5% sheep blood agar into brain heart infusion (BHI) broth and incubating the culture at 35 ± 2°C for 24 ± 2 h. Following incubation, the BHI was diluted and the diluted culture used to inoculate a bulk sample of food matrixes. The bulk portions were spiked and blended in large sterile stainless steel containers by using sterile spatulas to equilibrate the organism within the matrix. Prior to the inoculation of the cooked ham, the broth culture inoculum was heat-stressed for 10 ± 1 min at 50 ± 1°C. The degree of injury of the culture was estimated using the above formula by plating an aliquot of diluted culture onto MacConkey's agar and TSA. The agars were incubated at 35 ± 1°C for 24 ± 2 h and the colonies counted. The reference method and Medi·Ca EC method were performed as described above. A final biochemical confirmation was achieved by VITEK 2 GN Biochemical Identification (AOAC *Official Methods of Analysis*SM **2011.17**).

Results

Method Developer Validation Studies

Inclusivity and exclusivity study.—The 51 coliform strains, including 25 *E. coli* strains, were tested in the inclusivity study. Twenty-four *E. coli* strains formed navy blue/blue–purple colonies, and only *E. coli* O157 formed red–purple colonies (Table 1). Of 26 non-*E. coli* coliform strains, 25 were detected and one was not (Table 1). The undetected strain was *Escherichia blattae* (NBRC 105725). Forty-one noncoliform strains were tested in the exclusivity study of which 38 were not detected and 3 detected as non-*E. coli* coliform (Table 2). The strains detected as non-*E. coli* coliform were *Aeromonas hydrophila* (NBRC 12658), *Serratia marcescens* (NBRC102204), and *S. rubidaea* (NBRC 12973).

Matrix study.—Five 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 for the 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 0.32 $\log_{10}$, 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 –0.5 to 0.5. Most s_r and RSD_r values for the Medi·Ca EC method were lower than those for the reference method. The r^2 value obtained for each matrix was over 0.91. The r^2 values for total coliform and *E. coli* across all matrixes were both 0.96 (Figures 3 and 4).

Table 5. Inoculum heat-stress result

Matrix	Inoculation microorganism	Injury, %
Frankfurter sausage[a]	*E. coli* NBRC 12433	79.1
	E. cloacae ATCC 222	70.9
Cooked ham[a]	*E. coli* NBRC 13500	76.1
	E. cloacae NBRC 13536	74.1
Cooked ham[b]	*E. coli* NBRC 13500	66.0
	E. cloacae NBRC 13536	69.6

[a] Method developer study.

[b] Independent laboratory study.

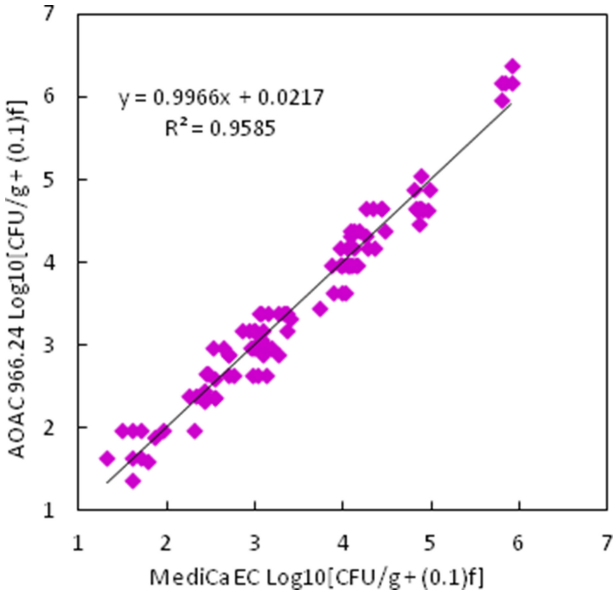

Figure 3. Linear regression analysis for all raw data in the matrix study (total coliform).

Product consistency and stability study.—No significant difference in *E. coli* and coliform counts between production lots was, nor was there a significant time slope (Table 6). These results indicated that the lot-to-lot variability of the Medi·Ca EC medium was very low and that the shelf-life of the medium was at least 20 months.

Robustness study.—No significant difference between combinations 1 and 2 or between combinations 3 and 4 was observed. It was indicated that an incubation time within the range of 22 and 26 h did not adversely affect *E. coli* and coliform count results (Table 7). On the other hand, variations

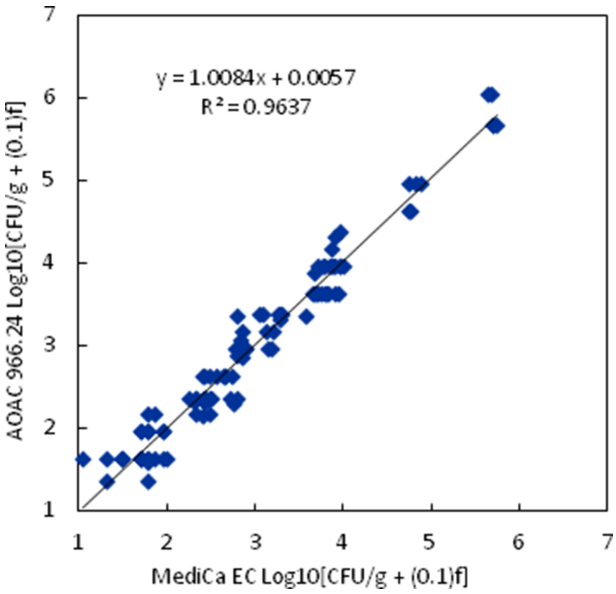

Figure 4. Linear regression analysis for all raw data in the matrix study (*E. coli*).

Table 6. Product consistency and stability study

			December 20, 2013		January 6, 2015		September 4, 2015	
Matrix	Inoculation microorganism	Contamination level	Mean[a]	s_r	Mean	s_r	Mean	s_r
Cooked ham	*E. cloacae* NBRC 13536	Uninoculated	<1.00	—	<1.00	—	<1.00	—
		Low	2.33	0.10	2.42	0.07	2.33	0.09
		High	4.22	0.14	4.28	0.03	4.30	0.12
Cooked ham	*E .coli* NBRC13500	Uninoculated	<1.00	—	<1.00	—	<1.00	—
		Low	2.79	0.05	2.82	0.12	2.83	0.07
		High	4.46	0.07	4.57	0.11	4.54	0.09

[a] Mean of five replicates after the logarithmic transformation, $\log_{10}$[CFU/g + (0.1)*f*].

in the sample volume within the range of 0.9–1.1 mL slightly affected counts. The difference was less than 0.25 $\log_{10}$.

Independent Laboratory Validation Study

Matrix study.—Artificially contaminated raw beef and cooked ham were evaluated by the two methods (Tables 3 and 4). Table 5 presents the results of the heat-stressed cultures for cooked ham. As in the method developer study, the mean differences between the two methods at each contamination level for each matrix were less than 0.32 $\log_{10}$, and much smaller in most cases. There were a few cases where the 95% CIs for the mean difference did not fall within the range of –0.5 to 0.5. In the result for total coliform count in raw beef, the 95% CI for the mean difference at the medium level was (0.01, 0.55). In the result for *E. coli* in raw beef, the 95% CI for the mean difference at the high level was (–0.14, 0.54). In the result for the total coliform count in cooked ham, the 95% CI for the mean difference at the high level was (–0.27, 0.66) and the medium level was (−0.06, 0.52). In the result for *E. coli* in cooked ham, the 95% CI for the mean difference at the high level was (–0.04, 0.68). For raw beef, r^2 values for total coliform and *E. coli* were 0.95 and 0.93, respectively, and both 0.93 for cooked ham.

Discussion

In the inclusivity study, *E. blattae* (NBRC105725) was not detected. The reason for this was that production of β-galactosidase by *E. blattae* did not occur (8). In the exclusivity study, Medi·Ca EC detected some *Serratia* and *Aeromonas* as non-*E. coli* coliform. Generally, most of these species produce β-galactosidase, but some do not have the ability to ferment lactose (2, 8). Therefore, those species were classified as noncoliform. This showed that Medi·Ca EC had the ability to detect coliform-related bacteria, such as *Serratia*.

In the results of the matrix study conducted by the independent laboratory, some of the 95% CIs for the mean differences fell outside of –0.5 to 0.5, but all mean differences were <0.5 $\log_{10}$. In each instance, a difference in means with a positive numerical value indicated higher recovery of the target analyte for the alternative method. In addition, most s_r and RSD_r values in the Medi·Ca EC method were lower than those for the reference method. Statistical differences may have been the result of comparing direct plate count to an MPN estimate, which limits the numerical values that can be generated. The test principle of the MPN method is inherently more variable than a direct plate count method.

Overall, it was generally observed that the Medi·Ca EC method produced statistically similar results compared with the reference method. This rapid method makes it possible to simultaneously detect and enumerate *E. coli* and coliform in only 24 h, whereas the reference method requires 7 to 10 days.

Conclusions

It can be concluded that the Medi·Ca EC method is a reasonable alternative to the AOAC **966.24** reference method for the selected food matrixes analyzed.

Table 7. Robustness study

			0.9 mL sample; 22 h		0.9 mL sample; 26 h		1.1 mL sample; 22 h		1.1 mL sample; 26 h		1.0 mL sample; 24 h	
Matrix	Inoculation microorganism	Contamination level	Mean[a]	s_r	Mean	s_r	Mean	s_r	Mean	s_r	Mean	s_r
Cooked ham	*E. cloacae* NBRC 13536	Uninoculated	<1.00	—	<1.00	—	<1.00	—	<1.00	—	<1.00	—
		Low	2.30	0.07	2.27	0.08	2.47	0.04	2.46	0.10	2.43	0.13
		High	4.13	0.11	4.18	0.11	4.38	0.06	4.28	0.10	4.13	0.17
Cooked ham	*E. coli* NBRC13500	Uninoculated	<1.00	—	<1.00	—	<1.00	—	<1.00	—	<1.00	—
		Low	2.76	0.07	2.76	0.03	2.91	0.07	2.91	0.04	2.82	0.05
		High	4.43	0.05	4.46	0.08	4.63	0.04	4.59	0.07	4.53	0.09

[a] Mean of five replicates after the logarithmic transformation, $\log_{10}$[CFU/g + (0.1)*f*].

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