FOOD BIOLOGICAL CONTAMINANTS

Dai Nippon Printing Co., Ltd, Medi·Ca AC for Enumeration of Aerobic Bacteria

Performance Tested Method[SM] 041302

Abstract

A ready-made dry medium method for aerobic count, the Medi·Ca AC method, was compared to the AOAC Official Method **966.23**, Microbiological Methods, for seven different heat-processed meat matrixes: cooked roast beef, Chinese barbecued pork (barbecued pork seasoned with honey-based sauce), bacon, cooked ham, frankfurter (made from beef and pork), and boiled and cooked pork sausage. The 95% confidence interval 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 five matrixes. These results demonstrate that the Medi·Ca AC method is a reasonable alternative to the AOAC **966.23** method for cooked meat products.

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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.*—Aerobic bacteria.

(**b**) *Matrixes.*—Cooked roast beef, Chinese barbecued pork (barbecued pork seasoned with honey-based sauce), bacon, cooked ham, frankfurter (made from beef and pork), and boiled and cooked pork sausage.

(**c**) *Summary of validated performance claims.*—The Medi·Ca AC method is a reasonable alternative to AOAC Official Method **966.23,** Microbiological Methods (1), for cooked meat products.

Principle of the Method

Medi·Ca AC is a ready-made dry medium for aerobic count made up of four components: a waterproof sheet, a dry medium containing a gelling agent, 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 AC method. The cover is lifted, sample suspension is placed on the center of the medium, and the cover is dropped onto the sample. The sample soaks into the medium and turns into a gel in 3 min. The gelled medium contains the redox indicator 2,3,5-triphenyl tetrazolium chloride derived from a coating on the cover. The incubation of the sheet at 35 ± 1°C for 48 ± 2 h develops red colonies because of the redox reaction involving the indicator.

Materials and Methods

Test Kit Information

(**a**) *Kit name.—Medi·Ca AC.*

(**b**) *Cat. No.*—AC-01.

(**c**) *Ordering information.*—Dai Nippon Printing Co., Ltd, 2nd Sales Department, Medical Healthcare Headquarters, Packaging Division, 1-17-28, Minamihorie, Nishi-ku, Osaka, 550-8505, Japan, Tel: +81-(0)6-6110-4043, URL: https://www.dnp.co.jp/CGI/inquiry_eng/form.cgi.

Media and Reagents

Media and reagents were prepared according to the U.S. Food and Drug Administration *Bacteriological Analytical Manual* (2).

(**a**) *Plate Count Agar (PCA).*—Dissolve 5 g tryptone, 2.5 g yeast extract, 1 g dextrose, 15 g agar in 1 L distilled water. Heat to dissolve ingredients and dispense into 500 mL Erlenmeyer flasks. Autoclave 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 sodium chloride (NaCl), 1.5 g bile salts, 10 g lactose, 0.03 g neutral red, 0.002 g crystal violet, 15 g agar in 1 L distilled water. Mix thoroughly and adjust to

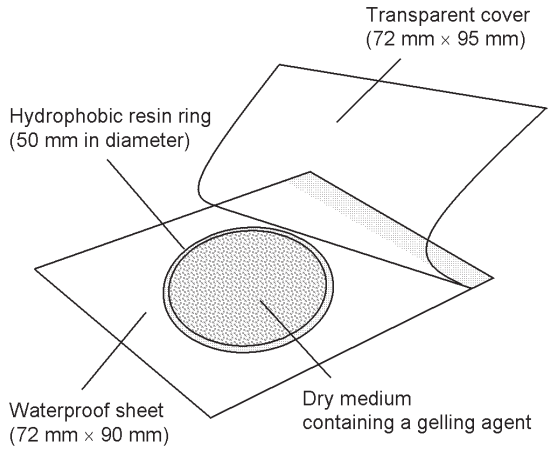

Figure 1. Structure of a Medi·Ca AC sheet.

pH 7.4 ± 0.2. Heat with agitation and boil for 2 min. Do not autoclave.

(**c**) *Tryptic Soy Agar (TSA).*—Dissolve 15 g trypticase peptone, 5 g phytone peptone, 5 g NaCl, 15 g agar in 1 L distilled water. Heat with agitation to dissolve agar and boil for 1 min. Dispense into 500 mL Erlenmeyer flasks. Autoclave 15 min at 121°C. Final pH, 7.3 ± 0.2.

(**d**) *Eosin-Methylene Blue Agar (EMBA).*—Dissolve 10 g peptone, 10 g lactose, 2 g K_2HPO_4, 15 g agar, 0.4 g eosin Y, 0.065 g methylene blue in 1 L distilled water. Boil to dissolve peptone, phosphate, and agar in 1 L of water. Add water to make original volume. Dispense in 100 or 200 mL portions and autoclave 15 min at not over 121°C. Final pH, 7.1 ± 0.2. Before use, melt, 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.

(**e**) *Tryptic Soy Broth (TSB).*—Dissolve 17 g trypticase peptone, 3 g phytone peptone, 5 g NaCl, 2.5 g KH_2PO_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.

(**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 pipet with 0.1 mL graduations.

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

Reference Materials

(**a**) *Enterobacter cloacae* subsp. *cloacae* (Jordan) Hormaeche and Edwards, subsp. nov. (ATCC 222).—Obtained from American Type Culture Collection (ATCC) Manassas, VA.

(**b**) *Escherichia coli* (Migula) Castellani and Chalmers (ATCC 25922).—Obtained from ATCC.

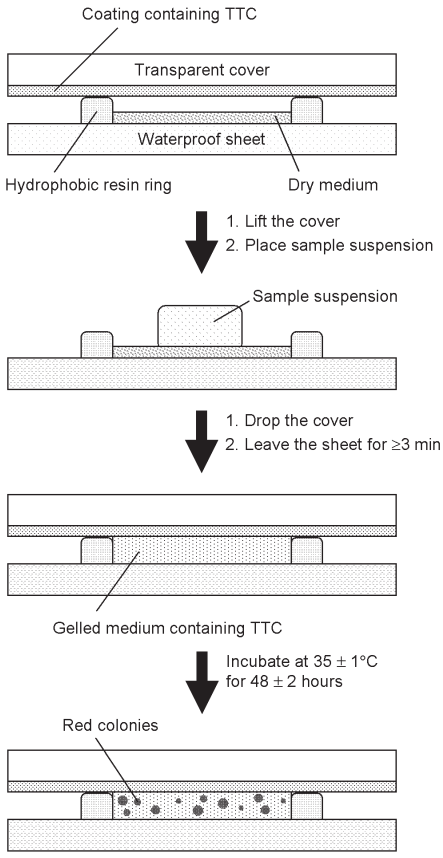

Figure 2. Principle of the Medi·Ca AC method.

(**c**) *Enterobacter cloacae* (Jordan 1890) Hormaeche and Edwards 1960 (NBRC 13536).—Obtained from NITE Biological Resource Center (Chiba, 292-0818, Japan).

Safety Precautions

If medium or reagent gets into eyes or mouth, rinse immediately with plenty of water and consult a doctor. Use of the product should be practiced under the supervision of a laboratory analyst with biohazard protection measures due to risks of laboratory-acquired infections. Inoculated product should be regarded as infectious in the laboratory. Any and all media, supplements, and reagents must be sterilized by autoclaving after use.

Sample Preparation

Sample preparation procedure is followed as described in AOAC **966.23**. Weigh each 50 g test portion into a blender jar, add 450 mL BPD, and blend for 2 min. Prepare all decimal dilutions with 90 mL BPD plus 10 mL previous dilution, and shake 25 times in a 30 cm arc.

Analysis

Place each Medi·Ca AC 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

Table 1. Matrix study (method developer)

Matrix	Inoculation microorganism	Contamination level	Medi·Ca AC			AOAC **966.23**			Mean difference	95% CI[d]		r^{2g}
			Mean[a]	s_r[b]	RSD_r[c]	Mean	s_r	RSD_r		LCL[e]	UCL[f]	
Cooked roast beef	NA[h]	Low	3.51	0.03	0.89	3.44	0.10	2.97	–0.07	–0.23	0.10	0.99
		Medium	6.20	0.14	2.31	6.28	0.08	1.26	0.08	–0.04	0.19	
		High	8.59	0.15	1.69	8.63	0.14	1.67	0.04	–0.31	0.39	
Chinese barbecued pork	NA	Low	4.61	0.07	1.58	4.56	0.03	0.61	–0.05	–0.13	0.04	1.00
		Medium	7.93	0.05	0.64	8.00	0.04	0.52	0.07	0.00	0.14	
		High	8.56	0.06	0.65	8.64	0.06	0.64	0.08	0.00	0.16	
Bacon	NA	Low	4.34	0.03	0.66	4.34	0.05	1.13	0.01	–0.05	0.07	0.99
		Medium	6.35	0.02	0.32	6.30	0.04	0.60	–0.04	–0.11	0.03	
		High	7.43	0.08	1.03	7.57	0.06	0.74	0.14	–0.01	0.28	
Cooked ham	NA	Low	2.61	0.04	1.57	2.60	0.04	1.40	–0.01	–0.10	0.08	0.99
		Medium	7.09[i]	0.04	0.54	7.48	0.04	0.54	0.39	0.34	0.43	
		High	9.26[i]	0.03	0.35	9.12	0.06	0.70	–0.14	–0.25	–0.03	
Frankfurter	NA	Low	4.88	0.05	0.93	4.91	0.04	0.78	0.04	–0.04	0.11	0.99
		Medium	5.74	0.04	0.70	5.71	0.05	0.82	–0.03	–0.07	0.01	
		High	6.12	0.04	0.59	6.13	0.03	0.55	0.02	–0.04	0.08	
Boiled pork sausage	*E. cloacae*[j] ATCC 222	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	
		Low	2.60[i]	0.06	2.18	2.97	0.04	1.47	0.37	0.27	0.47	0.99
		Medium	3.58[i]	0.04	1.11	3.81	0.05	1.40	0.24	0.12	0.35	
		High	4.55[i]	0.09	1.87	4.74	0.05	0.96	0.19	0.05	0.33	

[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 ($P < 0.05$).

[j] A heat-stressed culture with 71% injury was used.

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 carry it into an incubator. Incubate the sheets at 35 ± 1°C for 48 ± 2 h. It is possible to stack up to 25 sheets.

Interpretation and Test Result Report

Count all red colonies regardless of size or intensity. The suitable colony counting range is 25–250. See the following troubleshooting for the interpretation and test result report:

(**a**) When a 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 the estimated count since the circular growth area is approximately 20 cm^2.

(**b**) When the entire growth area become red or pink, 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 is spreading, 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.

Validation Study

The *Performance Tested Method*[SM] validation study was performed according to the AOAC INTERNATIONAL *Methods Committee Guidelines for Validation of Microbiological*

Table 2. Product consistency and stability study

Matrix	Inoculation microorganism	Contamination level	Oct. 6, 2011		June 25, 2012		Jan. 23, 2013	
			Mean[a]	s_r[b]	Mean	s_r	Mean	s_r
Boiled pork sausage	*E. coli* ATCC 25922	Uninoculated	<1.00	—	<1.00	—	<1.00	—
		Low	2.68	0.04	2.68	0.07	2.65	0.10
		High	4.43	0.10	4.41	0.12	4.47	0.09

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

[b] s_r = Standard deviation.

Methods for Food and Environmental Surfaces (3). Method developer studies were conducted in the laboratories of Dai Nippon Printing Co., Ltd, and included the matrix study 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 two of the claimed food matrixes.

Matrix Study

The Medi·Ca AC method was compared to the AOAC **966.23** method for six different heat-processed meat matrixes: cooked roast beef, Chinese barbecued pork, bacon, cooked ham, frankfurter, and boiled pork sausage. 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, cooked roast beef, cooked ham and frankfurter were stored at 35°C for 12–24 h, and Chinese barbecued pork and bacon were stored at 22°C for 3 days to achieve three different contamination levels so that each level was approximately one log higher than the previous. For artificial contamination, boiled pork sausage samples were inoculated with *E. cloacae* (ATCC 222). A 24 h TSB culture was heated at 50°C for 10 min, added to a bulk sample of boiled pork sausage, and allowed to equilibrate with the food environment at 4°C for 48–72 h. 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.

VRBA and PCA were used for selective and nonselective agar, respectively.

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) versus 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 AC sheets were examined for lot-to-lot variability and product stability. Production lots that were near the expiration date (October 6, 2011), near the middle of the expiration period (June 25, 2012), and recently manufactured (January 23, 2013) were selected. Boiled pork sausage samples were inoculated with *E. coli* (ATCC 25922). A 24 h TSB culture was added to a bulk sample of boiled pork sausage and allowed to equilibrate with the food environment at 4°C for 72 h. Each production lot of Medi·Ca AC sheets with five replicates of target at the high level, five replicates of target at the low level, and five replicates

Table 3. Robustness study

Matrix	Inoculation microorganism	Contamination level	0.9 mL sample; 44 h		0.9 mL sample; 52 h		1.1 mL sample; 44 h		1.1 mL sample; 52 h		1.0 mL sample; 48 h	
			Mean[a]	s_r[b]	Mean	s_r	Mean	s_r	Mean	s_r	Mean	s_r
Boiled pork sausage	*E. coli* ATCC 25922	Uninoculated	<1.00	—	<1.00	—	<1.00	—	<1.00	—	<1.00	—
		Low	2.94	0.04	2.97	0.06	3.04	0.05	3.06	0.06	3.02	0.05
		High	4.18	0.02	4.17	0.05	4.28	0.02	4.26	0.02	4.23	0.02

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

[b] s_r = Standard deviation.

Table 4. Matrix study (independent laboratory)

Matrix	Inoculation microorganism	Contamination level	Medi·Ca AC			AOAC **966.23**			Mean difference	95% CI[d]		
			Mean[a]	s_r[b]	RSD_r[c]	Mean	s_r	RSD_r		LCL[e]	UCL[f]	r^{2g}
Cooked roast beef	NA[h]	Low	2.79	0.07	2.59	2.78	0.10	3.46	0.00	–0.07	0.07	0.95
		Medium	3.33[i]	0.07	2.18	3.60	0.14	3.84	0.28	0.08	0.47	
		High	4.53	0.12	2.76	4.55	0.08	1.78	0.02	–0.10	0.14	
Cooked pork sausage	*E. cloacae*[j] NBRC 13536	Uninoculated	<1.00	—	—	<1.00	—	—	—	—	—	
		Low	2.81	0.10	3.43	2.84	0.08	2.85	0.03	–0.12	0.17	0.99
		Medium	3.91	0.06	1.47	3.98	0.11	2.83	0.07	–0.03	0.17	
		High	4.83	0.14	2.80	4.88	0.15	3.09	0.05	–0.03	0.13	

[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 ($P < 0.05$).

[j] A heat-stressed culture with 58% injury was used.

of uninoculated level was tested in a randomized blind-coded fashion.

Robustness Study

Volumes of sample and incubation time were varied to evaluate the ability of the Medi·Ca AC method to remain unaffected by small variations. A factorial study design was used testing five different combinations of sample volume and incubation time.

Boiled pork sausage samples were inoculated with *E. coli* (ATCC 25922). A 24 h TSB culture was added to a bulk sample of boiled pork sausage and allowed to equilibrate with the food environment at 4°C for 72 h. Medi·Ca AC 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.

Independent Laboratory Study

The Medi·Ca AC method was compared to AOAC **966.23** for two matrixes, cooked roast beef and cooked pork sausage. The study included five replicate test portions at each contamination level for each matrix. Cooked roast beef was analyzed for natural contamination of aerobic bacteria. Cooked pork sausage samples were inoculated with *E. cloacae* (NBRC 13536). A 24 h TSB culture was heated at 50°C for 10 min, added to a bulk sample of cooked pork sausage, and allowed to equilibrate with the food environment at 2–5°C for 48–72 h. The degree of injury of the culture was estimated using the above formula by plating an aliquot of diluted culture onto EMBA and TSA. The agars were incubated at 35 ± 1°C for 24 ± 2 h, and the colonies were counted. Data analysis was performed as described above.

Results

Matrix Study

Five different naturally contaminated matrixes and one artificially contaminated matrix were tested by the two methods (Table 1). A heat-stressed culture with 71% injury was used for artificial contamination of food samples. 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 cooked roast beef, Chinese barbecue pork, bacon, and frankfurter. However, the mean log_{10} counts of the Medi·Ca AC method at all three contamination levels for boiled pork sausage and at the medium level for cooked ham were significantly lower than those of the AOAC **966.23** method. The s_r and RSD_r values of the Medi·Ca AC method were equal to or lower than those of the AOAC **966.23** method for seven out of 18 samples. A square of the correlation coefficient (r^2) obtained for each matrix was 0.99 or more.

Product Consistency and Stability Study

No significant difference in aerobic counts between production lots and no significant time slope were observed (Table 2). These results indicated that the lot-to-lot variability

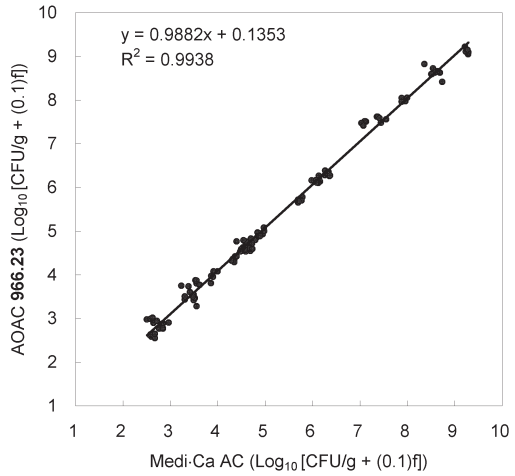

Figure 3. Linear regression analysis for all data of the two matrix studies.

of Medi·Ca AC sheets is very low and the shelf-life of the sheets is at least 16 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 44 to 52 h does not adversely affect aerobic count results (Table 3). However, the variation in volume of sample within the range of 0.9 to 1.1 mL slightly affected the aerobic counts.

Independent Laboratory Study

Naturally contaminated cooked roast beef and artificially contaminated cooked pork sausage were evaluated by the two methods (Table 4). A heat-stressed culture with 58% injury was used for artificial contamination of food samples. 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 the cooked pork sausage. However, the mean $\log_{10}$ count of the Medi·Ca AC method at the medium level for cooked roast beef was significantly lower than that of the AOAC **966.23** method. The s_r and RSD_r values of the Medi·Ca AC method were lower than those of the AOAC **966.23** method for four out of six samples. 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 AC method was compared to the AOAC **966.23** method for seven different heat-processed meat matrixes in the two matrix studies. 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 five matrixes (Tables 1 and 4). In addition, the repeatability of the Medi·Ca AC method was overall similar to that of the AOAC **966.23** method. These results demonstrate that the Medi·Ca AC method is a reasonable alternative to the AOAC **966.23** method for cooked meat products.

The mean $\log_{10}$ counts of the Medi·Ca AC method for boiled pork sausage contaminated with the heat-stressed strain were significantly lower than those of the AOAC **966.23** method (Table 1). Interestingly, the same matrix contaminated with the same strain without any heat treatment provided no significant difference (data not shown). These results suggest that the Medi·Ca AC medium cannot grow heat-stressed microorganisms as vigorously as the PCA, depending on the microorganisms.

The Medi·Ca AC method is similar to the Aerobic Plate Count in Foods (AOAC Official Method **990.12**), also known as the Petrifilm™ Aerobic Count Plate method (4). Morita et al. (5) pointed out that liquefaction of the gel by bacteria which caused diffusion of colonies was observed on the Petrifilm Aerobic Count plates. The same phenomenon, which sometimes interfered with counting, was also observed for Chinese barbecued pork, cooked ham, and frankfurter in this study (data not shown). On the other hand, Medi·Ca AC medium did not appear to be subject to the liquefaction by bacteria for all the matrixes, which made counting easier.

Conclusions

We concluded that the Medi·Ca AC method is a reasonable alternative to the AOAC **966.23** method for cooked meat products.

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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