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=Short Paper=

# Efficacy of Compact Dry EC for Coliform Detection in Seafood

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The Compact Dry EC (CD-EC) plate is ready-to-use test method for the enumeration of *Escherichia coli* and coliform bacteria in food. The plates are pre-sterilized and contained culture medium with a cold water-soluble gelling agent. The medium is re-hydrated with 1 m*l* of diluted sample inoculated onto the center of the self-diffusible medium, allowing the solution to diffuse by capillary action. The plate can be incubated at 35℃ for 20–24hr and the colonies counted without any further working steps. The CD-EC plate method was compared with the violet red bile agar (VRBA) and MPN of the FDA-BAM methods. The correlation coefficients were 0.96 between the CD-EC plate and VRBA methods, and 0.85 between the CD-EC plate and MPN methods. Of the 142 red/pink-colony-forming isolates from the CD-EC plate method, 116 isolates were identified as *Enterobacteriaceae*. Blue/blue purple-colonies on the CD-EC plate method were found in 8 samples. *E. coli* was detected in 5 samples, however the isolates from 3 samples were identified as *Escherichia vulneris*. These results suggest that the CD-EC plate method is useful for screening test on the spot for *E. coli* and coliform bacteria in seafood.

**Key words:** Compact Dry EC, Coliform, Seafood

## Introduction

The ability to rapidly and accurately detect *Escherichia coli* and coliform is important to any food safety program. *E. coli* and coliform are important indicators of the safety of processed foods[4], raw foods[5, 9] and water quality[1, 3]. In Japan, the standards of *E. coli* and coliform for seafood are as follows: coliform should be absent in frozen seafood intended for raw consumption and boiled octopus and crabs after freezing; for *E. coli* the MPN should be under 230 MPN per 100 g in oysters intended for raw consumption. Hence, it is important to test *E. coli* and coliform bacteria in the field for seafood.

Current classical methodologies for the detection of *E. coli* and coliform are labor intensive, time consuming and costly with respect to equipment, media, and personnel. The 3 M Petrifilm *E. coli*/Coliform Count (EC) plate method is the AOAC Official Method 998.08 for poultry, meats, and seafood. Evaluations of the Petrifilm plate in variety of foods have been reported [4, 9]. However, the Petrifilm plate is too soft and supple to inoculate the liquid samples without a hard flat table.

The Compact Dry EC (CD-EC) plate[7] is a detection method for *E. coli* and coliform that can yield a result in 20–24 hr. The plate is hard like plastic dish. Therefore, it is easy to inoculate the liquid samples at any places. Diluted samples are inoculated onto the CD-EC plate and incubated at 35℃. The CD-EC plate uses the Compact Dry system[8] and contains the nutrients developed primarily for the rapid growth of coliform[6], including 5-bromo-6-chloro-3-indoxyl-β-D-galactopyranoside (Magenta-Gal) for coliform and 5-bromo-4-chrolo-3-indoxyl-β-D-glucuronic acid (X-Gluc) for *E. coli*.

The colonies of *E. coli* and coliform on the CD-EC plate are blue to blue-purple and red to

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Table 1. Sea food (107 samples) used for this evaluation

| English name | Japanse name | Latin name | No. of samples |
|---|---|---|---|
| Abalone | Awabi | *Haliotis discus discus* | 1 |
| Albacore tuna | Bin-naga-maguro | *Thunnus alaunga* | 1 |
| Amberstripe scad | Muro-aji | *Depapterus muroadsi* | 1 |
| Bigeye tuna | Mebachi-maguro | *Thunnus obesus* | 19 |
| Black tiger prawn | Burakkutaiga | *Penaeus monodon* | 6 |
| Blue shark | Yoshikiri-zama | *Prionace glauca* | 2 |
| Chum salmon | Sake | *Oncorhynchus keta* | 8 |
| Common octopus | Ma-dako | *Octopus vulgaris* | 3 |
| Crucian carp | Funa | *Carassius auratus* | 1 |
| Flying fish | Tobi-uo | *Crpselurus agoo agoo* | 1 |
| Freshwater clams | Shijimi | *Corbicula japonica* | 3 |
| Greeneye | Mehikari | *Chlorophthalmus borealis* | 1 |
| Ice gody | Shiro-uo | *Leucopsarion petersii* | 2 |
| Japanese common squid | Surume-ika | *Todarodes pacificus* | 3 |
| Japanese littleneck | Asari | *Ruditapes philippinarum* | 4 |
| Japanese horse mackerel | Ma-aji | *Trachurus japonicus* | 4 |
| Japanese oyster[a] | Ma-gaki | *Crassostrea gigas* | 5 |
| Japanese scallop | Hotate-gai | *Patinopecten yessoensis* | 5 |
| Keen7s gaper | miru-gai | *Tresus keenae* | 1 |
| Lake prawn | Suji-ebi | *Palaemon paucidens* | 1 |
| Pacific cod | Ma-dara | *Gadus macrocephalus* | 3 |
| Pacific herring | Nishin | *Clupea pallasii* | 2 |
| Pacific saury | Sanma | *Cololabis saira* | 5 |
| Pen shell | Taira-gai | *Atrina pectinata* | 1 |
| Rainbow trout | Niji-masu | *Oncorhynchus mykiss* | 1 |
| Red sea bream | Ma-dai | *Pagrus major* | 1 |
| Rock oyster | Iwa-gaki | *Crassostrean nippona* | 1 |
| Sakhalin surf clam | Uba-gai | *Pseudocardium sachalinense* | 3 |
| Sea pineapple | Hoya | *Halocynthia roretzi* | 1 |
| Skipjack | Katsuo | *Katsuwonus pelamis* | 5 |
| Snow crab | Zuwai-gani | *Chionocetes opilio* | 3 |
| Spear squid | Yari-ika | *Loligo bleekeri* | 2 |
| Swordfish | Me-kajiki | *Xipbias gladius* | 1 |
| Turban shell | Sazae | *Batillus cornutus* | 1 |
| Whelk | Tubu-gai | *Neptunea* spp. | 4 |

[a] Japanese oysters werre used for cooking not for raw-eating.

pink, respectively. The combined total number of red/pink with blue/blue purple colonies gives in the total number of coliform. The selectivity of the CD-EC plate may be less inhibitory than that of the Petrifilm EC plate, allowing for greater recovery of *E. coli* and coliform. Gram-positive bacteria are inhibited by bile salts, and Gram-negative bacteria other than coliform do not form the typical blue to blue-purple or red to pink color colonies.

The comparison of the CD-EC plate method with the FDA-BAM methods (FDA Bacteriological Analytical Manual http://www.fda.gov/Food / ScienceResearch / LaboratoryMethods / BacteriologicalAnalyticalManualBAM / default.htm.) using the MPN method (AOAC Official Method 966.24) for enumeration of *E. coli* and coliform in raw meats has been reported[5]. In this study, we evaluated the CD-EC plate for seafood by comparison with FDA-BAM methods.

## Materials and Methods

### 1. Sample preparation

The total of 88 seafood samples (Table 1) consisting of 29 kinds of seafood were purchased from retail stores in Japan. The samples were transported at 4℃ and transportation time to the laboratory did not exceed 1 hr. The samples were stored at 4℃ until examination and examined within one day of purchase. Each 50 g test portion was weighed aseptically (using sterile forceps or spatulas) into a sterile high-speed blender cup. Then 450 m*l* sterile Butterfield's phosphate buffer (pH 7.2) was added into the cup and blended to disperse the material at 8,000 rpm (HOMOGENIZER CM-100, AS ONE Co., Ltd., Osaka, Japan) for 2 min.

Table 1. (continued) Sea food (88 samples) used for this evaluation

| *Seafood* | English name | Japanse name | Latin name | No. of samples | No. of samples isolated *E coli* |
|---|---|---|---|---|---|
| Molluscan*1 | Abalone | Awabi | *Haliotis discus discus* | 1 | 0 |
| | Common octopus | Ma-dako | *Octopus vulgaris* | 3 | 0 |
| | Freshwater clams | Shijimi | *Corbicula japonica* | 3 | 3 |
| | Japanese common squid | Surume-ika | *Todarodes pacificus* | 3 | 0 |
| | Japanese littleneck | Asari | *Ruditapes philippinarum* | 4 | 2 |
| | Japanese oyster*2 | Ma-gaki | *Crassostrea gigas* | 5 | 0 |
| | Japanese scallop | Hotate-gai | *Patinopecten yessoensis* | 5 | 0 |
| | Pen shell | Taira-gai | *Atrina pectinata* | 1 | 0 |
| | Rock oyster | Iwa-gaki | *Crassostrea nippona* | 1 | 0 |
| | Sakhalin surf clam | Uba-gai | *Pseudocardium sachalinense* | 3 | 0 |
| | Sea pineapple | Hoya | Halocynthia roretzi | 1 | 0 |
| | Spear squid | Yari-ika | *Loligo bleekeri* | 2 | 0 |
| | Turban shell | Sazae | *Batillus cornutus* | 1 | 0 |
| | Whelk | Tubu-gai | *Neptunea* spp. | 3 | 0 |
| | | | Sub-total | 36 | 5 |
| Crustaceans*3 | Black tiger prawn | Burakkutaiga | *Penaeus monodon* | 6 | 0 |
| | Snow crab | Zuwai-gani | *Chionocetes opilio* | 3 | 0 |
| | | | Sub-total | 9 | 0 |
| Finfish | Albacore tuna | Bin-naga-maguro | *Thunnus alalunga* | 1 | 0 |
| | Bigeye tuna | Mebachi-maguro | *Thunnus obesus* | 13 | 0 |
| | Chum salmon | Sake | *Oncorhynchus keta* | 4 | 0 |
| | Crucian carp | Funa | *Carassius auratus* | 1 | 0 |
| | Greeneye*4 | Mehikari | *Chlorophthalmus borealis* | 1 | 0 |
| | Ice gody*4 | Shiro-uo | *Leucopsarion petersii* | 2 | 0 |
| | Japanese horse mackerel | Ma-aji | *Trachurus japonicus* | 4 | 0 |
| | Japanese sardine | Ma-iwashi | *Sardinops melanostictus* | 1 | 0 |
| | Pacific cod | Ma-dara | *Gadus macrocephalus* | 3 | 0 |
| | Pacific herring | Nishin | *Clupea pallasii* | 2 | 0 |
| | Pacific saury | Sanma | *Cololabis saira* | 5 | 0 |
| | Red sea bream | Ma-dai | *Pagrus major* | 1 | 0 |
| | Skipjack | Katsuo | *Katsuwonus pelamis* | 5 | 0 |
| | | | Sub-total | 43 | 0 |
| | | | Ground total | 88 | 5 |

*1 Shellfish were removed shells before blending.
*2 Japanese oysters were used for cooking not for raw-eating.
*3 Crustaceans were examined just meat.
*4 Whole fish comprising skin, bones, and viscera was used.

Serial dilutions were made in sterile Butterfield's phosphate buffer.

## 2. CD-EC plate method

Two 1 m*l* aliquots of each dilution of 1 : 10, 1 : $10^2$, 1 : $10^3$, 1 : $10^4$, 1 : $10^5$ was inoculated onto the CD-EC plates according to manufacturer's instruction. After 20–24 hr of incubation at 35℃, *E. coli* and coliform counts (CFU/g) were calculated by counting the total colonies eliciting the obligatory color production on each medium multiplied by the dilution factor. Each well-isolated typical blue/blue purple colonies and typical red/pink colonies were picked and re-streaked onto plate count agar (PCA) plates to confirm pure cultures. The pure cultures were identified by Gram stain and biochemical tests including an oxidase test, catalase tests, IMViC test, and API 20E (SYSMEX bioMérieux Co., Ltd., Tokyo, Japan).

## 3. FDA-BAM solid medium method

Each 1 m*l* from 1 : 10, 1 : $10^2$, 1 : $10^3$, 1 : $10^4$, 1 : $10^5$ dilutions was inoculated in duplicate Petri dishes, and then 10 m*l* violet red bile agar (VRBA) tempered to 48℃ was poured in to these plates. The plates were swirled to mix and left to solidify. To prevent surface growth and spreading of colonies, the plates were then overlaid with 5 m*l* VRBA and left to solidify. The solidified plates were inverted and incubated at 35℃ for 18–24 hr. The plates were examined under magnifying lens and with illumination. The purple-red colonies that were 0.5 mm or larger in diameter and surrounded by a zone of precipitated bile acids were counted within the range of 25–250 colonies. To confirm that the colonies were coliform, at least

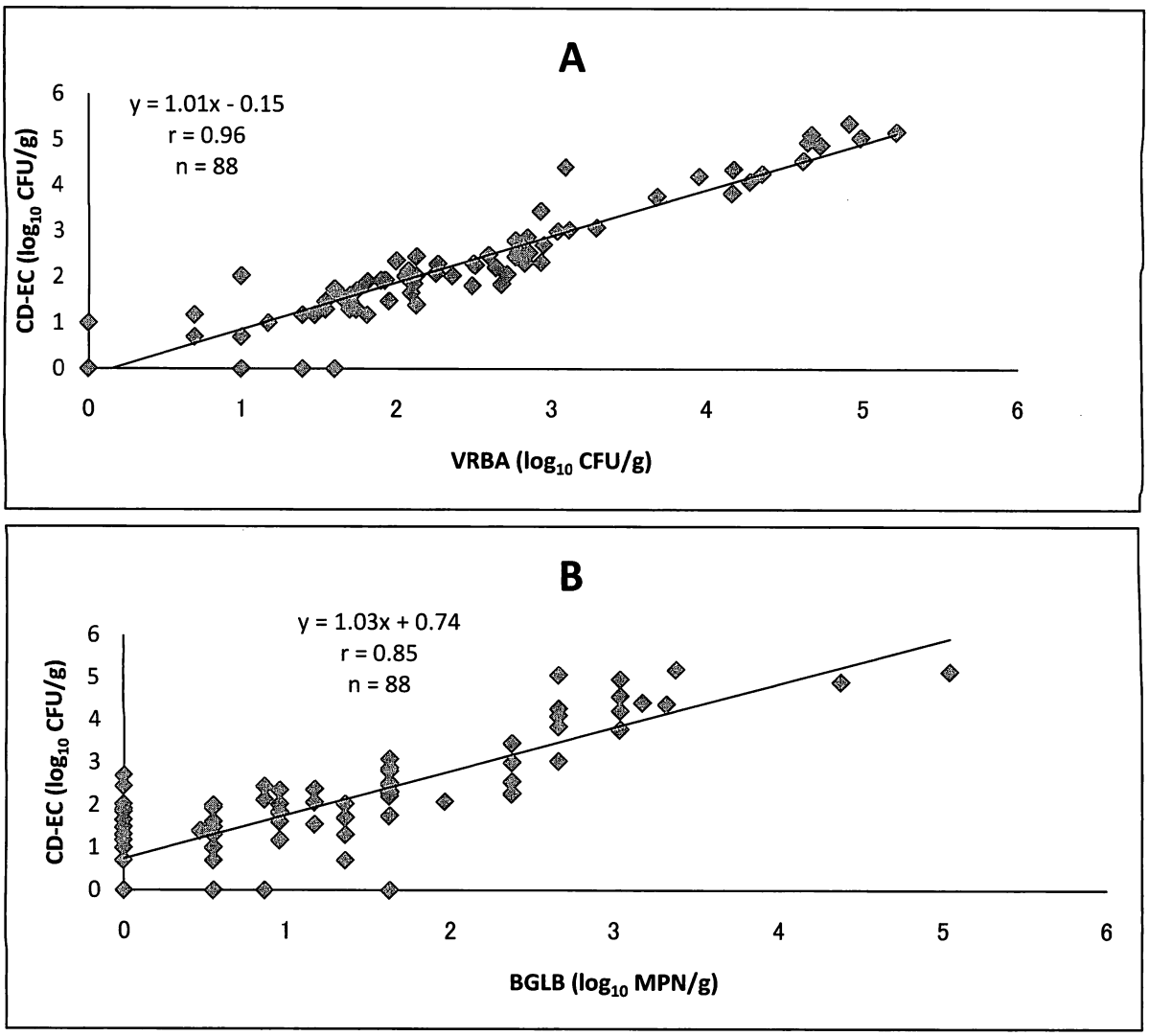


Fig. 1. Regression line for data from CD-EC plate method plotted against VRBA (A) and MPN (B) methods for determinig the population of coliforms in seafood.

10 representative colonies and transferred each to a tube of brilliant green lactose bile (BGLB) broth. The tubes were incubated at 35℃ and examined at 24 and 48 hr for gas production.

**4. FDA-BAM 5-tube MPN method**

The method completely followed the AOAC Official Method 966.24. Each 1 m*l* of 1 : 10, 1 : $10^2$, 1 : $10^3$, 1 : $10^4$, 1 : $10^5$ dilutions was inoculated into 5 lauryl sulfate tryptose (LST) tubes for 5 consecutive dilutions. They were incubated for 48±2 hr at 35℃ and examined for gas formation (as evidenced by displacement of liquid in an inverted tube). The tubes were examined for gas formation at 24 and 48 hr intervals. Cultures producing gas were transferred to BGLB broth and EC broth by using a 3 mm loop. Each BGLB broth was incubated at 35℃ for 48±2 hr. The MPN of coliform was calculated on basis of number of tubes of BGLB broth producing gas by the end of the incubation period. Each EC broth was incubated for 48±2 hr at 44.5±0.2℃. The tubes were examined for gas formation at 24 and 48 hr intervals. Cultures producing gas were streaked on Levine's eosin-methylene blue (L-EMB) agar and incubated for 24±2 hr at 35℃. Two or more well-isolated typical colonies from L-EMB agar plates were picked and transferred to PCA. The plates were incubated for 18–24 hr at 35℃. The cultures were identified by Gram stain and biochemical tests including an oxidase test, catalase tests, IMViC test, and API 20E.

**5. Data analysis**

The results for the different methods were compared for each of the 88 seafood samples examined. Results were calculated as $\log_{10}$ CFU of coliform per gram of test seafood. A one way

analysis of variance (ANOVA) for coliform was performed. The slope, intercept and the linear correlation coefficient ($r$) were calculated by plotting the CD-EC results on the $y$-axis *vs.* the FDA-BAM method results on the $x$-axis. Statistical calculations were carried out with the Microsoft Excel 2000 statistics package using default 95% confidence level. All statistical analyses were performed with a level of significance of 0.05 which the probability of obtaining a result ($p$ value) of $< 0.05$ was considered to be statistically significant.

## Results and Discussion

The CD-EC plate method was compared with two FDA-BAM methods using 88 samples consisting of 29 kinds of seafood (Table 1) in order to evaluate the performance of the CD-EC plate method. Regression analysis of 88 seafood samples on CD-EC versus VRBA (A) and CD-EC versus 5-tube MPN (B) are shown in Fig. 1. The scatter plot and regression analysis shows a positive correlation between the CD-EC plate and VRBA methods (Fig. 1, A). The differences for coliform results were shown between the CD-EC plate and VRBA methods. It was thought that the differences of inhibitory agents and inoculating method for two methods yield such results. With a correlation coefficient ($r$) of 0.96, a slope of 1.01, and an intercept of $-0.15$, a linear relationship is indicated over a wide range of CFU/g values. Likewise, the regression analysis of the data obtained with the CD-EC and 5-tube MPN methods (Fig. 1, B) yielded a correlation coefficient of 0.85 with a slope of 1.03 and an intercept of 0.74, again indicating a linear relationship between the two methods for coliform. For the CD-EC plate method, the mean $\log_{10}$ difference was $-0.12$ as compared with the VRBA method and 0.81 as compared with the MPN method, respectively. The mean $\log_{10}$ difference was 0.93 as compared with the MPN method for VRBA method. There was no significant difference ($p > 0.05$) between the CD-EC pate and VRBA methods. However, there were significant differences ($p < 0.05$) between the CD-EC plate and MPN methods and between the VRBA and MPN methods. The MPN values have a range associated with them and calculations for confidence limits[4] associated with them. It is important to keep in mind while the MPN tables are as accurate as they can be, this range still lends itself to some difficulty in doing statistical analysis between two methods, one of which gives a precise number and the other which yields a most probable number with a range attached.

Table 2. Identification of 142 red/pink-colony-forming isolates from the CD-EC plates

| Identification | No. of isolates |
|---|---|
| *Enterobacteriaceae* | |
| *Budvicia aquatica* | 1 |
| *Buttiauxella agrestis* | 1 |
| *Cedecea lapagei* | 1 |
| *Citrobacter freundii* | 8 |
| *Enterobacter agglomerans* | 12 |
| *E. amnigenus* | 12 |
| *E. asburiae* | 3 |
| *E. cloacae* | 6 |
| *E. sakazakii* | 9 |
| *Erwinia* spp. | 2 |
| *Hafnia alvei* | 5 |
| *Klebsiella pneumoniae* | 4 |
| *K. oxytoca* | 1 |
| *Kluyvera* spp. | 2 |
| *Lecleria adecarboxylata* | 1 |
| *Rahnella aquatilis* | 6 |
| *Serratia liquefaciens* | 25 |
| *S. marcescens* | 5 |
| *S. odorifera* | 4 |
| *S. plymnthica* | 8 |
| Sub-total | 116 |
| Non-*Enterobacteriaceae* | |
| *Aeromonas hydrophila* | 7 |
| *A. caviae* | 4 |
| *A. sobria* | 1 |
| Un-identified Gram-negative bacteria | 14 |
| Sub-total | 26 |
| Ground total | 142 |

Table 2 shows the identification results by API 20E for 142 red/pink-colony-forming isolates (presumptive coliforms). One hundred sixteen isolates were *Enterobacteriaceae*, 12 isolates were *Aeromonas* spp., and 14 isolates were not identified as Gram negative bacteria for the limit in the ability of API 20E to identify.

The statistical analysis of the data for *E. coli* was not carried out because *E. coli* positive samples (Table 1) were low in the naturally contaminated seafood tested. *E. coli* was detected in 5 samples (freshwater clams and Japanese littleneck) by the FDA-BAM methods. On the other hand, blue/blue purple colonies on the CD-EC plate method was found in 8 samples. It was *Escherichia vulneris* as a result of identifying the isolates from three samples

(Black tiger prawn, Greeneye, and Japanese horse mackerel) that showed the false positive. Therefore, it is needed to examine blue/blue purple colonies on the CD-EC plate method additional indole test to confirm *E. coli*. Because *E. vulneris* strains do not produce indole[2].

Overall, this study demonstrated that the CD-EC plate and conventional culture methods produced comparable coliform count results and *E. coli* detection in seafood. The CD-EC plate method provides many advantages compared to the FDA-BAM methods for *E. coli* and coliform analysis including that it is easy to inoculate samples using no special technique at any place. In addition, there is no media preparation required, it is a self-diffusible medium, it is a smaller size than conventional plates and it is easier to discard.

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# 生鮮魚介類の大腸菌群検査における Compact Dry EC の有用性の検討

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生鮮魚介類の細菌学的な検査を現場で実行するために有用な検査方法を提供する目的で，コンパクトドライ EC (CD-EC) を使って大腸菌群数の検査を実施したところ，以下の成績が得られた．

1. 生鮮魚介類 88 例について米国 FDA-BAM 法である混釈平板培養法と MPN5 本法を対照にして CD-EC で大腸菌群数を検査したところ，相関係数はそれぞれ 0.96 および 0.85 であった．

2. CD-EC の赤色集落（推定大腸菌群）142 株を同定した結果，*Citrobacter* 属 8 株，*Enterobacter* 属 42 株，*Hafnia* 属 5 株，*Klebsiella* 属 5 株，*Serratia* 属 42 株，その他の腸内細菌 15 株，腸内細菌以外の菌 26 株であった．

3. 大腸菌は米国 FDA-BAM 法でシジミとアサリの 5 例で認めた．

4. CD-EC で青いコロニー（推定大腸菌）を認めた検体はシジミとアサリの 5 例に加えて，ブラックタイガー，メヒカリ，アジであったが，分離した菌株は *Escherichia vulneris* で偽陽性であった．