Evaluation of the Novel Dry Sheet Culture Method for the Enumeration of Enterobacteriaceae

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MC-Media Pad™ EB (MMP-EB), a novel sheet culture method for the enumeration of Enterobacteriaceae, has been evaluated. When both inclusivity and exclusivity of MMP-EB were assessed using 104 microbes including 51 Enterobacteriaceae strains, all tested Enterobacteriaceae strains grew and formed obvious red-colored colonies and all tested non-Enterobacteriaceae strains were shown different appearance from Enterobacteriaceae strains. For the comparison study of the method, MMP-EB was compared with violet red bile glucose agar (VRBG) according to ISO 21528-2:2017 and Petriﬁlm™ Enterobacteriaceae Count Plate (Petrifilm EB) method using 100 naturally contaminated food samples. The correlation coefﬁcients between MMP-EB and VRBG, and MMP-EB and Petrifilm EB were 0.940 and 0.972, respectively. Furthermore, there were no statistically signiﬁcant difference between MMP-EB and both reference methods. Our results demonstrated that MMP-EB was a suitable alternative method for the enumeration of Enterobacteriaceae in food samples.

Key words: Enterobacteriaceae / Dry culture medium / ISO 21528-2:2017.

Enterobacteriaceae is known as facultative anaerobic gram-negative bacteria which ferment glucose to acid (Kornacki et al., 2015). Also, Enterobacteriaceae includes important foodborne pathogen such as Salmonella, Shigella and Yersinia, in addition to total coliform bacteria. Hence, Enterobacteriaceae have been used for years as a well-known hygiene indicator in maintenance of food quality and safety (Kornacki et al., 2015).

The ISO 21528-2:2017 recommends to use violet red bile glucose agar (VRBG) for the enumeration of Enterobacteriaceae (International Organization for Standardization, 2017; Mossel et al., 1962 and 1978). However, the test procedure of this method is time-consuming and labor-intensive since it needs preparation, pouring and overlay of medium. In the present day, several ready-to-use culture methods, such as the 3M Petrifilm™ Enterobacteriaceae Count Plate (Petrifilm EB; 3M Company, Microbiology Products, St. Paul, MN), have been evaluated as suitable alternatives (AOAC International, 2006; Silbernagel and Lindberg, 2002 and 2003). These quantitative dry culture methods eliminate burdensome operations of the conventional pour plate techniques since they need not preparation and overlay of medium. However, Petrifilm EB requires not only the spreader device when inoculation of sample but also conﬁrmation of the presence of yellow-colored zone and/or production of gas bubbles around red-colored colonies to count according to its instruction manual.

In view of this fact, the MC-Media Pad™ EB (MMP-EB; JNC Corporation, Tokyo, Japan) method, a novel dry sheet culture method for the enumeration of Enterobacteriaceae, has been developed. This re-hydrated quantitative culture system has multilayered structure which is comprised of a transparent polypropylene cover film, a non-woven nylon fabric layer, a

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medium layer with polyvinyl alcohol and guar gum, and an adhesive bottom sheet (Teramura et al., 2017). Once 1 ml aliquot sample is inoculated on MMP-EB, sample is automatically diffused to the entire pad through the capillary action of non-woven fabric. After 24 h of incubation at 37°C, Enterobacteriaceae grow and form red-colored colonies on the MMP-EB (Figure 1). MMP-EB method eases not only test operation including medium preparation but also interpretation of target colony appearance. The purpose of this study was to evaluate the performance of MMP-EB for the enumeration of Enterobacteriaceae using pure culture and naturally contaminated food samples.

For the inclusivity and exclusivity studies, 51 strains of Enterobacteriaceae, 18 gram-negative non-Enterobacteriaceae bacteria, 33 gram-positive bacteria and 2 yeast strains were used. Tested strains were cultured on Tryptic Soy Agar (TSA; Difco, Becton Dickinson, Sparks, MD, USA) at 37°C for 24 h and then suspended in sterile Butterfield’s phosphate buffer (BPB) according to the FDA-Bacteriological Analytical Manual to obtain a turbidity equivalent to No.1 McFarland standard (3.0 x 10^8 CFU/ml; McFarland, 1907). After initial suspension was subjected to 10-fold serial dilutions in BPB, 1 ml of each suspension was inoculated onto MMP-EB, Petrifilm EB and VRBG (Oxoid, Basingstoke, Hampshire, UK) with pour plate technique. The appearance and number of colony on each medium were observed and read after 24 h of incubation at 37°C. At same time, the inoculated microbial number of each strain tested was enumerated using standard plate count agar (SPC; Difco).

The results from the inclusivity and exclusivity studies are shown in Table 1. A total of 51 Enterobacteriaceae strains grew and formed red-colored and violet-colored colonies on MMP-EB and VRBG, respectively. Thirty-eight out of 51 Enterobacteriaceae strains tested formed red-colored colonies with both yellow-colored zone and gas bubbles, and the others formed red-colored colonies with only yellow-colored zone on Petrifilm EB. Of the 18 gram-negative non-Enterobacteriaceae strains, 16 strains failed to grow on MMP-EB. Achromobacter denitrificans NBRC 15125 and Stenotrophomonas ginsengisoli NBRC 101154 grew and formed very small pale pink-colored colonies on MMP-EB. However, these grown 2 strains were different from typical Enterobacteriaceae colonies on MMP-EB. For Petrifilm EB, 14 out of 18 gram-negative non-Enterobacteriaceae strains failed to grow, whereas A. denitrificans NBRC 15125, Pseudomonas aeruginosa IFO 3446, P. aerugiosa NBRC 12689 and P. aeruginosa NBRC 13275 grew as small pale pink-colored colonies. These grown 4 strains could be also distinguished from typical Enterobacteriaceae colonies on Petrifilm EB as well as MMP-EB. A total of 18 gram-negative non-Enterobacteriaceae strains grew as colorless colonies on VRBG. All tested gram-positive bacteria and yeast strains failed to grow on these 3 media.

As explained above, MMP-EB was more selective for the growth of non-Enterobacteriaceae strains than Petrifilm EB and VRBG. Since Enterobacteriaceae colonies needed to confirm the presence of yellow-colored zone and/or gas bubbles in addition of red-colored colonies on Petrifilm EB, to distinguish Enterobacteriaceae colonies is burdensome task to count compared to MMP-EB. Additionally, it is difficult to discriminate purple-red-colored Enterobacteriaceae colonies from colorless non-Enterobacteriaceae gram-negative bacteria colonies on VRBG, because medium color of VRBG is dark red to violet due to contamination of pigments. Thus it should be necessary to count Enterobacteriaceae colonies by experienced examiner.

According to ISO 16140-2:2016, at least 50 pure cultures of target microorganisms and at least 30 pure cultures of non-target microorganisms shall be tested for inclusivity and exclusivity studies (International Organization for Standardization, 2016). Our results showed that both inclusivity and exclusivity of MMP-EB met the criteria of ISO 16140-2:2016.

For the comparison study of the method, 100 naturally contaminated samples (34 meat samples, 33 seafood samples and 33 vegetable samples) were obtained at retail stores in Yokohama city. The comparison study of the method was conducted following procedure. Each 10 g of sample was added to a 9-fold volume of BPB and then homogenized for 90 sec. by homogenizer (MASTICATOR 400S, IUL, S. A., Barcelona, Spain). Samples were then subjected to 10-fold serial dilutions in BPB and 1 ml aliquot of sample was inoculated onto

FIG. 1. The colony appearance of Enterobacteriaceae on MMP-EB. Enterobacter cloacae NBRC 13536 grew and formed red-colored colonies on MMP-EB after 24 h of incubation at 37°C. There were 42 CFU on MMP-EB.
## TABLE 1. Growth and colony appearance of microbes tested on various media.  

| Microbes tested | Colony appearance<sup>c</sup> / log CFU/ml | MMP-EB | Petrifilm EB | VRBG | SPC |
|-----------------|------------------------------------------|--------|--------------|------|-----|
| **Enterobacteriaceae** | | | | | |
| **Cedecea lapagei JCM 1684** | | R 8.29 | R, y 8.48 | V 8.11 | 8.47 |
| **Citrobacter amalonaticus IFO 13547** | | R 8.86 | R, y 8.76 | V 8.05 | 8.91 |
| **C. freundii IFO 12681** | | R 8.64 | R, y, g 8.39 | V 7.83 | 8.59 |
| **C. koseri JCM 1659** | | R 8.90 | R, y, g 8.69 | V 8.20 | 8.87 |
| **C. koseri NBRC 105690** | | R 8.66 | R, y 8.68 | V 8.59 | 8.68 |
| **Cronobacter sakazakii ATCC 12868** | | R 8.65 | R, y, g 8.74 | V 8.31 | 8.73 |
| **C. sakazakii ATCC 29544** | | R 9.26 | R, y, g 9.31 | V 8.83 | 9.23 |
| **Edwardsiella tarda JCM 1656** | | R 9.17 | R, y, g 9.23 | V 8.63 | 9.18 |
| **Enterobacter aerogenes IFO 13534** | | R 9.03 | R, y, g 9.04 | V 9.08 | 9.07 |
| **E. cloacae IID 977** | | R 9.04 | R, y 8.79 | V 6.70 | 8.48 |
| **E. cloacae JCM 1232** | | R 8.88 | R, y 8.64 | V 7.98 | 8.56 |
| **E. cloacae NBRC 102198** | | R 8.30 | R, y 8.27 | V 6.30 | 7.56 |
| **E. cloacae NBRC 13536** | | R 8.63 | R, y, g 8.72 | V 8.31 | 8.76 |
| **E. gergoviae JCM 1234** | | R 8.81 | R, y, g 8.57 | V 8.24 | 8.78 |
| **Escherichia coli NBRC 3543** | | R 8.67 | R, y, g 8.72 | V 8.54 | 8.63 |
| **E. coli NBRC 3546** | | R 8.79 | R, y, g 7.68 | V 7.34 | 8.79 |
| **E. coli NBRC 3806** | | R 8.68 | R, y, g 8.64 | V 8.64 | 8.83 |
| **E. coli NBRC 3891** | | R 8.72 | R, y, g 8.49 | V 8.28 | 8.63 |
| **E. coli NBRC 3972** | | R 8.86 | R, y, g 8.85 | V 8.75 | 8.94 |
| **E. coli NBRC 12602** | | R 9.07 | R, y, g 9.08 | V 8.95 | 9.12 |
| **E. coli NBRC 12433** | | R 8.90 | R, y, g 8.80 | V 8.87 | 9.03 |
| **E. coli NBRC 13500** | | R 9.20 | R, y, g 9.25 | V 9.22 | 9.24 |
| **E. coli NBRC 13891** | | R 7.92 | R, y 6.95 | V 7.04 | 8.01 |
| **E. coli NBRC 13892** | | R 8.93 | R, y 8.72 | V 6.95 | 7.23 |
| **E. coli NBRC 14129** | | R 8.51 | R, y, g 8.85 | V 8.63 | 8.89 |
| **E. coli NBRC 15034** | | R 8.74 | R, y, g 8.76 | V 8.78 | 8.75 |
| **E. coli O157 ATCC 35150** | | R 8.61 | R, y, g 8.54 | V 8.52 | 8.53 |
| **E. coli O157 ATCC 43890** | | R 8.76 | R, y, g 8.74 | V 8.53 | 8.89 |
| **E. fergusonii NBRC 120419** | | R 9.05 | R, y, g 8.96 | V 8.86 | 9.08 |
| **E. hermannii ATCC 33650** | | R 8.93 | R, y, g 8.79 | V 8.96 | 9.00 |
| **E. hermannii JCM 1296** | | R 8.91 | R, y, g 8.86 | V 8.95 | 8.92 |
| **E. vulneris NBRC 102420** | | R 8.63 | R, y, g 8.67 | V 8.48 | 8.53 |
| **Haemophilus alvei JCM 1666** | | R 8.48 | R, y, g 8.58 | V 8.30 | 8.54 |
| **Klebsiella oxytoca JCM 1665** | | R 8.80 | R, y, g 8.88 | V 8.71 | 8.89 |
| **K. pneumoniae JCM 1662** | | R 8.78 | R, y, g 8.74 | V 8.59 | 8.72 |
| **Kluyvera ascorbata JCM 1681** | | R 8.61 | R, y, g 8.66 | V 8.54 | 8.71 |
| **K. intermedia JCM 1238** | | R 7.84 | R, y 7.61 | V 6.60 | 7.53 |
| **Leclercia adecarboxylata NBRC 102595** | | R 8.58 | R, y, g 8.58 | V 8.39 | 8.53 |
| **Morganella morgani iFO 3848** | | R 9.01 | R, y, g 8.94 | V 8.88 | 7.58 |
| **Proteus mirabilis JCM 1669** | | R 8.92 | R, y 9.09 | V 9.02 | 9.15 |
| **P. mirabilis NBRC 13300** | | R 7.00 | R, y 8.61 | V 6.95 | 7.00 |
| **P. vulgaris NBRC 3851** | | R 8.85 | R, y 8.52 | V 7.59 | 8.48 |
| **Providencia alcalifaciens IFO 12931** | | R 7.40 | R, y 7.08 | V 7.08 | 7.23 |
| **Rahnella aquatilis IFO 13544** | | R 8.48 | R, y 6.00 | V 6.90 | 7.40 |
| **Raoultella planticola IFO 14939** | | R 8.75 | R, y 8.54 | V 8.44 | 8.76 |
| **R. terrigena NBRC 14941** | | R 8.37 | R, y 8.35 | V 7.43 | 8.53 |
| **Salmonella Choleraesuis NBRC 15183** | | R 8.82 | R, y 8.59 | V 6.60 | 8.81 |
| **S. Enteritidis NBRC 3313** | | R 8.86 | R, y, g 8.69 | V 8.84 | 8.74 |
| **S. Typhimurium JCM 1652** | | R 8.72 | R, y 8.17 | V 8.28 | 8.68 |
| **Serratia marcescens JCM 1239** | | R 8.62 | R, y 8.68 | V 8.27 | 8.61 |
| **S. rubidaea NBRC 12973** | | R 8.49 | R, y 7.49 | V 8.38 | 7.00 |

**Gram negative, non-Enterobacteriaceae**

| Achromobacter xylosoxidans NBRC 15126 | not grown | not grown | C 8.68 | 9.08 |
| A. denitrificans NBRC 15125 | sp<sup>c</sup> | sp<sup>c</sup> | 8.59 | 8.73 | 9.29 |
| Acinetobacter lwoffi NBRC 109760 | not grown | not grown | C 7.55 | 7.53 |
| Aeromonas hydrophila JCM 1027 | not grown | not grown | C 7.81 | 8.28 |
| Burkholderia cepacia NBRC 15124 | not grown | not grown | C 7.81 | 8.28 |

subtotal number of strains tested: 51
| Species                                      | ATCC / NBRC / JCM / IID / IFO | Growth | Growth | Growth | Log CFU/ml | Log CFU/ml | Log CFU/ml |
|---------------------------------------------|------------------------------|--------|--------|--------|------------|------------|------------|
| B. mimosarum NBRC 106338                    | not grown                   | not grown | C     | 8.80   | 7.98       |
| Chryseobacterium balustinum NBRC 15053      | not grown                   | not grown | C     | 8.73   | 8.23       |
| C. indologenes NBRC 14944                   | not grown                   | not grown | C     | 8.48   | 7.95       |
| Pseudomonas aeruginosa IFO 3446             | not grown                   | sP     | 9.09  | C      | 9.03       | 9.20       |
| P. aeruginosa NBRC 12689                    | not grown                   | sP     | 8.80  | C      | 8.79       | 8.83       |
| P. aeruginosa NBRC 13275                    | not grown                   | sP     | 9.07  | C      | 8.72       | 9.10       |
| P. fluorescens NBRC 15842                   | not grown                   | not grown | C     | 8.74   | 7.20       |
| P. putida NBRC 14164                        | not grown                   | not grown | C     | 8.82   | 8.34       |
| P. stutzeri NBRC 103163                     | not grown                   | not grown | C     | 7.88   | 8.11       |
| Stenotrophomonas ginsengisoli NBRC 101154   | sP                           | 7.91   | not grown | C      | 8.85       | 7.90       |
| S. maltophilia NBRC 14161                   | not grown                   | not grown | C     | 7.79   | 9.34       |
| Sphingomonas paucimobilis NBRC 13935        | not grown                   | not grown | C     | 8.70   | 8.71       |

**Gram positive bacteria**

Bacillus cereus NBRC 13494: not grown not grown not grown 7.08
B. licheniformis NBRC 12200: not grown not grown not grown 7.78
B. subtilis NBRC 3134: not grown not grown not grown 7.40
Corynebacterium ammoniagenes NBRC 12612: not grown not grown not grown 7.34
Enterococcus faecalis JCM 7783: not grown not grown not grown 8.56
E. faecalis JCM 8726: not grown not grown not grown 8.78
E. hirae IFO 3181: not grown not grown not grown 7.81
Lactobacillus lactis IFO 3376: not grown not grown not grown 7.26
L. plantarum IFO 12519: not grown not grown not grown 7.85
Leuconostoc citreum JCM 9698: not grown not grown not grown 7.23
L. mesenteroides NBRC 100496: not grown not grown not grown 8.03
Listeria ivanovii JCM 7681: not grown not grown not grown 7.40
L. monocytogenes JCM 7680: not grown not grown not grown 8.99
Micrococcus luteus NBRC 12708: not grown not grown not grown 7.51
Staphylococcus aureus NBRC 14462: not grown not grown not grown 8.31
S. aureus NBRC 15035: not grown not grown not grown 8.51
S. aureus NBRC 100910: not grown not grown not grown 8.39
S. auricularis ATCC 33753: not grown not grown not grown 7.81
S. capitis JCM 2420: not grown not grown not grown 7.94
S. caprae JCM 3573: not grown not grown not grown 8.76
S. epidermidis NBRC 12993: not grown not grown not grown 8.26
S. epidermidis NBRC 100911: not grown not grown not grown 7.86
S. haemolyticus JCM 2416: not grown not grown not grown 6.60
S. hominis JCM 2419: not grown not grown not grown 8.00
S. hyicus JCM 2423: not grown not grown not grown 8.37
S. intermedius ATCC 29663: not grown not grown not grown 8.48
S. lentus ATCC 20970: not grown not grown not grown 8.71
S. saprophyticus JCM 2427: not grown not grown not grown 8.58
S. schleiferi ATCC 43808: not grown not grown not grown 8.79
S. sciuri ATCC 29062: not grown not grown not grown 7.80
S. simulans JCM 2424: not grown not grown not grown 8.34
S. xylosus JCM 2418: not grown not grown not grown 8.47
Streptococcus thermophilus ATCC 14485: not grown not grown not grown 6.85

**Yeasts**

Candida albicans NBRC 1594: not grown not grown not grown 7.23
Saccharomyces cerevisiae NBRC 100929: not grown not grown not grown 7.49

**Total number of strains tested:** 104

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Each strain was cultured on MMP-EB, Petrifilm EB, VRBG and SPC at 37°C for 24 h.

*Strains were derived from ATCC (American Type Culture Collection, VA, USA), IFO (Institute for Fermentation Osaka, Japan), IID (International Research Center for Infectious Diseases, Institute for Medical Science, The University of Tokyo), JCM (Japan Collection of Microorganisms) and NBRC (NITE Biological Resource Center, Japan).

*Characteristics indicate colony appearance: R, red; V, violet; C, colorless; sP, small pink; g, gas production; y, yellow zone.

*Numbers (log CFU/ml) were microbe number of initial suspension obtained from each tested method.
MMP-EB, Petrifilm EB and VRBG. After 24 h of incubation at 37°C, the number of typical Enterobacteriaceae colonies on each medium was counted. Numbers of colony obtained from each method were converted to log_{10} CFU of Enterobacteriaceae per gram of each tested food. To determine statistically significant between MMP-EB and reference methods, a one-way analysis of variance (ANOVA) was carried out using Microsoft Excel 2013. The linear correlation coefficients (r), slopes, intercepts and 95 % confidence limits among the 3 methods were calculated.

The results of the comparison study of the method are given in Table 2. The MMP-EB, Petrifilm EB and VRBG recovered Enterobacteriaceae from all of 100 tested samples. The ranges of Enterobacteriaceae numbers (log_{10} CFU per gram) for 34 meat samples, 33 seafood samples and 33 vegetable samples obtained from these 3 methods were 1.00-6.88 (MMP-EB), 1.00-6.77 (Petrifilm EB) and 1.18-6.60 (VRBG); 1.48-7.67 (MMP-EB), 1.40-7.71 (Petrifilm EB) and 1.40-7.54 (VRBG); and 1.18-7.60 (MMP-EB), 1.00-7.49 (Petrifilm EB) and 1.00-7.38 (VRBG), respectively. The ranges of Enterobacteriaceae numbers obtained from MMP-EB were similar to those of Petrifilm EB and VRBG in each food category. The means log_{10} CFU ± standard deviation (SD) obtained from MMP-EB, Petrifilm EB and VRBG in all 100 food samples were 4.61±1.59, 4.48±1.59 and 4.19±1.43, respectively. The correlation coefficients between MMP-EB and VRBG, MMP-EB and Petrifilm EB, Petrifilm EB and VRBG were 0.940, 0.972 and 0.948, respectively. The slopes and intercepts determined by liner regression analysis between MMP-EB and VRBG, MMP-EB and Petrifilm EB, Petrifilm EB and VRBG were 0.940, 0.977 and 0.948, respectively. The 95% confidence limits and 95% confidence limits among the 3 methods were calculated.

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**TABLE 2** Statistical relationship of MMP-EB with reference methods for the enumeration of Enterobacteriaceae in food samples.

| Parameter                      | MMP-EB vs VRBG | MMP-EB vs Petrifilm EB | Petrifilm EB vs VRBG |
|-------------------------------|----------------|------------------------|----------------------|
| No. of samples                | 100            | 100                    | 100                  |
| Correlation coefficient (r)   | 0.940          | 0.972                  | 0.948                |
| Slope                         | 1.045          | 0.977                  | 1.049                |
| Intercept                     | 0.228          | 0.231                  | 0.086                |
| 95% confidence limits         | ± 0.301        | ± 0.313                | ± 0.298              |
| P value (ANOVA)²             | 0.055          | 0.576                  | 0.179                |

Range (mean) of log CFU/g for 34 meat samples
MMP-EB 1.00 - 6.88 (4.30) 1.00 - 6.88 (4.30) 1.00 - 6.88 (4.30)
VRBG 1.18 - 6.60 (3.73) 1.18 - 6.60 (3.73) 1.18 - 6.60 (3.73)
Petrifilm EB 1.00 - 6.77 (4.23) 1.00 - 6.77 (4.23)

Range (mean) of log CFU/g for 33 seafood samples
MMP-EB 1.48 - 7.67 (3.99) 1.48 - 7.67 (3.99) 1.40 - 7.54 (3.94)
VRBG 1.40 - 7.54 (3.94) 1.40 - 7.54 (3.94) 1.40 - 7.54 (3.94)
Petrifilm EB 1.40 - 7.71 (3.98) 1.40 - 7.71 (3.98)

Range (mean) of log CFU/g for 33 vegetable samples
MMP-EB 1.18 - 7.60 (5.55) 1.18 - 7.60 (5.55) 1.00 - 7.38 (4.92)
VRBG 1.00 - 7.38 (4.92) 1.00 - 7.38 (4.92) 1.00 - 7.38 (4.92)
Petrifilm EB 1.00 - 7.49 (5.26) 1.00 - 7.49 (5.26)

Mean log CFU/g ± SD (overall)
MMP-EB ± SD 4.61 ± 1.59 4.61 ± 1.59 4.61 ± 1.59
VRBG ± SD 4.19 ± 1.43 4.19 ± 1.43 4.19 ± 1.43
Petrifilm EB ± SD 4.48 ± 1.59 4.48 ± 1.59

MMP-EB was compared with Petrifilm EB and VRBG using 100 naturally contaminated food samples.
All tested media were cultured for 24 h at 37°C.
²P > 0.05 indicates no significant difference between both methods.
In general, naturally contaminated food samples are contaminated not only *Enterobacteriaceae* but also non-*Enterobacteriaceae* strains. In cases where there are lots of gram-negative non-*Enterobacteriaceae* bacteria in food sample, these bacteria can grow and make interpretation of target *Enterobacteriaceae* colonies difficult to count in conventional methods. For Petrifilm EB, even though *Enterobacteriaceae* colonies need to confirm the presence of yellow-colored zone and/or gas bubbles adjacent to red-colored colonies, these yellow-colored zone is hard to distinguish since it tends to diffuse due to strength of acid production. Furthermore, it is cumbersome to discriminate gas bubbles due to *Enterobacteriaceae* from intrinsic air bubbles when film was closed. For VRBG, even though *Enterobacteriaceae* forms purple-red-colored colonies, it needs to confirm size (more than 0.5 mm) and presence of precipitation from complex comprised of neutral red and bile salts to count (Kornacki et al., 2015). Furthermore, violet-colored precipitate tends to spread to whole VRBG plate in case that there are lots of *Enterobacteriaceae*, which has high activity of acid production in VRBG plate. Hence, counting target *Enterobacteriaceae* colonies needs experience of examiner. By contrast, MMP-EB could be easily counted colonies, because all red-colored colonies on the MMP-EB can be regarded as *Enterobacteriaceae* colonies.

In these days, several methods for the enumeration of *Enterobacteriaceae* in food, which include biochemical methods like the TEMPO EB automated most probable number (MPN) system (Owen et al., 2010; Paulsen et al., 2008), fluorescence in situ hybridization (FISH) method (Yamaguchi et al., 2012) and quantitative real-time PCR method (Takahashi et al., 2017), have been developed. Although these methods can enumerate *Enterobacteriaceae* faster than culture based methods, these methods require specific devices, technical expertise and high initial cost. On the other hand, ready-to-use dry culture media including MMP-EB need no specific devices and technical expertise. Especially, MMP-EB eases to count in addition to easy operation. In conclusion, we demonstrated that MMP-EB had enough inclusivity and exclusivity, and excellent correlation with reference methods. It suggested that the MC-Media Pad EB method is a convenient alternative for the enumeration of *Enterobacteriaceae*.

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