Research Article

Matching the results of *Escherichia coli* analysis in pure culture and food models obtained through the Most Probable Number and through Tryptone Bile X-Glucuronide chromogenic plate count methods

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Abstract

Escherichia coli is widely used as a bacterial indicator for faecal contamination in food. While the chromogenic plate count is increasingly used for analysis of this microorganism, the traditional Most Probable Number (MPN) method is still applied in small/medium food industries, especially in Asian developing countries. This article deals with the problem of whether the results obtained from these two methods can be mutually converted for the purpose of evaluating the wholesomeness of food and for microbiological criterion setting. We studied the levels of *E. coli* in the pure culture, uncooked naturally contaminated food, and frozen naturally contaminated food. Two methods of evaluation were used: 9-tube MPN estimation and the chromogenic Tryptone Bile X-Glucuronide (TBX) plate count. The results obtained by these two different menthods were compared using correlation graphs. The results from the two methods showed high correlation ($r^2 = 0.7978$) with the pure culture, and were less correlated with uncooked and frozen foods ($r^2 = 0.6657$ and 0.6744, respectively). However, the regression trends of the uncooked and frozen food groups were not matching with each other. Further, they significantly deviated from that of the pure culture (at a 99 % confidence limit). Thus, should there be any attempt to convert an MPN value of *E. coli* to a TBX value, and *vise versa*, the pure culture model should not be used as the generalised predictive correlation model for a specific food. Instead, specific graphs should be consracted for specific food groups. We also suggest the use of upper correlating values, rather than the average, in relating the values between the two methods since this is more relevant concerning food safety, yet it cannot be applied without precautions. The results from this study also point that in establishing the microbiological standards of *E. coli* in food to be relevant for a chromogenic plate count method which is increasingly used, it would best be done through assessment and evaluation based on refered plate count values rather than by attempting to convert values from the existing MPN-based criteria.

Introduction

Estimation of indicator microorganisms in food which has been done through the most probable number (MPN) method for many decades [1] is under transition to more rapid enumeration methods. One disadvantage of the MPN method is the processing time, especially when identification of species is required as in the case of *Escherichia coli*, the most common bacterial indicator of faecal contamination [2]. The rapid plate count methods using microbiological media incorporated with a chromogenic substrate [3, 4, 5, 6] have increasingly been used in place of the MPN method since they are rapid, simple, and reliable [7, 8]. At present, the *E. coli* chromogenic plate count method relies on detection of the enzyme β-glucuronidase, using media incorporated with chromogenic glucuronide media such as p-nitrophenol-β-D-glucuronide (PNPG), 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-GLUC or BCIG), and 8-hydroxyquinoline-β-D-glucuronide (HQG) [5]. This enzyme is specific to the organism [9, 10, 11] with only a few exceptions to some strains of *Salmonella*, *Shigella*, *Yersinia*, *Citrobacter*, *Klebsiella*, and *Serratia* [5, 11, 12, 13] Many methods based on this principle have been accepted as "official methods" in the microbiological analysis of food, such as the method employing Tryptone Bile X-Glucuronide agar (TBX) [14] or 3M Petrifilm *E. coli* count plate [15].

In the transition period when the use of a traditional method is being replaced by a rapid method, there often is a need among the food industries and/or governmental food agencies to relate values obtained from one method to another for the purpose of communication or evaluation of food quality (Ruengprapun, N., Regional Medical Sciences Centre, Chiang Mai, Thailand; personal communication). This is a problem particularly in countries where adoption of microbiological techniques by food industries may be at unequal stages. Another problem is about establishment of food microbiological criteria on the basis of a new method. In case of *E. coli*, the food groups that are facing this particular problem are those required to comply with non zero-tolerance criteria such as uncooked food, raw or partly cooked frozen meat and fish products, and unpasteurised dairy products (most cooked or processed food are required to contain no *E. coli*). Although standards and guidelines that are based on colony count method for acceptable contamination levels of *E. coli* in some types of food have been established [16, 17, 18], they are limited to certain types of potentially hazardous food common in certain geographical areas. For indigenous foods, relying only on such standard would be of great limitation. Moreover, the adoption of the rapid colony count method by private organisations in newly industrialised countries, such as Thailand, can often be more advanced than the official establishment of microbiological criteria. Microbiological criteria that correspond to the colony count methods are therefore urgently needed. Since the criteria based on MPN have been well established, one approach towards such criterion setting is to convert the presently available criteria for *E. coli* based on MPN values into colony forming units (cfu), provided that the analytical results obtained from the two methods are precisely related. The other approach is to establish criteria in colony forming unit values based solely on the plate count method, which involves extensive evaluation [19].

In response to the problems indicated, we therefore aimed to explore how the numbers of *E. coli* obtained from MPN and plate count methods are related. The specific methods selected in this study are the 9-tube MPN and the Tryptone Bile X-Glucuronide (TBX) chromogenic plate count methods, which would be employed to pure *E. coli* culture and selected food

groups. The results of this study would point to possibility and/or limitations of relating the MPN of *E. coli* into colony forming units, and *vise versa*, for communication of the result and microbiological criterion establishment.

Materials and Methods

Preparation of pure culture inoculum

An early stationary phase culture ($OD_{600} \sim 0.8$) of *E. coli* (strain FT20, originally isolated from swine excrement) was prepared in one large batch in Nutrient Broth and stored in aliquots (each concentrated from 1.5 ml culture) at -70°C in 50% (v/v) glycerol. When preparing inocula for enumeration, an aliquot of the frozen culture was grown in 250 ml Nutrient Broth at 37°C in agitating conditions (130 rpm) until reaching the level of approximately 10^9 cfu/ml ($OD_{600} \sim 0.8$). The active culture was then serially diluted by 10-fold to obtain samples with cell density ranging from 10 to 10^8 cfu/ml. Portions from the tubes containing 10 to 10^4 cfu/ml were further diluted to obtain cell concentrations (in estimated number) in finer scales within this range, as shown in Table 1.

Food samples and preparation of food homogenates

Naturally contaminated food samples (144 in total) analysed in this study consist of uncooked foods (including minced pork, minced fish fillet, and beef), frozen foods (including frozen seafood products, frozen minced pork, and ice deserts), and processed/heat-treated foods (including sausages, fish balls, and meat balls). These foods are commercially available. When preparing a food homogenate for enumeration, a twenty-five gram portion of each food sample was homogenised in 225 ml sterile Peptone Water using a food homogeniser (Seward Stomacher 400, Brinkmann, Canada) for 1 min, giving a 10^{-1} dilution. The food homogenate was further diluted by a 10-fold serial dilution in 9 ml Peptone Water until the required dilutions were obtained.

Enumeration of E. coli *using the Most Probable Number (MPN) and the Tryptone Bile X-Glucuronide (TBX) chromogenic plate count methods*

The number of *E. coli* in each sample was estimated using Most Probable Number (MPN) and a chromogenic plate count methods. The MPN method used was the 9-tube MPN (three sets of three tubes containing 0.1, 0.01, 0.001 g inocula in each set). MPN values for the samples were deduced from the MPN table [20] after confirming *E. coli* through the set of biochemical tests including Indole production, Methyl Red, Voges-Proskauer, and citrate utilisation (IMViC) tests. For the chromogenic plate count method, Tryptone Bile X-Glucuronide (TBX) agar (20 g/L Peptone, 1.5 g/L bile salt, 0.075 g/L 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-GLUC, Fluka, U.S.A.), specific to β-glucuronidase [4], 15 g/L agar), was used. Culturing of *E. coli* from pure culture and food samples was carried out by spreading 0.1 ml suspension on TBX agar plate (duplicate) and incubated at 37°C for 6 h and subsequent incubation at 45°C for 18-24 h for maximum recovery of injured cells as a result of food processing [14]. For pure culture, blue *E. coli* colonies were enumerated directly. For food samples, presumptive *E. coli* (blue colonies) were overlayed with Kovac's reagent (3 ml) on the surface of the TBX agar. Colonies of which the colour changed from blue to red or pink were confirmed as *E. coli* [15]. Correlation between data (log_{10} cfu/g or ml) obtained by the two methods for each set of samples was analysed using the Excel programme (Microsoft, USA).

Table 1. Dilution of *E. coli* culture to obtain approximate required concentrations within the range of 10-10^4 cells per millilitre

| culture to diluent ratio	volume (ml)		estimated concentration (cells/ml) obtained from further dilution of tubes containing approximate cell density of			
	culture	diluent[a]	10/ml	10^2/ml	10^3/ml	10^4/ml
1/9	1	9	1	10	100	1000
1/8	1	8	1.11	11.11	111.11	1111.11
1/7	1	7	1.25	12.5	125	1250
1/6	1	6	1.42	14.28	142.85	1428.57
1/5	1	5	1.66	16.66	166.66	1666.66
¼	2	8	2	20	200	2000
1/3	2	6	2.5	25	250	2500
½	3	6	3.33	33.33	333.33	3333.33
1/1	5	5	5	50	500	5000
2/1	6	3	6.66	66.66	666.66	6666.66
3/1	6	2	7.5	75	750	7500
4/1	8	2	8	80	800	8000
5/1	5	1	8.33	83.33	833.33	8333.33
6/1	6	1	8.57	85.71	857.14	8571.42
7/1	7	1	8.75	87.5	875	8750
8/1	8	1	8.88	88.88	888.88	8888.88
9/1	9	1	9	90	900	9000

[a]Peptone water was used as diluent.

Results and Discussion

Relating the MPN estimates and the TBX counts of **E. coli** *observed from the pure culture model*

E. coli cultures of different concentrations were exposed to the 9-tube MPN estimation and the TBX count. Log_{10} values of the results obtained from the two methods were plotted against each other, excluding the MPN values below 3 and above 1100, which were the lower and upper limits of detection of the MPN method used in this study. A linear regression was deduced from the plot (Figure 1) with a high correlation between the results obtained by the two methods ($r^2 = 0.7978$). It was noted, nevertheless, that one value obtained from one

method correlated to varied ranges of values obtained from the other. This can be expected from the nature of the MPN method [1].
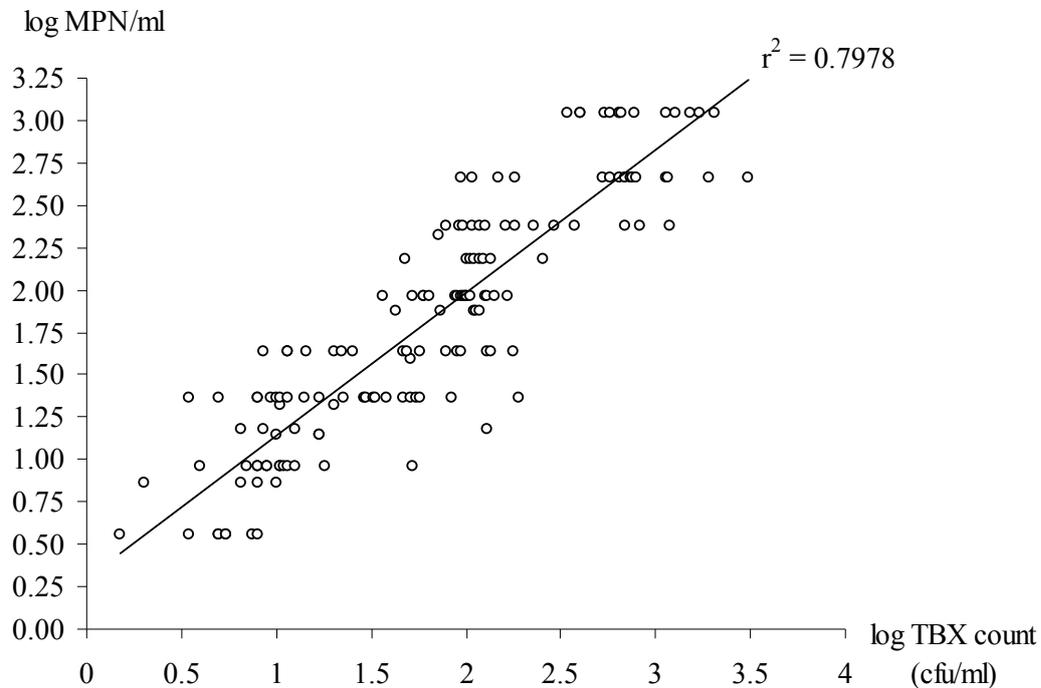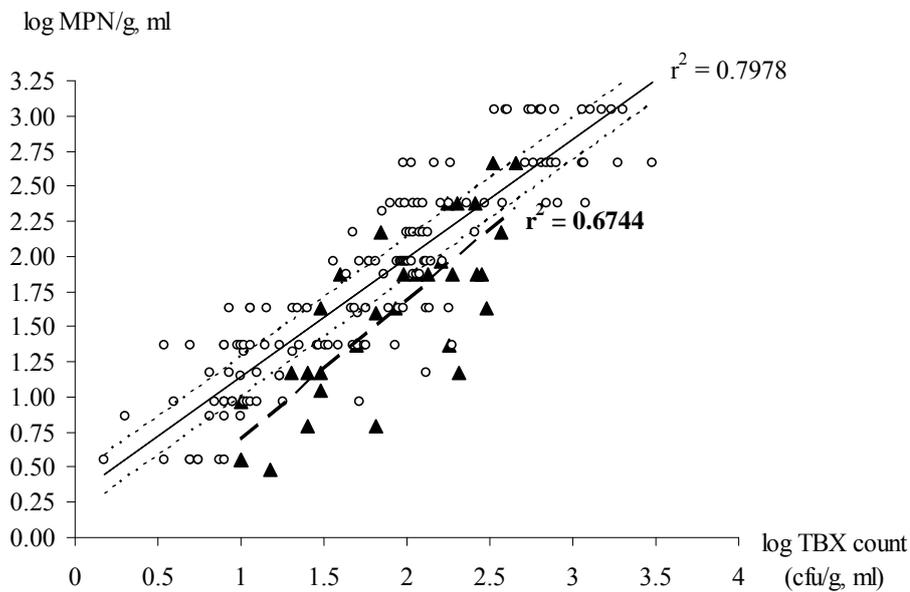

Figure 1. Correlation between log values of MPN and TBX count of *E. coli* observed from the pure culture model.

Relating the MPN estimates and the TBX counts of* E. coli *observed from the food models
We examined naturally contaminated *E. coli* in two food types: raw/uncooked foods and frozen foods to investigate whether the correlation between the MPN and TBX count of *E. coli* in food would follow the pattern of the pure culture. Correlation between the results for these food groups obtained from the two methods and that from the pure culture are demonstrated in Figures 2 and 3. It can be seen that the results obtained by the two methods in raw/uncooked food and frozen food were less correlated (r^2 = 0.6657 and 0.6744, respectively) than it was with the pure culture (r^2 = 0.7978). The patterns of correlation (slope of the regression line) of the two food types were also different from each other. This pointed to inconsistencies of correlation in relation to the type of food and the level of *E. coli*.
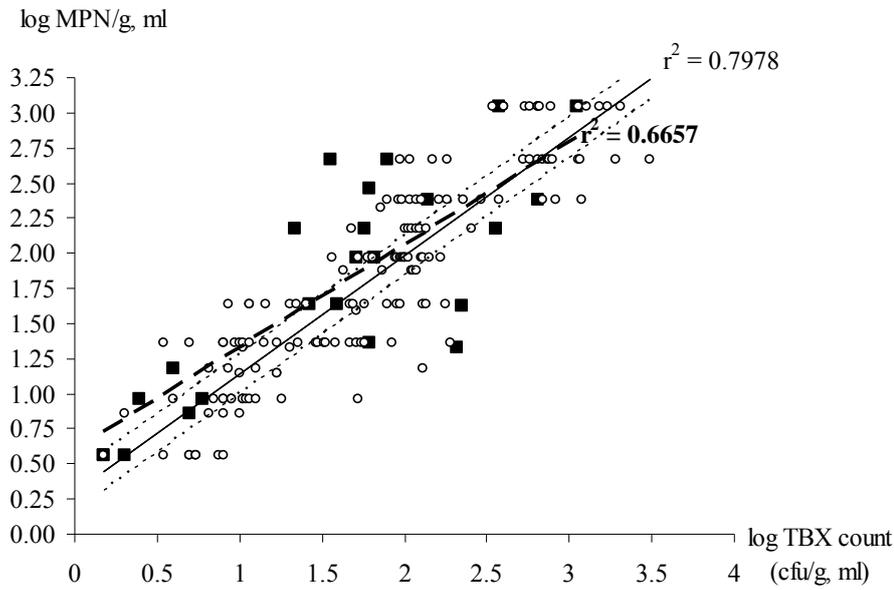
Figure 2. Correlation between log values of MPN and TBX count of *E. coli* in raw/uncooked food (dotted line with square symbols) compared with that observed in the pure culture model (continuous line with circle symbols). The dotted line paralleled to the curve of the pure culture represents the standard deviation at a 99% confidence level.

Figure 3. Correlation between log values of MPN and TBX count of *E. coli* in frozen food (dotted line with triangle symbols) compared with that observed in the pure culture model (continuous line with circle symbols). The dotted line paralleled to the curve of the pure culture represents the standard deviation at a 99% confidence level.

From Figures 2 and 3, the regression trend of both food groups deviated (at 99% confidence limit) from that of the pure culture. The deviation was more significant in frozen food than in raw/uncooked food. In other words, at this confidence limit, the pattern of correlation of the MPN and the TBX count of the pure culture model was quite different from those of the food models to be generally applicable to them.

Lower and upper correlating values of MPN and TBX count from the pure culture and food models

Should there be any attempt to convert an MPN value of *E. coli* to a TBX value, and *vise versa*, the upper correlating value, rather than the average, should be considered since this is more relevant concerning food safety. The correlating ranges of values of the TBX counts and MPNs are listed in Tables 2 and 3. It can be seen from these tables that although many correlating MPN values of most food samples were within the range of those of the pure culture, there were also many exceptional cases. Some correlating upper values from food samples can be higher than that of the pure culture by one log. Therefore, even the upper correlating values of the pure culture model could not be applied to food without precautions. A validation should be carried out for the particular type of food, and should involve a sufficiently large number of samples having different levels of contamination, before conversion between the MPN and the colony count can be made accurately. Nevertheless, such tables should not be treated as a standard, but rather, as a guideline where there is a necessity to communicate or convert results obtained from one method in light of the other. They should be used with care, taking into account the inconsistency of the curve pattern that may be obtained for each food type and the possibility that the   upper value(s) of a sample being analysed may extend beyond the upper values observed for the samples used when constructing the curve.

Table 2. Lower and upper values of the MPN that correlated with ranges of the TBX counts observed from the pure *E. coli* culture, raw/uncooked food samples and frozen food samples.

TBX count (cfu/ml)	correlating MPN value per g or ml for								
	pure culture			raw/uncooked food			frozen food		
	no. of samples	lower value	upper value	no. of samples	lower value	upper value	no. of samples	lower value	upper value
0-10	51	<3	43	8	3.6	15	60	<3	9.1
11-50	33	9.1	150	5	9.1	460[a]	11	<3[a]	75
51-100	27	9.1	460	6	23	460	5	6.2[a]	150
101-500	34	15	1100	6	21	1100	15	15	460
501-1000	20	240	>1100	1	240	240	ND	ND	ND
1001-5000	27	240	>1100	8	1100	>1100	ND	ND	ND
>5000	40	>1100	>1100	ND	ND	ND	ND	ND	ND

[a]lower or upper correlating MPN values that extended beyond the range observed in the pure culture;
ND: no data available.

Table 3. Lower and upper values of TBX counts that correlated to MPN values observed from the pure *E. coli* culture, raw/uncooked food samples, and frozen food samples

MPN values	correlating TBX values (cfu/ml)								
	pure *E. coli* culture			raw/uncooked food			frozen food		
	no. of samples	lower value	upper value	no. of samples	lower value	upper value	no. of samples	lower value	upper value
<3	19	0	5.5	ND	ND	ND	55	0	15
3	1	1	1	ND	ND	ND	1	15	15
3.6	11	0	8	4	0	2	6	0	10^{a}
7.2	1	6.5	6.5	ND	ND	ND	2	25	65^{a}
7.4	3	2	10	1	5	5	ND	ND	ND
9.2	13	4	52	3	2.5^{a}	18	1	10	10
11	ND	ND	ND	ND	ND	ND	1	30	30
14	3	10	17	ND	ND	ND	ND	ND	ND
15	4	6.5	1.30×10^{2}	1	4	4	4	20	2.05×10^{2a}
21	2	10.5	20.5	1	2.10×10^{2}	2.10×10^{2a}	ND	ND	ND
23	22	3.5	1.90×10^{2}	1	61.5	61.5	2	50	1.80×10^{2}
38	1	51	51	ND	ND	ND	1	65	65^{a}
43	16	8.5	1.80×10^{2}	2	26.5	2.21×10^{2}	3	30	3.05×10^{2a}
75	5	43	1.20×10^{2}	ND	ND	ND	7	40	2.80×10^{2a}
93	17	36.5	1.66×10^{2}	2	51	66	1	1.60×10^{2}	1.60×10^{2}
150	8	48	2.56×10^{2}	4	21.5^{a}	3.60×10^{2a}	2	70	3.70×10^{2a}
210	1	71.5	71.5	ND	ND	ND	ND	ND	ND
240	14	79.5	1.19×10^{3}	2	1.40×10^{2}	6.40×10^{2}	3	1.75×10^{2}	2.60×10^{2}
290	ND	ND	ND	1	62	62	ND	ND	ND
460	15	95	3.05×10^{3}	2	36^{a}	79.5	2	3.30×10^{2}	4.55×10^{2}
1100	14	3.40×10^{2}	2.03×10^{3}	2	3.79×10^{2}	1.12×10^{3}	ND	ND	ND
>1100	62	6.50×10^{2}	1.05×10^{8}	7	1.14×10^{3}	2.16×10^{3}	ND	ND	ND

[a] the lower or upper correlating MPN values that extended beyond the range observed in the pure culture;
ND: No data available.

Sensitivity of the MPN and TBX plate count methods when applied to food samples with low levels of E. coli

The MPN method has a presumptive step that enriches the organisms of interest, therefore, it is presumed to have a higher sensitivity than the plate count method. This could be another reason that prevents many food industries from switching from MPN methods to a rapid chromogenic plate count method apart from the cost. We analysed data from food samples containing low levels of *E. coli*, including frozen and heat treated foods, yielded by the MPN and the TBX plate count (pour plate, having limit of detection of 10 cfu/g) methods. Interestingly, those samples revealed to be positive by the TBX plate count method were of greater number than those revealed to be positive by the MPN method (Table 4).

Table 4. Number of food samples revealed to be positive for *E. coli* by the MPN or TBX plate count methods

food category	no. of samples positive by one of the methods used	
	MPN method (MPN/g)	TBX plate count method (CFU/g)
frozen food	3 (3.6[a])	3 (5)
		1 (10)
		2 (15)
heat-treated food	2 (3.6[a])	1 (5)
		3 (10)
		1 (20)
		1 (55)
Total number of samples	5	12

[a] reading from the positive tube set 1-0-0

It should be noted that all samples that were positive according to the TBX count method while negative according to the MPN estimation contained *E. coli* at levels lower than 100 cfu/g. A chromogenic plate count method that has detection limit of 100 cfu/g, therefore, would fail to detect *E. coli* in such samples. Matner *et al.* [21] also demonstrates higher sensitivity of a chromogenic plate count, which is based on the same principle as the TBX plate count, over the MPN method when analysing food samples with low levels of *E. coli*.

Conclusions

The problem underlying this study is whether or not it would be possible to convert analytical values of *E. coli* obtained by a chromogenic plate count method to MPN values or *vise versa*. The results from this study indicated that there are great limitations, since there were inconsistencies in the correlation of values obtained from the two methods and the additional variation due to the type of food. It is strongly adviced that food industries take precautions when attempting to relate data obtained from one method to the other. In countries where food authorities still rely on the MPN values, it is suggested that priority should be given to establishing criteria based on the chromogenic plate count methods whichare increasingly used by food industry to cover major types of food relevant to the country. In addition, establishing these criteria would best be done through assessment and evaluation based on the use of chromogenic plate count methods rather than by converting from the existing MPN values. The higher sensitivity of the TBX count compared to the MPN method in revealing low level contamination in the majority of the samples should also further encourage the use of this method by the food industry in place of the MPN estimation for quantitative analysis of *E. coli* in foods that should comply with a zero-tolerance criterion. The observations and deduction made in this study, though limited to *E. coli*, may be useful when considering other indicator microorganisms or pathogens, especially at this transition period when there is an urgent need for revision and establishment of food microbiological criteria in response to the rapid, continual development of microbiological methods.

Acknowledgements

References

1.   Oblinger, J.L. and Koburger, J.A. (1975). Understanding and teaching the most probable number technique. **Journal of Milk and Food Technology.** 38, 540-545.

2.   Mossel, D.A.A., Corry, J.E.L., Struijk, C.B. and Baird, R.M. (1995). Essentials of the microbiology of foods, a textbook for advanced studies. John Wiley & Sons, Chishester, UK, pp 294-295.

3.   Robison, B.J. (1984). Evaluation of a fluorogenic assay for detection of *Escherichia coli* in food. **Applied and Environmental Microbiology.** 48, 285-288.

4.   Frampton, E.W., Restaino, L. and Blaszko, N. (1988). Evaluation of the β-glucuronidase substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-GLUC) in a 24-hour direct plating method for *Escherichia coli*. **Journal of Food Protection.** 51, 402-404.

5.   Manafi, M. (2000). New developments in chromogenic and fluorogenic culture media. **International Journal of Food Microbiology.** 60, 205-218.

6.   Manafi, M., Kneifel, W. and Bascomb, S. (1991). Fluorogenic and chromogenic substrates used in bacterial diagnostics. **Microbiology and Molecular Biology Reviews.** 55, 335-348.

7.   Suwansonthichai, S. and Rengpipat, S. (2003). Enumeration of coliforms and *Escherichia coli* in frozen black tiger shrimp *Penaeus monodon* by conventional and rapid methods. **International Journal of Food Microbiology.** 81, 113-121.

8.   González, R.D., Tamagnini, L.M., Olmos, P.D. and de Sousa, G.B. (2003). Evaluation of a chromogenic medium for total coliforms and *Escherichia coli* determination in ready-to-eat foods. **Food Microbiology.** 20, 601-604.

9.   Kilian, M. and Bülow, P. (1976). Rapid diagnosis of Enterobacteriaceae. I. Detection of bacterial glycosidases. **Acta Pathologica Microbiologica Scandinavica.** 84B, 245–251.

10.   Restaino, L., Frampton, E.W. and Lyon, R.H. (1990). Use of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-GLUC) for enumerating *Escherichia coli* in 24 h from ground beef. **Journal of Food Protection.** 53, 508-510.

11.   Gauthier, M.J., Torregrossa, V.M., Babelona, M.C., Cornax, R. and Borrego, J.J. (1991). An intercalibration study of the use of 4-methylumbelliferyl-β-D-glucuronide for the specific enumeration of *Escherichia coli* in seawater and marine sediments. **Systematic and Applied Microbiology.** 14, 183-189.

12.   Geissler, K., Manafi, M., Amorós, I. and Alonso, J.L. (2000). Quantitative determination of total coliforms and *E. coli* in marine waters with chromogenic and fluorogenic media. **Journal of Applied Microbiology.** 88, 280-285.

13.   Finney, M., Smullen, J., Foster, H.A., Brokx, S. and Storey, D.M. (2003). Evaluation of Chromocult coliform agar for the detection and enumeration of Enterobacteriaceae from faecal samples from healthy subjects. **Journal of Microbiological Methods.** 54, 353-358.

14.   ISO (2001). Standard 16649-2, Microbiology of food and animal feeding stuffs--Horizontal method for the enumeration of presumptive *Escherichia coli*, Part 2: Colony-count technique at 44 degrees C using 5-bromo-4-chloro-3-indolyl-beta-D-glucoronide. International organisation for standardisation.

15.   Andrews, H.W. (1995). Microbiological Methods. In: Cunniff P (ed.). Official methods of analysis of AOAC International. AOAC International, 16th edn. Virginia, USA, pp 13-15.

16.   FSANZ (2001). User guide to standard 1.6.1 − Microbiological limits for food with additional guideline criteria. Food Standards Australia New Zealand, pp 1-22.

17.   European Commission (2001). Overview of microbiological criteria for foodstuffs in community legislation in force, updated June 2001.
(ec.europa.eu/food/fs/sfp/mr/mr_crit_en.pdf).

18.   Gilbert, R.J., de Louvois, J., Donovan, T., Little, C., Nye, K., Ribeiro, C.D., Richards, J., Roberts, D. and Bolton, F.J. (2000). Guidelines for the microbiological quality of some ready-to-eat foods sampled at the point of sale. **Communicable Disease and Public Health.** 3, 163-167.

19.   CAC (1997). Principles for the establishment and application of microbiological criteria for foods (CAC/GL 21, 1997). Codex Alimentarius Commission, Agriculture and Consumer Protection Department, Food and Agriculture Organisation of the United Nations. (http://www.fao.org/docrep/w6419e/w6419e04.htm).

20.   Blodgett, R. (2006). Most probable number from serial dilutions. In: Jackson GJ, Merker RI, Bandler R (eds.). Bacteriological analytical manual online, Center for Food Safety and Applied Nutrition, US Food and Drug Administration. (http://www.cfsan.fda.gov/~ebam/bam-a2.html).

21.   Matner, R.R., Fox, T.L., McIver, D.E. and Curiale, M.S. (1990). Efficacy of petrifilm^{TM} *E. coli* count plates for *E. coli* and coliform enumeration. **Journal of Food Protection.** 53, 145-150.