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Practical coliforms and *Escherichia coli* detection and enumeration for industrial food samples using low-cost digital microscopy

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Abstract

This research explored several alternative coliforms and *E. coli* detection strategies proposed for industrial application especially low-resource settings and less advanced food manufacturers. The colony count using 2 industrial favourites (i.e., Petrifilm™ by 3M and regular pour plate techniques) were contrasted to 4 alternative low-cost strategies. Two modified conventional protocol (i.e., pour and spread plate techniques) in standard mini Petri dishes and two drop plate techniques in microtiter plate formats (i.e., 24 - and 96-well plate) were applied to count industrial frozen food samples. The colony detection in all treatments was visually facilitated by low-cost digital microscopy technique comparing colony count, the detection time and the colony area in pixels. All experiments except for the Petrifilm™ *E. coli*/Coliform (EC) Plate utilized Chromocult® Coliform Agar (CCA). The inoculum sizes were varied depending on the cell count technique used; 10 µl and 5 µl for the 24- and 96-well microtiter plates, 50 µl for the mini-plate (both pour and spread plate techniques), and 1000 µl for the full-size Petri dishes and Petrifilm™ *E. coli*/Coliform (EC) Plate. The incubation temperature was fixed at 35±2°C. The number of colonies from the conventional pour plate technique was plotted against those of the other techniques. In all treatments, the relationship plots showed highly linearity from the lower detection limit (100 CFU/ml depending on technique used) to the upper detection limit (10,000 CFU/ml). The slopes of all regression lines were close to unity showing very high correlation of the values of colony counts from different techniques. All techniques were applied to evaluate actual swap samples from the production line and returned highly consistent colony count numbers and good separation of *E. coli* from coliforms. The colony counts from the ready-to-eat product samples showed similar and comparable results to the two routine factory techniques. The results indicated that all six colony count methods were simply interchangeable to perform colony enumeration in the low-resource industrial setting.

Keywords: Chromocult® Coliform Agar; Coliforms; Enumeration; *Escherichia coil*; Spread plate; Pour plate; Micro inoculation; Petrifilm™ E. coli/Coliform Count (EC) Plate

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1. Introduction

Microbiological quality is among key attributes to indicate integrity of food products and hygiene of food processing. Coliforms and *E. coli* Detection has been adopted by world-class food industry to determine microbiological quality of food products [1, 2]. Detection of *E. coli* suggests contaminations from direct or indirect fecal origins of humans and warm-blooded animals [3]. High counts of *E. coli* and total coliform (TC) in food samples directly imply poor practices in food handling and production operations in the manufacturing chain [4, 5, 6, 7]. Most of classical techniques to determine the presence of coliforms and *E. coli* are dependent on their unique biochemical properties, including the most probable number technique (MPN), Petrifilm™ *E. coli*/Coliform Count (EC) and Chromocult® Coliform agar (CCA) [8, 9, 10, 11]. By far, Petrifilm™ Plate (3M Center, St. Paul, MN, USA) and CCA (Merck, Germany) are among the most favorite protocols for local Thai food industry, since it helps simplify the laborious and complex protocol of MPN and minimize analytical time to identify *E. coli*/coliforms contamination [12, 13].

As opposed to the general recommendations from food industrial literatures and manufacturer's publication, these chromogenic methods were no longer cost-effective when they applied to developing world settings. Import tax and overhead cost applied by local distributors contribute to price hike and the saving benefited from minimal medium preparation was marginally advantageous in developing world application where labor cost was relatively inexpensive. However, most local food processors still apply these protocols due to customer's requirement and universal acceptance on their accuracy and high specificity to detect *E. coli* and coliforms. Generally both protocols call for 24 to 48 h at 35-37°C for colony detection differentiating red colonies for coliforms from dark blue to violet colonies for *E. coli* [14, 15]. For CCA, the manufacturer's standard protocol frequently recommends using pour plate technique to carry out the detection. The restriction of oxygen transfer slows down cell growth and colony expansion; as a result, it delays colony detection. Same situation applies to Petrifilm™ EC Plate where thin polymer film was utilized to cover the medium surface.

The principle of chromogenic media for *E. coli*/coliforms detection is based on the use of substrate material designed to react with specific microorganism enzyme producing unique colony color [16, 17]. Any barrier restricted the essential oxygen supply for colony growth may retard the colony growth and detection sensitivity. Submerged colonies proliferated inside the agar layer or polymer film of the Petrifilm™ application can limit the rate of colonies expansion; hence, it deteriorates colonies growth kinetics [14]. These industrial routine techniques and other similar methods have defects. The conventional methods, on the other hand, require several days to show results because they rely on the ability of microorganisms to multiply to visible colonies. It is no longer applicable for modern day food industry. There is an emerging need for more fast detection methods to provide prompt and critical information on the possibility of pathogen contamination in raw materials and finished food products as well as monitoring of cleaning and hygiene. This paper focused on finding alternative solution to replace the commercial methods with same accuracy but less cost for industrial purpose. Several common protocols to estimate coliforms and *E. coli* were evaluated. The proposed alternatives were also experimented by miniaturizing the cultivation volume using the conventional CCA. Low-cost, commercial digital microscope was used to assist the detections of *E. coli*/coliform colonies.

2. Materials and methods

2.1. Industrial processing swabs and food product samples

The industrial samples used in this study included the green papaya salad and swab samples from production lines from a local food factory. They were routinely collected to evaluate coliforms and *E. coli* contamination according to the factory work instruction. Several techniques were applied on these samples, including Petrifilm™ *E. coli*/Coliform Count (EC) Plate, CCA using full – size and mini Petri

dish pour plate techniques, CCA using full – size and mini Petri dish spread plate techniques, and CCA in microtiter formats (i.e., 24-well and 96-well microtiter plates). Bacteriological analysis was initiated within 3 h after sampling. A 25 g analytical unit of each food was homogenized with 225 ml of sterile water-peptone (0.1% w/v) for 2 min and then serial 10 fold dilutions were prepared with sterile water-peptone [19].

2.2 Detection protocols

2.2.1 Petrifilm™ E. coli/Coliform Count (EC) Plate

Sample suspension (1 ml) at appropriate dilution was pipetted onto the surface of a Petrifilm™ *E. coli*/Coliform Count (EC) plate. Slowly apply the cover film on the plate and incubate the plate at 35 °C for 24 h. According to the manufacturer's manual, red colonies surrounded with trapped gas were coliforms and blue colonies with trapped gas were *E. coli*. Duplicate trials were performed per dilution. Coliforms and *E. coli* were reconfirmed using EMB agar and IMViC testing. Positive control using *E. coli* was included in the experiment.

2.2.2 Regular and Mini standard Petri dish Chromocult® pour plate cultivation

Again, serially dilute the stomacher sample to proper dilution. Apply 1 ml of samples mixed well with 14 and 4 ml of CCA to form the full-size and mini Petri dish pour plates, respectively. The temperature of melting CCA was controlled at 45ºC. The pour plate cultivation was incubated at 35±2ºC for 24 h. Each dilution was tested on duplicate CCA repetitions. A well-distributed colony plate with 15–150 colonies was digitized using a low-cost digital microscope (Dino BW - 908). Chromocult® Coliform Agar (CCA) was supplied by Merck, Germany.

2.2.3 Regular and Mini standard Petri dish Chromocult® spread plate technique

Similar to the pour plate experiment, CCA was utilized to form solid agar plates using full-size and mini Petri dish formats. However, only 0.1 and 0.02 ml of sample suspensions were pipetted onto the pre-fabricated plate. A sterile spreader was applied to disperse the sample until the agar surface was dried out. The growing colonies were monitored over 24 h at 35±2ºC. The rest of the protocol was similar to the pour plate technique.

2.2.4 Chromocult® Micro Inoculation Culture (i.e., 24-well and 96-well microtiter plates)

Cultivation volumes were fixed at 5 and 10 µl onto the top of 24, 96-well microtiter plates CCA agar surface and incubated at 35±2ºC. Then later normally 12 – 15 h will be detected and captured using a low-cost digital microscopy. A constructed prototype of digital image analysis protocol was implemented to evaluate the area that each colony occupied on the agar surface. The experimental assumption was that the colony only expanded horizontally and the area of expansion was highly correlated with the growth of pathogens on the solid medium. The areas of colony growths were digitized every hour.

2.2.5 Statistical analysis

All CFU counts per ml or gram were transformed to log CFU/g counts before statistical analysis. All data were analyzed at $p<0.05$ for significant values by ANOVA and Duncan's multiple range tests.

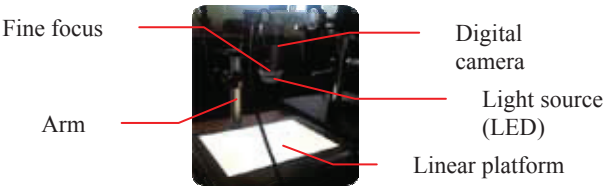


Fig. 1. Image acquisition prototype of digital microscopy

3. Results and discussion

Several alternative protocols for coliforms and *E. coli* detection strategies were investigated and proposed for industrial application, especially low-resource settings and less advanced food manufacturers. Two most common routines (i.e., Petrifilm™ by 3M and regular pour plate CCA) were

compared to five alternative low-cost alternatives. Basically, pour and spread plate CCA techniques were conducted on two formats of Petri dishes (i.e., standard 15x100 mm and mini 15x60 mm petri dishes). The underlining concept was to reduce the expense from CCA medium usage. The reduction of CCA was extended to the application of microtiter plates (i.e., 24- and 96-well microplates). By doing so, the expensive CCA usage was diminished as follows; 15 ml per regular Petri dish, 4 ml per mini Petri dish, 1.45 ml per well in 24-well microtiter plate and 0.36 ml per well in 96-well microtiter plate. First, the comparison of CCA cultivation results using these different formats of detection was conducted. After the method validation, the real industrial samples were used to re-validate these *E. coli* detection kits once more.

3.1 Validation of Coliforms and E. coli detection protocols

The standard plots comparing the pure culture *E. coli* counts on CCA using different techniques was compiled in Fig.2. Essentially, all techniques returned practically same readings pending that the sample inoculum was prepared with proper dilution for colony evaluation. All standard slopes were close to unity with fairly good correlation coefficients. Noticeably, most slopes extracted from the data using pre-fabricated CCA techniques showed values slightly higher than one. Only treatment that had slope value less than one was from the mini Petri dish pour plate technique. From the experimental point of view, the mixing of sample inoculum with lukewarm CCA was to some degree detrimental to viable cell counts. Many literatures reported no colony survival of *E. coli* at 40-50°C incubation [19]. The use of pour plate colony count to enumerate predominantly injured cells from industrial samples was prone to underestimation and to detect critical pathogens usually present in low quantity was questionable. The use of pre-fabricated CCA kits seemed more appropriate for industrial *E.coli*/coliforms enumeration.

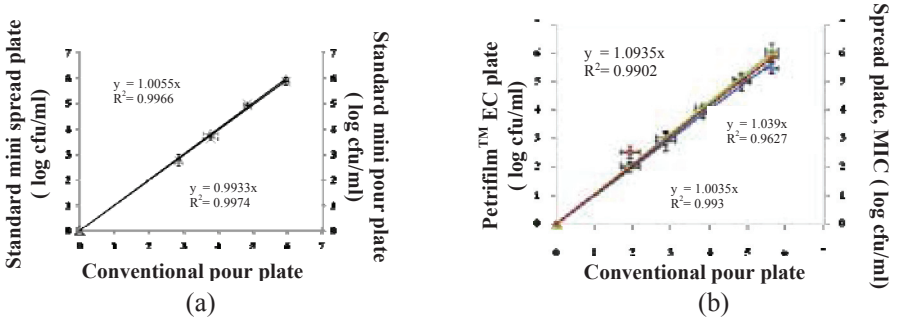
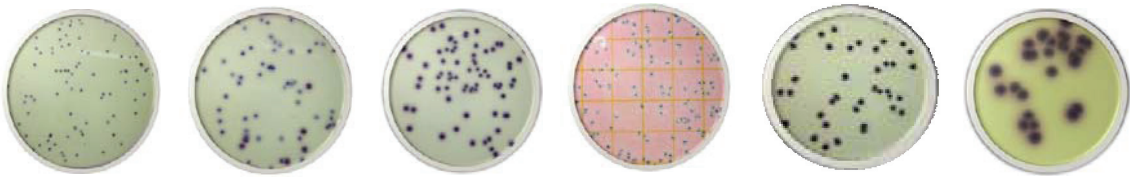


Fig. 2. (a) Validation of *E. coli* enumeration comparing between standard mini spread plate, standard mini pour plate and regular pour plate. (b) Validation of *E. coli* enumeration comparing Petrifilm™ EC plate , regular spread plate, regular pour plate and MIC

The use of miniaturized formats either the mini Petri dishes or 24- and 96-well microtiter plates calls for higher magnification detection system to assist the enumeration process. As opposed to the traditional regular Petri dish format which normally uses human visual detection, a low-cost digital microscopy scheme was established for high throughput industrial application. This prototype detection system also worked well with the Petrifilm™ EC plate format. The sample images of the *E. coli* colonies grown on CCA at different formats were shown in Fig. 3. This digital colony imagery system allows efficient data storage for future reference and facilitates automatic colony enumeration and morphological detection in other chromogenic agars.



(a) (b) (c) (d) (e) (f)

Fig. 3. Digitized images of *E. coli* colony from pure culture after 12 h of incubation at 35±2°C. (a) Regular pour plate; (b) Mini Petri dish pour plate; (c) Mini Petri dish spread plate; (d) Petrifilm™ EC plate; (e) Regular spread plate; (f) Spread plate in 24-well microtiter plate format.

The images of *E. coli* colonies in Fig. 3. were taken at the same incubation time (approximately 12 h); however, the colony size and color morphology varied significantly depending on the magnification of the microscope and the intrinsic growth of *E. coli* from different format of cultivation. Clearly, the regular Petri dish possesses a large agar surface to cover and allows only low-magnification image acquisition to cover the entire plate see Fig. 3a. The smaller mini Petri dish facilitates higher magnification power of microscope and the colony size was enlarged significantly see Fig. 3b. In Fig. 3b, the non-uniformity of colony size and color was the result of submerged colonies proliferated inside the agar layer where oxygen supply was limited and affected the rate of colony expansion and color development. Only with higher magnification that the differences of colony characteristics were observed and this finding would be irrelevant for human visual inspection.

The expansion of *E. coli* colony area using different detection protocols in Fig. 4. demonstrated significant slower colony growth kinetics for both pour plate treatments (i.e., standard Petri dish and mini Petri dish). The *E. coli* colony in all spread plate techniques not only expanded at a much faster rate but also grew to much larger colonies. The cultivation volume and formats of the spread plate technique didn't affect the colony expansion kinetics despite the variation of medium layer thickness in each format. They directly affected the volume of CCA used and the less medium usage produced a cost efficient protocol for industrial practice. Only one drawback from using the smaller cultivation format was that the restricted agar surface limit the amount of sample used; hence, it reduced the detection limit of the methodology. Only certain concentration can be detected when the sample inoculum size was in the order of microliters [18].

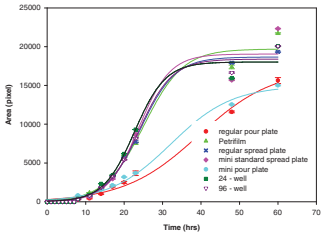


Fig. 4. *E.Coli* colony expansion profiles using various detection protocols

3.2 Application of microbial detection for food samples and production line swabs

All protocols were repeated and applied to two types of industrial samples (i.e., food samples and production line swabs). The use of the regular pour plate and Petrifilm™ tests were to confirm the implementation of the three modified conventional protocol (i.e., pour and spread plate techniques) in standard mini Petri dishes and regular spread plate.

3.2.1 Food samples

For rather critical samples where the potential contaminant was diluted and the sensitivity of detection was crucial, very small cultivation volumes as in the 24- and 96-well microtiter plates were not applicable. To perform colony count for food products, the mini Petri dish format and spread plate technique were compared to the Petrifilm™ EC plates and the conventional pour plate routine. As concluded in the protocol validation experiments, the implementation of pour plate and spread plate

techniques or the Petri dish formats did not alter the final colony counts of the real food product samples see in Table 1. All protocol resulted in statistically same final colony readings. There were only coliforms contamination and no *E. coli* colony.

Table 1. Coliforms enumeration in food samples using various techniques.

Samples	Coliforms (log CFU/ml)				
	Regular Pour plate	Petrifilm^{TM} EC plate	Mini Standard Pour plate	Regular Spread Plate	Mini Standard Spread plate
1	3.24±0.03[a]	3.12±0.03[a]	3.27±0.02[a]	3.85±0.07[a]	3.70±0.10[a]
2	2.51±0.02[a]	2.18±0.07[a]	2.55±0.02[a]	2.63±0.24[a]	2.60±0.10[a]
3	3.60±0.05[a]	3.15±0.18[a]	3.55±0.04[a]	3.72±0.07[a]	3.65±0.15[a]
4	3.57±0.08[a]	3.35±0.06[a]	3.47±0.07[a]	3.89±0.24[a]	3.73±0.23[a]
5	2.71±0.10[a]	2.57±0.05[a]	2.80±0.01[a]	2.91±0.06[a]	2.82±0.04[a]
6	3.78±0.07[a]	3.52±0.07[a]	3.69±0.04[a]	3.86±0.06[a]	3.77±0.10[a]

[a] values in a row with different superscripts are significantly difference at $P<0.05$.

3.2.2 Production line swabs

The routine measure of processing and equipment cleanliness requires frequent and large numbers of swab samples. The evaluation of processing environment calls for fast estimation of Coliforms/*E.coli* numbers. The slow evaluation of production facility hygiene can inflict serious damage to food manufacturers [19]. The frequency of production line swabs must be adequate to ensure good hygienic practice (GHP). However, most food factory abstains from more swab sampling merely because it increases the production cost. For this purpose, the cultivation volume of *E. coli*/coliforms detection was minimized. The use of the regular pour plate was to confirm the implementation of the three modified conventional protocols (i.e., mini standard pour plate, 24- and 96-well microtiter plate techniques). Table 2 showed that the coliform results of the proposed mini standard pour plate, 24- and 96-well formats, were agreeable with the regular pour plate technique. Again, there was no *E. coli* colony present. In all treatment, the spread plate technique returned higher colony count than the pour plate technique.

Table 2. Coliforms enumeration in production line swab using various techniques.

Samples	Coliforms (log CFU/ml)			
	Regular Pour plate	Mini Standard Pour plate	24-well Microtiter plates	96-well Microtiter plates
Shelf	3.30 ± 0.11[a]	3.27 ± 0.10[a]	3.76± 0.06[a]	3.91± 0.09[a]
Tray before freezing	2.54 ± 0.11[a]	2.41 ± 0.09[a]	2.80 ± 0.02[a]	2.89 ± 0.10[a]
Centrifuge	3.35 ± 0.10[a]	3.28 ± 0.08[a]	3.75 ± 0.05[a]	3.84 ± 0.07[a]
Weighing	3.41 ± 0.09[a]	3.38 ± 0.07[a]	3.51 ± 0.02[a]	3.68 ± 0.08[a]

[a] values in a row with different superscripts are significantly difference at $P<0.05$.

3.3.3 The detection time

The benefit of the miniaturized cultivation to detect *E. coli*/coliforms was not only the reduction of expensive CCA but also the use of digital microscopy allow higher magnification to observe suspected colonies when they were very small and hardly visible by naked eyes. Early detection of minute colonies allows significant analytical time reduction as shown in Table 3. The routine pour plate technique and Petrifilm^{TM} plate ask for 24-48 h where most of the proposed protocols need only 10 h owing to microscope-assisted detection system. The short incubation time should provide prompt and accurate routine industrial *E. coli*/coliforms detection. (i.e., high throughput capability, rapid response, and low cost per test)

Table 3. Detection time of coliforms enumeration using various techniques

Factor	Factory routine		Proposed industrial-oriented protocols				
	Regular pour plate	Petrifilm™	Regular spread plate	Mini standard pour plate	Mini standard spread plate	MIC 24 well	MIC 96 well
Detection Time (h)	24 – 48[a]		10[b]				

[a] values in a row with different superscripts are significantly difference at $P<0.05$

3.4 Conclusion

Alternative protocols to detect and enumerate *E. coli*/coliform in industrial food samples, as well as production line and swab samples, were proposed and validated. The use of miniaturized cultivation volume together with medium magnification microscopy was able to reduce expense of expensive CCA and incubation time significantly. The comparison of different detection techniques demonstrated high correlation between protocols and suggested the replacement of conventional routines (i.e., pour plate and Petrifilm™ techniques) by the proposed methods without compromising the detectability and accuracy. With the appropriate detection sensitivity depending on the cultivation volume used, food industry was able to benefit from low cost per sample and faster analytical time.

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