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ORIGINAL ARTICLE

Evaluation of Petrifilm™ EC method for enumeration of *E. coli* from soil

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Abstract

Aims: To evaluate the suitability of commercially available Petrifilm™ EC plates for enumeration of *Escherichia coli* from soil.
Methods and Results: A confirmed *E. coli* strain isolated from liquid swine manure was inoculated into sterilized sandy clay loam and loam soils at the concentrations of 10^2, 10^3, 10^5 CFU g^{-1} of soil. The efficiency of recovery on Petrifilm™ EC plates for soils spiked with *E. coli* was compared with standard membrane filtration techniques on m-FC basal medium supplemented with 3-bromo-4-chloro-5-indoyl-β-D-glucopyranoside (BCIG) and most probable numbers (MPN) techniques in *E. coli* medium with 4-methylumbelliferyl-β-D-glucuronide (EC-MUG) broth. Petrifilm™ EC and m-FC (BCIG) methods were then assessed for the ability to recover *E. coli* from field soils applied with swine manure. No significant differences ($P > 0{\cdot}05$) were observed between Petrifilm™ EC, m-FC (BCIG) and MPN methods for the recovery of *E. coli* from spiked samples, irrespective of soil type. However, recovery of *E. coli* from manure-applied field soil samples showed a significant difference ($P < 0{\cdot}05$) between the Petrifilm™ EC method and the m-FC method in enumerating *E. coli* possibly as a result of false positives on m-FC.
Conclusion: The Petrifilm™ EC method is suitable for the enumeration of *E. coli* from soil with a detection limit of 10 CFU g^{-1} soil.
Significance and Impact of the Study: The commercially available Petrifilm™ EC method is comparatively low cost, easy to use method for the enumeration of *E. coli* from soil without the need for further confirmation tests.

Introduction

Escherichia coli is a commonly used indicator for monitoring faecal contamination in the environment (Alonso *et al.* 1999). *Escherichia coli* has been adopted as an indicator because it is present in high numbers in the faeces of warm-blooded animals, persists in the environment and shows a strong correlation between the presence of *E. coli* in water and the occurrence of associated illnesses (Edberg *et al.* 2000; Leclerc *et al.* 2001; Anon 2004; Hamilton *et al.* 2005). A number of chromogenic and fluorogenic enumeration methods based on β-galactosidase and β-glucuronidase (GUD) activity have been developed in the past decade for enumeration of *E. coli* in water, as an alternative to traditional membrane filtration or multiple fermentation tube method based on most probable numbers (MPN) (Alonso *et al.* 1999; Manafi 2000; Niemela *et al.* 2003). Of those, m-Coli Blue (Hach, Loveland, CO, USA), Colilert-18®/Quanti-Tray® system (IDEXX Laboratories, Portland, ME, USA), m-TEC agar (Becton Dickinson, Sparks, MD, USA) and Petrifilm™ EC (3M, St Paul, MN, USA) are commercially available and widely used.

The application of manure to agricultural fields is a common practice for efficient use and disposal of manure in many farms across North America. The survival of manure-derived pathogenic bacteria in soil upon manure application increases the probability of contamination of source waters and re-infection of fresh farm produce (Franz *et al.* 2008; Holley *et al.* 2008). Monitoring of the survival of *E. coli* as an indicator for potential pathogenic bacteria in manure-applied soils can be used

to assess the potential health risks. The detection of *E. coli* in soil has been challenging because many of the commercially available methods were developed for water quality testing. However, a few methods have been tested for nonwater samples. The Colilert-18®/Quanti-Tray® system has been shown to be an appropriate method for the enumeration of *E. coli* from sewage sludge samples (Eccles *et al.* 2004). Furthermore, Muirhead *et al.* (2004) reported that the Colilert-18®/Quanti-Tray® system could be used for the enumeration of *E. coli* in soil. This method is, however, relatively expensive and needs some initial investment for required instrumentation. Alternatively, the Petrifilm™ EC method is widely used in food and dairy industries (Priego *et al.* 2000; Russell 2000; Gracias and McKillip 2004) and for environmental water samples (Vail *et al.* 2003). The detection method is based on GUD activity where blue-colored colonies associated with entrapped gas bubbles are confirmed as *E. coli*. The Petrifilm™ EC has been approved by the Association of Analytical Communities as an Official Methods of Analysis for the enumeration of *E. coli*; however, it has not been thoroughly assessed for its ability to enumerate *E. coli* from soil. Hence, this study evaluated the Petrifilm™ EC method for the enumeration of *E. coli* from soil.

Materials and methods

Soil preparation and inoculation

Soil was sampled at the University of Guelph Elora Research Station (ON, Canada) from research plots and immediately transported back to the laboratory. The soil samples were classified by texture as (i) sandy clay loam (50% sand, 24% clay, 26% silt) and (ii) loam (36% sand, 18% clay, 46% silt) using the hydrometer method (Kroetsch and Wang 2008). Each soil sample was sieved (2 mm) and sterilized in small samples (about 200 g) in 500-ml glass beakers at 121°C for 15 min. Ten grams of each soil sample was placed into nine sterilized Nalgene bottles (125 ml).

A confirmed *E. coli* strain isolated from liquid swine manure was grown to log phase in trypticase soy broth (Becton Dickinson), and the cell concentration was adjusted to 10^6 CFU ml^{-1} by optical density (600 nm). The cells were re-suspended in sterile water followed by serial dilutions (10^6–10^3 CFU ml^{-1}). Soils were spiked with 1 ml of the appropriate amount of *E. coli* to achieve the target concentrations; i.e. *c.* 10^2, 10^3, 10^5 colony-forming units (CFU) *E. coli* g^{-1} soil. Briefly, 10 g of soil was inoculated into 95 ml of diluent, followed by serial 1 : 10 dilution, where 10 g of mineral soil is assumed to occupy *c.* 5 cm^3 of volume (Zuberer 1994). The inoculated samples were shaken for 3 min on a bench top shaker (New Brunswick Scientific, Edison, NJ, USA) at 400 rev min^{-1} for homogenization. Each target concentration was prepared in triplicate.

Enumeration of *Escherichia coli* from inoculated soils

Escherichia coli was enumerated from inoculated test soils by three methods: (i) Petrifilm™ EC (3M); (ii) m-FC basal medium (Becton Dickinson) supplemented with BCIG (3-bromo-4-chloro-5-indoyl-β-D-glucopyranoside) (INALCO SPA, Milano, Italy; supplied by Med-OX Diagnostics Inc. Ottawa, ON, Canada) and (iii) MPN analysis (five tube) in *E. coli* medium with 4-methylumbelliferyl-β-D-glucuronide (EC-MUG) broth (Becton Dickinson). All three methods employed in this study were based on GUD activity of *E. coli*. Soils inoculated with target concentrations of *E. coli* (10^2, 10^3, 10^5 CFU g^{-1} soil) were serially diluted, and 1 ml of the appropriate dilution was plated onto triplicate Petrifilm™ EC plates. For the m-FC method, 1 ml of the appropriate dilution was filtered through a 0·45-μm filter (Fisher Scientific, Ottawa, ON, Canada) and the filters were placed on m-FC agar supplemented with BCIG. One ml of appropriate dilution was inoculated for each MPN tube along with a Durham tube to assess trapped gas. Petrifilm™ EC plates and m-FC plates were incubated at 44·5°C for 15–18 and 24 h, respectively. The MPN tubes were incubated at 44·5°C in a water bath for 24 h. Blue colonies with associated gas bubbles were counted as *E. coli* on Petrifilm™ EC plates (3M interpretation guide; 3M, London, ON, Canada). All blue colonies on m-FC plates were counted as presumptive *E. coli*. The number of positive tubes with growth at 44·5°C, gas production and fluorescence when exposed to UV (360 nm) were counted, and an MPN table was consulted for the enumeration of *E. coli* for MPN (Anon 1989).

Enumeration of *Escherichia coli* from manure-applied field soils

Petrifilm™ EC and m-FC methods were compared in a field experiment designed to evaluate the recovery of faecal indicator bacteria and pathogens under different manure application methods. The experiment was laid out in randomized complete block design ($n = 4$) with a plot size of 15 × 15m. Liquid swine manure used in this study had an initial *E. coli* concentration of 4·5 × 10^3 CFU ml^{-1}. Treatments were (i) no manure (Control – C), (ii) surface applied manure at the rate of 52 000 l ha^{-1} (SA), (iii) surface applied manure followed by incorporation 2 h after application with a C-tine cultivator (IN), (iv) surface applied manure on pretilled plots (S-tine cultivator imme-

diately prior to application) (PT) and (v) surface applied manure on pretilled plots followed by incorporation within 2 h of application (PT + IN). Soil was sampled within 24 h after the manure application to enumerate *E. coli*. Ten samples (25–50 g each) were taken randomly with sterilized hand shovels from each plot (0–2 cm), and composite samples (about 100 g) were prepared by mixing samples thoroughly in sterilized aluminium trays. The hand shovels and aluminium trays were sterilized by spraying 70% ethanol followed by brief flaming. The samples were stored on ice and transported back to the laboratory for analysis within 3 h. For recovery of *E. coli*, 10 g of soil was added to 95 ml of 0·1% sodium pyrophosphate according to Zuberer (1994). Sodium pyrophosphate was used as the diluent to enhance the release of bacteria attached to soil particles in manure-applied field soils (Ahn *et al.* 2005). The samples were shaken at 200 rev min^{-1} on incubator shaker (New Brunswick Scientific) for 20 min at room temperature (23°C). Plating and incubation were carried out on Petrifilm™ EC and m-FC (BCIG) as described elsewhere.

Statistical analysis

Data were recorded as CFU and were transformed to log 10 for analysis. The data generated using the MPN method was analysed along with two other methods for comparison. Statistical analysis was performed using SAS version 9.1.3 (SAS Institute Inc., Cary, NC, USA). Normality and homogeneity of variance of residuals (error) were tested using *proc univariate* and generating plot of residuals against predicted values in SAS. Outliers were assessed using Lund's test (Bowley 1999). *Proc GLM* procedure of SAS was used for variance partition and to generate *least square means*.

Results

Enumeration of *Escherichia coli* from inoculated soils

The MPN method tended to produce slightly higher numbers in comparison with the Petrifilm™ EC method and the m-FC (BCIG) method at spiked *E. coli* concentrations 10^2 and 10^3 CFU g^{-1} soil, although they were not significantly different (Table 1). Analysis of variances (ANOVA) indicated that there was a significant concentration × method interaction ($P < 0·05$) because of low MPN values recorded for the spiked concentration of 10^5 CFU g^{-1} soil, irrespective of soil type (Table 2). There was no significant difference ($P > 0·05$) between the Petrifilm™ EC method and the m-FC (BCIG) method for any of the spiked *E. coli* concentrations tested irrespective of soil type. Moreover, there were no significant differences between soil type × method × concentration interactions ($P > 0·05$), indicating that the three methods are comparable for the three concentrations tested for both soil types. The most critical dilution to be tested was 10^2 and 10^3 CFU g^{-1} as these concentrations result in relatively high amounts of soil particles in the culture media. The variance partition of the effects of different methods [MPN, m-FC (BCIG) and Petrifilm™ EC], soil types (sandy clay loam and loam), dilution (10^2,10^3 and 10^5 CFU g^{-1} soil) and their interactions are summarized in Table 2.

Table 1 Recovery of *Escherichia coli* from inoculated soil samples (mean ± SE) by Petrifilm™ EC, m-FC and most probable numbers (MPN) methods at three spiked *E. coli* concentrations (i.e. c. 10^2 g^{-1} soil; 10^3 g^{-1} soil; 10^5 g^{-1} soil) (n = 9)

Soil type	Enumeration method	Count (log_{10} CFU g^{-1} soil)		
		10^2*	10^3*	10^5*
Sandy clay loam	Petrifilm™ EC	2·03 ± 0·02**	2·96 ± 0·02	5·44 ± 0·03
	m-FC (BCIG)	1·99 ± 0·03	2·99 ± 0·02	5·48 ± 0·02
	MPN	2·06 ± 0·20	3·16 ± 0·06	5·39 ± 0·03
Loam	Petrifilm™ EC	1·95 ± 0·03	3·04 ± 0·02	5·30 ± 0·04
	m-FC (BCIG)	1·86 ± 0·04	3·02 ± 0·01	5·32 ± 0·03
	MPN	2·06 ± 0·02	3·23 ± 0·16	5·04 ± 0·26

*Target concentration.
***E. coli* count mean ± SE.

Table 2 The variance partition of the effects of different methods, soil types, target concentration and their interactions

Source of variation	df	SS	*F* value	*F* > *P*
Concentration	2	185·56	6224·40	<0·0001
Method	2	0·03	0·98	0·3780
Soil	1	0·14	9·37	0·0028
Concentration × soil	2	0·29	9·86	0·0001
Concentration × method	4	0·41	6·98	<0·0001
Soil × method	2	0·01	0·40	0·6722
Soil × method × concentration	4	0·06	1·08	0·3682
Residual	105	1·56		

Recovery of *Escherichia coli* from field soils

Recovery of *E. coli* from field soil samples using Petrifilm™ EC and m-FC (BCIG) methods showed that the m-FC method recorded significantly ($P < 0·05$) higher CFU counts in comparison with the Petrifilm™ EC method irrespective of individual treatments (Fig. 1).

Discussion

Petrifilm™ EC plates for *E. coli* and coliform bacteria have been previously validated for food (Priego *et al.* 2000) and for environmental water samples (Vail *et al.* 2003) but have

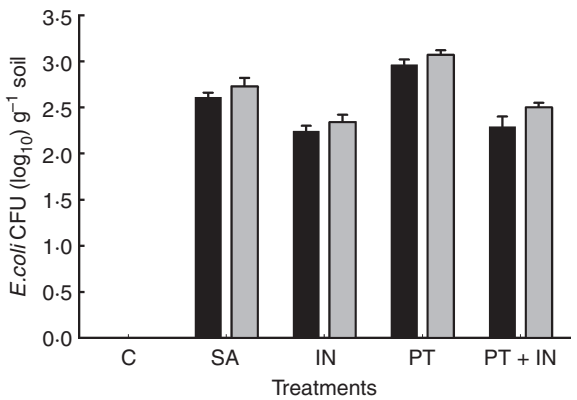

Figure 1 Comparison of Petrifilm™ EC (black bars) and m-FC (grey bars) methods for the recovery of *Escherichia coli* in field soils receiving swine manure (mean ± SE). Field experiment treatments are: No manure control (C), surface applied manure (SA), surface application manure with incorporation (IN), surface application manure on pre-tilled plots (PT), surface application manure on pretilled plots followed by incorporation (PT + IN) (n = 4).

not been tested for environmental samples with suspended soil particles. Vail *et al.* (2003) reported in their study that the Petrifilm™ EC method is comparable to membrane filtration and Quanti-Tray 2000 (based on MPN) for quantifying *E. coli* from water samples in the high-count range but variable when the cell density is low (<300 CFU per 100 ml). In this study, we evaluated the performance of Petrifilm™ EC plates to enumerate soil *E. coli* with the detection limit of 10 CFU g^{-1} of soil. The Petrifilm™ EC method and the two other reference methods we used, m-FC and MPN method in EC-MUG medium, are based on GUD activity that has been found over 94% of *E. coli* (Hansen and Yourassowsky 1984).

Muirhead *et al.* (2004) reported using 10 ml of 1 : 10 dilution in the Colilert Quanti-Tray 2000 method without affecting the reading, which led to the improvement of detection limit to 1 CFU g^{-1} of soil in Colilert Quanti-Tray 2000 method. The high detection limit (10 CFU g^{-1}) of the Petrifilm™ EC method in comparison with MPN-based methods was because of the limits in loading the inoculum (1 ml) on to Petrifilm™ EC plates. In this study, all Petrifilm™ EC plates were incubated at 44 ± 0·5°C to avoid the growth of total coliforms. The incubation at 37°C, as recommended by the manufacturer, resulted in excessive gas bubbles from total coliforms, which interfered with counting of *E. coli*. Suspended soil particles on the gel plate did not interfere with the formation of gas bubbles. Further, it is advisable to load the sample (1 ml) in the middle of the Petrifilm™ EC plate to ensure that it is evenly spread.

The results from the field soil samples indicated that the m-FC (BCIG) method recorded significantly higher CFUs in comparison with the Petrifilm™ EC method ($P < 0·05$). The reason for the difference between the two methods is unclear. One possibility is that the Petrifilm™ EC method includes two confirmation steps because only blue colonies with gas bubbles are counted as *E. coli* (Interpretation guide, 3M Canada); whereas for the m-FC method, all blue colonies were counted, without confirmation of gas production. Despite the fact that 94–96% of *E. coli* strains are found to be glucuronidase positive, some non-*E. coli* bacteria such as *Salmonella*, *Shigella* and *Yersinia* spp., *Citrobacter freundii*, some strains of *Klebsiella oxytoca*, *Serratia fonticola* and *Yersinia intermedia* have been reported as GUD positive (Manafi 2000). Therefore, higher CFUs, counted as blue colonies on m-FC (BCIG) medium, may be false positives for *E. coli*. In the series of samples tested in our laboratory, we observed that the percentage of production of false positives on m-FC (BCIG) media was between 10 and 20% (as detected by indole production of *E. coli*). The other possibility for the lower *E. coli* counts detected by Petrifilm™ EC plates could be because it has been reported that the ability of *E. coli* to produce gas from lactose may be retarded at 44·5°C in comparison with 37°C as a result of thermal stress (Jackson *et al.* 1992; Manafi 2000; Hamilton *et al.* 2005). In this study, all media including Petrifilm™ EC plates were incubated at 44 ± 0·5°C. It is therefore possible that some *E. coli* colonies did not produce gas bubbles, resulting in an underestimation of CFUs on Petrifilm™ EC plates. However, controlled laboratory testing with known concentrations of a known GUD-positive *E. coli* strain inoculated into sterile soil showed no significant difference between the two methods, despite incubation at 44 ± 0·5°C (Table 1). Therefore, it seems likely that incubation of Petrifilm™ EC plates at 44 ± 0·5°C is appropriate, and the presence of GUD-positive non-*E. coli* isolates has lead to false positives using the m-FC method, resulting in the difference between the two methods.

The sensitivity of the Petrifilm™ EC method is a drawback in using this method to count soil *E. coli*. However, the Petrifilm™ EC method can be effectively used for experiments where a detection limit of 10 CFU g^{-1} of soil is sufficient. Furthermore, this method is useful in isolating single colonies for further tests and for genomic studies. The simplicity and the relatively low cost of the Petrifilm™ EC method are additional advantages over other methods.

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