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Suitability of the traditional microbial indicators and their enumerating methods in the assessment of fecal pollution of subtropical freshwater environments

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Twenty-four freshwater sampling sites (11 river water, 6 spring water, and 7 groundwater) were selected from 4 sampling areas located in the northern and central parts of Taiwan. A total of 125 water samples were collected during a 5-month sampling period, and the numbers of total heterotrophic bacteria, total coliforms, fecal coliforms, enterococci, *Aeromonas hydrophila*, and *Salmonella* spp. were enumerated. Besides the traditional membrane filtration method, total coliforms and *Escherichia coli* were also simultaneously enumerated using the Colilert (Quanti-Tray/2000^{TM}) method. On average, 94% and 80% of the water samples assessed with the Colilert method had equal or higher total coliform and *E. coli* counts, respectively, as compared with the membrane filtration method. Furthermore, when m-FC agar was used to enumerate fecal coliforms, 18% of the samples failed to yield the typical bluish colonies, while *E. coli* were counted in the same samples using the Colilert method. The data indicate that the m-FC agar culture method is inadequate for the enumeration of fecal coliforms in subtropical water samples. Significant correlations were observed between the total number of bacteria and various indicator bacteria in river water samples, but no such correlations were found for groundwater and spring water. This finding suggested that the river water was polluted by anthropogenic sources. The counts of total coliforms, *E. coli*, and other indicator bacteria were significantly correlated in all river water samples, while in groundwater and spring water, significant (p<0.01) correlation was only observed with enterococcal counts. The presence of total coliforms/*E. coli* generally implies the presence of fecal pollution possibly including pathogenic enteric bacteria. However, no *Salmonella* spp. were detected in any of the 107 water samples analyzed. The results of this study suggest that the use of these commonly employed microbial indicators for assessing subtropical water quality, especially in a pristine body of water (ie, mountain spring and groundwater), is highly questionable.

Key words: Colilert, fecal indicator bacteria, membrane filtration, subtropical water bodies

Fecal coliforms (or thermotolerant coliforms) are a subgroup of microorganisms that are capable of growth and fermenting lactose with the production of gas and acid at 44.5°C. Because they are almost exclusively found in the waste of warm-blooded animals, this group of bacteria more accurately reflects the presence of fecal contamination than does the total coliform group [1]. So far, countries located in the temperate zone have successfully used freshwater fecal coliforms or *Escherichia coli* as fecal pollution indicators [2]. However, the adequacy of fecal coliforms as indicators for fecal pollution in tropical and subtropical regions has not been properly demonstrated [3]. Several researchers have reported that in tropical water samples, the densities of fecal coliform or *E. coli* do not necessarily coincide with known sources of fecal contamination or the presence of enteric pathogens, such as *Salmonella* spp. [4-7]. Furthermore, it has been shown that in the tropical aquatic environments, fecal coliforms or *E. coli* may originate from non-fecal sources, such as soil, and these organisms can even become autochthonous members of the local microbial community [8-10]. Jen and Bell [11] reported that in tropical water samples, significantly higher numbers of bacterial colonies were able to develop on the plating media when they were incubated at an elevated temperature (ie, 30°C). It is known that a high density of background bacteria may affect the development of specific morphological characteristics of the target colony on selective medium, making the differentiation

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between target and non-target colonies difficult [12]. Santiago-Mercado and Hazen [13] showed that the use of the m-FC agar method to enumerate fecal coliforms in tropical waters resulted in higher recovery of non-target organisms, higher undetected-target error, higher false-positive error, and lower selectivity, than the worst method (ie, MacConkey broth [membrane filtration]) used for temperate waters. This may partially be due to the fact that besides *E. coli*, some non-*E. coli* thermophilic coliform isolates can also grow on plates at 44.5°C [14]. Therefore, it is generally accepted that when the usefulness of fecal coliforms is in doubt, *E. coli* should be used.

The Colilert system, a rapid detection method for waterborne coliform bacteria using o-nitrophenyl-β-D-galactopyranoside and 4-methylumbelliferyl-β-D-glucuronide as substrates, is able to simultaneously detect the presence of total coliform and *E. coli* in water samples [15-17]. Comparable results were obtained between Colilert and the classical membrane filtration and multiple-tube fermentation methods in several studies done in temperate regions [18,19]. However, similar evaluation of the Colilert system in subtropical and tropical regions has not been reported.

In Taiwan, which is located at the junction of tropical and subtropical zones (the Tropic of Cancer crosses the middle of the island), the official method for fecal coliform enumeration uses m-FC agar plates incubated at 44.5°C as described in Standard Methods for the Examination of Water and Wastewater [20]. In this study, we compared the efficiencies of the membrane filtration method and the Colilert method in enumerating total coliforms and fecal coliforms/*E. coli* in subtropical water samples, and evaluated the adequacy of fecal coliforms or *E. coli* as indicators of fecal pollution of river water, groundwater and spring water in the subtropical environment.

Materials and Methods

Locations and sampling

Twenty-four freshwater sampling sites (11 river water, 6 spring water, 7 groundwater) were selected from 4 sampling areas located in the northern and central parts of Taiwan (Fig. 1). From 8 water treatment plants along the Kee-Lung River (area 1), 2 sites along Ta-Chia Shi (area 3), and 3 sites along Ta-Tu Shi (area 4), water samples were collected at the inlet of the treatment plant. In the rural area of Taiwan, spring water or groundwater is frequently piped into a central storage tank and then distributed to the surrounding users without any treatment. In this study, 10 such systems, 4 located at

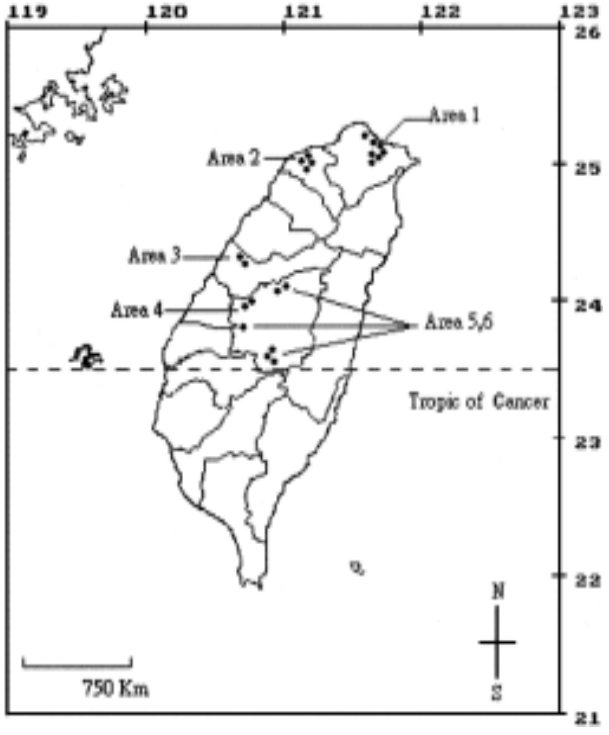

Fig. 1. Sampling sites located in northern and central parts of Taiwan.

Tao-Yuan plateau (area 2) and 7 located in the central mountain region (areas 5 and 6) were selected, and water samples were collected from storage tanks for further study.

At each sampling site, water samples were collected once per month, in some cases twice per month, for 5 consecutive months. From July 5 to November 13, 2001, a total of 55 river water, 30 spring water and 40 groundwater samples were collected. One-liter grab samples were collected at each site using two 500-mL autoclaved polypropylene bottles. After collection, these bottles were placed on ice and transported to the laboratory. A similar set of bottles containing sterile saline was also included in the transport process serving as shipping blanks. Water samples were analyzed immediately, and the entire process, from the moment the first sample was collected to the time the laboratory work was completed, took less than 24 h as specified by the regulations published by the Environmental Protection Agency of the Republic of China. When using the membrane filtration method, duplicate samples were analyzed for each water sample. Data on the daily water temperature, pH value and turbidity were collected from the record of the water treatment plant, while for groundwater and spring water only temperature and pH value data were collected.

Media and enumeration

When necessary, a series of 10-fold dilutions of the water sample were made using sterile phosphate buffer-magnesium chloride (KH_2PO_4, 42.5 mg/L; $MgCl_2$, 405. 5 mg/L) solution as dilution blanks. Total heterotrophic, total coliform, and fecal coliform counts were enumerated by following the official method published by the Environmental Protection Agency of the Republic of China. Heterotrophic plate counts were obtained by spreading 0.2 mL of diluted water sample on plate count agar (Difco Laboratories, Detroit, MI, US), and incubating at 35 ± 1°C for 48 ± 3 h (ROC-EPA Method NIEA E203.52B). For the enumeration of total coliforms, fecal coliforms, enterococci, and *Aeromonas* spp., water samples (minimum of 1 mL) were first filtered through 0.45 μm, 47 mm diameter, gridded membrane filters (ADVANTEC MFS Inc., Pleasanton, CA, US) as described previously [20]. For the enumeration of total coliforms, the membrane was then placed on m-ENDO agar (Difco) and incubated at 35 ± 1°C for 24 ± 2 h. All red colonies with metallic sheen were considered members of the coliform group (ROC-EPA Method NIEA E202.51B). For fecal coliforms, membranes containing m-FC agar (Difco) were incubated at 44.5 ± 0.5°C for 24 ± 2 h, and all blue or partially blue colonies were counted as fecal coliforms (ROC-EPA Method NIEA E214.00C). For enterococci, the filter was initially placed on m-E agar (Difco), incubated at 41 ± 1°C for 48 ± 3 h, and then transferred to EIA agar (esculin, 1.0 g/L; ferric citrate, 0.5 g/L; and agar, 15 g/L) and incubated at the same temperature for 20 min [20]. Red colonies with black or reddish brown precipitation underneath were considered as enterococci. For aeromonads, the filter was incubated on Ampicillin-Dextrin agar (USEPA Method 1605) at 35 ± 1°C for 24 ± 2 h, and colonies of various shades of yellow were marked. Based on the ratios of colonies showing different yellowish color, a minimum of 10 colonies were randomly selected from each of the same color category, and the presence of cytochrome c (ie, oxidase test) and the ability to ferment trehalose were tested. For aeromonads, both of these tests should be positive and the actual numbers of aeromonads were then calculated.

Salmonella spp. concentrations were enumerated by the multiple tube fermentation (MTF) method [20] with a triplicate series of 100-, 10- and 1-mL water samples. The 100- and 10-mL volumes were filtered through 0.45-μm membranes (ADVANTEC MFS), which were then placed into 10 mL of selenite brilliant green enrichment medium (Nissui Inc., Tokyo, Japan). The culture was incubated at 35 ± 1°C for 18 to 24 h. A drop of enriched sample was then added to the center of a Petri plate containing modified semi-solid Rappaport-Vassiliadis medium (Merck KGaA, Darmstadt, Germany), incubated at 42 ± 1°C for 24 h, and examined for an opaque halo indicating growth around the sample. A loopful of sample was taken from the opaque zone and inoculated on to the *Salmonella-Shigella* agar (Difco) and incubated at 37 ± 1°C for 24 h. Colorless colonies with a black center were selected, recultured on brain-heart infusion agar (Difco), and the bacteria were tested for flagella H agglutination (Seiken, Tokyo, Japan).

The Colilert (Quanti-Tray/2000™) method (IDEXX Laboratories, Inc., Westbrook, ME, US) was used according to the manufacturer's instructions. The prepacked, dehydrated Colilert medium was first dissolved in 100 mL of water sample, then poured into Quanti-Tray/2000™ and the trays were heat sealed using Quanti-Tray Sealer (IDEXX). The inoculated trays were incubated at 35 ± 1°C for 24 h, yellow wells were counted and the number of coliforms was calculated using a most probable number (MPN) table. The number of *E. coli* cells was similarly calculated from the number of fluorescing wells (366 nm).

Accuracy tests and positive control tests were done for *E. coli* ATCC 11775, *Enterococcus faecalis* ATCC 19433, and *Aeromonas hydrophila* ATCC 7966 *Salmonella choleraesuis* subsp. *choleraesuis* (serotype *enteritidis*) ATCC 13076.

Results and Discussion

In the present study, a total of 125 water samples were collected and analyzed by both the classical methods (ie, the membrane filtration and the multiple tube fermentation methods) and the Colilert method. Samples with counts exceeding detection limits or duplicate samples with log values of the calculated counts differing by more than 0.4 were considered invalid and eliminated from the analysis.

The presence of total coliforms is the universal microbial indicator for either drinking or source water. When total coliforms in river water, groundwater, and spring water were enumerated using both the membrane filtration (m-ENDO) and the Colilert method, 58%, 60% and 57% of the samples showed equivalent results, respectively (Table 1). In 36%, 38%, and 33% of the river water, groundwater, and spring water samples, respectively, higher (ie, by more than 0.5 log value) total coliform counts were detected by the Colilert method than by the membrane filtration method (Table 1). Fricker *et al* [19] also reported that the recovery of coliforms in potable water was higher using the Colilert

Table 1. Enumeration of coliform bacteria in source water by membrane filtration and Colilert methods

Water type	No. of samples	No. of samples with			
		Equivalent, positive results	Equivalent, negative results	Higher recovery by Colilert method	Higher recovery by standard method
All	125				
River water	55	31	1	20	3
Groundwater	40	12	12	15	1
Spring water	30	17	0	10	3

as compared with the membrane filtration method. Nevertheless, care should be taken in interpreting these data, since it has been shown that the presence of *Aeromonas* sp., an organism commonly found in aquatic environments, would lead an aged (ie, 4 weeks short of shelf-life expiration) Colilert reagent to overestimate coliforms [21]. However, not all coliforms are enteric, for example *Enterobacter cloacae* and *Citrobacter freundii* are both environmental coliforms. Hence, fecal coliforms or *E. coli* (in some countries) are frequently included in the monitoring process and their presence used to indicate fecal contamination. Researchers have shown that when detecting *E. coli* in water samples, the numbers obtained with the Colilert system were equivalent to those obtained by the membrane filtration method [18,19], or multiple-tube fermentation method [16,17]. However, in this study, when the membrane filtration and the Colilert methods were used to enumerate fecal coliform/*E. coli*, only 34%, 63% and 38% of the samples taken from river water, groundwater, and spring water, respectively, showed equivalent results (Table 2). On the other hand, 25%, 21%, and 23% of the samples from river water, groundwater, and spring water, respectively, yielded invalid numbers of fecal coliforms when measured by the membrane filtration method. Furthermore, out of 90 samples with valid data, 20 showed higher recovery of *E. coli* by the Colilert method. In 16 of these, no typical bluish colonies were detected on m-FC agar even though in the same samples *E. coli* was detected using the Colilert method. Elmund *et al* [22] reported that in wastewater effluent, thermotolerant *Klebsiella pneumoniae* would interfere with the recovery of fecal coliforms on m-FC media, while this interference was not observed in the enumeration of *E. coli* by the Colilert method. These data indicate that the m-FC agar medium does not allow satisfactory enumeration of fecal coliforms in subtropical water samples.

Generally speaking, a higher number of total bacteria in water samples does not necessarily mean that the water is polluted by fecal material. In this study, the numbers of total bacteria and numbers of various indicator bacteria in river water samples were significantly correlated which suggested that the river waters collected in this study were polluted by fecal materials (Table 3). However, in spring water and groundwater, although *E. coli* were detected in more than 60% of the samples, we failed to observe any correlation between total counts and the indicator bacteria (Table 4). Besides total coliforms and fecal coliforms, it has been proposed that enterococci [23] and *A. hydrophila* [24,25] be included as indicator bacteria in tropical water quality assays. Studies in mountain streams or in water samples that have been exposed to low levels of pollution found no correlations between aeromonads and fecal indicator organisms [26, 27]. Hence, these studies concluded that *A. hydrophila* was an indigenous bacterium to aquatic environments, with a distribution unrelated to human pollution. Similar results (ie, no correlations between aeromonads and fecal indicator bacteria) were also observed in the present study when groundwater and spring water were analyzed (Table 4). Significant correlations were observed between numbers of total coliforms, *E. coli*

Table 2. Enumeration of fecal coliforms/*E. coli* in source water by membrane filtration and Colilert methods

Water type	No. of samples	No. of samples with			
		Equivalent, positive results	Equivalent, negative results	Higher recovery by Colilert method	Higher recovery by standard method
All	117[a] (27)[b]				
River water	53 (13)	17	1	15	7
Groundwater	38 (8)	2	22	2	4
Spring water	26 (6)	8	2	3	7

[a]Out of 125 samples, 2 river water , 2 ground water, and 4 spring water samples were improperly diluted when using the Colilert method.
[b]Numbers in parenthesis are the number of samples yielding invalid data.

Table 3. Relationships between different indicator bacteria in subtropical river water

Indicator	*r*-value (level of significance)			
	Total bacterial count	Total coliforms		*E. coli*
		m-ENDO agar	Colilert method	(Colilert method)
Total coliforms (m-ENDO agar)	0.655 (p<0.01)	ND	0.662 (p<0.01)	0.711 (p<0.01)
Enterococci	0.756 (p<0.01)	0.563 (p<0.01)	0.501 (p<0.01)	0.591 (p<0.01)
Aeromonas hydrophila	0.642 (p<0.01)	0.351 (p<0.01)	0.267 (p<0.05)	0.248 (p<0.05)
E. coli (Colilert method)	0.763 (p<0.01)	ND	0.619 (p<0.01)	ND

Abbreviation: ND = not determined.

and other indicator bacteria tested in river water samples, whereas in groundwater and spring water, significant (p<0.01) correlation was only observed between total coliforms (Colilert)/*E. coli* and enterococci (Tables 3 and 4). It is not clear why in groundwater and spring water a positive correlation was not observed between the numbers of total coliforms (m-ENDO) and enterococci. The presence of *E. coli* or enterococci generally means fecal pollution in possible conjunction with the existence of pathogenic enteric bacteria in the respective environment [23]. Townsend [6] reported that in pools located in tropical northern Australia, significant correlations were observed between indicator organisms and *Salmonella*, while total coliform and enterococci counts were more consistent than fecal coliform counts. In this study, even though *E. coli* and enterococci were detected in 80% of the water samples, *Salmonella* spp. was not detected in any of the 107 water samples analyzed. Nevertheless, the absence of *Salmonella* spp. in the present samples does not necessarily imply the absence of other enteric pathogens, and the presence of *E. coli* does indicate that some of the water samples, especially spring water and groundwater, failed to comply with the existing portable water standard. In some of the studies dealing with the distribution of *E. coli* and enterococci in tropical and subtropical environments, the data seem to indicate that these organisms were autochthonous to the above environments, and this effectively excludes their use as indicator bacteria [8,9,28].

In the present study, water samples were collected in the summer and early fall, when surface water temperature varied by 3 to 5°C between different months and the average pH values of water samples was 7.1 ± 0.4 with the exception of samples taken from Tao-Yuan plateau (area 2) which had a mean pH value of 6.4 ± 0.2. Turbidities under the influence of weather (ie, rain) varied greatly between water samples collected at different months. However, no correlations were observed between the numbers of the indicator bacteria and the above water properties. When using the classical membrane filtration method and the Colilert method to enumerate total coliforms in subtropical river water samples, the counts obtained with the Colilert method were always similar or larger than those obtained with the membrane filtration method, which suggests that the classical method provides a better and more stable recovery. When the membrane filtration method was used to enumerate fecal coliforms in subtropical water samples, more than 20% of the samples either yielded invalid results or entirely failed to develop the typical bluish colony. It was also found that in subtropical groundwater and spring samples, the numbers of total coliforms obtained by the membrane filtration method

Table 4. Relationship between different indicator bacteria in subtropical spring, and ground waters

Indicator	*r*-value (level of significance)			
	Total bacterial count	Total coliforms		*E. coli*
		m-ENDO agar	Colilert method	(Colilert method)
Total coliforms (m-ENDO agar)	0.121	ND	0.611 (p<0.01)	0.007
Enterococci	0.114	0.104	0.707 (p<0.01)	0.802 (p<0.01)
Aeromonas hydrophila	0.150	0.073	0.136	0.226
E. coli (Colilert method)	0.057	ND	0.645 (p<0.01)	ND

Abbreviation: ND = not determined.

failed to show any positive correlations with the other indicator bacteria. All these results indicated that the adequacy of the membrane filtration method when used to assay subtropical water samples is in doubt. Although the Colilert method has shown better enumeration efficiency than the membrane filtration method, its accuracy when used to enumerate total coliforms and *E. coli* in tropical or subtropical water samples remains to be established. Total coliforms/*E. coli* have generally been considered as the most efficacious indicators for public health protection against waterborne pathogens. However, our data indicated that the suitability of these indicators in subtropical aquatic environments, especially in pristine bodies of water (ie, mountain springs and groundwater), needs careful re-evaluation.

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