COMPARISON OF THERMOTOLERANT COLIFORMS AND *ESCHERICHIA COLI* DENSITIES IN FRESHWATER BODIES

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ABSTRACT

Fecal bacterial indicator analyses have been widely used for monitoring the water quality. This study was designed to determine the ratio between the density of *Escherichia coli* and other Thermotolerant Coliforms (TtC) bacteria from freshwater samples collected for a two-year period of monitoring. TtC were enumerated by membrane filtration on mFC agar. *E. coli* enumeration was done by two methods: TtC colonies identified in mFC were inoculated in EC-MUG or water samples were filtered and inoculated in modified mTEC agar media, and both methods were compared for quantitative recovery of *E. coli.* The results pointed out a mean percentage of *E. coli* among other thermotolerant coliforms (*E. coli*/TtC ratio) of 84.3% in mFC media. Taking these results into account, a mandatory standard of 1000 thermotolerant coliforms would correspond to 800 *E. coli* and the adoption of these *E. coli* based standards will represent a major improvement for the monitoring of freshwater quality.

Key words: Thermotolerant Coliforms (TtC), *E. coli*, Water quality, microbiological standards

INTRODUCTION

Coliform bacteria are the commonly used bacterial indicator for sanitary quality of water (18, 19). They are defined as members of genera or species within the family Enterobacteriace capable of growth at 37º C (total coliforms) or 44º - 45º C (thermotolerant coliforms) that possess β-galactosidase (9). Coliform bacteria are abundant in the feces of warm-blooded animals but can also be found in soil, aquatic environments and vegetation. Unlike other coliform bacteria, *Escherichia coli* are almost exclusively of fecal origin and can be detected in elevated densities in human and animal feces, sewage and water subjected to recent fecal pollution. It is therefore considered the best fecal indicator microrganism (9, 24).

Fecal bacteria have been used as an indicator to the possible presence of pathogens in surface waters and the risk of disease based on epidemiological evidence of waterborne diseases. Consequently, because of the difficulties to detect the many possible pathogens (such as *Salmonella* sp, *Shigella* sp, diarrheogenic *E. coli*, *Giardia lamblia*, *Cryptosporidium parvum* and enteric viruses) concentrations of fecal bacteria including thermotolerant coliforms, enterococci and *E. coli*, are used as the primary indicators of fecal contamination (23).

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Studies suggest that *E. coli* is a more reliable indicator of fecal pollution and the occurrence of pathogens in water than total and thermotolerant coliforms (9, 16). Therefore, the use of *E. coli* as the main bacterial indicator instead of other coliform bacteria has been proposed in water quality monitoring programs which tailors the microbiological quality of water.

In fact, the United States Environmental Protection Agency recommends *E. coli* or enterococci to replace fecal coliform bacteria in state water quality standards based on the studies which showed a statistically significant relationship between *E. coli* and enterococci concentration in freshwater and rates of swimming-related illness. These studies, carried out from 1979 to 1982 in freshwater beaches of Lake Erie, Pennsylvania and Keystone Lake, Oklahoma, clearly demonstrated a higher risk of gastrointestinal illness at those beaches having the highest degree of fecal contamination. Among the fecal contamination indicators eveluted (fecal coliforms, *E. coli* and enterococci) *E. coli* and enterococci showed the best correlation with swimming-associated gastrointestinal symptons, whereas fecal coliform showed little or no correlation (20). The current EPA criteria for recreational water determines that the geometric mean of the indicated bacterial densities should not exceed 126 for *E. coli* or 33 for enterococci in 100 mL of freshwater, based on a statistically number of samples (generally not less than 5 samples equally spaced over a 30-day period) (21). Considering the previous criteria used by USEPA for TtC enumeration (geometric mean of 200/100 mL of freshwater), the current *E. coli* density of 126/100 mL corresponds to 63% of total TtC density (23).

Recently, a study performed by Garcia-Armisen et al. (12) showed a mean percentage of 77% of *E. coli* among other TtC in 166 samples collected from Sena river (France). The correlation found in this study suggests a scientifically based parameter that can be used to convert TtC historical data, since European Union (EU) will adopt the *E. coli* as the microbiological criteria to state recreational water quality (10).

Apart from that, many countries still use TtC enumeration to provide a legal basis to State water quality. For example, according to Canadian Legislation either *E. coli* or fecal coliforms can be used if experience shows that greater than 90% of the fecal coliforms are *E. coli* (15).

The surface water quality in Brazil is mainly regulated by federal laws that define water classification and guidelines based on water uses. The Rule 357/2005 from the National Council of the Environment (7) establishes water microbiological standards for different purposes based on thermotolerant coliforms but allows the State Environmental Agencies to adopt *E. coli* for such evaluation by setting their own criteria. Regarding recreational activities there is a specific regulation (6) that has already fixed standards for TtC, *E coli* and enterococci.

The present study was designed to evaluate the ratio between *E. coli* and TtC in water bodies as an effort to scientifically support a future redefinition on the current standards that define sanitary water quality. The knowledge of *E. coli*/TtC ratio suitable for freshwater sites will allow the conversion of historical microbiological records expressed in TtC, into *E. coli*, providing a comparison parameter for present and future water monitoring. It will also be possible to standardize more stringent criteria to monitor the water quality throughout the water system supply.

MATERIALS AND METHODS

Sampling

This study was carried out during two years, from January 2004 to December 2005, in 25 sites from different water bodies across Sao Paulo State (Table 1). Sampling was performed bimonthly in 18 sites in rivers and monthly in seven sites used as recreational areas, located in two reservoirs, amounting to 380 samples. These water bodies were selected based on their level of contamination and sources of pollution.

The water samples were collected in sterile 500 mL, wide mouth, plastic bottles, according to American Public Health Association (2), kept on ice for transportation and processed within 24 h.

Table 1. Geographical location of the 25 collection sites

Water body	Collection site	Geographical location	
		Latitude	Longitude
Paraiba river	1	23 18 48	45 58 20
Grande river	2	23 24 42	45 06 39
Pardo river	3	21 06 00	47 45 44
	4	22 57 14	49 52 02
Atibaia river	5	23 06 12	46 32 42
Jaguari river	6	22 41 56	47 09 07
Corumbataí river	7	22 38 01	47 40 58
Capivari river	8	23 00 22	47 06 00
	9	23 31 11	46 44 47
Tietê river	10	23 32 55	46 08 09
	11	22 57 25	47 49 23
Baquirivú-Guaçú river	12	23 24 50	46 23 05
Jundiaí river	13	23 38 56	46 11 48
Mogi river	14	23 51 08	46 22 41
Mogi-Guaçú river	15	21 00 44	48 10 20
Sorocaba river	16	23 10 21	47 47 47
Preto river	17	20 37 40	49 21 18
Aguapeí river	18	21 40 35	50 35 21
	19	23 46 37	46 32 01
Billings reservoir	20	23 46 18	46 30 50
	21	23 46 37	46 37 09
	22	23 40 30	46 43 51
	23	23 41 57	46 44 41
Guarapiranga reservoir	24	23 41 48	46 43 11
	25	23 42 53	46 42 58

Bacteriological Analysis

Throughout the monitoring period 380 water samples were analyzed by the membrane filtration technique. The enumeration of thermotolerant coliforms was performed using a lactose-based agar (mFC: membrane fecal coliform) recommended by the American Public Health Association (2) since 1971. Typical colonies on mFC agar were differentiated to *E. coli* with EC-MUG (*Escherichia coli*-methylumbelliferyl-β-D-glucuronide) medium.

The 166 samples collected during 2005 were also analyzed directly for *E. coli* using modified mTEC (membrane Thermotolerant *Escherichia coli*) agar (4, 22). This enzymatic based medium can provide reliable confirmed results in 24 h (8) and has been employed by others authors to study the *E. coli*/thermotolerant coliform ratio (11, 14).

According to the expected degree of fecal contamination different volumes of each sample (70, 25 and 5 mL) or decimal dilutions of 1 mL were filtered in 4.5 cm diameter, 0.45 μm-pore-size, gridded, mixed ester membrane filters, in order to achieve a range of 20 to 60 countable TtC or *E. coli* Colony Forming Units (CFU). The filters containing different volumes of the samples were transferred to the surface of the mFC or mTEC plates. mFC plates were incubated at $44.5 \pm 0.2°$ C for 20 ± 2 h. Modified mTEC plates were pre-incubated at $35 \pm 0.5°$ C for 2 h and then incubated at $44.5 \pm 0.2°$ C for 22 h. Blue colonies on mFC were counted as TtC. According to the classical procedures stated for verification or differentiation of colonies in the membrane filtration technique (2), ten typical blue coliform colonies on mFC medium were randomly selected from membrane filter for each tested sample. Each selected colony was inoculated in EC-MUG medium and incubated at $44.5 \pm 0.2°$ C for 24 h. Simultaneously, colonies submitted to differentiation on EC-MUG were inoculated in eosine-methylene blue agar (EMB agar). Coliform-typical

colonies on EMB agar, originated from EC-MUG-negative colonies, were identified with API 20E (Biomérieux, France). Growth in the EC-MUG was examined for fluorescence under long-wavelength (365 nm) UV light to detect β-glucuronidase activity. Colonies that were positive for β-glucuronidase activity were considered *E. coli* colonies. With the modified mTEC media, red-magenta colonies were identified as *E. coli* CFU without further confirmative tests.

Statistical Analysis

Thermotolerant coliform (mFC agar) and *E. coli* colony counts from both media (mFC + EC-MUG and modified mTEC) were converted to log 10 values to ensure data normality. In order to compare the analysis methods, mFC + EC-MUG vs. modified mTEC, a paired t-test was performed. Linear regressions between TtC counts on mFC agar and *E. coli* counts with EC-MUG as well as with E. *coli* counts obtained with the modified mTEC agar were performed in order to determine the proportion *E. coli*/ TtC obtained by

these two methods.

RESULTS AND DISCUSSION

The mean percentage of *E. coli* isolated from mFC media and further confirmed with the EC-MUG test was 84.3%. Figure 1 shows the regression line depicting a strong positive correlation between TtC counts on mFC agar and *E. coli* counts with EC-MUG with a correlation coefficient (r) of 0.996 suggesting that near of 80% of TtC colonies isolated from mFC media are *E. coli* isolates in the analyzed water samples.

When the results obtained with both methodologies used to quantify *E. coli* CFU were compared, the paired t-test demonstrated that there were no significant differences between colonies counts obtained with the mFC + EC-MUG technique and the counts obtained with mTEC isolation. Therefore, the membrane filtration method using modified mTEC agar can be used to monitor *E. coli* for freshwater quality.
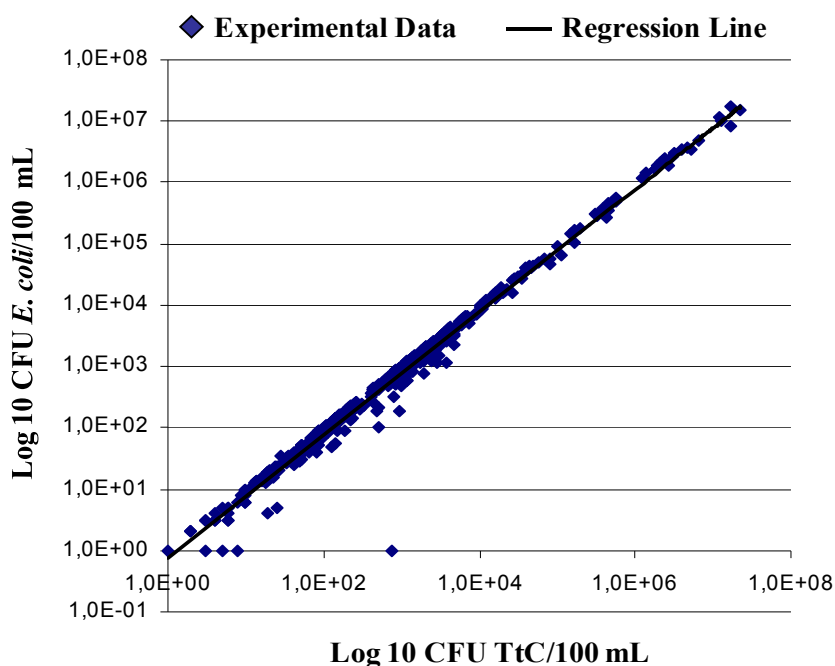

Figure 1. Log regression line between thermotolerant coliform concentrations on mFC agar and *E. coli* concentrations on EC-MUG

Isolated colonies that displayed a negative β-glucuronidase activity with EC-MUG medium were tested for enteric bacteria with the API-20E system (BioMérieux, France).

The distribution of MUG-negative coliforms isolated from mFC media is presented in Table 2. It was observed a predominance of the *Klebsiella* genre (60.6% of *K. pneumoniae*). Also, 25.9% of the colonies were identified as MUG-negative *E.*

coli bacteria. These data are in accordance with Bordalo (5) who demonstrated a predominance of *Klebsiella* genre bacteria among MUG-negative bacteria isolated from aquatic environments. Alonso *et al*. (1) compared the performance of chromogenic culture media for the recovery of *E. coli* and TtC and found that *Citrobacter freundii* was the dominant species in river and marine water samples.

Table 2. Number, percentage and species of thermotolerant coliforms isolated on mFC agar and negative on EC-MUG

SPECIES	NUMBER OF ISOLATE/PERCENTAGE (%)
Klebsiella pneumoniae pneumoniae	354/60.6
Escherichia coli (MUG -*)*	151/25.9
Enterobacter cloacae	20/3.4
Klebsiella terrigena	20/3.4
Enterobacter aerogenes	15/2.6
Citrobacter freundii	14/2.4
Pantoea sp	2/0.3
Klebsiella pneumoniae ozaenae	2/0.3
Klebsiella ornithinolytica	1/0.2
Stenotrophomonas maltophila	1/0.2
Citrobacter youngae	1/0.2
Kluyvera sp	1/0.2
Leclercia adecarboxylata	1/0.2
Serratia liquefaciens	1/0.2
TOTAL	584/100

Twelve samples displayed a higher concentration of *E. coli* in mTEC media than the TtC concentration found in mFC media, which means that for these samples the proportion *E. coli*/TtC ranged from 1.3 to 3.19 (data not shown). Since *E. coli* is a member of the thermotolerant coliform group, this ratio should not be superior to 1.0. These results can be explained by the greater sensitivity of modified mTEC agar (a chromogenic method based on the detection of β-glucuronidase) over mFC agar (a lactose based method).

In a recent methodology review, Hamilton et al. (14) discussed the *E. coli*/TtC ratio proportion found in several studies that analyzed water samples from different environments, showing that this proportion could overpass the ideal limit of 1.0 in many cases. In fact, the authors conclude that the differences observed could be due to the recovery range obtained with different methodologies, and also, that

media containing chromogenic substrates are usually more efficient in *E.coli* recovery. The relationship between enzymatic and traditional methods to detect coliforms in freshwaters was studied by George et al. (13). They used the fluorogenic substrates MUGal (4 methylumbelliferyl-β-D-galactoside) and MUGlu (4 methylumbelliferyl-β-D-glucuronide) and verified a significative correlation between both methods. According to their study viable but not culturable *E. coli* could be detected by the enzymatic method. However, Francy and Darner (11) compared *E. coli* concentrations in recreational freshwaters using traditional and modified mTEC agar and concluded that the chromogenic medium recovered less bacteria.

Other authors have also studied the proportion of these fecal contamination indicators aiming to convert historic fecal coliform bacteria data to estimate *E. coli* densities or to

establish *E. coli* values to replace thermotolerant coliforms criteria. Rasmussen and Ziegler (17) observed an *E.coli*/TtC ratio of 0.77 during a study performed to evaluate the sanitary quality of selected Kansas streams. Garcia Armisen et al. (12) reported values of 77% for *E.coli*/TtC ratios in differently contaminated freshwater samples.

The linear regression models applied to the results obtained in the present study demonstrated an *E. coli*/TtC proportion of 80%. As an example, taking this proportion into account, mandatory standards of 1000 thermotolerant coliforms for freshwater would correspond to approximately 800 *E. coli*. In order to use more stringent criteria, the inferior confidence level of the linear regression model (620 *E. coli*) could be adopted instead.

As *E. coli* is recommended by many studies (9, 12, 14, 16) and publications (3, 23, 24) as a better indicator to protect public health than thermotolerant coliforms, the adoption of *E. coli* based standards will represent a major improvement for the microbiological monitoring of water quality intended to be used as drinking water source, irrigation of crops and aquaculture.

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