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Comparison and Recovery of *Escherichia coli* and Thermotolerant Coliforms in Water with a Chromogenic Medium Incubated at 41 and 44.5°C

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This study compared the performance of a commercial chromogenic medium, CHROMagarECC (CECC), and CECC supplemented with sodium pyruvate (CECCP) with the membrane filtration lauryl sulfate-based medium (mLSA) for enumeration of *Escherichia coli* and non-*E. coli* thermotolerant coliforms (KEC). To establish that we could recover the maximum KEC and *E. coli* population, we compared two incubation temperature regimens, 41 and 44.5°C. Statistical analysis by the Fisher test of data did not demonstrate any statistically significant differences ($P = 0.05$) in the enumeration of *E. coli* for the different media (CECC and CECCP) and incubation temperatures. Variance analysis of data performed on KEC counts showed significant differences ($P = 0.01$) between KEC counts at 41 and 44.5°C on both CECC and CECCP. Analysis of variance demonstrated statistically significant differences ($P = 0.05$) in the enumeration of total thermotolerant coliforms (TTCs) on CECC and CECCP compared with mLSA. Target colonies were confirmed to be *E. coli* at a rate of 91.5% and KEC of likely fecal origin at a rate of 77.4% when using CECCP incubated at 41°C. The results of this study showed that CECCP agar incubated at 41°C is efficient for the simultaneous enumeration of *E. coli* and KEC from river and marine waters.

The significance of various coliform organisms in water has been and remains an extensively studied subject. Since fecal coliforms are not defined taxonomically, *Escherichia coli* is the only member species for which standardized data exists. According to Leclerc et al. (16), the coliform species of fecal origin and their isolation frequency in human feces (15) are as follows: *E. coli*, 100%; *Citrobacter diversus*, 70%; *Citrobacter amalonaticus*, 70%; *Citrobacter freundii*, 70%; *Klebsiella pneumoniae*, 49%; *Klebsiella oxytoca*, 49%; *Enterobacter cloacae*, 9%; and *Enterobacter aerogenes*, 9%. The following species will probably be of nonfecal origin (16): *Klebsiella trevisamii*, *Enterobacter agglomerans*, *E. gergoviae*, *E. sakazakii*, *Hafnia alvei*, *Serratia marcescens*, *S. liquefaciens*, *S. marinorubra*, and *S. odorifera*. Unfortunately, the specificity of fecal coliforms as indicators of fecal pollution varies considerably depending on the environmental conditions and the presence of industrial effluent (3). Some authors (7, 19) have suggested that the term "fecal coliforms" should be excluded from microbiology. "Thermotolerant coliforms" (TTC) is considered to be a more appropriate description of these organisms (4). The acronym KEC is introduced in this study to describe the β-galactosidase positive thermotolerant coliforms other than *E. coli*. On the other hand, *E. coli* is generally considered a more reliable sanitary indicator (8). Therefore a direct enumeration of *E. coli* is needed to monitor fecal contamination in surface waters (20).

Conventional procedures for verification of fecal coliforms as *E. coli* when fecal contamination is questionable are very laborious and time-consuming (11). The use of media containing chromogenic and fluorogenic substrates for the enzymes β-galactosidase (LAC) and β-glucuronidase (GUD) for simultaneous detection of coliforms and *E. coli* is increasing (23). One such medium, CHROMagarECC, developed by CHROMagar, simultaneously detects coliforms and *E. coli*.

Addition of pyruvate to media improves the recovery of injured bacteria through its action in degrading peroxides and promoting cell recovery (18). Incubation temperature should also be considered in an effort to recover the maximum number of fecal coliform strains. Some authors (17) have indicated that the optimal temperature for incubation of all fecal coliform strains is 41°C.

This study was designed to evaluate CHROMagarECC (CECC), modified by the addition of sodium pyruvate (CECCP), for the simultaneous enumeration of *E. coli* and KEC by the membrane filtration technique in river and marine waters. To help maximize the recovery of both *E. coli* and KEC populations, we incubated the bacteria at 41 and 44.5°C.

Sampling. A total of 50 water samples were collected from different environmental sources in the area of Valencia, Spain. The water samples were as follows: 7 samples from Turia River (site TR) near the Valencia drinking-water treatment plant, 3 samples from the Jucar River (site JR), 20 samples of seawater from Malvarrosa beach (sites M1 and M2), and 20 samples of seawater from Alboraya beach (sites A1 and A2). All samples were collected in sterile glass bottles, refrigerated, and assayed within 2 h after collection.

Medium comparison. All membrane filtration analyses were carried out in duplicate, and bacterial concentrations were reported as the mean of these replicates. Specific incubation and enumeration procedures for each test medium were as follows. The water samples were diluted, and duplicates of each dilution were filtered through sterile 0.45-μm-pore-size membranes (Whatman, Maidstone, England). One membrane

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of each set of duplicates was placed on a pre-treated layer of CECC agar (CHROMagar Microbiology, Paris, France) in a 47-mm-diameter petri dish. The second membrane of each duplicate pair was placed on CECC supplemented with 0.05% (wt/vol) sodium pyruvate (Sigma Chemical Co., St. Louis, Mo.) (CECCP agar). A set of duplicate membranes placed on CECC and CECCP media was incubated at 41°C, and a second set of membranes was incubated at 44.5°C. All blue colonies (LAC^+ GUD^+) were counted as *E. coli*, and all red colonies (LAC^+ GUD^-) were counted as KEC. The number of TTCs was calculated as the sum of blue and red colonies. For comparison, duplicates of each dilution were processed by a standard membrane filtration method for fecal coliforms (24). The membranes were placed onto membrane filtration lauryl sulfate-based medium (mLSB; Oxoid, UNIPATH Ltd., Basingstoke, England) solidified by adding 1.2% agar (mLSA). The membranes placed on mLSA were incubated in a water bath at 44.5°C for 24 h, and duplicate membranes were incubated for 30°C for 4 h (resuscitation) followed by 44.5°C for 20 h. Fecal coliforms, if present, appeared as yellow colonies.

Biochemical identification. A total of 521 colonies from the most appropriate dilutions of CECCP agar incubated at 41°C were identified. This medium and the 41°C incubation temperature was selected for the specificity study because these conditions gave the best overall recovery of *E. coli*, KEC, and TTC in our initial experiments. *E. coli* colonies (blue), KEC colonies (red), and nontarget colonies (white) were tested for cytochrome oxidase activity. *E. coli* colonies were identified by using the Microbact 12E system (Medvet Sxcience Pty Ltd., Adelaide, Australia). KEC and nontarget colonies were identified by using the API 20E system (BioMérieux, Marcy l'Etoile, France).

Statistical analysis. Results were analyzed by linear regression to verify the linearity of the relationship between *E. coli* and KEC isolated on CECCP agar incubated at 41°C. To examine the medium performance (CECCP agar incubated at 41°C) over a range of sample types and concentrations, the samples were grouped by sample site; by *E. coli*, KEC, and TTC counts; and by incubation temperatures. Overall significant effects and interaction effects were tested by two-way analysis of variance ANOVA. Each procedure category was further separated by one-way ANOVA. When the *P* value of the F-test was less than 0.05, a multiple-range test was used to find which means were significantly different. All *E. coli*, KEC, and TTC counts were converted to $\log_{10}$ values for statistical analysis. All statistical methods were performed with Statgraphics Plus 2.1 software.

Enumeration of *E. coli*, KEC, and TTC. The 10 river water samples tested had *E. coli* counts ranging from 10 to 2.9×10^3 CFU/100 ml, with an arithmetic mean of 3.3×10^2 CFU/100 ml (CECCP incubated at 41°C). The 40 marine samples analyzed had *E. coli* counts ranging from 5 to 7.2×10^3 CFU/100 ml with an arithmetic mean of 1.2×10^3 CFU/100 ml. Analysis of variance of the *E. coli* data demonstrated no difference between temperatures and media, with the F test yielding a *P* value of 0.994 for river samples and a *P* value of 0.666 for marine samples. It should, however, be noted that CECCP incubated at 41°C exhibited the highest *E. coli* counts. Ho and Tam (14) found that the performance of CHROMagar Liquid ECC was comparable to mLSB plus urea but the chromogenic medium was superior to the conventional medium in sensitivity and specificity. Sartory (24) reported that incorporation of sodium pyruvate into mLSA resulted in significant improvements on membrane filtration recovery of *E. coli* from chlorinated drinking-water samples.

Positive correlations ($P = 0.01$) between the concentration of *E. coli* and KEC were found at sites TR ($r = 0.99$), M1 ($r = 0.96$), M2 ($r = 0.88$), A1 ($r = 0.97$), and A2 ($r = 0.98$). At these sites, *E. coli* represented on average 22.0 to 35.2% of the TTC population. At site JR, there was no correlation ($r = -0.15$) between *E. coli* and KEC. The significant correlation coefficients obtained between *E. coli* and KEC at sites TR, M1, M2, A1, and A2 confirm the fecal origin of KEC at these sites, although KEC at site JR were probably of nonfecal origin. In our study, *E. coli* represented a range of 8.8 to 35.2% of the TTC population. Bordalo (5), studying different types of water ranging from polluted seawater to unpolluted freshwater, found that *E. coli* made up 82% of the TTC. However, in this study, *E. coli* level was compared with TTC counts in samples incubated at 44.5°C. This temperature inhibited the growth of all the coliforms of nonfecal origin as well as a large number of fecal coliforms (16). A temperature of 41°C is near the optimum temperature of fecal coliform growth (16, 17).

The 10 river water samples had KEC counts ranging from 10 to 5.1×10^3 CFU/100 ml. The 40 marine water samples analyzed had KEC counts ranging from 5 to 1.9×10^4 CFU/100 ml. KEC counts on CECC were compared with KEC counts on CECCP incubated at both incubation temperatures (41 and 44.5°C). Data from the ANOVA performed on KEC counts

TABLE 1. Summary of enumeration results for TTC by medium and incubation temperature

Medium and temp (°C)	No. of samples	TTC counts (CFU/100 ml)			
		Mean	SD	Minimum	Maximum
River water					
CECCP, 41	10	1.2×10^3	2.4×10^3	2.7×10^2	7.9×10^3
CECC, 41	10	1.1×10^3	2.3×10^3	2.3×10^2	7.6×10^3
CECCP, 44.5	10	4.8×10^2	1.0×10^3	8.4×10	3.3×10^3
CECC, 44.5	10	4.2×10^2	9.2×10^2	7.0×10	3.0×10^3
mLSB, 44.5	10	3.9×10^2	1.0×10^3	2.5×10	3.3×10^3
Marine water					
CECCP, 41	40	4.6×10^3	7.3×10^3	6.5×10	2.6×10^4
CECC, 41	40	3.7×10^3	6.1×10^3	4.0×10	2.1×10^4
mLSB, 44.5	40	1.7×10^3	2.9×10^3	1.5×10	1.0×10^4
mLSB, 30 and 44.5	40	1.6×10^3	2.8×10^3	2.0×10	1.0×10^4
CECCP, 44.5	40	1.5×10^3	2.4×10^3	1.3×10	9.3×10^3
CECC, 44.5	40	1.3×10^3	2.2×10^3	1.0×10	9.3×10^3

[a] Listed in ascending order according to mean. For a complete description of media and incubation temperatures, see the text.

TABLE 2. Identification of colonies picked from river water samples incubated on CECCP at 41°C

Species	No. of isolates identified in each category (% of total no. in the category)			Total no. of isolates (% of total)
	Blue colonies (*E. coli*)	Red colonies (KEC[a])	Other colonies (background[b])	
Escherichia coli	95 (91.3)	3 (2.4)	2 (2.6)	100 (32.7)
Enterobacter agglomerans	1 (1.0)	21 (16.8)		22 (7.2)
Enterobacter sakazakii		2 (1.6)	1 (1.3)	3 (1.0)
Enterobacter cloacae		18 (14.4)	1 (1.3)	19 (6.2)
Enterobacter aerogenes		4 (3.2)		4 (1.3)
Klebsiella pneumoniae	2 (1.9)	22 (17.6)		24 (7.8)
Klebsiella oxytoca		20 (16.0)		20 (6.5)
Citrobacter freundii	6 (5.8)	24 (19.2)	2 (2.6)	32 (10.5)
Citrobacter diversus		2 (1.6)		2 (0.7)
Hafnia alvei		2 (1.6)		2 (0.7)
Serratia liquefaciens		2 (1.6)		2 (0.7)
Serratia marcescens		1 (0.8)		1 (0.3)
Salmonella spp.			1 (1.3)	1 (0.3)
Aeromonas spp.			54 (70.1)	54 (17.6)
Pseudomonas spp.			12 (15.6)	12 (3.9)
Vibrio spp.			2 (2.6)	2 (0.7)
Morganella morganii			1 (1.3)	1 (0.3)
Unidentified		4[c] (3.2)	1[d] (1.3)	5 (1.6)
Total[e]	104	125	77	306

[a] KEC, thermotolerant coliforms other than *E. coli*.
[b] Nonblue, nonred colonies.
[c] Oxidase negative.
[d] Oxidase positive.
[e] The percentages of the total numbers of isolates in the three categories were as follows: blue colonies, 33.9%; red colonies, 40.8%; and other colonies, 25.1%.

showed significant differences ($P = 0.01$) between KEC counts at 41 and 44.5°C both on CECC and on CECCP, with the F test yielding a P value of 0.00001 for river and marine samples. The mean KEC counts obtained with CECCP at 41°C was 6.2 times the mean KEC counts at 44.5°C. Geldreich (13) suggested that an effort should be made to optimize the recovery of coliforms while excluding environmental strains of no significance by investigating incubation temperatures between 39 and 42°C.

Counts of TTC on CECC and CECCP were compared with counts on mLSA. A summary of the TTC counts by medium and incubation temperatures is given in Table 1. The concentration of TTC in the marine water samples was higher; the range of TTC for marine samples was 10 to 2.6×10^4 CFU/100 ml, whereas the range of TTC for the river samples was 70 to 7.9×10^3 CFU/100 ml. The differences between TTC counts obtained with the two chromogenic media incubated at 41°C and mLSA at two incubation temperatures (4 h at 30°C plus 20 h at 44.5°C, and incubation at only 44.5°C) were statistically different as determined by ANOVA and the multiple-range test. The use of CECCP and incubation at 41°C resulted in significantly greater detection of TTC in river and marine water samples compared with the use of mLSA in two incubation procedures.

Characterization of *E. coli*, KEC, and nontarget isolates. The identities of the three types of colonies (LAC^+ GUD^-, LAC^+ GUD^+, and LAC^- GUD^-) on CECCP agar incubated

TABLE 3. Identification of colonies picked from marine water samples incubated on CECCP at 41°C

Species	No. of isolates identified in each category (% of total no. in the category)			Total no. of isolates (% of total)
	Blue colonies (*E. coli*)	Red colonies (KEC[a])	Other colonies (background[b])	
Escherichia coli	81 (92.0)		4 (5.8)	85 (39.5)
Enterobacter agglomerans	1 (1.1)	3 (5.1)		4 (1.9)
Enterobacter sakazakii		7 (11.8)		7 (3.3)
Enterobacter cloacae		15 (25.4)		15 (7.0)
Klebsiella pneumoniae		8 (13.5)		8 (32.7)
Klebsiella oxytoca		3 (5.1)		3 (1.4)
Citrobacter freundii	6 (6.8)	20 (33.9)		26 (12.1)
Citrobacter diversus		2 (3.3)		2 (0.9)
Aeromonas spp.			3 (4.3)	3 (1.4)
Pseudomonas spp.			61 (88.4)	61 (28.4)
Providencia alcalifaciens			1 (1.4)	1 (0.5)
Total[c]	88	58	69	215

[a] KEC, thermotolerant coliforms other than *E. coli*.
[b] Nonblue, nonred colonies.
[c] The percentages of the total numbers of isolates in the three categories were as follows: blue colonies, 40.9%; red colonies, 26.9%; and other colonies, 32.1%.

at 41°C are shown in Table 2 (river samples) and Table 3 (marine samples).

The mean confirmation rate of target blue colonies was 91.3% in river water samples and 92% in marine water samples. Other investigators (6) have shown a good correlation between GUD detection and *E. coli* when using chromogenic media with indoxyl derivatives. The *E. coli* false-positive rate was 8.7% (9 of 104 colonies) in river samples and 8.0% (7 of 88 colonies) in marine samples. In our study, GUD activity was found in some strains of *Citrobacter freundii*, *Enterobacter agglomerans*, and *Klebsiella pneumoniae*. The GUD^- colonies which were confirmed to be *E. coli* were found at rates of 2.5 and 3.1% for river and marine waters, respectively. Four LAC^- GUD^- *E. coli* strains at 41°C were further cultured in CECCP at 37°C for 24 h to determine whether the expression of LAC and GUD was temperature dependent. The four *E. coli* strains showed LAC production but not GUD production at 37°C (LAC^+ GUD^-). Schets and Havelaar (25) found that although the GUD reaction was specific for *E. coli* with 4-methylumbelliferyl-β-D-glucuronide (MUG) substrate, an average of 14% of these strains were GUD negative at 44°C; of these strains, 24% showed GUD activity at 37°C. Alonso et al. (2) found that false-negative *E. coli* colonies occurred less frequently at 37°C than at 44.5°C. However, other authors (10) found more false-negative colonies at the lower incubation temperature of 35 to 37°C. Olson et al. (21) suggested that injury, impermeability, lack of gene expression, or nonutilization of the GUD substrate may all account for the GUD^- phenotype in *E. coli*.

The criteria for the identification of KEC of likely fecal origin used for the evaluation of CECCP in this study is based on the work by Leclerc et al. (16). The percentage of KEC of likely fecal origin isolated on CECCP agar incubated at 41°C was higher in marine water than in river water. Of the 125 LAC^+ GUD^- colonies, 90 (72.0%) were confirmed as KEC of likely fecal origin (21 *E. agglomerans* strains, 2 *E. sakazakii* strains, 2 *Hafnia alvei* strains, 1 *Serratia liquefaciens* strain, and 1 *S. marcescens* strain were not included) in river waters, and of the 58 LAC^+ GUD^- colonies, 48 (82.7%) were confirmed as KEC coliforms of likely fecal origin (3 *E. agglomerans* strains and 7 *E. sakazakii* strains were not included) in marine waters. The dominating KEC species of likely fecal origin was *Citrobacter freundii* in river (19.2%) and marine (33.9%) water samples. A total of 9 LAC^+ GUD^+ colonies and 4 LAC^- GUD^- colonies in river water samples and 7 LAC^+ GUD^+ colonies in marine water samples were KEC coliforms, resulting in a false-negative rate of 7.2% (13 of 181 colonies) in river water samples and 4.5% (7 of 157 colonies) in marine water samples. None of the *Aeromonas* or *Vibrio* strains showed LAC activity at 41°C. It is evident that this incubation temperature is useful for the elimination of background interference due to *Aeromonas* and helps to avoid other LAC^+ noncoliform bacteria belonging to the genus *Vibrio*. The main sources of false-positive results in other types of coliform chromogenic media are *Vibrio* and *Aeromonas* sp. (1, 22).

In summary, the results of this study showed that CECCP incubated at 41°C is efficient for the simultaneous enumeration of *E. coli* and KEC from river and marine waters.

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