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# Quantitative determination of *Escherichia coli* in water using CHROMagar® *E. coli*

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# Quantitative determination of *Escherichia coli* in water using CHROMagar® *E. coli*

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**Abstract**

A new medium, CHROMagar® *E. coli* (CAEC), containing a combination of X-glucuronide and methyl-glucuronide for the detection of $\beta$-glucuronidase activity of *Escherichia coli* has been evaluated by the membrane filtration (MF) technique in fresh water samples. The CAEC agar was compared with conventional media, mFC agar and mLSB, for the enumeration of faecal coliforms. The variance analysis showed that CAEC was as sensitive as mFC agar and mLSB. A good correlation was found between *E. coli* versus those from faecal coliforms in the water sampling areas tested. Of 321 presumptively positive *E. coli* colonies (blue) and 154 presumptively negative *E. coli* colonies (white), only 8 (2.5%) false positive and 19 (12.4%) false negative colonies were found. Specificity of the CEAC agar in spanish samples was temperature dependent, false negative *E. coli* colonies occurred less frequently at 37°C (2.3%) than at 44.5°C (18.8%). The results of this study indicate that CAEC agar is efficient for the enumeration of *E. coli* from a wide range of environmental freshwater samples.

*Keywords:* *Escherichia coli*; Faecal pollution; Freshwater; GUD; Membrane filtration

## 1. Introduction

The detection and enumeration of indicator organisms are of primary importance for the monitoring of sanitary and microbiological quality of water. Faecal coliforms have long used as indicators of faecal contamination in water and food. Among these, *Escherichia coli* is generally considered the most reliable, since its presence directly relates to faecal contamination with its implied threat of the presence of enteric disease agents [1].

A major limitation of the standard methods available for the enumeration of *E. coli* in water is the length of time required to complete the testing [2]. To overcome this problem, new enzymatic methods incorporating fluorogenic and chromogenic substrates have been developed for the presumptive detection of *E. coli*. $\beta$-glucuronidase (GUD) (E.C. 3.2.1.31) is an enzyme that catalyzes the hydrolysis of $\beta$-D-glucopyranosiduronic (GLR) derivatives into their corresponding aglycons and D-glucuronic acid [3], 94–97% of *E. coli* were found to have positive GUD-activity [4], which is not expressed by other species of *Escherichia* [5]. Other members of the Enterobacteriaceae producing GUD activity include

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17–29% of *Salmonella* sp. [6,7], 50% of *Shigella* [8] and a minority of *Yersinia* sp. [9]. Chromogenic and fluorogenic $\beta$-glucuronides incorporated in various liquid and solid media have served as indicators of GUD activity. The fluorogencic substrate 4-methylumbelliferyl-$\beta$-D-glucuronide (MUG) has been used in the detection of *E. coli* in water by membrane filtration (MF) technique [10–15]. However, MUG analysis requires the use of fluorescence light rendering it less suitable for using in membrane filtration [14,16]. The chromogenic substrate *p*-nitrophenol-$\beta$-D-glucuronide (PNPG) is also used to detect *E. coli*, but PNPG is unable to enumerate distinct *E. coli* colonies because the colour that develops during bacterial growth is not retained within the bacterial colony [17]. Recently, other chromogens, such as indoxyl derivatives, 5-bromo-4-chloro-3-indolyl-$\beta$-D-glucuronide (X-Gluc) and indoxyl-$\beta$-D-glucuronide (IBDG) have also been used to detect or enumerate *E. coli* in water [18–20]. These substrates release indoxyl (or halogenated indoxyl) which is rapidly oxidized to the insoluble indigo or its analogue, providing a very distinct differentiation of GUD-positive *E. coli* as blue colonies on agar plates or membranes [14].

This study was designed to evaluate CHROMagar® *E. coli*, containing a combination of X-glucuronide and methyl-glucuronide, as a new medium for the enumeration of *E. coli* by the membrane filtration technique in a variety of freshwater samples from Spain and England.

## 2. Materials and methods

### 2.1. Sampling

A series of freshwater samples from England and Spain were used to evaluate the ability of CAEC agar to enumerate *E. coli*. Locations of sampling sites are indicated in Table 1. All samples were collected in sterile glass bottles, refrigerated and assayed within 3 h after collection.

### 2.2. Media

CHROMagar® *E. coli* (CAEC agar was obtained from CHROMagar (Paris, France). The composition of the powdered medium is in $mg \cdot l^{-1}$: peptone 5, yeast and meat extracts 3, sodium chloride 5, selective chromogenic mix 9, agar 15 (pH 6.0).

Membrane lauryl sulphate broth (mLSB) (Oxoid, Basingstoke, UK) and mFC broth (Difco, MI, USA) were prepared according to the manufacturer's instructions. In the case of mFC broth was solidified by adding 1.2% agar before boiling [21].

### 2.3. Media comparison

Dilutions of the water samples were made and duplicates of each dilution filtered through sterile 0.45-$\mu$m porosity membranes (Millipore, Bedford, MA, USA) using the standard membrane filtration technique [21].

One of each set of duplicate membranes was placed onto a pre-prepared layer of CAEC agar in a 47 mm petri-dish. These were then incubated at 44.5°C for 24 h and all blue colonies (GUD+) were identified as presumptive *E. coli*. Non-blue colonies (GUD−) were counted as presumptive non-*E. coli* isolates.

For comparison, the second duplicate membrane of each duplicate pair was processed by a standard method for faecal coliforms. The membranes containing the English samples were placed onto sterile absorbent pads soaked with mLSB in 47 mm Petridishes. These were then incubated at 44.5°C for 24 h. All yellow colonies of a diameter greater than 1 mm

Table 1
Locations of sampling sites in England and Spain

| Sampling site | Pollution sources | Site code |
|---|---|---|
| Hadley woods, England (Pymmes Brook Source) | Wildfowl, fish, animals | S |
| Osidge Lane, England (Pymmes Brook-Waterfall Walk) | Sanitary wastewater, surface water sewers | OL |
| Waterfall Road, England (Pymmes Brook-Arnos Park) | Combined sewerage, surface water sewers | WR |
| Telford Road, England (Bounds Green Brook) | Surface water sewers | TR |
| Irrigation channel, Spain | Sewage | S1 |
| Vera irrigation channel, Spain (Alboraya village) | Sewage | S2 |

were counted as presumptive faecal coliforms [22]. The membranes containing the Spanish samples were layered onto mFC agar and were incubated at 44.5°C for 24 h. All blue colonies were counted as faecal coliforms [21].

### 2.4. Colony verification and identification

Isolated colonies were picked from the most appropriate dilution of CAEC agar. For each sample, 2 to 5 $GUD^+$ (blue colonies) and 2 to 5 $GUD^-$ (white colonies) were randomly picked and subcultured on nutrient agar (Oxoid). Colonies picked were then confirmed as *E. coli* by gas production in EC broth (Oxoid) and indole production in tryptone water following incubation at 44.5°C for 24 h as recommended for the standard methods [21,22]. Isolates were further identified using the API 20E system (bioMerieux, Marcy-l'Etoile, France).

The results of specificity testing were expressed as two individual terms; the error introduced by false positive colonies and the error resulting from undetected target colonies.

### 2.5. Accuracy of CAEC agar

The accuracy, defined as a measure of the closeness of observed values to a known true value, was determined using two reference capsules of *E. coli* WR1 (prepared at the SVM, RIVM, Bilthoven, The Netherlands). The mean bacterial numbers of reference capsules of the batches used in England and Spain were 37.00±5.40 cfu per 100 $ml^{-1}$ and 35.70±9.28 cfu per 100 $ml^{-1}$, respectively. The content of each capsule was reconstituted in a tube with 10 ml of peptone saline solution (PS), ingredients ($g \cdot l^{-1}$): peptone 1, NaCl 8.5, as recommended by SVM. One ml of the resulting *E. coli* suspension was then added to 99 ml of PS and filtered through a 0.45-$\mu$m membrane. The membranes were then layered onto CAEC agar and incubated at 44.5°C for 24 h as described for the environmental water samples. Five replicates per capsule were done.

### 2.6. Regression analysis and statistical evaluation

Bacterial counts were logarithmically transformed prior to statistical treatment. Results were analyzed by linear regression to verify the linearity of the relationship between *E. coli* and faecal indicators obtained with different media. A unifactorial variance analysis was performed on the means of the data. To examine the medium performance over a range of samples types and concentrations, the samples were grouped by sample site and by *E. coli* counts. All statistics were obtained using Statgraphics software.

## 3. Results and discussion

A series of 60 water samples was investigated in England and Spain for *E. coli* and faecal coliforms. The 12 water samples tested in Spain had *E. coli* counts ranging from $5.8\times10^5$ cfu·100 $ml^{-1}$ to $4\times10^6$ cfu·100 $ml^{-1}$ (Table 2). The 48 water samples analyzed in England had *E. coli* counts ranging from 160 cfu·100 $ml^{-1}$ to $1.8\times10^6$ cfu·100 $ml^{-1}$ (Table 3). The data presented in Table 4 show very good correlation ($P\leq0.01$) between *E. coli* and faecal coliforms counts at the six sites tested. A total of 88 out of 98 faecal coliform colonies (89.8%) on mLSB were *E. coli*. Ten yellow colonies out of 98 on mLSB were not *E. coli*, with 8 colonies being identified as *Klebsiella pneumoniae* (8.2%), 1 colony as *Citrobacter freundii* (1%) and 1 colony as *Pasteurella* sp. (1%). Bordalo [23] investigating the recovery of faecal coliforms on mFC agar in various types of water, ranging from polluted seawater to unpolluted freshwater found that *E. coli* represent, on average 50.0–70.8% of the faecal coliform population. Faecal coliforms are widely used as indicators of faecal contamination of water [21]), although some authors questioned the usefulness of such

Table 2
Counts of *Escherichia coli* and faecal coliforms at sampling sites of Spain

| Date | Site S1 (cfu·100 $ml^{-1}$)[a] | | Site S2 (cfu·100 $ml^{-1}$) | |
|---|---|---|---|---|
| (1994) | *E. coli* | F. coliforms[b] | *E. coli* | F. coliforms |
| 7 March | $1.9\times10^6$ | $2.6\times10^6$ | $8.0\times10^5$ | - |
| 8 March | $2.3\times10^6$ | $3.7\times10^6$ | $5.8\times10^5$ | $7.2\times10^5$ |
| 9 March | $1.8\times10^6$ | $2.7\times10^6$ | $7.8\times10^5$ | $8.9\times10^5$ |
| 10 March | $1.1\times10^6$ | $1.5\times10^6$ | $1.2\times10^6$ | $1.4\times10^6$ |
| 26 April | $4.0\times10^6$ | $4.5\times10^6$ | $2.8\times10^6$ | $3.2\times10^6$ |
| 27 April | $3.8\times10^6$ | $4.4\times10^6$ | $2.0\times10^6$ | $2.6\times10^6$ |

[a]Values are the mean of two replicates.
[b]Faecal coliforms.

Table 3
Counts of *Escherichia coli* and faecal coliforms at sampling sites of England

| Date (1994) | Site S (cfu 100 $ml^{-1}$)[a] | | Site OL (cfu 100 $ml^{-1}$) | | Site WR (cfu 100 $ml^{-1}$) | | Site TR (cfu 100 $ml^{-1}$) | |
|---|---|---|---|---|---|---|---|---|
| | *E. coli* | Faecal coliforms | *E. coli* | Faecal coliforms | *E. coli* | Faecal coliforms | *E. coli* | Faecal coliforms |
| 3 May | 820 | 700 | $1.2\times10^4$ | $1.9\times10^4$ | $1.4\times10^4$ | $2.3\times10^4$ | $1.5\times10^5$ | $1.4\times10^4$ |
| 10 May | 2000 | 600 | $2.3\times10^4$ | $6.1\times10^4$ | $2.4\times10^4$ | $1.8\times10^4$ | $1.6\times10^5$ | $1.6\times10^5$ |
| 17 May | 1800 | 2100 | $1.7\times10^5$ | $7.2\times10^5$ | $1.8\times10^6$ | $5.0\times10^5$ | $2.6\times10^5$ | $3.6\times10^5$ |
| 24 May | 1900 | 1700 | $2.8\times10^4$ | $6.0\times10^4$ | $8.7\times10^4$ | $1.0\times10^4$ | $2.1\times10^5$ | $1.7\times10^5$ |
| 31 May | 1900 | 1800 | $1.3\times10^4$ | $2.1\times10^4$ | $2.8\times10^4$ | $2.9\times10^4$ | $3.6\times10^4$ | $3.8\times10^4$ |
| 21 June | 1500 | 1350 | $8.9\times10^4$ | $1.2\times10^4$ | $1.1\times10^4$ | $8.1\times10^4$ | $1.4\times10^4$ | $1.6\times10^4$ |
| 5 July | 1000 | 1330 | $1.4\times10^4$ | $1.4\times10^4$ | $2.5\times10^4$ | $5.8\times10^4$ | $1.5\times10^5$ | $1.7\times10^5$ |
| 12 July | 1100 | 1200 | $1.5\times10^5$ | $5.2\times10^5$ | $6.1\times10^4$ | $4.6\times10^4$ | $1.0\times10^4$ | $8.0\times10^3$ |
| 19 July | 400 | 500 | $7.9\times10^4$ | $5.4\times10^4$ | $1.1\times10^4$ | $1.1\times10^4$ | $4.0\times10^4$ | $4.4\times10^4$ |
| 26 July | 2500 | 1800 | $1.4\times10^5$ | $1.3\times10^5$ | $3.0\times10^4$ | $2.0\times10^4$ | $2.1\times10^4$ | $7.0\times10^3$ |
| 2 August | 320 | 290 | $4.8\times10^4$ | $5.6\times10^4$ | $3.7\times10^4$ | $3.9\times10^4$ | $1.0\times10^4$ | $1.0\times10^4$ |
| 9 August | 160 | 190 | $4.3\times10^4$ | $4.0\times10^4$ | $4.0\times10^4$ | $2.3\times10^4$ | $4.0\times10^4$ | $4.4\times10^4$ |

[a] Values are the mean of two replicates.

thermotolerant oragnisms as indicators [24,25]. *E. coli* is preferred because it is specific and most reliably reflects the faecal origin [13].

Counts of *E. coli* on CAEC agar from water samples in Spain and England were compared with faecal coliform counts obtained with the comparison media, mFC agar and mLSB. The overall ANOVA test revealed no significant differences with the *E. coli* counts on CAEC agar and faecal coliforms on mFC agar and mLSB. For purposes of analysis, the abundance range was fractionated into five intervals (101–1000; 1001–10 000; 10 001–100 000; 100 001–1 000 000; ≥1 000 000). Considering the results of the ANOVA test for each interval it was found a good level of agreement between *E. coli* and faecal coliform counts.

The mean bacterial numbers of *E. coli* WR 1 in capsules tested in Spain and in England were found to be 36.30±4.45 cfu·100 $ml^{-1}$ and 34.80±2.89 cfu·100 $ml^{-1}$, respectively. Recovery of the *E. coli* reference strain was satisfactory on CAEC agar. An average of 101.6% in Spain and 94.1% in England was obtained. These results showed a good accuracy for the CAEC agar.

Apparent *E. coli* colonies on CAEC agar were blue and all other colonies were white. The identities of the two types of colonies are shown in Table 5. Of the 321 blue colonies 313 (95.7%) were confirmed as *E. coli* (Table 6). This confirmation rate is similar to that reported for other media with indoxyl derivatives [19,20]. Some investigators [20,26] found that media with indoxyl derivatives (IBDG, BCIG) proved the easiest to read and produced the least number of false positives and false negatives than media with MUG or PNPG. False positive results on CAEC agar were due to strains of *Klebsiella oxytoca*, *Enterobacter intermedium*, *Serratia fonticola*, *Pasteurella multocida* and *Yersinia intermedia*. Al-

Table 4
Regression and correlation parameters from data obtained using chromogenic and reference media

| Sample source | Media[a] | CC[b] | *P* | Intercept (a) | Slope (b) |
|---|---|---|---|---|---|
| S1 | CAEC-mFC | 0.963 | ≤0.01 | −1.029 | 1.141 |
| S2 | CAEC-mFC | 0.996 | ≤0.01 | −0.052 | 0.996 |
| S | CAEC-mLSB | 0.893 | ≤0.01 | 0.081 | 0.987 |
| OL | CAEC-mLSB | 0.856 | ≤0.01 | 1.126 | 0.733 |
| WR | CAEC-mLSB | 0.930 | ≤0.01 | −0.915 | 1.210 |
| TR | CAEC-mLSB | 0.971 | ≤0.01 | 0.794 | 0.839 |

[a] CAEC (*E. coli*): dependent variable; mFC and mLSB (Faecal coliforms): independent variable.
[b] Correlation coefficient.

Table 5
Identification of colonies picked from CAEC agar using the API 20e system

| Species isolated | GUD+[a] | Species isolated | GUD−[b] |
|---|---|---|---|
| *Escherichia coli* | 119 | *Escherichia coli* | 16 |
| *Pasteurella multocida* | 2 | *Klebsiella pneumoniae* | 48 |
| *Enterobacter intermedium* | 2 | *Aeromonas hydrophila* | 14 |
| *Klebsiella oxytoca* | 2 | *Enterobacter cloacae* | 9 |
| *Serratia fonticola* | 1 | *Citrobacter freundii* | 9 |
| *Yersinia intermedia* | 1 | *Klebsiella oxytoca* | 7 |
| | | *Enterobacter agglomerans* | 4 |
| | | *Pasteurella multocida* | 3 |
| | | *Salmonella* sp | 1 |
| | | *Salmonella paratyphi* A | 1 |
| | | *Serratia rubidaea* | 1 |
| | | *Pseudomonas putrefaciens* | 1 |
| | | *Enterobacter sakazakii* | 1 |
| Total | 127 | | 115 |

[a] Number of blue colonies.
[b] Number of non-blue colonies.

though β-glucuronidase activity have been reported [5,18,27,28] in some strains of *Enterobacter agglomerans*, *Enterobacter amnigenus*, *Citrobacter freundii*, *Citrobacter amalonaticus*, *Escherichia vulneris*, *Yersinia kristensenii*, *Hafnia alvei*, *Aeromonas* sp. and *Acinetobacter* sp., their occurrence appears to be very infrequent [18]. The reason for the production of β-glucuronidase by these organisms is not known, but other investigators [19] have suggested that the reaction may be plasmid mediated.

There seems to be some controversy over the number of GUD false negative *E. coli* and therefore questions have been raised on its effectiveness, as GUD-based tests may underestimate the presence of *E. coli*. In the present investigation we have found *E. coli* false negative occurrences of 18.8% in Spain in comparison with 6% in England (Table 6). These differences may have been based on differences in samples, and therefore the physiological condition of the bacteria could be responsible for the non-expression of enzyme activity as suggested by different investigators [2,29–31]. The higher frequency of false *E. coli* strains in more polluted sites of Spain could be accounted for on the basis of different survival rate. Coyne and Schuler [32] suggested that if GUD positive *E. coli* survive longer in the environment than GUD negative *E. coli*, a higher frequency of GUD negative would be expected in freshly contaminated sites. Some investigators [30,33–37] observed that part of the genetic sequences of the *uid*A gene, which encodes for the GUD enzyme, was present in most if not all *E. coli* isolates, regardless of the GUD-phenotype and the absence of GUD enzyme activity may be attributed to physiological state of the isolates. We have incubated the CAEC agar at 37°C in some spanish

Table 6
Verification of colonies observed on CAEC agar[a]

| Sampling country | $T$[b] | No. of blue colonies ($GUD^+$) | | No. of non-blue colonies ($GUD^-$) | |
|---|---|---|---|---|---|
| | | *E. coli* | non-*E.coli* | *E. coli* | non-*E. coli* |
| Spain | 44°C | 218 (98.2) | 4 (1.8) | 19 (18.8) | 82 (81.2) |
| | 37°C | – | – | 3 (2.3) | 129 (97.7) |
| England | 44°C | 95 (95.9) | 4 (4.1) | 6 (6.0) | 94 (94.0) |
| Total | 44°C | 313 (97.5) | 8 (2.5) | 25 (12.4) | 176 (87.6) |

[a] The percentage of isolates which were picked at random, tested and confirmed as *E. coli* or non-*E. coli* is in parentheses.
[b] Incubation temperature of CAEC agar.

samples to determine whether the expression of GUD production was temperature dependent. *E. coli* false negative colonies occurred less frequently at 37°C (2.3%) than at the higher temperature, 44.5°C (18.8%) (Table 6). From the data, we can assume that specificity of the CAEC agar in spanish samples could be related to the physiological condition of *E. coli* isolates.

The results of this study indicate that the CAEC agar with the membrane filtration technique is satisfactory for the selective enumeration of *E. coli* in freshwater samples with different degree of contamination. Although, it is recommended that before its use elsewhere, a minimal number of samples should be examined locally to determine whether the specificity is affected by local environments.

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