IDEXX
Literature Cover Sheet

IDEXX #: 10A

Title: Colorimetric Enumeration of *Escherichia coli* Based on *B*-Glucuronidase Activity

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Topic: Non-Fluorescing *E. coli*

Highlights:

- Approximately 95% of the *E. coli* strains have been shown to produce the enzyme *B*-Glucuronidase.

- A small percent of *E coli* are *B*-glucuronidase negative yet environmental samples are likely to contain a mixture of strains, thus reducing the chances of false-negative results.

* See page 2021 & page 2024.

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Colorimetric Enumeration of *Escherichia coli* Based on β-Glucuronidase Activity

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A medium containing a chromogenic substrate was developed for the enumeration of *Escherichia coli* on the basis of β-glucuronidase activity. In this medium there was an inverse linear relationship between the log initial *E. coli* concentration and the time taken for the color to reach a threshold optical density of 0.05. This relationship applied even when the *E. coli* population contained 5% β-glucuronidase-negative cells. Incubation at 44°C reduced the time taken for color development and allowed the procedure to be used in the presence of a competitive microflora that outnumbered the *E. coli* population by a factor of 10^4. Sodium lauryl sulfate as an additional selective agent gave no significant improvement. In the analysis of environmental water samples, the technique gave a good correlation with a standard cultural method. The procedure shows promise as a simple method for testing the compliance of environmental samples with microbiological criteria for *E. coli*.

Established techniques for the enumeration of *Escherichia coli* in environmental samples such as food and water are based on most probable number or direct plate counting procedures. These can be labor intensive and require at least 24 h to obtain a result.

Approximately 95% of the *E. coli* strains investigated have been shown to produce the enzyme β-glucuronidase (7, 8). ecies of the closely related genus *Shigella* also show a ..gh incidence of β-glucuronidase production (40%), but a far lower frequency is reported for other gram-negative bacteria (5, 8, 11, 15). Among the gram-positive bacteria, only an occasional incidence of β-glucuronidase-positive streptococci has been reported (12, 17). Production of β-glucuronidase is used as a feature in the identification of *E. coli*, and chromogenic and fluorogenic enzyme substrates have been incorporated into diagnostic enumeration media (6, 10, 14, 16).

Microbiological criteria for environmental samples frequently set threshold levels for *E. coli*. A rapid, specific colorimetric assay would facilitate testing for compliance with such limits. Here we report an investigation into the suitability of β-glucuronidase activity measurement as the basis for such an assay.

MATERIALS AND METHODS

Bacterial strains. Single-strain experiments used *E. coli* ATCC 25922. The multiple-strain mixture comprised 19 β-glucuronidase positive strains isolated from food, water, or feces and a β-glucuronidase-negative strain (VTEC O:157). All were supplied by T. Donovan, Ashford Public Health Laboratory, Kent, United Kingdom. *Citrobacter diversus*, *Staphylococcus aureus*, *Bacillus cereus*, *Lactobacillus plantarum*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* were obtained from the culture collection of the Microbiology Department. Cultures were maintained on nutrient agar slopes stored at 4°C.

Inoculum preparation. Test organisms were grown up ..ividually in broth culture for 18 h. *P. aeruginosa* was grown in nutrient broth (Oxoid Ltd.) at 30°C, *L. plantarum* was grown in MRS broth (Oxoid) at 30°C, and all others were grown in nutrient broth at 37°C.

The mixture of *E. coli* strains was prepared by adding an 18-h broth culture (0.5 ml) of each strain to a sterile bottle and mixing for 2 s on a Vortex mixer before enumeration. Components of the competitor microflora were enumerated individually before broth cultures (1 ml) were mixed in a sterile bottle.

Inoculum levels were determined by preparing a decimal serial dilution of broth cultures in quarter-strength Ringer solution and enumerating by the Miles and Misra technique (9) on nutrient agar (Oxoid) incubated for 24 h at 30°C for *P. aeruginosa* and at 37°C for other species. *L. plantarum* was enumerated by the same technique on MRS agar incubated at 30°C for 2 days.

β-Glucuronidase assay. β-Glucuronidase–tryptone (GT) medium containing *p*-nitrophenol-β-D-glucuronide (0.03 mg/ml; Sigma Chemical Co.) tryptone (25 mg/ml; Oxoid), and sodium chloride (5 mg/ml) was filter sterilized (0.45-μm-pore-size filter; Millipore Corp.) and aliquoted (4.5 ml) into sterile bottles. GT medium was inoculated with a premixed broth culture or dilution (0.5 ml) containing the organisms under test and incubated at 37 or 44°C. At each sampling time the contents of a bottle were filtered (0.45-μm pore size), and the filter membrane was washed with 1 ml of carbonate buffer (14 mg of anhydrous Na_2CO_3 per ml and 6 mg of $NaHCO_3$ per ml [pH 9.3]). The optical density (OD) of the filtrate was measured at 400 nm in a Pye Unicam SP6-450 spectrophotometer against a reagent blank. For each inoculum level, two bottles were sampled to obtain duplicate readings. The detection time corresponding to an OD of 0.05 was read from a graph of OD against time.

Environmental samples. Local pond water samples (100 or 250 ml), some of which had been spiked with settled raw sewage, were filtered through a 0.22-μm-pore-size Millipore membrane filter. The membrane was placed in GT medium (90 ml) with 9 ml of maximum recovery diluent (Oxoid) pre-equilibrated to 44°C. The mixture was incubated at 44°C and sampled (5 ml) periodically to determine β-glucuronidase activity. *E. coli* concentration was determined from the standard curve. The enumeration technique of Anderson and Baird-Parker was used as a reference method (1).

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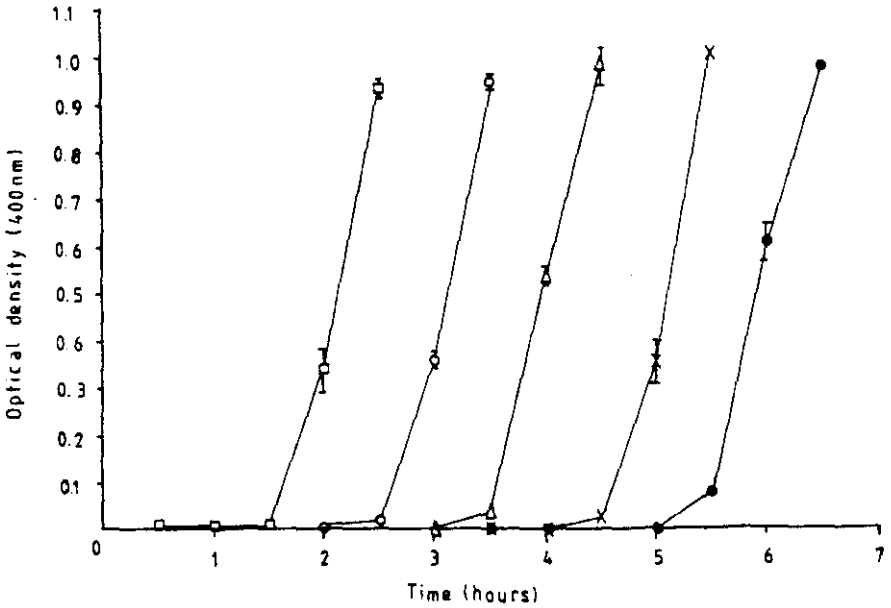

FIG. 1. Hydrolysis of 4-nitrophenol-β-D-glucuronide in GT medium by a mixture of 20 *E. coli* strains (including 1 β-glucuronidase-negative strain). Graph indicates the OD at 400 nm against period of incubation at 44°C with five inoculum concentrations (CFU per milliliter): □, 5.3×10^7; ○, 5.1×10^6; △, 5.3×10^5; X, 5.1×10^4; ●, 5.3×10^3. Ranges of duplicate samples are indicated.

RESULTS

Cultures of *E. coli* in media containing the chromogen p-nitrophenol-β-D-glucuronide showed a lag period followed by a phase of rapid color development (Fig. 1). Accurate determination of the extent of chromogen hydrolysis required filtration of the culture and adjustment of the filtrate pH by addition of carbonate buffer (pH 9.3). The culture incubation period for filtrates to register an OD of 0.05 at 400 nm was taken as the detection time.

Supplementation of a basal medium of tryptone (15 mg/ml) and chromogen (0.3 mg/ml) with tryptone and sodium chloride (10 and 5 mg/ml, respectively) reduced the detection time by about 25 min. The chromogen concentration could be reduced to 0.03 mg/ml with little change in detection time but significant savings in medium cost. At this lower concentration the detection time corresponds to hydrolysis of 2.8% of the chromogen present. These modifications to the basal medium were incorporated to produce the GT medium used in all work reported here. Glucose (10 mg/ml) did not reduce the detection time and was not included.

Detection was most rapid when cultures in GT medium were incubated at 44°C (Table 1). With a mixed population of *E. coli* comprised of 19 environmental isolates and a β-glucuronidase-negative strain, the detection time corresponded to an *E. coli* population in the GT medium of 7.4 to 7.6 log CFU/ml at 44°C (Fig. 2) or 7.6 to 8.1 log CFU/ml at 37°C.

Detection time increased with decreasing concentration of *E. coli* in the inoculum (Fig. 1). With both a single β-glucuronidase-positive strain and the mixture of 20 strains, there was an inverse linear relationship between the log of the inoculum concentration of *E. coli* and the detection time. This was true for incubation at 37 or 44°C and enabled calibration curves relating detection time to log inoculum *E. coli* concentration to be drawn. The correlation for the mixed strains at 44°C is presented as Fig. 3 along with the equations of the linear regression lines for mixed strains and the single strain at 37°C.

Using the respective calibration curves, initial concentrations of the mixed *E. coli* culture were determined from the detection times at 37 and 44°C in the presence of a mixed competitive microflora. This microflora comprised a mixture of five different species of β-glucuronidase-negative bacteria likely to occur in food or environmental samples. Dilutions of these were added to give several different *E. coli*/competitor ratios in the inocula. The results and conventional plate count data on initial numbers are presented as Table 2. At 37°C, there was reasonable agreement between the two methods when the inoculum population of *E. coli* was comparable to or exceeded the level of competitors. As the ratio of competitor to *E. coli* population in the inoculum increased, the detection time decreased and the colorimetric method overestimated the numbers of *E. coli* by a half log cycle at a ratio of 10:1 and a full log cycle at 1,000:1. Agreement was much better at 44°C over a wider range of inoculum population ratios with two different levels of *E. coli*. With an inoculum *E. coli* concentration of 2.62 log CFU/ml, competitors had to outnumber *E. coli* by a factor greater than 10^4 before the colorimetric assay was more than a half log cycle greater than the plate count.

Species of *Enterobacter* and *Klebsiella* that were able to

TABLE 1. OD (400 nm) of *E. coli* culture filtrates (pH 9.3) after growth in GT medium incubated at 30, 37, or 44°C for various periods

Time (h)	OD after growth at:		
	30°C	37°C	44°C
1.0	0	0	0
2.0	0	0	0
2.5			0.01
3.0	0	0.03	0.32
3.5		0.53	0.99
4.0	0	1.03	
5.0	0.17		
5.5	0.64		

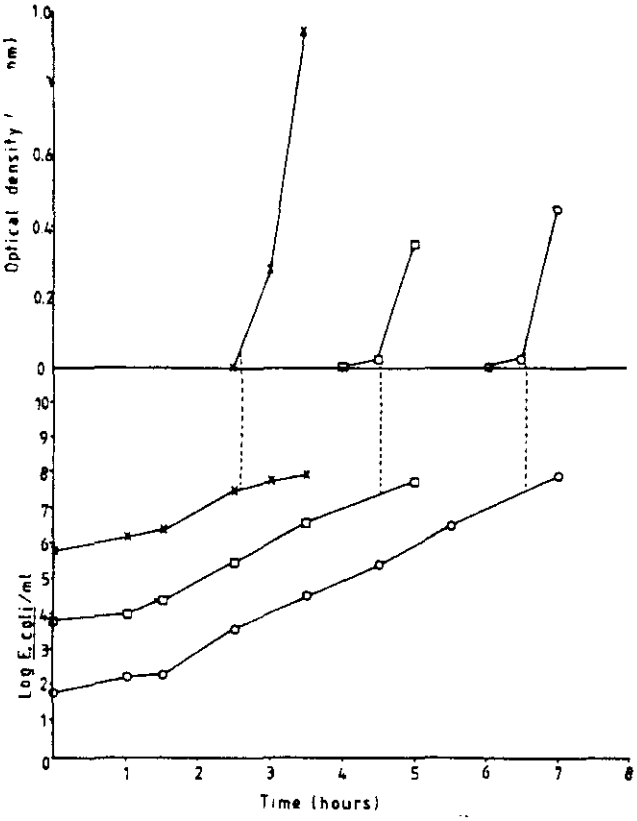

FIG. 2. Hydrolysis of 4-nitrophenol-β-D-glucuronide and growth of *E. coli* in GT medium from a mixed inoculum comprised of 20 *E.* strains (including 1 β-glucuronidase-negative strain). Graph ..icates the OD at 400 nm and log *E. coli* CFU per milliliter against period of incubation at 44°C for three inoculum concentrations (CFU per milliliter): X, 7.6×10^6; □, 7.6×10^4; ○, 7.6×10^2.

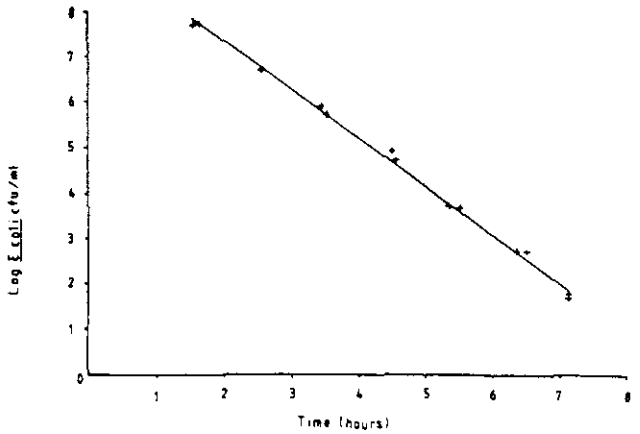

FIG. 3. Relationship between log concentration of *E. coli* (mixture of 20 strains, including 1 β-glucuronidase-negative strain) and detection time in GT medium at 44°C. Linear regression equation for the 20 strains at 44°C: $y = -1.07x + 9.48$ ($r = 0.99$). Linear regression equation for the single strain at 37°C: $y = -0.88x + 9.46$ ($r = 0.99$). Linear regression equation for the 20 strains at 37°C: $y = -0.99x + 10.02$ ($r = 0.99$).

grow at 44°C interfered more with *E. coli* enumeration. When *E. coli* were outnumbered by the *Enterobacter* species by a factor of 10^2 or by the *Klebsiella* species by a factor of 10, the concentration of *E. coli* was overestimated by a half log cycle.

Inclusion of sodium lauryl sulfate (0.2 mg/ml) as a selective agent in GT medium had no effect on the detection time of pure cultures of *E. coli*. In experiments with mixed competitors, it gave no significant improvement over standard GT medium incubated at 44°C.

E. coli were enumerated by using the β-glucuronidase assay and by a direct plate count method in 22 natural water samples, some spiked with settled raw sewage to obtain a range of natural microbial population sizes (3). The correlation curve (Fig. 4) shows good agreement between the two methods.

DISCUSSION

Although the curve of OD against time resembles a bacterial growth curve, the apparent lag phase includes a period of exponential growth (Fig. 2) and must include the period required for induction of the permease–β-glucuronidase enzyme system (13).

Incubation of the test medium at 44°C gave a more rapid ... accurate estimate of *E. coli* concentration than did ...ubation at 37°C. It reduced interference from competing organisms, and inclusion of lauryl sulfate in the medium produced no further improvement. When the result was adversely affected by competition, the detection time was reduced and the *E. coli* concentration was overestimated. Since the competitor organisms were all β-glucuronidase negative, the reduced detection time is probably due to enhanced enzyme production by *E. coli* in competition for nutrients.

The same inoculum gave a shorter detection time at 44°C than at 37°C, despite the presence of lower numbers of *E. coli* in the medium at the detection time. The rate of glucuronide hydrolysis was more rapid at the higher temperature.

The value of β-glucuronidase activity as a quantitative

TABLE 2. *E. coli* enumeration by β-glucuronidase activity at two incubation temperatures in the presence of a mixed competitive microflora

Temp (°C)	*E. coli*[a] (log CFU/ml)	Competitors[a,b] (log CFU/ml)	Detection time (h)	*E. coli* (log CFU/ml) from detection time
37	4.39	7.55	4.65	5.45
	4.39	6.55	4.90	5.20
	4.39	5.55	5.30	4.80
	4.39	4.55	5.53	4.60
	4.39	3.55	5.53	4.60
44	4.48	7.39	4.40	4.85
	4.48	6.39	4.58	4.65
	4.48	5.39	4.65	4.55
	4.48	4.39	4.65	4.55
	4.48	3.39	4.70	4.50
	4.48	2.39	4.70	4.50
44	2.62	7.80	5.80	3.40
	2.62	6.80	6.00	3.18
	2.62	5.80	6.30	2.85
	2.62	4.80	6.53	2.65
	2.62	3.80	6.53	2.65
	2.62	2.80	6.53	2.65

[a] Determined by plate count.
[b] Equal volumes of overnight broth cultures of *C. diversus*, *S. aureus*, *L. plantarum*, *P. aeruginosa*, and *B. cereus*.

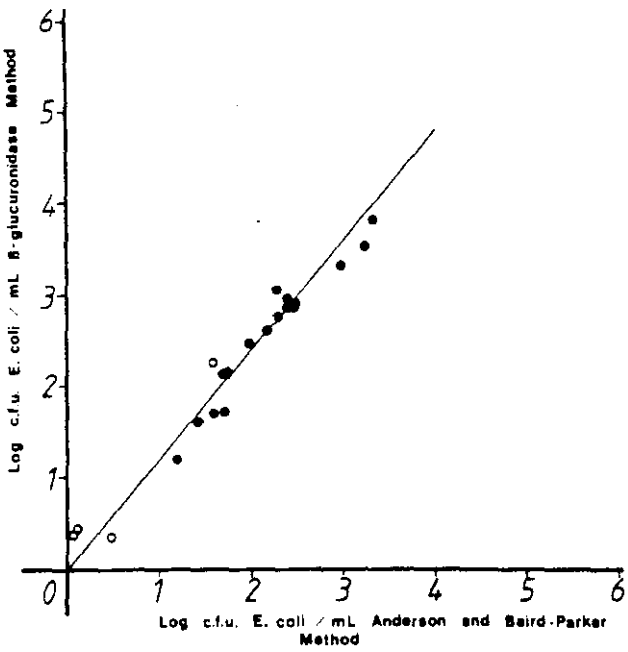

FIG. 4. *E. coli* enumeration in environmental samples by β-glucuronidase assay and Anderson Baird-Parker cultural method (1). Graph indicates correlation between two methods using natural water samples (○) and natural water samples spiked with settled raw sewage (●). Linear regression equation for 22 observations: $y = 1.17x$ ($r = 0.98$).

index of *E. coli* depends critically on the variability of enzyme activity within the species. The similarity of the correlation between detection time and inoculum concentration for a single *E. coli* strain and for a mixture of 20 unrelated strains (95% β-glucuronidase positive) suggests that this need not be a problem, particularly if an appropriate set of strains is used for calibration.

Although a small percentage of *E. coli* are β-glucuronidase negative, environmental samples are likely to contain a mixture of strains, thus considerably reducing the chances of false-negative results (12; W. E. Hill, J. L. Ferreira, W. L. Payne, V. M. Jones, C. L. Carlisle, and R. B. Read, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, P1, p. 199). False-positives may also be significant, since most other β-glucuronidase-positive bacteria are undesirable in food or water samples.

Some interference has been demonstrated, particularly with certain *Enterobacter* and *Klebsiella* spp., but only when these are present in excess. The good correlation between the β-glucuronidase assay and a traditional method with environmental samples suggests this need not be a significant problem with natural samples.

A similar approach to *E. coli* enumeration with a fluorogenic substrate was reported as too insensitive for rapid analysis of water, although no data were presented (2). Edberg and co-workers have successfully used β-glucuronidase activity for this purpose, but their technique employs a multiple-tube most-probable-number approach and requires 24 h of incubation to obtain a result (3, 4). The protocol described here is too labor intensive for routine enumeration of *E. coli*, although it is amenable to automation and more rapid than current techniques. In its present form, however, it could serve as the basis of a presence-absence test in water or, in foods, as a simple pass-fail test relating to predetermined acceptable threshold levels of *E. coli*.

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