Fluorogenic Assay for Rapid Detection of *Escherichia coli* in Food

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An assay procedure to screen for *Escherichia coli* in foods by using 4-methylumbelliferyl-β-D-glucuronide (MUG) incorporated into lauryl tryptose (LST) broth was evaluated. The β-glucuronidase produced by *E. coli* cleaves the MUG substrate to yield a fluorescent end product. *E. coli*-negative samples can be identified by lack of fluorescence in LST-MUG within 24 h. MUG was not inhibitory to coliforms and *E. coli*. Over 1,400 food and dairy samples were tested to compare the standard three-tube most-probable-number procedure with the MUG-containing or non-MUG-containing LST procedure. LST-MUG testing detected a greater number of *E. coli*, with a lower false-positive rate (1.4%) and in a shorter time, than did the standard procedure. All false-positive results in the LST-MUG testing were attributable to β-glucuronidase-producing staphylococci. No false-negative result was encountered. Use of MUG in LST broth obviates the EC broth step, allowing a 2.5-day procedure to a completed *E. coli* test versus the present 4- to 6-day standard most-probable-number method.

The various methods accepted by the Association of Official Analytical Chemists for the detection of *Escherichia coli* in food and dairy samples rely on the ability of the coliform to ferment lactose. In the commonly used most-probable-number (MPN) method (1), this lactose fermentation is identified by gas production in lauryl tryptose (LST; Difco Laboratories, Detroit, Mich.) broth. Subsequent transfers from gas-positive LST broth tubes to EC broth, with similar gas production, confirm the presence of this microbe. Confirmation of *E. coli* is completed by streaking samples from gas-positive EC broth tubes onto Levine eosin-methylene blue (EMB) agar and by biochemically characterizing the *E. coli*-typical isolates (1).

A serious limitation of the MPN method is the time required to complete the testing. Since other coliforms also produce gas in LST broth, gas production only presumptively indicates *E. coli* presence. Discrimination of *E. coli* beyond this initial step requires additional testing. In total, this standard method requires 4 to 6 days before *E. coli* presence can be declared. At the minimum, 4 days are necessary to prove its absence.

A more rapid and sensitive assay has been developed (4) which allows the presumptive identification of *E. coli* within 24 h. Its presence can be confirmed within an additional 1.5 days. This new method relies on the almost exclusive presence of the enzyme β-glucuronidase in *E. coli*. This enzyme cleaves the substrate, 4-methylumbelliferyl-β-D-glucuronide (MUG; no. M9130, Sigma Chemical Co., St. Louis, Mo.; no. 21844-21, Hach Co., Ames, Iowa), to yield a fluorescent end product. When MUG is incorporated into LST broth, fluorescence of the end product under long-wavelength UV light, regardless of gas production, indicates the probable presence of *E. coli*. After streaking the presumptive culture onto EMB agar, the characteristic colony is picked, and the microbe is biochemically confirmed. Absence of fluorescence in LST-MUG, regardless of gas production, indicates the absence of *E. coli*.

Of all of the *E. coli* strains examined, 97% produce this enzyme (5). In the family *Enterobacteriaceae*, the only other

microbes reportedly able to produce β-glucuronidase are some *Salmonella* spp. (4, 6), some *Shigella* spp. (4, 5), and some *Yersinia* spp. (7).

This study was undertaken to verify the capability of the LST-MUG medium to detect *E. coli*. The first phase examined the reproducibility of the procedure (4). The second phase compared the LST-MUG procedure with the current MPN procedure by using a wide variety of products routinely submitted to a food microbiology laboratory over a 2-year period.

MATERIALS AND METHODS

Stock cultures and media. Microorganisms included in this study were *E. coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 23355), and *Klebsiella pneumoniae* (ATCC 13883). All test organisms were grown in Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md.) or LST at 35°C for 24 h before their use. The overnight cultures were serially diluted with sterile 0.1% peptone water. Inoculum levels were determined by using a Petroff-Hausser counting chamber, with verification by plating onto plate count agar (Difco). All other bacteriological media used in these studies were from Difco Laboratories. Biochemical characterization of microbial isolates was done by using the Micro-ID enteric identification system (General Diagnostics, Div. of Warner-Lambert Co., Morris Plains, N.J.). MUG was incorporated into LST broth at the levels specified. Slight heating with agitation of the rehydrated medium helped dissolve the MUG before autoclaving at 121°C for 15 min.

Samples. A wide variety of samples were tested by the LST-MUG assay. Most ingredients and finished product samples were routine food microbiology laboratory submissions requiring clearance for *E. coli* and other food-borne pathogens. Samples included frozen dinners, pastas, fillers and stabilizers, water, raw meat, sauces and vegetables, spices, and dairy products (Table 1). In a few instances, product samples were purchased from local supermarkets for this testing.

Food samples were prepared by initially blending 25-g samples in 225 ml of sterile lactose broth. Serial dilutions were then made by using sterile 0.1% peptone water. Inoculation of the three-tube MPN procedure was as described

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TABLE 1. Variety of the 1,297 samples tested with LST broth and LST-MUG

Food type	Variety	No. of samples
Frozen food	Ethnic foods, one-dish meals, and side dishes	985
Ingredients	Pasta, tortillas, and fillers and stabilizers	67
Water	Well water	32
Raw meat	Ground beef, pork log, and beef log	22
Sauces and vegetables	Frozen entree sauces, beans, broccoli, and peas	105
Spices	Onion, garlic, oregano, and miscellaneous items	28
Dairy products	Pasteurized milk, whey, and cheese	58

before (1); this method was modified in some testing by incorporating MUG into the LST broth.

Experimental approach. The experimental approach was divided into two phases. Phase 1 examined the reproducibility of the procedure (4). Pure culture strains of *E. coli*, *K. pneumoniae*, and *E. cloacae* were used in this testing. Phase 2 verified the noninhibitory nature of MUG and examined the capability of this method to detect *E. coli*.

Phase 1—specificity studies. Testing was undertaken to determine the possible interference of *E. coli* detection by competitors. This would include suppression of *E. coli* growth as well as the determination of the fluorescence-producing ability of the other enterics. *K. pneumoniae* and *E. cloacae* were used as competitors in this testing. LST broth containing 100 μg of MUG per ml (LST-MUG) was inoculated with *E. coli* alone, *E. coli* plus enterics, or enterics alone. Each species was used at an inoculum level resulting in a final concentration of 30,000 organisms per ml. After inoculation, the tubes were incubated for 48 ± 2 h at 35°C. Fluorescence was detected by holding the tubes under a long-wavelength (366 nm) UV lamp.

Sensitivity of assay. A pure culture of *E. coli* was used to determine the minimum cell numbers and shortest time required to detect fluorescence under optimum conditions. An overnight culture of *E. coli* grown in LST broth was serially diluted to extinction. Samples of 0.1 ml each of each dilution were inoculated in triplicate into LST-MUG (100 μg of MUG per ml of LST broth) tubes, which were then incubated at 35°C. LST-MUG tubes were checked hourly to determine onset of fluorescence. Samples were also plated on trypticase soy agar at the time of initial inoculation and when fluorescence was detected. Initial cell counts were correlated with time to fluorescence to determine the sensitivity of the assay.

Optimizing MUG concentration in LST broth. Different concentrations of MUG were incorporated into LST broth to determine the minimum acceptable level to detect fluorescence. Triplicate samples of LST broth containing 10, 25, 50, 75, and 100 μg of MUG per ml were each inoculated with 10^6 *E. coli* per ml and were examined after 24 h at 35°C.

Phase 2—examination of inhibitory potential of MUG. The inhibitory potential of MUG was evaluated by two methods. The first method compared the growth rates of *E. coli* in different concentrations of MUG incorporated into LST broth. Erlenmeyer flasks containing 200 ml of sterile LST broth with 0, 50, 100, or 200 μg of MUG per ml were inoculated with approximately 100 CFU of *E. coli*. The cell population of all cultures was measured from the time of inoculation to stationary phase. Each level of MUG was represented by triplicate flasks. *E. coli* populations were

enumerated by duplicate platings onto plate count agar at each time reading. Plate count agar plates were counted after 24 h of incubation at 35°C.

The second method to evaluate the inhibitory potential of MUG compared the *E. coli* detection and recovery of LST broth versus that of LST-MUG. Parallel tests were conducted on 144 naturally contaminated frozen foods or their ingredients. By using the standard MPN procedure for *E. coli* detection, one set of LST broth tubes contained 50 μg of MUG per ml, but the other set had no MUG. Figure 1 presents a flow diagram of the testing procedure. All gas-positive or fluorescence-positive tubes in both sets were transferred into EC broth, followed by EMB isolation of characteristic colonies and biochemical characterization to confirm the presence of *E. coli*.

LST-MUG testing of routine food sample submissions. To examine the performance of the LST-MUG assay on a routine basis, all samples submitted to our food microbiology laboratory requiring *E. coli* analysis were examined with this method. The standard three-tube MPN procedure used in this testing had the LST broth supplemented with 50 μg of MUG per ml.

RESULTS

Phase 1. Our results in phase 1 of the study (data not shown) verified the reproducibility of this method for *E. coli* detection (4). In several repetitions of this experiment, *E. coli* alone or in a mixture with other enteric bacteria produced sufficient β-glucuronidase to enable detection by this method (Fig. 2). *E. cloacae* and *K. pneumoniae* failed to elicit any detectable fluorescence by this method; this conforms with their inability to produce β-glucuronidase. Even though these competitors initially outnumbered *E. coli* by
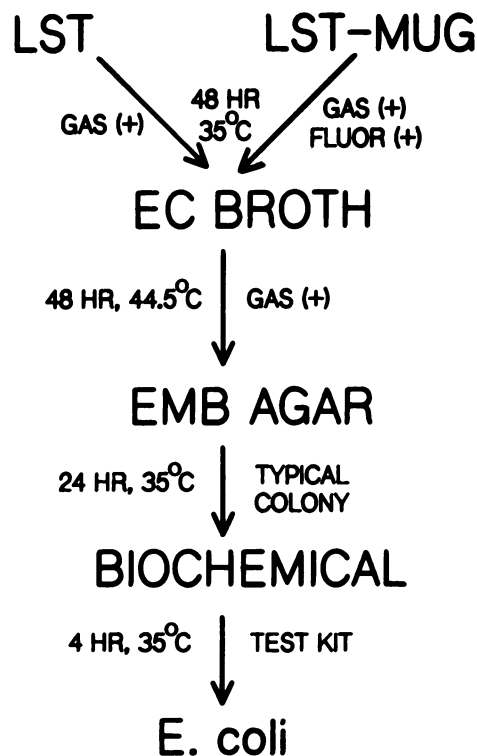

FIG. 1. Flow diagram illustrating parallel testing procedure of 144 food samples by using LST broth with and without MUG.
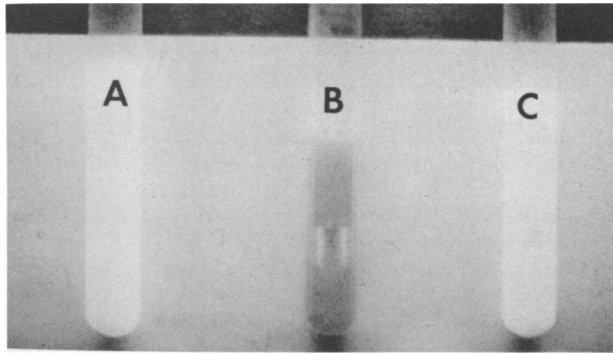
FIG. 2. Fluorescence production in LST-MUG inoculated with *E. coli* and other enteric bacteria. (A) *E. coli* alone; (B) mixture of *K. pneumoniae* and *E. aerogenes*; (C) mixture of *E. coli*, *K. pneumoniae*, and *E. aerogenes*.

TABLE 2. *E. coli* population levels in LST containing different concentrations of MUG

Time (h)[b]	Mean log$_{10}$ population levels at MUG concn (μg/ml)[a]:			
	0	50	100	200
0	3.08	3.10	3.07	3.08
0.75	3.36	3.24	3.31	3.28
3.0	3.30	3.32	3.40	3.41
5.0	4.88	4.92	4.97	5.18
7.0	8.47	8.30	8.42	8.45
9.0	8.72	8.77	8.79	8.79

[a] Duplicate readings of three repetitions.
[b] After inoculation of LST or LST-MUG with *E. coli*.

2:1, neither was able to suppress the β-glucuronidase production and thus prevent the detection of *E. coli*.

The sensitivity of the assay was also confirmed. An initial *E. coli* cell population of 1 organism per ml was detected in our testing within 12 h after LST-MUG inoculation. Higher initial cell populations required shorter times for fluorescence detection. Initial *E. coli* populations higher than 10^4 cells per ml were detected in less than 6 h. The *E. coli* population when fluorescence was first detectable was approximately 10^8 cells per ml. The two repetitions of this experiment agree with the previous research (4).

Although Feng and Hartman (4) recommend using MUG at the 100-μg/ml concentration, our study indicates that concentrations as low as 50 μg/ml provide sufficient intensity to detect fluorescence (Fig. 3).

Phase 2. Growth rate studies detected no inhibition of *E. coli* by MUG concentrations as high as 200 μg/ml. The results show no significant population difference from the control when testing used 50, 100, and 200 μg of MUG per ml of LST broth (Table 2). Each reading represents the mean of the duplicate samplings of the three repetitions. In tests of 144 naturally contaminated food samples, no evidence of any inhibitory effect of MUG was observed in either coliform or *E. coli* detection (Table 3). MUG had no adverse effect on gas production in LST broth. Both LST broth and LST-MUG identified the same 67 of the 144 total samples as being positive for coliforms. Similarly, the MPN level of coliforms

was not significantly different ($P > 0.05$) in either medium (LST broth, 306 ± 534 versus LST-MUG, 317 ± 548; mean microbes per gram of sample ± standard deviation).

The fluorescence in LST-MUG more accurately and quickly identified *E. coli* presence than did the gas production; 23 samples were presumptively identified as being *E. coli* positive within 24 h (Table 3). When transferred to EC broth, the 67 gas-positive LST broth tubes identified 27 of the samples as being positive for *E. coli*. When the source was the 67 gas-positive LST-MUG broth tubes, the same 27 samples were EC broth positive. The MPN level of gas-positive EC broth tubes was not significantly different ($P > 0.05$) in the two sets of samples (LST broth, 262 ± 458 versus LST-MUG, 306 ± 543; mean microbes per gram of sample ± standard deviation). However, further confirmation by EMB plating and biochemical testing revealed that only 23 of the 27 samples contained *E. coli*. Other enterics accounted for the false-positive reactions. Although the 23 samples containing *E. coli* were accurately identified in 24 h by LST-MUG, 5 days were required to confirm its presence by the standard procedure. Neither method detected any false-negative sample.

Of the 1,297 routine submission samples tested, both the LST-MUG assay and the conventional method identified the same 117 samples (9.0%) that contained *E. coli* (Table 4). I detected 18 false-positive samples (1.4%) by using fluorescence as an indicator of *E. coli*; all were identified as *Staphylococcus* spp. (Table 5). Specific *Staphylococcus* spp. were identified by use of the Staph-Ident staphylococcal system (Analytab Products, Plainview, N.Y.). I detected 35 false-positive samples (2.7%) by using gas production in EC broth as an indicator in the conventional scheme; all were either *Klebsiella* spp. or *Enterobacter* spp. Neither method detected any false-negative sample.
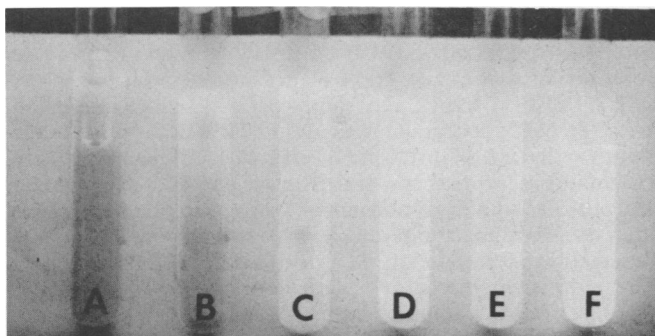

FIG. 3. Fluorescence production by *E. coli* inoculated in LST broth containing MUG at various concentrations (in micrograms per milliliter): A, 0; B, 10; C, 25; D, 50; E, 75; F, 100.

TABLE 3. Results of testing 144 naturally contaminated food samples to determine the inhibitory potential of MUG

Test	No. of food samples showing positive reactions with the following steps			
	LST broth		EC broth gas production[a]	Biochemical indication of *E. coli*
	Gas production	Fluorescence		
LST[b]	67	NA[c]	27	23
LST-MUG	67	23	27	23

[a] Four samples were false-positive (two *Serratia liquefaciens*, one *Klebsiella oxytoca*, and one *Klebsiella* sp.).
[b] MPN.
[c] NA, Not applicable.

TABLE 4. Comparative three-tube MPN testing of 1,297 food samples by using the conventional MPN method versus the proposed LST-MUG method of *E. coli* detection

Sample type	No. tested	No. of samples showing positive reactions					
		Conventional MPN assay			LST-MUG assay		Biochemical assay
		LST	EC broth	False[a]	Fluorescence	False[b]	*E. coli*
Frozen food	985	330	122	16	117	11	106
Ingredients	67	12	4	4	7	7	0
Spices	28	10	6	6	0	0	0
Water	32	0	0	0	0	0	0
Raw meat	22	14	10	0	10	0	10
Sauces and vegetables	105	10	0	0	0	0	0
Dairy products	58	16	10	9	1	0	1

[a] These 35 false-positive isolates were all enterics: 26 *K. pneumoniae*, 8 *K. oxytoca*, and 1 *Enterobacter agglomerans* isolate.

[b] These 18 false-positive isolates were all staphylococci.

A statistical analysis of the 117 positive samples showed that fluorescence was more efficient than gas production in the detection of *E. coli*. The paired *t* test found that by using fluorescence in LST-MUG as an indicator of *E. coli*, the mean number of *E. coli* recovered per gram sample (144 ± 282; mean $\pm$ standard deviation) was significantly greater ($P < 0.05$) than that recovered by using gas production in EC broth (113 ± 243; mean $\pm$ standard deviation) as an indicator.

DISCUSSION

Samples that contain coliforms but are free of *E. coli* comprise the majority of samples encountered in a food microbiology laboratory. Incorporation of the MUG substrate into LST broth provides the capability of a rapid screening procedure for *E. coli* detection. *E. coli*-negative samples are identified by lack of fluorescence within 24 h and require no further testing. *E. coli*-positive samples can be identified by fluorescence development in LST-MUG within 24 h. In this study, even though all LST-MUG tubes were held for 48 h, no further fluorescent reactions had developed beyond the 24-h period. Confirmation of *E. coli* then requires an additional 1.5 days for selective isolation on EMB agar and biochemical characterization. The 2.5 total days required for *E. coli* identification is more rapid than the 4 to 6 days required by the current MPN procedure.

Although some species of *Salmonella*, *Shigella*, and *Yersinia* reportedly have the enzyme β-glucuronidase and therefore the ability to produce fluorescence, none was encountered in the >1,400 samples evaluated by this study. With the proposed testing procedure, had these pathogens been present, they would have been identified during the

TABLE 5. *Staphylococcus* species which yielded false-positive reactions in LST-MUG

Organism	No. of samples yielding organism	Sample yielding organism
S. haemolyticus[a]	1	Pasta
S. simulans	6	Pasta, soy flour
S. cohnii[a]	7	Frozen food
S. xylosus[a]	3	Frozen food
S. warneri	1	Frozen food

[a] Indicates species are associated with the normal flora of human skin (8).

attempted *E. coli* isolation and biochemical characterization. In the most conservative of interpretations, strong fluorescence in LST-MUG could be indicative of pathogens or potential problems with a product. Confirmatory tests using selective isolation (EMB agar) and biochemical characterization would then identify the responsible microbe. In a more liberal approach, as proposed by Feng and Hartman (4) and Robison (7), fluorescence in LST-MUG is sufficient to confirm the presence of *E. coli*. Data of this study certainly support the latter viewpoint. No false-negative sample was detected in the study by either the LST-MUG or the standard MPN procedure. The LST-MUG procedure would have detected those *E. coli* which are gas negative but fluorescence positive. Anaerogenic *E. coli* represent approximately 5% of the known strains (3). The standard MPN procedure would have detected those *E. coli* which are gas positive but fluorescence negative. Only 3% of known *E. coli* strains do not produce β-glucuronidase (5) and thus fluorescence. The LST-MUG procedure relying on fluorescence is more sensitive to *E. coli* detection than are those procedures relying on gas production.

The 18 false-positive samples (1.4%) encountered with the LST-MUG method were all due to *Staphylococcus* species. All of these species reportedly produce β-glucuronidase. Typically, they showed poor growth in LST-MUG, but their fluorescent reaction was still of sufficient intensity to warrant further testing. The presence of these staphylococci in the individually handled frozen food and pasta products is not unusual; most of the isolates are associated with the normal flora of human skin (8). Their presence was easily discerned by selective plating on EMB agar. Robison (7) detected a 4.8% false-positive rate in LST-MUG due to streptococci. She also suggested the use of EMB agar to confirm the presence of non-*E. coli* fluorescence-producing isolates.

The 2.7% false-positive samples (35 samples) detected with the MPN procedure was almost twice that percentage found with the LST-MUG method (1.4%). All microbes were common enteric bacteria which grew and produced gas in EC broth at 44.5°C. Biochemical testing provided their true identity. A similar rate of false-positive samples (2.8%) detected with the standard MPN procedure was encountered when the two procedures were compared in the parallel testing; 4 of 144 samples were identified as false-positive non-*E. coli* samples. In all of these instances, the LST-MUG system correctly identified these samples (no fluorescence) as containing no *E. coli*.

The higher population level of *E. coli* detected by fluores-

cence versus gas formation in the 117 positive samples may be due to the increased sensitivity of fluorescence detection as compared with gas accumulation. MUG was found to be neither inhibitory nor stimulatory to *E. coli* when its effect on the microbial growth rate was evaluated; no growth rate difference was detected when cell population levels with MUG concentrations from 0 to 200 μg/ml were monitored.

The minimum amount of the MUG substrate needed to detect fluorescence was found to be lower than that previously reported. A MUG concentration of 50 μg/ml provided the same visual intensity as did the higher MUG levels. MUG concentrations below 50 μg/ml showed weak fluorescent reactions; such reactions may be subjectively rated as negative by untrained observers. These lower MUG levels may inadvertently result from weighing errors due to excessive moisture in the hygroscopic compound. Likewise, the commercial MUG preparations from different manufacturers may vary in moles of water, thus necessitating a weight correction for proper MUG concentration in the final medium. Positive and negative controls should be run with every batch of LST-MUG produced to assure the adequacy of the fluorescent reaction. Several manufacturers are now commercially marketing LST broth into which MUG has been incorporated.

Although this study was limited predominantly to frozen foods and their components, no interference of the food products was found in the fluorescence assay. Other reports (4, 7) on LST-MUG have similarly identified no food interference with the fluorescence testing. However, a recent report (S.R. Rippey and L.A. Chandler, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, N20, p. 220) has noted an interference with the use of the assay in testing shellfish. The tissues of raw oysters, mussels, and hard- and soft-shelled clams apparently contain sufficient β-glucuronidase to interfere with the reaction. This problem was solved by incorporating the MUG into the EC broth instead of into the LST broth. A positive fluorescent reaction then confirms the presence of *E. coli* in the EC broth-MUG after transfer from gas-positive LST broth samples. Use of MUG enables a more rapid detection of *E. coli* in shellfish than does the present method (2).

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