APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1993, p. 4347–4349
0099-2240/93/124347-03$02.00/0

Vol. 59, No. 12

Rapid Glutamate Decarboxylase Assay for Detection of *Escherichia coli*

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Received 22 June 1993/Accepted 12 October 1993

A rapid test procedure for the enzyme glutamate decarboxylase was developed for detection of *Escherichia coli*. The assay procedure was able to confirm the presence of *E. coli* in enteric broth cultures with 95% specificity for both pure cultures and environmental samples. The procedure was capable of detecting survivors among chlorine-exposed cells.

Escherichia coli is an important indicator organism used to assess the sanitary quality of various environmental water samples. Under recently enacted federal legislation, all drinking water samples shown to contain total coliform bacteria must be further analyzed for the presence of fecal coliforms or *E. coli* (3, 4). All of the approved methods for detection of *E. coli* in drinking water rely upon an assay for the enzyme β-glucuronidase with the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide (MUG).

Assays for the enzyme glutamate decarboxylase (GAD) (EC 4.1.1.15) have been reported to be very selective for *E. coli*, with specificity rates ranging from 97 to 99% (5, 6, 10, 12, 13). The enzyme catalyzes the α-decarboxylation of glutamic acid to yield γ-aminobutyric acid and carbon dioxide. Some form of lytic agent is necessary for liberation of the enzyme (7). Several methods have been proposed which use toluene for cell lysis and detection of GAD by a change in pH brought about by alkalinization of the test reagent (6, 9, 13). Recently, a method was developed in which toluene was replaced by a hypertonic sodium chloride solution (5) to release the enzyme. Sodium chloride was utilized previously in assays designed for detection of GAD in anaerobic, gram-positive bacteria (9). This report describes a rapid GAD assay, based on the procedure of Fiedler and Reiske (5), designed to verify the presence of *E. coli* initially isolated in two enteric broth media routinely used in water analysis.

The GAD reagent consisted of 1 g of L-glutamic acid (Sigma, St. Louis, Mo.), 0.05 g of bromocresol green (Eastman Kodak, Rochester, N.Y.), 90 g of NaCl, and 3 ml of Triton X-100 (Baker, Philipsburg, Pa.) per liter of distilled water (pH 3.4). The reagent was thoroughly mixed until all ingredients were dissolved. The reagent was stable for 2 months when stored at 5°C and can be filter sterilized and treated as a sterile solution. Five-milliliter samples of 20 to 24-h cultures grown in lauryl tryptose broth (LTB; Difco, Detroit, Mich.) or presence-absence (PA) broth (Difco) were transferred from the culture tubes to 15-ml conical screw-cap test tubes, and the bacteria were concentrated by centrifugation at 500 × *g* for 10 min. The supernatant was discarded, and the bacterial cells were washed by resuspension in 5 ml of phosphate buffer (1) and then centrifugation at 500 × *g* for 10 min. After the wash step, the supernatant was discarded and the bacteria were resuspended by vigorous vortexing followed by addition of 1.0 ml of the GAD reagent. The tubes were then incubated at 35°C and observed hourly for 4 h. A distinct change from yellow to blue was considered a positive response for *E. coli*. An *E. coli* culture and a fecal coliform-positive *Klebsiella pneumoniae* culture served as GAD-positive and -negative controls, respectively.

Pure-culture studies were conducted with strains from the stock culture collection of the U.S. Environmental Protection Agency Drinking Water Research Division. Cultures were grown for 18 to 20 h at 35°C in LTB. Tubes exhibiting growth were assayed for GAD as described above. Aliquots of a 20-h *E. coli* culture grown in LTB were assayed to determine the density of cells required for a positive GAD reaction within a given time period. The number of cells present in the culture was determined by assaying serial dilutions of the culture by the spread plate technique on plate count agar (Difco) and incubating the plates at 35°C for 24 h.

Six types of environmental waters were sampled and analyzed for total coliform bacteria by the multiple-tube fermentation procedure with both LTB and PA media. Appropriate decimal dilutions of each sample were prepared on the basis of probable coliform density (1). When shipment was required, the samples were placed on ice, shipped by overnight carrier, and examined within 24 h of collection. All other samples were analyzed on the day of collection.

Each total coliform-positive LTB and PA tube was transferred to EC-MUG medium (11) and incubated for 24 ± 2 h in a water bath at 44.5 ± 0.2°C. The tubes were exposed to a long-wavelength (366-nm) UV light source to determine fluorescence (11). The presence of fluorescence indicated a positive response for *E. coli*. Control cultures consisting of a known MUG-positive *E. coli* culture and fecal coliform-positive, MUG-negative *K. pneumoniae* culture were incorporated in each experiment.

Each culture which yielded a positive coliform response was examined for the presence of *E. coli*. When the culture exhibited a positive MUG reaction, a loopful of the EC-MUG culture was streaked onto MacConkey agar and incubated at 35°C for 24 h. Any total coliform-positive culture which did not produce a positive MUG reaction was also subcultured from the positive LTB or PA tube to MacConkey agar and incubated in the same manner. Target coliform colonies were picked from the MacConkey plates and identified to the species level with the API 20E (Analytab, Plainview, N.Y.) system. At least five colonies were

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TABLE 1. GAD assay results for the bacterial taxa tested

Organism(s) (no. of strains)	No. (%) of strains positive
Aeromonas hydrophila (8)	0 (0)
Citrobacter freundii (33)	0 (0)
Enterobacter aerogenes (2)	0 (0)
Enterobacter agglomerans (2)	0 (0)
Enterobacter amnigenus (2)	0 (0)
Enterobacter cloacae (15)	0 (0)
Enterobacter sakazakii (3)	0 (0)
Enterobacter taylorae (4)	0 (0)
Escherichia coli (305)	291 (95)
Inactive *Escherichia coli* (5)	5 (100)
Escherichia fergusonii (5)	0 (0)
Escherichia hermannii (7)	0 (0)
Escherichia vulneris (7)	0 (0)
Flavobacterium odoratum (1)	0 (0)
Hafnia alvei (9)	0 (0)
Kluyvera spp. (15)	0 (0)
Morganella morganii (2)	0 (0)
Proteus mirabilis (3)	0 (0)
Proteus vulgaris (1)	0 (0)
Pseudomonas aeruginosa (1)	0 (0)
Salmonella arizonae (1)	0 (0)
Salmonella enteritidis (1)	0 (0)
Salmonella schottmuelleri (1)	0 (0)
Serratia marcescens (1)	0 (0)
Serratia odorifera (1)	0 (0)
Shigella boydii (7)	1 (14)
Shigella dysenteriae (4)	1 (25)
Shigella flexneri (6)	5 (83)
Shigella sonnei (2)	0 (0)
Vibrio fluvialis (1)	0 (0)
Yersinia enterocolitica (1)	0 (0)

picked from every total coliform-positive, EC-MUG-negative culture. Target colonies from each MUG-positive, EC-MUG culture were picked until an *E. coli* culture was identified, up to a maximum of 10 colonies. Five hundred fifteen isolates were examined. The results were compared on a tube-per-tube basis for each sample for the two procedures. A quantitative estimate of the number of *E. coli* organisms present was made by the most-probable-number (MPN) technique (1). The mean MPN values for the EC-MUG and GAD-positive responses were compared for each sample, and the correlation coefficient was determined (8).

A consortium of 20 *E. coli* isolates was used as the inoculum for the inactivation experiments. All cultures had been isolated from various environmental sources and stored on heart infusion agar slants for no longer than 2 months. Individual cultures were grown in LTB at 35°C for 18 to 20 h. The cultures were pooled by combining 2 ml of each culture. The pooled consortium was concentrated and washed three times by centrifugation at 1,000 × *g* for 10 min with phosphate buffer (1). The initial level of organisms exposed to the disinfectants was approximately 1.7×10^6 CFU/ml. Chlorine solutions were prepared with sodium hypochlorite (Fisher, Pittsburgh, Pa.) in chlorine demand-free 0.05 M KH_2PO_4 buffer at pH 7.0. Ammonium sulfate was used as the nitrogen source for monochloramine solutions at a chlorine-to-nitrogen ratio of 3:1 (wt/wt). Controls consisted of chlorine demand-free buffer without the disinfectant. Inactivation experiments were conducted as previously described at 20°C (2). Control and chlorine-exposed cells were assayed by the multiple-tube fermentation technique with five tubes per dilution series and both LTB and PA media (1). All presumptively gas-positive tubes were assayed for *E. coli* by transfer to EC-MUG medium and the GAD reagent as described above.

TABLE 2. Comparison of environmental sample EC-MUG- and GAD-positive reactions on the basis of a tube-per-tube comparison

Sample (no.)	Presumptive medium	No. of positive reactions			No. of confirmed *E. coli* cultures
		Total coliforms	EC-MUG	GAD	
River (1)	LTB	20	15	15	15
	PA	19	14	14	14
Lake (1)	LTB	18	14	14	15
	PA	15	13	13	13
Creek (1)	LTB	16	8	8	8
	PA	14	10	7	10
Sewage (1)	LTB	21	19	18	18
	PA	22	20	19	20
Seawater (3)	LTB	11	5	4	5
	PA	11	7	7	8
Drinking water (2)	LTB	14	6[a]	7	7
	PA	17	3	3	3
Total		198	134[b]	129[c]	136

[a] Two weak MUG reactions.
[b] 98.5% were *E. coli* positive.
[c] 94.8% were *E. coli* positive.

A total of 454 isolates comprising 30 species from 15 genera were examined for GAD activity (Table 1). Of the 310 *E. coli* cultures tested, 296 (95.5%), including five inactive *E. coli* (Alkalescens-Dispar) isolates, yielded positive responses. All of the other *Escherichia* species (*E. fergusonii, E. hermannii, and E. vulneris*) were negative in the GAD reaction. *Shigella* was the only other genus that produced a positive GAD reaction. Five of six strains of *Shigella flexerni* and one each of seven strains of *S. boydii* and six strains of *S. dysenteriae* were GAD positive.

The GAD assay, performed on various volumes of a 20-h culture of *E. coli*, indicated that 3.0 ml of the culture (2.5×10^9 CFU) was required to produce a positive GAD response in 1 h. A positive response was achieved within 2 h with a volume of 2.5 ml (2.1×10^9 CFU) and within 3 h with a volume of 2.0 ml (1.7×10^9 CFU). On the basis of these results and the potential for *E. coli* growth in LTB or PA broth in mixed cultures, a 5-ml aliquot was chosen as the standard volume of presumptive medium for concentration in the GAD assay.

The results of the environmental sample analyses are shown in Table 2. *E. coli*, identified by the API 20E system from the MacConkey agar streak plates, was found in 136 (68.7%) of the 198 cultures examined. The EC-MUG procedure detected 134 (98.5%) and the GAD reaction detected 129 (94.8%) of these *E. coli* cultures. The GAD reaction occurred within 1 h in 122 (94.6%) of the 129 positive responses.

The mean $\log_{10}$ MPN values for the EC-MUG and GAD reactions for each sample are graphically presented in Fig. 1. The data covered a wide range of values, with one of the drinking water samples giving an MPN of 2/100 ml while the MPN value for the sewage sample was 14,000/100 ml. The correlation coefficient (r) for these data was 0.98.

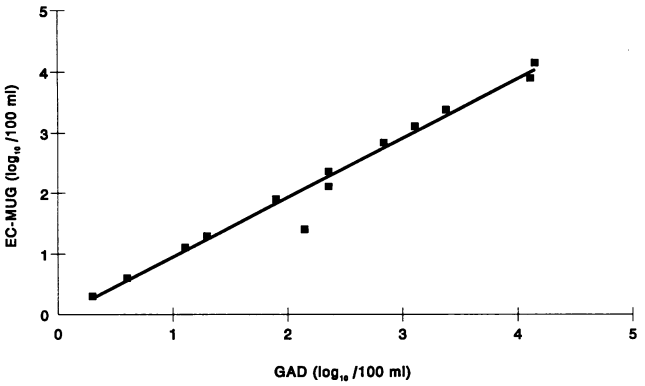

FIG. 1. Comparison of *E. coli* EC-MUG and GAD densities for natural samples obtained by the multiple-tube fermentation procedure.

The results of the chlorine inactivation experiments are listed in Table 3. There was no difference in the GAD and EC-MUG responses for the free-chlorine inactivation experiments. For *E. coli* cells exposed to monochloramine inactivation, the results showed only a 1-tube difference between GAD and EC-MUG procedures. These results indicate that the GAD assay is equivalent to EC-MUG medium in the ability to detect the survivors of an *E. coli* population exposed to these two disinfectants.

The approved method for confirmation of the presence of *E. coli* in total-coliform-positive broth cultures requires transfer to EC-MUG medium with an additional 24 ± 2 h of incubation at the elevated temperature of 44.5 ± 0.2°C (11). While this method has been shown to have good specificity, it is not a rapid procedure and calls for interpretation of a positive result on the basis of fluorescence observed under a long-wavelength UV light source.

Preliminary experiments incorporating various concentrations of EDTA into the GAD reagent did not increase the sensitivity of the assay. Similarly, inclusion of the cosubstrate pyridoxal-5′-phosphate did not enhance the reaction. While this procedure offers many advantages, it may be possible to simplify the procedure further by using better methods for cell concentration or lysis.

TABLE 3. Consortium of 20 strains of *E. coli* exposed to 0.5 mg of free chlorine or monochloramine per liter at pH 7.0 and 20°C

Disinfectant	Exposure time (min)	Presumptive medium	*E. coli* survival			
			EC-MUG[a]	GAD[a]	EC-MUG[b]	GAD[b]
Free chlorine	2	LTB	7,200	7,200	33	33
		PA	3,600	3,600	29	29
Monochloramine	30	LTB	30,000	30,000	33[c]	33
		PA	13,000	18,000	30	31

[a] Mean MPN index per 100 ml (mean of three experiments, rounded to two significant figures).
[b] No. of positive tubes.
[c] One weak positive MUG reaction.

The GAD reagent is very inexpensive to prepare compared with the cost of EC-MUG medium. The need for centrifugation in the GAD procedure is easily offset by the rapidity of the test and the requirement for other special equipment, such as the elevated-temperature water bath and long-wavelength UV light source needed for the EC-MUG method.

The GAD procedure enables an analyst to provide rapid confirmation of the presence of *E. coli*. In greater than 90% of the cases, a positive response can be detected within 1 h of assay initiation. The assay readily lends itself to use with the multiple-tube fermentation procedure or the single-bottle cultures used in the PA concept for drinking water analysis. Use of this procedure would allow public health authorities a more expeditious time frame in which to respond to a fecal contamination event.

We thank A. P. Dufour for insightful guidance in reference to the initiation of this project. We also thank Elizabeth A. Creamer, Paralee J. Mason, and Carol J. Palmer for assistance.

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