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Comparison of β-Glucuronidase-Based Substrate Systems for Identification of *Escherichia coli*

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Methods based on the measurement of β-glucuronidase have been shown to be specific and inexpensive for the identification of *Escherichia coli* from bacterial colonies within 1 h. Recently, commercial systems incorporating β-glucuronidase substrates were introduced. Rapid Identification Method *E. coli* (Austin Biological Laboratories, Curtin Matheson Scientific, Inc., Houston, Tex.) and Rapid Detect *E. coli* (Organon Teknika, Morris Plains, N.J.) are single-tube test combinations to simultaneously measure β-glucuronidase (fluorescence at 366 nm), *o*-nitrophenyl-β-D-galactopyranoside (yellow), and indole (red). To determine the accuracy and utility of these two systems, we used them to test 169 *E. coli* and 150 non-*E. coli* and compared them with conventional substrate tests. The Rapid Detect test was more efficient than the Rapid Identification Method in demonstrating β-glucuronidase activity, but the commercial systems were equal to each other and to the conventional tests for *o*-nitrophenyl-β-D-galactopyranoside and indole. There were no false reactions by either system.

Nationally, 95% of urinary tract infections are caused by a single infecting microbe, *Escherichia coli* being responsible for approximately 80% of all nosocomial and more than 90% of community-acquired infections (1).

In 1976 Kilian and Bulow (3), in a survey of the activity of bacterial glycosidases, found that β-glucuronidase activity was confined to the genera *Escherichia*, *Shigella*, and *Salmonella*. Based on this enzyme characteristic, procedures have been developed for the specific detection of *E. coli* from water (2), food (4, 6), and biologic fluids (1, 7). A rapid and economical system for the identification of *E. coli* utilizing the β-glucuronidase test as the pivotal decision point in an algorithm is commonly used and has led to commercial production of β-glucuronidase substrates.

The β-glucuronidase substrate originally described for the identification of *E. coli* was a *p*-nitrophenyl derivative. Now the fluorogen methylumbelliferyl-β-D- linked to glucuronic acid and combined with other substrates has become commercially available. Manufacturers have provided a test battery for the identification of *E. coli* that employs a single inoculation for simultaneous detection of β-glucuronidase (fluorescence at 366 nm), *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (yellow), and indole (red).

Two of these commercial β-glucuronidase-based substrate systems for the rapid identification of *E. coli* were examined in this study. The Rapid Identification Method (RIM; Austin Biological Laboratories, Curtin Matheson Scientific, Inc., Houston, Tex.) employs the three substrates on a cotton-tipped swab. The Rapid Detect *E. coli* (RDE) system (General Diagnostics, Div. Organon Teknika, West Orange, Morris Plains, N.J.) provides the same substrates on paper disks. These commercial *E. coli* identification substrate systems were compared with conventional substrate formulations to determine their accuracy.

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MATERIALS AND METHODS

Bacterial isolates. All isolates were from patient specimens identified in the Clinical Microbiology Laboratory, Yale-New Haven Hospital, New Haven, Conn. Analyses were conducted with 169 *E. coli* and 150 non-*E. coli Enterobacteriaceae*. All but 16 isolates were freshly obtained specimens from patients; 16 came from the laboratory culture collection. Bacteria were first identified by the Micro-ID system (Organon Teknika). Only organisms demonstrating a normalized probability of 99% or above were accepted. Isolates showing a lower than 99% normalized probability were further analyzed in the API-20E System (Analytab Products, Plainview, N.Y.) supplemented by conventional tests when necessary.

Comparative test procedures. All tests were conducted on isolates grown in pure culture on MacConkey (MAC) agar and 5% sheep blood agar plates (BAP) (BBL Microbiology Systems, Cockeysville, Md.) for 18 to 24 h. In addition, 30 isolates of *E. coli* and 30 of non-*E. coli Enterobacteriaceae* were tested after 18 to 24 h on eosin-methylene blue (EMB) agar.

Conventional test procedures. The β-glucuronidase test reagent consisted of 0.5 mg of *p*-nitrophenyl-β-D-glucuronide(Sigma Chemical Co., St. Louis, Mo.) per ml in 0.05 M Sorensen phosphate buffer, pH 7.5. The reagent was dispensed into test tubes (12 by 75 mm; 0.25 ml per tube). Several colonies from each test isolate were inoculated from each test medium and incubated for 1 h at 35°C. Development of a yellow color indicated the presence of β-glucuronidase; no color change in the original colorless solution was read as negative (1). Indole and ONPG tests were performed by conventional procedures (5, 8).

RIM *E. coli* test. The RIM system consisted of reagent-impregnated cotton swabs on wooden sticks. The test was performed in accordance with manufacturer instructions. One to three colonies were touched with a single reagent-impregnated swab. The swab containing the bacterial inoculum was placed in 0.5 ml of RIM buffer, incubated at 35°C, and examined at 30 and 60 min. A positive ONPG

reaction was noted with the appearance of a yellow color. If positive, the test was subsequently examined for the presence of β-glucuronidase activity by placing the tube over a longwave (366 nm) UV light source. Fluorescence indicated β-glucuronidase activity; no fluorescence was read as negative. The RIM β-glucuronidase was enhanced by addition of Na_2CO_3. If the test was positive for β-glucuronidase activity, five drops of Kovac reagent were added, and the tube was gently shaken. The appearance of a red color indicated the presence of indole.

RDE test. The RDE system consisted of substrates impregnated on paper disks. Test procedure followed the manufacturer-supplied protocol. Bacterial colonies were taken from agar and inoculated into 0.2 ml of distilled water to yield a suspension equal to a 4.0 McFarland turbidity standard. A disk containing both β-glucuronidase and ONPG substrates was added to this tube which was then incubated at 35°C in ambient air. The reactions were noted at 30 and 60 min. Development of a yellow color indicated the presence of β-galactosidase. If no yellow color occurs at 30 min, the manufacturer recommends reincubation for an additional 30 min. If positive, the tube was examined with a 366-nm UV light source. Fluorescence indicated the presence of β-glucuronidase; if there was no fluorescence, the test was negative. If negative for β-glucuronidase, the test was reincubated for an additional 30 min. If the β-glucuronidase test was negative, an indole indicator disk was added to test for indole production. A red color indicated the presence of indole.

Interpretation of results. Interpretation of the β-glucuronidase, ONPG, and indole tests was the same for the commercial and conventional systems. An organism demonstrating positivity in all three tests was *E. coli*. An organism demonstrating negative ONPG, β-glucuronidase, or indole was considered to have a low enough probability of being *E. coli* to warrant an alternative procedure for confirmatory identification.

RESULTS

The number of positive β-glucuronidase tests for each of the three substrate systems tested from BAP, MAC agar, and EMB agar are presented in the table. ONPG and indole tests agreed in all systems. In no case was there either a false-positive enzyme assay or identification. Therefore, the primary purpose of the substrate combination was fulfilled in each system.

BAP. β-Glucuronidase activity was demonstrated in 150 of 169 *E. coli* isolates by the conventional method. The sole *Salmonella arizonae* isolate tested was positive. No non-*E. coli Enterobacteriaceae* showed β-glucuronidase activity. The RDE procedure demonstrated 95% correlation and the RIM test showed 46% correlation with the conventional test. In both systems a significantly higher number of *E. coli* isolates were positive at 60 min compared with 30 min of incubation at 35°C. Both the RDE and RIM systems showed complete correspondence with conventional tests for ONPG and indole for BAP colonies.

MAC agar. β-Glucuronidase fluorescence was muted when taken from MAC agar by all tests. This appeared to be due to both the red color imparted to the colonies by neutral red after lactose fermentation and the decrease in pH caused by fermentation of the carbohydrate. β-Glucuronidase tests taken from MAC agar should be incubated for the complete 60 min (Table 1). Addition of Na_2CO_3 clarified the endpoint. ONPG reactions were weaker when taken from MAC agar than when from BAP by all systems; however, the percent positivity was unchanged for MAC agar colonies. Indole reactions, in keeping with results previously reported in the literature, were also weak in all systems using MAC agar colonies.

EMB agar. Colorimetric endpoints from EMB agar were difficult to ascertain. *E. coli* often had a fluorescent sheen on EMB agar. This fluorescence resulted in a green-pink color when the indole test was performed. The fluorescence from the β-glucuronidase test was very difficult to interpret because a mixture of yellow-green colors was observed together with fluorescence. The indole test color was also significantly changed so that its interpretation was unreliable.

DISCUSSION

E. coli is the gram-negative bacterium most commonly isolated from human urinary tract infections. A rapid and economical means to identify *E. coli* has been developed based on β-glucuronidase assay. The test also served as the starting point in an algorithm for processing urine cultures. This bacterium can be identified by the following characteristics: growth and lactose fermentation on MAC agar, production of β-glucuronidase, production of indole, and lack of production of oxidase (1). Recently, several commercial companies have modified these enzyme assays and packaged them in multitest substrate assays in a single-delivery mode. The value of the β-glucuronidase-based rapid *E. coli* tests lies in their specificity (all tested in this study) (1, 4) and low cost to the laboratory.

Of the 169 *E. coli* strains from human clinical specimens analyzed here, there were no false-positive tests. Reactions for all three enzyme substrate tests for both commercial systems and the conventional enzyme assays were clearer from BAP than from MAC agar. It has previously been shown that enzyme assays from MAC agar may be muted or falsely negative owing to the red color imparted from neutral red during lactose fermentation and the decrease in pH produced as a result of lactose fermentation (1). These factors are particularly important when utilizing hydrolyzable substrates for which pK_a values of the released moiety must be alkaline to yield color. The supplier of the RIM system recommends adding sodium carbonate to the β-glucuronidase test to enhance fluorescence clarity. The supplier of the RDE system recommends dropping the indole disk into the inoculum to test for indole. It was found more easily readable, however, if the inoculum was tilted onto the disk, which had been placed on the side of the glass test tube.

The RIM system has its three substrates impregnated on fiber-tipped wooden applicator sticks. The RDE system employs two disks; the first contains β-glucuronidase and β-galactose substrates, and the second contains the indole substrate. The RDE disk system was more convenient to handle and use on a routine basis than the RIM stick system. This was especially noticeable during batch processing, when many tubes were arranged in a test tube rack. The need to keep the swab in the tube while testing by the RIM system made it difficult to observe β-glucuronidase reactions at times. It eliminated some of the viewing area of the reaction mixture as well as enhancing fluorescence from the colored reaction products from MAC agar. Furthermore, the RDE system requires distilled water rather than buffer, which is handier on a routine basis.

Reactions from EMB agar for all systems were difficult to read and EMB agar is not recommended. Often, *E. coli* had

TABLE 1. Comparison of commercial β-glucuronidase systems for the identification of *E. coli*

Species	No. of strains tested	No. of strains classified by RDE on:												No. of strains classified by RIM on:												No. of strains positive by conventional β-glucuronidase test
		BAP				MAC agar				EMB agar[a]				BAP				MAC agar				EMB agar[a]				
		30 min		60 min		30 min		60 min		30 min		60 min		30 min		60 min		30 min		60 min		30 min		60 min		
		+	−	+	−	+	−	+	−	+[b]	−	+[b]	−	+	−	+	−	+[b]	−	+[b]	−	+[b]	−	+[b]	+	
Escherichia coli	169	109	60	143	26	30	139	117	52	30	0	30	0	38	131	68	101	13	156	32	137	29	1	29	1	150
Salmonella arizonae	1	1	0	1	0	1	0	1	0					1	0	1	0	1	0	1	0					1
Citrobacter diversus	4	0	4	0	4	0	4	0	4	2	)	2	0	0	4	0	4	0	4	0	4	1	1	2	0	0
Citrobacter freundii	3	0	3	0	3	0	3	0	3	1	0	1	0	0	3	0	3	0	3	0	3	1	0	1	0	0
Enterobacter aerogenes	14	0	14	0	14	0	14	0	14	4	0	4	0	0	14	0	14	0	14	0	14	4	0	4	0	0
Enterobacter cloacae	11	0	11	0	11	0	11	0	11	5	0	5	0	0	11	0	11	0	11	0	11	5	0	5	0	0
Klebsiella oxytoca	6	0	6	0	6	0	6	0	6					0	6	0	6	0	6	0	6					0
Klebsiella pneumoniae	59	0	59	0	59	0	59	0	59	8	0	8	0	0	59	0	59	2	57	2	57	8	0	8	0	0
Morganella morganii	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0
Proteus mirabilis	18	0	18	0	18	0	18	0	18	0	4	0	4	0	18	0	18	0	18	0	18	0	4	0	4	0
Proteus rettgeri	1	0	1	0	1	0	1	0	1					0	1	0	1	0	1	0	1					0
Proteus vulgaris	5	0	5	0	5	0	5	0	5	0	1	0	1	0	5	0	5	0	5	0	5	0	1	0	1	0
Salmonella enteritidis	10	0	10	0	10	0	10	0	10	0	1	0	1	0	10	0	10	0	10	0	10	0	1	0	1	0
Streptococcus liquefaciens	1	0	1	0	1	0	1	0	1					0	1	0	1	0	1	0	1					0
Serratia marcescens	14	0	14	0	14	0	14	0	14	2	0	2	0	0	14	0	14	0	14	0	14	1	1	1	1	0
Serratia sonnei	1	2	0	2	0	2	0	2	0					2	0	2	0	2	0	2	0					0

[a] Fewer strains were tested on EMB agar than on MAC agar or BAP.
[b] Some strains which fluoresced yellow owing to ingredients in the media were called positive.

a green fluorescent sheen from this medium. This green color, when mixed with the indole reagent, resulted in an indistinguishable blend of blue-green-pink. Negative reactions appeared as a pale to lime green color. The β-glucuronidase tests demonstrated yellow or yellow-orange instead of blue fluorescence when positive.

Commercial products have become available that utilize a rapid β-glucuronidase assay as a pivotal test for identification of *E. coli*. Overall, the RDE test was more efficient in determining β-glucuronidase activity than was the RIM, whereas both commercial systems were equally accurate, corresponding completely with conventional tests for indole and ONPG. In no case was there a false-positive reaction. The production of inexpensive rapid tests for identification of commonly isolated bacteria is welcome and should reduce costs by providing flexibility for the laboratory.

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