Enzyme Capture Assay for Rapid Identification of *Escherichia coli* in Blood Cultures

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An enzyme capture assay (ECA) for rapid identification of *Escherichia coli* in blood cultures by using \(\beta\)-D-glucuronidase as a marker was developed. Microdilution plates coated with antigucluronidase were used to capture this enzyme from the cell lysates of blood cultures which showed growth of gram-negative bacteria. The assay, using 4-methylumbelliferyl-\(\beta\)-D-glucuronide as a fluorogenic substrate, had a detection limit of 0.1 ng/ml (3 \(\times\) 10\(^{-15}\) M) for the enzyme; this was approximately equal to a cell concentration of 10\(^6\) CFU of *E. coli* per ml. Among 212 blood cultures showing growth of gram-negative bacteria, 77 specimens were found to contain *E. coli* by conventional culture procedures and 73 samples were positive by ECA. Among the 135 blood cultures from which *E. coli* was not isolated, ECA gave one false-positive (*Salmonella enteritidis*) reaction. Thus, the sensitivity and specificity for the identification of *E. coli* in blood cultures by ECA were 94.8% (73/77) and 99.3% (134/135), respectively. From the finding of positive growth in the culture bottle, the assay can be completed within 4 h. In view of the high rate of isolation of *E. coli* from bacteremic patients, the test can be performed in parallel with conventional culture protocols; this may shorten the identification time for *E. coli*, and proper antimicrobial treatments may be started 24 h earlier than when results of conventional identification systems are used.

The isolation of any significant microorganism from a blood culture is an occurrence that requires careful evaluation by the clinicians, and prompt action is usually necessary. The incidence of bacteremia and fungemia has been reported to be 3-4 to 28 per 1,000 hospital admissions and was estimated to average 10 per 1,000 admissions (1%) in the United States (31). The five most common isolates from blood cultures were *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (2). *E. coli* is one of the more important gram-negative bacteria recovered in clinical microbiology laboratories. It is isolated frequently from specimens from bacteremic episodes (2, 10, 19, 21, 25), either as a pure culture or as a part of a mixed culture. The isolation rate for *E. coli* can be as high as 40% of the gram-negative bacteria found in bacteremia (25). During 1991 and 1992, isolates of *E. coli* accounted for 21.7% of all bacteria causing bloodstream infections at National Cheng-Kung University Hospital, Tainan, Taiwan (unpublished data). In another study, *E. coli* was found to account for 28.4% of all isolates from bacteremic episodes in the pediatric department of another hospital (5). In view of the high incidence and mortality (2), a rapid identification method for *E. coli* in blood specimens is of clinical importance.

Since 1976, when Kilian and Bülow (17) described the activity of a \(\beta\)-D-glucuronidase (GUD) as being restricted to *Escherichia*, *Shigella*, and *Salmonella* spp., this property has been widely used for the detection and identification of *E. coli* (6, 9, 11, 16, 30) in food and in clinical microbiology laboratories. Most studies incorporated 4-methylumbelliferyl-\(\beta\)-D-glucuronide (MUG) as a fluorogenic substrate of GUD in the medium. The native GUD of *E. coli* may be a tetramer having a molecular weight of 296,000 (1). Some strains of *Yersinia*, *Flavobacterium*, staphylococci, and streptococci are also positive for this enzyme (12, 22, 26), and false-positive reactions are sometimes caused by animal tissues which contain the enzyme. For this reason, an enzyme capture assay (ECA) for detection of *E. coli* in oysters was developed (12). Anti-GUD antibodies coated on microdilution plates were used to capture the enzyme produced by *E. coli* present in food samples; this was followed by the addition of fluorogenic or chromogenic substrates to demonstrate GUD activity. Under these conditions, cross-reactions are caused only by members of the family *Enterobacteriaceae*.

Although the direct incorporation of a fluorogenic substrate of GUD in the culture medium is a convenient and fast way to detect *E. coli* present in specimens (9, 30), the practice is impossible for blood cultures because of the deep red color of blood, which masks the fluorescence generated by GUD on the substrate. ECA seems to be a good choice for solving this problem. Hussin et al. (14) have described an alkaline phosphatase capture test for the identification of *E. coli* and *Shigella* species in blood cultures and urine specimens. The sensitivity of the alkaline phosphatase capture assay for detecting *E. coli* in blood cultures is 91%.

We describe here a rapid and sensitive ECA to identify *E. coli* in positive blood cultures showing growth of gram-negative bacteria, including mixed cultures containing gram-negative bacteria. For the preparation of anti-GUD used in the ECA, a rapid procedure for the purification of GUD by preparative polyacylamide gel electrophoresis is also described.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** Among the 108 strains of *E. coli* tested for the production of GUD by ECA, 102 were clinical isolates obtained from the Triservice General Hospital (Taipei, Taiwan). The remaining 6 strains of *E. coli* and 60 other strains of *Enterobacteriaceae* were from the Culture Collection and Research Center (CCRC), Hsinchu,
Taiwan). The identities of all *E. coli* isolates were reconfirmed by the Micro-ID diagnostic kit (Organon Teknika Corp., Durham, N.C.). To test for the production of GUD by various bacteria under blood culture conditions, a single colony of each strain grown overnight on tryptic soy agar was suspended in 0.5 ml of sterile saline. Following the injection (with a syringe) of this bacterial suspension into a BACTEC NR6A (Becton Dickinson, Sparks, Md.) aerobic blood culture vial, 3 ml of human blood (from healthy donors) was added to the culture bottle. The culture bottles were incubated at 35°C for 24 h before the ECA was performed.

**Preparation and purification of antibody.** The gel strip containing GUD was crushed to fine particles and used for immunizing rabbits according to the procedure of Chang et al. (4). The specificities of the antisera were tested by Ouchterlony double diffusion (24), while the titers of the antisera were determined by an enzyme-linked immunosorbent assay (4) using microdilution plates coated with GUD. The immunoglobulin G fractions of the antisera were purified by DEAE ion-exchange chromatography (18) and were further purified by affinity chromatography (13).

**ECA.** The ECA was performed as previously described (16) with some modifications. Wells of microdilution plates (MicroFluor; Dynatech, Alexandria, Va.) were coated with affinity-purified anti-GUD and blocked with bovine serum albumin. Serially diluted GUD solution or cell lysate was then added to the wells and incubated at 37°C for 1.5 h. After the wells were washed, MUG solution (200 μg/ml) was added and incubated at 37°C for 1.5 h. The reaction was stopped with 0.2 N NaOH, and the results were read with a MicroFluor reader (Dynatech) at a wavelength of 365 nm. As a negative control, phosphate-buffered saline was used instead of GUD. A reading of fluorescence units greater than that of the negative control plus three standard errors was considered positive.

Culture broth (0.5 ml) obtained after 24 h of growth for pure cultures or obtained from blood cultures positive for gram-negative bacteria in the BACTEC NR vials was aseptically drawn into to a microcentrifuge tube, which was capped and centrifuged at 5,000 × g for 10 min. The cell pellet was lysed at 35°C for 15 min in 0.5 ml of 0.1 M phosphate buffer containing 8% (wt/vol) sucrose, 25 mM EDTA, 0.05% Triton X-100, and lysozyme (Sigma) (1.2 mg/ml), pH 7.0. The activity of GUD in the lysate was assayed by ECA.

**Detection limit for *E. coli*.** To determine the minimal number of *E. coli* cells in the blood culture vials necessary to give a positive ECA result, 3 ml of blood and 0.1 ml of a diluted suspension of *E. coli* (O5-8 and CCRC 10316) were injected into the BACTEC NR6A bottle to give an inoculum level of approximately 2 to 10 CFU/ml. The vials were incubated at 35°C, sampled at intervals to determine the cell numbers by plate count, and tested for GUD activity by ECA.

**Clinical specimens.** Blood specimens were collected at the National Cheng-Kung University Hospital. The BACTEC NR6A and NR7A vials were normally inoculated with 3 to 5 ml of blood from the patients, inserted into a BACTEC NR600 instrument (Johnson Laboratories Inc., Towson, Md.), and incubated at 35°C. Broth from positive vials was Gram stained and subcultured onto MacConkey, chocolate, and blood agar plates. All isolates were identified by conventional microbiological techniques. The culture vials showing growth of gram-negative bacteria, including mixed cultures containing gram-negative bacteria, were tested by ECA according to the procedures described above.

**Sensitivity and specificity.** Sensitivity and specificity were determined as described by McClure (20).

**Induction of GUD.** Since GUD is an inducible enzyme (23), four β-glucuronidases were compared for their abilities to induce the enzyme in two strains of *E. coli* (O5-8 and CCRC 10316). After the addition of 0.5 ml of the bacterial suspension and 3 ml of blood to the BACTEC NR6A vial, each stock solution (16.5 mg/ml) of β-glucuronidases (5-bromo-4-chloro-3-indolyl-β-D-glucuronide, methyl-β-D-glucuronide, MUG, and p-nitrophenyl-β-D-glucuronide [all from Sigma]) was added to each culture vial to reach a final concentration of 50 μg/ml. The culture vials were then incubated at 35°C for 24 h before the ECA was performed.

**RESULTS**

**Preparation of anti-GUD.** The antisera were specific to GUD, since only one precipitation line was found in the Ouchterlony double diffusion test (data not shown). The titers of the antisera were found to be around 10^7 as determined by an enzyme-linked immunosorbent assay (4).

**Bacteria producing GUD.** Among the 108 strains of *E. coli* tested, 104 (96.3%) produced GUD as detected by ECA (Table 1). Of the 28 strains of *Salmonella*, two strains (7%) gave positive ECA results. However, among the eight strains of *Shigella*, two strains (25%) were ECA positive. None of the other 24 strains of *Enterobacteriaceae* produced GUD as determined by ECA.

**Detection limit for GUD and *E. coli*.** For microdilution plates coated with affinity-purified anti-GUD, the detection limit for GUD by ECA was about 0.1 ng/ml (3 × 10^-13 M) (Fig. 1). This enzyme concentration was approximately equal to a cell density of about 10^6 CFU/ml, as determined with two *E. coli* strains (O5-8 and CCRC 10316) in the inoculation experiments. The assay was about 5 to 10 times less sensitive when DEAE-purified anti-GUD was used for the coating of microdilution plates (data not shown).

**Detection of *E. coli* in blood cultures.** The bacteria isolated from the 212 blood cultures which showed growth of gram-negative bacteria were *E. coli* (77 isolates), *K. pneumoniae* (33 isolates), *P. aeruginosa* (26 isolates), *Acinetobacter anitratus* (11 isolates), *Enterobacter cloacae* (10 isolates), and other bacteria, with a total of 247 isolates. Of the 212 positive blood cultures,
was 1446 infections of 15 positive (Table 1). Therefore, MUG compound was more sensitive, and MUG was reported as confirmed by subculturing the isolates in tryptic soy broth containing MUG (9) and retesting with ECA. The fourth false negative was a blood sample from which only E. coli was isolated, and this isolate was confirmed to be GUD negative.

Induction of GUD. Although most strains of E. coli can produce GUD in the BACTEC NR6A vials without added inducers, the captured GUD activity was about 5 to 64 times higher in the presence of inducers added at a concentration of 50 μg/ml (Fig. 2). Both E. coli CCRC 10316 and O5-8 were induced to approximately the same extent by each β-glucuronidase. However, p-nitrophenyl-β-D-glucuronide was found to be the best inducer among the four compounds tested; the captured enzyme activity was 45 and 64 times higher in E. coli CCRC 10316 and O5-8, respectively, in the presence of this compound than in vials without any inducers added. Methyl- β-D-glucuronidase was the second most effective inducer among the four β-glucuronidases (Fig. 2).

**DISCUSSION**

Of 108 isolates of E. coli tested with ECA, 104 (96.3%) were positive (Table 1). This value is slightly higher than that (94%) reported by Holt et al. (12) and is very close to that (97%) reported by Kilian and Bülow (17). False positives caused by pure cultures of Shigella and Salmonella spp. were 25 and 7% (Table 1), respectively. However, only a small percentage of bloodstream infections are caused by Salmonella spp., and bacteremia caused by Shigella spp. is rarely found (10, 19, 25, 27, 31). In this study, among the 212 blood cultures, containing gram-negative bacteria, Salmonella spp. were found in six specimens (2.8%), and only one (S. enteritidis) of the six isolates displayed an ECA-positive result. However, Salmonella bacteremia is being identified with increasing frequency in AIDS patients (3, 7, 28, 29). For this reason, the ECA may be not suitable for rapid identification of E. coli in AIDS patients having bloodstream infections. No Shigella spp. were isolated from the 212 positive blood samples.

The sensitivity (94.8%) and specificity (99.3%) of ECA for identifying E. coli in blood samples were high. Of the four false-negative results by ECA, three were polymicrobial infections (E. coli and K. pneumoniae, E. coli and P. aeruginosa, and E. coli, K. pneumoniae, and P. aeruginosa). The three E. coli isolates from the polymicrobial bacteremia were GUD positive as confirmed by subculturing these isolates in tryptic soy broth containing MUG (9) and retesting with ECA. The fourth false-negative specimen was unimicrobial, with the isolated E. coli being GUD negative. Therefore, most false-negative reactions were due to mixed cultures present in the blood samples.

The reason why ECA could not detect GUD in a polymicrobial environment is not clear at present; one possibility might be that the cell density of E. coli was not high enough to produce a detectable amount (0.1 ng/ml or 10⁻¹⁵ M [Fig. 1]) of GUD with the competition of other bacteria in the blood broth environment.
cause of decreased *E. coli* growth. A possible method to reduce false negatives is to include an inducer (β-D-glucuronidase) in the culture broth to enhance the production of GUD by *E. coli*. Among the four inducers tested, *p*-nitrophenyl-β-D-glucuronide had the ability to strongly promote enzyme production, by a factor of 45 to 64 times (Fig. 2). The effect of *p*-nitrophenyl-β-D-glucuronide on the growth of nine strains of gram-positive and gram-negative bacteria was tested, and no inhibition was found (data not shown). The feasibility of adding any inducer, which may cause contamination, before or after blood is introduced into the culture bottles needs further study.

Edberg and Trepeta (8) reported a high sensitivity (93%) for identification of *E. coli* isolates within 1 h by GUD assay in a buffer containing *p*-nitrophenyl-β-D-glucuronide. However, Jackson et al. (15) recently found that some *E. coli* isolates need 24 to 48 h to develop visible fluorescence in a broth containing MUG. We had similar findings in tests of more than 100 pure cultures of *E. coli* from food and clinical sources (data not shown). In addition, to obtain pure cultures, a subculturing step which normally requires overnight incubation is needed. Some commercial products (e.g., RAPIDEC coli [Analytab Products, Montalieu Vercieu, France] and MUG Disk [Remel, Lenexa, Kans.]) are GUD-based rapid identification tests for *E. coli*, but pure cultures are required for these tests. Another advantage of the ECA would be that GUD cross-reactions caused by gram-positive bacteria (staphylococci or streptococci) (12, 22, 26) other than *Enterobacteriaceae* are not likely to occur. False positives caused by gram-positive bacteria present in the 12 mixed cultures were not found in this study.

Husson et al. (14) reported an alkaline phosphatase ECA for the detection of *E. coli* in urine and blood cultures. Since the substrate (*p*-nitrophenyl phosphate) used was a chromogenic compound, a lower sensitivity of the assay could be anticipated. The detection limit of the assay for *E. coli* was about 10⁷ CFU/ml, which is about 10 times less sensitive than the present ECA using MUG as a fluorogenic substrate. In addition, a concentrating step using 5 ml of the cultured blood is needed in the alkaline phosphatase immunocapture test, and to separate bacterial cells from blood cells, differential centrifugation steps were used in that study (14). However, the ECA described in the present report requires only 0.5 ml of the culture broth, and after a simple lysis step with lysozyme, the sample can be directly analyzed.

In view of the high mortality and high isolation rate for *E. coli* from bacteremic episodes, a rapid identification method for this bacterium is important. The common practice used in hospitals is to subculture blood specimens at intervals after an incubation period of 12 h to 2 weeks (10, 32) and at the time when Gram stain is positive or bacterial growth is apparent (e.g., by turbidity, gas production, or hemolysis). The subculture and identification steps normally take at least 24 h. Therefore, in addition to the conventional culture protocols, we propose that when growth of gram-negative bacteria is found, the immunocapture assay for GUD be performed in parallel to rapidly identify *E. coli* in the blood cultures. The time required for ECA is 4 h, and multiple samples can be semi-automatically analyzed in microdilution plates. This could allow proper antimicrobial treatment 24 h before results of other commonly used systems are available and may help reduce the mortality from bacteremia caused by *E. coli*.

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