Urinary tract infection: detection of *Escherichia coli* antigens in human urine with an ELIEDA immunoenzymatic assay

Myriam T. N. Campanhã, Sumie Hoshino-Shimizu, and Marina Baquerizo Martinez

**ABSTRACT**

Escherichia coli is the most common causative agent of urinary tract infection (UTI), and diagnosing this infection usually relies on bacteriologic methods. Nevertheless, screening methods can be useful for a rapid presumptive diagnosis even though some of these screening methods have low sensitivity or are expensive. To investigate a possible new alternative approach, an antigen-based immunosassay—enzyme-linked immunoelectrodiffusion assay (ELIEDA)—was standardized for screening for this bacterial infection. Combining counterimmunoelectrophoresis with an immunoenzymatic assay, the ELIEDA requires concentrated urine specimens, a cellulose acetate membrane, polyclonal antibodies to *E. coli* raised in rabbits, and peroxidase-labeled sheep antibodies to rabbit immunoglobulin G (IgG). This ELIEDA technique was evaluated using 244 urine specimens, 76 of them with *E. coli*, 47 with heterologous bacteria, and 121 without bacteria. In comparison to bacteriologic methods, the sensitivity, specificity, and positive and negative predictive values for the ELIEDA were 93.4%, 98.2%, 95.9%, and 97.1%, respectively. The data obtained suggest that this assay is useful for routine diagnostic screening for UTI caused by *E. coli*. In addition, since the ELIEDA stained membranes can be stored, this assay makes retrospective studies possible.

Urinary tract infections (UTIs) are among the most common infections in humans. The majority of cases are caused by a limited number of bacterial genera; *E. coli* strains in particular are responsible for 80% of the UTI cases seen in outpatient clinics (1). These strains are found in the normal flora of the intestinal tract, skin, and vagina. However, under individual predisposing conditions they can multiply rapidly and are capable of adhering to uroepithelial cells, producing infection (1, 2).

The diagnosis of bacterial UTI has usually been made by bacteriologic methods of isolation and identification in voided urine. Nevertheless, the use of other screening methods can have advantages. Results can be obtained more quickly than with the standard culture method, and most of these other methods are simple and easy to perform. While biochemical and automated methods are often used for UTI screening, they are either insensitive (2) or costly (3). In Brazil, automated methods are too expensive for many clinical laboratories.

Immunologic methods are less frequently used to diagnose bacterial infections. However, bacterial antigens or antibodies can be detected in the biologic fluids of infected patients through such immunologic assays as counterimmunoelectrophoresis, coagglutination, latex agglutination, and immunoenzymatic assays (4–16). Hospital and public health laboratories in developing countries usually use electrophoresis or counterimmunoelectrophoresis systems for diagnostic purposes (15, 16).

Pinon and Dropsy (14) combined counterimmunoelectrophoresis (CIE) with immunoenzymatic staining to create the so-called enzyme-linked immunoelectrodiffusion assay (ELIEDA).
This assay was shown to be more sensitive than CIE in the serodiagnosis of parasitic and fungal infections (17). Before this study, the ELIEDA had not been applied for antigen detection in bacterial infectious diseases. In the present study, this immunoenzymatic assay was standardized to detect \textit{E. coli}-group antigens in urine specimens from outpatients with \textit{E. coli} UTI, using a specific polyclonal antibody. This work also compared the ELIEDA to conventional bacteriologic methods.

**MATERIALS AND METHODS**

**Urine specimens**

A total of 244 urine specimens were collected and analyzed between February 1993 and May 1994 from outpatients who were clinically suspected of urinary tract infection. The specimens were collected at two locations in the city of São Paulo, Brazil, the Laboratory of Clinical Microbiology of the Department of Clinical Analysis of the University of São Paulo and the Hospital of the University of São Paulo. The outpatients ranged in age from 2 to 90; 75% of them were females and 25% were males. The specimens were examined by standard quantitative culture methods (2), and the results were compared with those obtained by ELIEDA. The urine specimens that were negative or positive from the quantitative urine culture were used as controls for the ELIEDA tests. The urine specimens used as positive controls were positive for \textit{E. coli}; no microorganisms were isolated in the negative controls.

**Hyperimmune serum**

\textit{E. coli} serogroup O6 was grown in tryptic soy broth (Difco Laboratories, Detroit, MI, United States of America) overnight at 37 °C. In three inoculations containing 5% formalin (first inoculation, 0.3 mL; second, 0.5 mL; third, 1.0 mL), a 3-month-old New Zealand male rabbit received whole culture medium bacteria and their extracellular products. In three subsequent inoculations of 2.0 mL each, no formalin was used. Ewing’s (18) inoculation approach was utilized, and blood was collected 7 days after the last inoculation. The sera obtained were stored at −20 °C. The antibody titers were determined by an agglutination test on glass plate (18). The stability of the hyperimmune serum was checked throughout the experimental period, with the same quantitative agglutination test.

Immunoblot assay (IBA) (19, 20) was used to characterize the polyclonal serum, using urine specimens from seven of the outpatients included in the study group. \textit{E. coli} was isolated from two of them; three others presented \textit{Klebsiella pneumoniae}, \textit{Proteus mirabilis}, and \textit{Streptococcus sp.}; and the remaining two specimens were fully negative. For the IBA, 20 μL of sample buffer (Tris 0.0625M, pH 6.8; SDS 2.3% w/v; glycerol 10% v/v; β-mercaptoethanol 5% v/v) was added to 20 μL of centrifuged urine, and the preparation was boiled for 5 min. Electrophoresis was carried out on 12% SDS-polyacrilamide gel, the antigenic bands were transblotted to a 0.45 μm nitrocellulose membrane (Millipore, Bedford, MA, United States), and the membrane was incubated with hyperimmune serum (1:2 000 to \textit{E. coli}). After washing, the membrane was incubated with peroxidase-conjugate antibody (1:2 000) to rabbit immunoglobulin G (IgG) (Cappel-Organon Teknika Corp., West Chester, PA, United States) diluted to 1:500 in PBS, 0.05% Tween 20, for 30 min, with shaking. To obtain more specific results, the strip was treated with 5% skim milk in 0.15 M phosphate buffer saline (PBS), pH 7.2, containing 0.05% Tween 20, for 30 min, with shaking. After washing in PBS three times for 15 min each, the strip was incubated with sheep anti-rabbit IgG peroxidase conjugate (Cappel-Organon Teknika Corp., West Chester, PA, United States) diluted to 1:500 in PBS, 0.15% Tween 20, for 30 min. The strip was then incubated in a mixture of 0.5 mg/mL DAB and 1 μL/mL of 30% H2O2 in 0.1 M Tris-HCL buffer, pH 7.6, for 5 min, with shaking. Distilled water was added to stop the reaction. After being dried and made transparent, the strip was stored.

In this assay, the urine specimens with several \textit{E. coli} serogroups developed one to four precipitating lines. The first line close to the hyperimmune serum spot was consistently present in urine from patients with \textit{E. coli} UTI, so this line was considered to be specific. Precipitating lines more distant from the hyperimmune serum spot were interpreted as corresponding to cross-reactive or nonspecific lines (Figure 1).

**Enzyme-Linked Immunoelectrodiffusion Assay (ELIEDA)**

The ELIEDA was performed as described previously (14, 21), with some modifications. For each assay, 5 mL of recently collected urine was centrifuged in a conic glass tube at 1 500 × g for 30 min. The resulting pellet (± 100 μL) was processed immediately or stored at −20 °C. A Cellogel cellulose acetate strip (Chemometron, Milan, Italy) was immersed in 0.1 M Tris-glycine-phosphate buffer, pH 8.5, for 15 min. The acetate strip was then removed from the buffer and dried with filter paper. A volume of 10 μL of the urine concentrate was applied to the cellulose acetate strip, and 5 μL of undiluted rabbit hyperimmune serum to \textit{E. coli} was added to the strip at a distance of 1.5 cm from the urine.

Using the same Tris-glycine-phosphate buffer, the electrophoretic run was carried out for 60 min at 110 V and 1 mA/cm width. The strip was washed in 0.85% NaCl containing 1% Tween 80 for 30 min, with shaking. To obtain more specific results, the strip was treated with 5% skim milk in 0.15 M phosphate buffer saline (PBS), pH 7.2, containing 0.05% Tween 20, for 30 min, with shaking. After washing in PBS three times for 15 min each, the strip was incubated with sheep anti-rabbit IgG peroxidase conjugate (Cappel-Organon Teknika Corp., West Chester, PA, United States) diluted to 1:500 in PBS, 30 min at 37 °C. After three more washings of 15 min each in PBS, the strip was then incubated in a mixture of 0.5 mg/mL DAB and 1 μL/mL of 30% H2O2 in 0.1 M Tris-HCL buffer, pH 7.6, for 5 min, with shaking. Distilled water was added to stop the reaction. After being dried and made transparent, the strip was stored.

Diagnosis performance of the ELIEDA

The ELIEDA was evaluated in terms of sensitivity, specificity, agreement, and positive and negative predictive value by comparing its results with those obtained by urine culture, which
was used as the gold standard (22). We determined inter- and intra-assay reproducibility, as well as the stability of the bacterial antigens in urine that had been stored at 220 °C.

RESULTS

Urine culture

Through the urine cultures, bacteria were isolated from 123 urine specimens; no microorganism was found in the remaining 121 specimens. A urine culture with ≥10^5 colony-forming units/mL (CFU/mL) was considered to be positive, a culture with ≥10^4 but < 10^5 CFU/mL was considered to be borderline, and a culture with <10^4 CFU/mL was considered to be negative. All the microorganisms grown in the culture were identified by previously described methods (23).

Of the 123 urine specimens infected with bacteria, 71 were positive for *E. coli*, and 5 more were borderline for *E. coli*. From the remaining 47 infected urine specimens, 44 of them contained a single heterologous bacteria and 3 showed an association of two different bacterial species. The concentrations of the 50 heterologous bacteria varied from 1.3 x 10^4 CFU/mL to ≥10^5 CFU/mL. Of the 50, 35 of the heterologous bacteria were found at the positive level and 15 at the borderline level.

ELIEDA assay

The ELIEDA was positive for all 71 of the urine specimens in which *E. coli* had been identified at a bacterial concentration higher than 10^5 CFU/mL. However, the ELIEDA assay gave negative results for the 5 urine specimens with *E. coli* concentrations ranging from 1.2 x 10^4 to 5 x 10^4 CFU/mL. These values below 10^5 CFU/mL are conventionally considered to be non-significant from a clinical standpoint (10, 24). In one of these 5 urine specimens an association of *E. coli* with *P. mirabilis* was observed.

The ELIEDA was negative for all 121 of the urine specimens for which the cultures had given negative results. The ELIEDA was also negative for the 44 urine specimens from which heterologous bacterial species were isolated by quantitative urine culture. The ELIEDA was positive for 3 urine specimens from which heterologous bacterial species were isolated by quantitative urine culture. The three bacteria that were cross-reactive with anti-*E. coli* serum were *K. pneumoniae*, *Klebsiella oxytoca*, and *P. mirabilis*.

A comparison of the results in detecting *E. coli* antigens by quantitative urine culture and by ELIEDA is given in Table 1.

The diagnostic features of the ELIEDA can be expressed in terms of sensitivity, specificity, agreement, and positive and negative predictive value. The values found in this study were: 93.4% (95% CI = 85.0%–97.2%), 98.2% (95% CI = 94.9%–99.4%), 96.7% (95% CI = 93.7%–98.3%), 95.9 (95% CI = 88.7%–98.6%), and 97.1% (95% CI = 93.3%–98.7%), respectively.

A group of 29 urine specimens stored at −20 °C showed no difference in their results even after two to five consecutive freeze-thawings over a period of 10 months. The hyperimmune serum did not change in agglutination titer (1:4 096) during this study period.

The reactivity of the rabbit hyperimmune serum (anti-*E. coli* O6) was characterized by IBA so as to determine the specificity and cross-reactivity among urine-excreted *E. coli* antigens, other enterobacterial (*K. pneumoniae* and *P. mirabilis*) antigens, and Gram-positive (*Streptococcus* sp.) bacterial antigens. The hyperimmune serum reacted with all urine specimens studied. However, bands of 77, 66, 51, 43, and 21 kDa were detected in urine specimens positive for *E. coli*. The urine specimens that were positive for heterologous bacteria showed one to four bands, which were combination of bands 77, 66, 51, 24, and 22 kDa. In two negative urine specimens, a band of 77 kDa was visualized. To identify which of these bands correspond to the ELIEDA precipitating lines, the hyperimmune serum must be absorbed with concentrated *E. coli* urine-excreted antigens and retested in the IBA. It is difficult to make a direct correlation between ELIEDA precipitating lines and IBA stained bands since in the ELIEDA the
bacterial antigens were crude, whereas in the IBA these antigens were treated with β-mercaptoethanol.

**DISCUSSION**

In urinary tract infections, screening tests can provide a more rapid presumptive diagnosis than the conventional bacteriological methods. The ELIEDA for *E. coli* antigen detection has some interesting features. For example, results are available within 4 hours of urine collection. In addition, the stained cellulose acetate strips can be stored for a long period of time after being made transparent. This allows results to be rechecked and retrospective studies to be carried out.

The diagnostic features of the ELIEDA are promising because of the test’s high sensitivity and specificity and other diagnostic parameters. When the concentration of the *E. coli* was higher than 105 CFU/mL, i.e., clinically significant, 100% agreement was observed between ELIEDA and the quantitative urine culture method.

The reproducibility of the ELIEDA was considered to be satisfactory since 10 urine specimens from UTI patients gave similar positive results when tested twice on the same day, and 17 other urine specimens from UTI patients gave similar positive results when tested on three different days.

The data obtained in the present study are better than those obtained with the Bac-T-Screen, a rapid screening method frequently used to determine nonspecific bacteriuria and/or pyuria. In a study conducted by Davis et al. (25), the sensitivity, specificity, and positive and negative predictive value of this method were found to be 93.2%, 77.2%, 69.2%, and 95.5%, respectively, for ≥ 10^4 CFU/mL. New screening methods recently reported (26, 27) have lower sensitivity than the ELIEDA but similar specificity. These methods, however, generally take 18 to 24 hours to provide results, much longer than the 4 hours the ELIEDA requires.

As standardized here, the ELIEDA provides data that reflect both bacterial identification and significant bacteriuria. The most accepted criterion for bacteriuria, ≥ 10^5 CFU/mL, was taken as the reference in the present study, although this value may vary according to different individual conditions (1, 2, 24).

Five urine specimens in which *E. coli* was identified by the bacteriologic method but at concentrations ≤ 5 × 10^4 CFU/mL were negative with the ELIEDA. These results imply that these persons were either in the initial stage of *E. coli* infection or that their urine samples were contaminated. The significance of low *E. coli* counts could be elucidated only when new urine specimens are collected from the same outpatients.

In three urine specimens an association of two bacteria was seen in the cultures. However, the *P. mirabilis*, *S. saprophyticus*, and *S. aureus* found were probably contaminants, since their counts were much lower than 10^5 CFU/mL. As a concomitant infection by two bacteria is infrequent in UTI, the associated bacteria with low counts were interpreted as contaminants of the urine specimens.

UTI with heterologous bacteria was observed in 35 outpatients. The remaining 12 patients were borderline cases; UTI might or might not have developed, so that these outpatients should have been followed up.

The ELIEDA showed high specificity but cross-reactivity was observed with three enterobacteria: *K. pneumoniae*, *K. oxytoca*, and *P. mirabilis*. Apparently, cross-reactivity seems to be related to bacterial strains rather than species.

Absorptions of the hyperimmune serum were not carried out with cross-reactive bacterial strains. Since cross-reactivity occurs only in some bacterial strains and not in species, the absorptions are thought to be difficult. The bacterial antigens of urine specimens proved to be resistant to several freeze-thawing cycles and appear to be polysaccharides. This aspect is now under investigation.

The reactivity of the rabbit antiserum with *E. coli* O6 antigens and the IBA cross-reactivity with other unrelated bacteria may provide a foundation to produce other monoclonal antibodies for immunoassays designed for bacterial antigen captures.

In spite of the fact that ELIEDA is not yet automated, it is suitable for developing countries since it can easily be adapted for any laboratory in which the usual serum electrophoresis is carried out. For a public health laboratory, the cost would be about US$ 0.70 per urine specimen. Another ELIEDA advantage is the possibility of storing stained strips for a long period of time, thus allowing retrospective studies.

The present findings suggest that the ELIEDA is useful for laboratory diagnostic screening for UTI caused by *E. coli*. However, this assay could be improved by concentrating urine, to obtain more sensitive results; preparing a mixture of hyperimmune serum specific to different bacterial species; and

**TABLE 1. Comparison of detection of *Escherichia coli* infections by quantitative culture and by ELIEDA in 244 urine specimens, São Paulo, SP, Brazil, 1993–1994**

<table>
<thead>
<tr>
<th></th>
<th>Quantitative Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive or borderline</td>
</tr>
<tr>
<td><strong>ELIEDA</strong></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>71</td>
</tr>
<tr>
<td>Negative</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>76</td>
</tr>
</tbody>
</table>

^a Those “positive” by the culture method have ≥ 10^5 colony-forming units/mL.

^b Those “borderline” by the culture method have 1.2 × 10^4 to 5 × 10^4 colony-forming units/mL.

^c These 168 specimens in which the quantitative culture was found to be negative include 121 without any bacteria and 47 samples with heterologous bacteria.
identifying and characterizing bacterial antigens in urinary excretions.

**Acknowledgments.** This work was supported by the Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and partially by the Conselho Nacional de Pesquisa e Desenvolvimento (CNPq). We wish to thank Sonia R.C. Buratto and Sueli Nonogaki, of the Faculdade de Ciências Farmacêuticas, University of São Paulo, SP, Brazil, for their technical assistance with the quantitative urine cultures and also the University Hospital and the Laboratory of Clinical Microbiology of the Department of Clinical Analysis of the University of São Paulo, SP, Brazil, for the urine specimens.

**REFERENCES**


Manuscript received on 21 November 1997. Revised version accepted for publication on 4 January 1999.
Escherichia coli es el agente causal más frecuente de las infecciones urinarias (IU), cuyo diagnóstico suele basarse en métodos bacteriológicos. No obstante, los métodos de tamizaje pueden ser útiles para hacer un diagnóstico preliminar con rapidez, pese a que algunos de ellos tienen poca sensibilidad y son caros. Con el fin de investigar la posibilidad de usar otra técnica de diagnóstico, se estandarizó un inmunoensayo de tipo antigénico—ensayo inmunoenzimático por electrodifusión (ELIEDA, por *enzyme-linked immunoelectrodiffusion assay*)—para hacer el tamizaje de este tipo de infección. El ELIEDA, que consiste en el uso combinado de contrainmunoelectroforesis y un ensayo inmunoenzimático, requiere muestras de orina concentrada, una membrana celulosa con acetato, anticuerpos policlonales contra *E. coli* formados en conejos, y anticuerpos de cordero marcados con peroxidasa contra inmunoglobulinas G (IgG) de conejo. Esta técnica de ELIEDA se evaluó con 244 especímenes urinarios: 76 tenían *E. coli*; 47 tenían bacterias heterólogas y 121 carecían de bacterias. Al compararlo con los métodos bacteriológicos, el ELIEDA mostró una sensibilidad, especificidad y valores predictivos positivo y negativo de 93,4%, 95,9% y 97,1%, respectivamente. Los resultados obtenidos indican que este ensayo es útil para el tamizaje diagnóstico de rutina de las IU causadas por *E. coli*. Además, el ensayo facilita la realización de estudios retrospectivos, ya que las membranas teñidas usadas para el ELIEDA son almacenables.