Enzyme Immunoassay for Detection of Antibody-Coated Bacteria

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To quantitatively evaluate factors potentially affecting antibody coating of bacteria in urine, we developed an assay with enzyme-linked rather than fluorescein-conjugated immunoglobulin. Using the enzyme immunoassay (EIA) in an in vitro system in which concentrations of serotype O44 *Escherichia coli* and antibody titer to *E. coli* O44 O antigen were known, we compared specimens run in parallel with a fluorescent antibody (FA) assay. At $\geq 10^5$ bacteria per ml, antibody titer to homologous O antigen correlated directly with absorbance in the EIA. Both tests had sensitivities exceeding 95% in specimens containing $\geq 10^5$ bacteria per ml, but the FA test detected 23 of 27 positive specimens with $< 10^5$ bacteria per ml compared with 21 of 43 detected by EIA ($P = 0.002$). However, nonspecific fluorescence caused false positives in 8% of negative tests run by FA compared with 1% of simultaneous EIA tests ($P = 0.05$). pH alterations and pretreatment of bacteria with antibiotics did not affect either test. Heterologous *E. coli* strains showed no cross-reactivity with O44 antiserum, but all *Staphylococcus aureus* isolates tested caused false positives in both assays, and one *Klebsiella* strain repeatedly caused a false-positive FA assay. The EIA appears to be a simple, quantitative, and specific technique for detection of antibody-coated bacteria in this experimental system.

Detection of antibody-coated bacteria (ACB) in urine by the fluorescent-antibody (FA) technique has been used widely for differentiating upper and lower tract urinary infections in epidemiological studies (4-8) and has been used to identify patients most likely to respond to single-dose antimicrobial therapy (2). However, in studies that have directly compared the ACB test with other means of localizing urinary tract infections, up to 33% false positives and 27% false negatives have been reported (4-8). Furthermore, because the ACB test is qualitative rather than quantitative, various factors potentially affecting it have not been evaluated in detail. We have developed an assay for ACB with enzyme-linked rather than fluorescein-conjugated immunoglobulin and found that this method resulted in quantitative and reproducible detection of ACB. Using this assay in parallel with the FA technique, we evaluated five factors potentially affecting the outcome of both tests: (i) concentration of bacteria; (ii) antibody titer; (iii) urinary pH; (iv) pretreatment of bacteria with antibiotics; and (v) cross-reactivity with heterologous *Escherichia coli* strains and other genera.

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MATERIALS AND METHODS

Preparation of ACB. ACB were prepared artificially by suspending colonies from an 18-h culture of *E. coli* O44 in 1 ml of phosphate-buffered saline (PBS, pH 7.3) with 0.05% Tween (PBST), adding 0.1 ml of *E. coli* O44 antiserum, and incubating for 30 min. At bacterial concentrations $\geq 10^5$ /ml and antiserum dilutions of $< 1/1,000$, this technique produced uniform, strong fluorescence in the FA test, indicating that most bacteria were antibody coated. Rabbit antiserum to *E. coli* O44 O antigen used in all experiments was obtained from the Center for Disease Control (lot 1128, undiluted titer 1:1,280) and was diluted 1:100 in PBST for all experiments unless otherwise specified. Bacterial concentrations were adjusted by measuring absorbance at 340 nm and by using serial 10-fold dilutions in Trypticase soy broth (BBL Microbiology Systems), with aliquots subsequently placed on blood agar plates for colony count determination.

EIA method. For the enzyme immunoassay (EIA) method, previously prepared ACB were washed three times in 1 ml of PBST and were incubated with 1 ml of peroxidase-conjugated goat-anti-rabbit antiserum (Cappel Laboratories, Inc., Downingtown, Pa.), diluted 1:1,000 in PBST (pH 7.3) with purified bovine serum albumin in a concentration of 0.5%. After 30 min of incubation at 37°C, the ACB were washed twice in PBST. After washing, 1 ml of freshly prepared

substrate (1 ml of *O*-phenylenediamine, 1% [wt/vol] in methanol, 0.1 ml of 3% hydrogen peroxide, and 99 ml of PBST) was added, and the tubes were incubated for 30 min in the dark at room temperature. After incubation, the reaction was stopped with 0.125 ml of eight normal sulphuric acid and absorbance was read at 490 nm. Control specimens containing bacteria but no antiserum, antiserum but no bacteria, and a reagent blank were prepared in all experiments. Results were expressed as sample absorbance minus absorbance of the simultaneously run control containing bacteria only, and positive tests were considered those where absorbance exceeded that of the control by ≥ 0.02 U. All specimens were coded and run blindly.

FA method. The FA test was performed with slides prepared from washed sediments of artificially coated bacteria. One drop of a 1:20 dilution of fluorescein-conjugated goat-anti-rabbit antiserum (Cappel Laboratories, Inc., Downingtown, Pa.) was placed on each slide and incubated for 30 min at room temperature in the dark and then rinsed with 0.02 M PBS (pH 7.3). Slides were then soaked in the same PBS solution for 15 min, dried, and read in a fluorescent microscope. Specimens showing five or more fluorescing bacteria per 100 fields examined were considered positive.

Conditions for individual experiments. (i) Effects of bacterial concentration. *E. coli* O44 organisms were coated with antibody (1:100 dilution) as outlined above and were diluted with PBST to concentrations of 10^2 to 10^8 bacteria per ml as determined by measuring absorbance at 340 nm. Control specimens consisted of *E. coli* O44 without antiserum exposure and *E. coli* O44 antiserum (dilution 1:100 to 1:1,000) in PBST. After preparation, specimens and controls were tested in the EIA and the FA assay as described above.

(ii) Effects of antiserum dilution. Serial 10-fold dilutions of *E. coli* O44 antiserum were incubated with 10^8 *E. coli* O44 per ml to prepare ACB. After preparation, specimens and controls were tested in the EIA and the FA assay as described above.

(iii) Effects of pH or pretreatment of bacteria with antibiotics. *E. coli* O44 (10^7 bacteria per ml) and O44 antiserum (1:100 dilution) were incubated in PBST previously adjusted to pH 4, 5, 6, 7, or 8 by addition of 0.1 N hydrochloric acid or 0.1 N sodium hydroxide. After 30 min of incubation, the FA assay and the EIA were performed. To test the effect of antibiotic exposure upon antibody coating, *E. coli* O44 were exposed to sublethal doses of gentamicin or ampicillin (0.5, 1.0, and 1.5 μ g of each drug per ml) for 30 min before incubation with antiserum.

(iv) Heterologous *E. coli* serotypes and non-*E. coli* strains. Individual *E. coli* strains of serotypes O1, O4, O6, O7, O25, O75, O50, O15, O77, O126, O115, O102, O137, and three rough strains (10^8 /ml) were tested in the EIA and FA systems after incubation with 1:100 dilution of *E. coli* O44 antiserum for 30 min and after incubation without antiserum. All strains were obtained from patients with urinary tract infections. Similarly, strains of *Klebsiella* (six), *Staphylococcus aureus* (three), *Staphylococcus epidermidis* (two), *Pseudomonas aeruginosa* (two), *Proteus mirabilis* (one), *Enterobacter* (one), and enterococci

(one) were tested with and without incubation in *E. coli* O44 antiserum.

RESULTS

Effects of bacterial concentration. Increasing concentrations of *E. coli* O44 from 10^2 to 10^8 bacteria per ml produced increasing absorbance in the EIA (Fig. 1). Comparison of the FA and EIA tests with respect to concentrations of bacteria in the prepared suspension showed that both tests were highly sensitive in specimens containing $\geq 10^5$ bacteria per ml, but in specimens with lower concentrations of bacteria, the FA test detected 23 of 27 positives, whereas the EIA detected only 21 of 43 ($P = 0.002$; Table 1). In tests with known negative controls (either bacteria without antibody or antibody without bacteria), nonspecific fluorescence caused false positives in 5 of 60 specimens tested by FA but only 1 of 80 in the EIA ($P = 0.05$, Fisher's exact test, one tailed). Four of the five false positives with the FA test were in specimens containing antibody alone.

Effects of antiserum dilution. Using 10^8 bacteria per ml, dilution of antiserum to the *E. coli* O44 antigen correlated directly with absorbance in the EIA (Fig. 2). FA results paralleled those of the EIA, with both tests becoming negative at antiserum dilutions of between 1:1,000 and 1:10,000 (Fig. 2).

Effects of pH or pretreatment of bacteria with antibiotics. Incubation of *E. coli* O44 and antiserum was carried out at pH values ranging from 4 to 8. No significant difference in absorbance obtained with the EIA was found over this range of pH values, and FA tests remained positive at all pH's tested.

Experiments performed after pretreating bacteria with 0.5, 1.0, and 1.5 μ g of gentamicin or ampicillin per ml before their exposure to antiserum had no effect upon either test.

Heterologous *E. coli* serotypes and non-*E. coli* strains. We evaluated 16 *E. coli* strains other than serotype O44 and 16 non-*E. coli* strains in both the FA and EIA tests. No cross-reactivity with heterologous *E. coli* strains and O44 antiserum was observed. All three *S. aureus* strains tested caused positive results in both assays irrespective of prior treatment with *E. coli* O44 antiserum. Other strains tested (two strains of *S. epidermidis* and *P. aeruginosa*, and one strain each of *P. mirabilis*, *Enterobacter*, and enterococci) did not cause false positives, with the exception of one of six *Klebsiella* strains. This strain was repeatedly positive in the FA but not the EIA test and, like the *S. aureus* strains, was positive with and without addition of *E. coli* antiserum.

TABLE 1. Comparison of FA and EIA in positive specimens (*E. coli* O44 plus homologous antibody)

Concn	FA			EIA		
	No. positive	No. negative	% Negative	No. positive	No. negative	% Negative
$\geq 10^5$	52	3	5	68	2	3
$< 10^5$	23	4	17 ^a	21	22	51 ^a

^a $P = 0.002$, Fisher's exact test, FA versus EIA.

FIG. 1. Effect of increasing bacterial concentration on absorbance in the EIA.

DISCUSSION

EIA has been widely used for detection of soluble antigens and antibodies in biological fluids, but has not often been employed for detection of antibody attached to bacteria. In the classic EIA technique, antigen is fixed or bound to a plastic support medium, and antibody attached to the bound antigen is detected by enzyme-linked anti-human globulin (1). In our assay, rabbit immunoglobulin attached to bacteria was incubated with enzyme-linked goat-anti-rabbit immunoglobulin, and bound enzyme-linked immunoglobulin was separated from unbound by centrifugation. This method was simple to perform, quantitative, and more specific than the FA assay in detecting *E. coli* artificially coated with antibody. False positives with the FA test occurred mainly in specimens with antibody but no bacteria and probably resulted from nonspecific adherence of antibody to bacterial contaminants or to debris closely resembling bacteria. Unfortunately, the sensitivity of

FIG. 2. Effect of decreasing antiserum dilution on absorbance in the EIA.

the EIA fell below 50% in specimens containing $< 10^5$ bacteria per ml, whereas the FA test detected 85% of positives in this group. We suspect that the EIA requires a substantial proportion of bacteria to be antibody coated before enough peroxidase-conjugated immunoglobulin is retained to result in a positive test, whereas the FA test can identify antibody-coated cells even when only a small number are present. Most infected urines actually contain 10^8 to 10^{10} bacteria per ml (3), so lack of sensitivity in specimens with $< 10^5$ bacteria per ml may not preclude use of the EIA in clinical specimens. However, the percentage of bacteria coated with antibody in actual upper tract infections may be lower than in our artificial system where at antiserum dilutions of 1:100, most bacteria exhibited fluorescence. We are now examining urines from infected patients with both assays to determine their sensitivity and specificity in

clinical use.

Since the EIA provides quantitative results, we assessed factors that may cause either false positives or false negatives in the FA test by running both assays in parallel. The direct correlation of EIA absorbance with dilution of antiserum suggests that insufficient antibody will probably cause false-negative tests. In a rabbit model, Smith demonstrated that positive FA tests did not occur until 10 to 14 days after experimental pyelonephritis when sufficient antibody titers had developed (9). Concentration of bacteria in the assay also directly correlated with EIA absorbance. In infections with lower counts of *E. coli*, false-negative tests may result from this phenomenon. Urinary pH and pre-treatment with antibiotics had no apparent effect upon the ACB test or the EIA. However, we examined only brief exposure of bacteria to antibiotics. Longer exposures, as could occur in patients, might produce different results.

Of particular interest were the false-positive FA tests observed with both *S. aureus* and *Klebsiella* strains. All *S. aureus* strains tested caused false-positive tests in both assays with or without the addition of *E. coli* antiserum. Presumably this phenomenon results from attachment of fluorescein-conjugated or enzyme-linked immunoglobulin to staphylococcal protein A via the FC receptor. False-positive results with one of six *Klebsiella* strains were unexpected but occurred repeatedly in this strain when tested by FA. This strain did not result in false positives using the EIA. We cannot explain this false-positive test at the present time, but the ACB test may not be as reliable for *Klebsiella* as for *E. coli*.

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LITERATURE CITED

1. Engvall, E., and P. Perlmann. 1972. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-linked antiimmunoglobulin in antigen-coated tubes. *J. Immunol.* **109**:129-135.
2. Fang, L. S. T., N. E. Tolkoff-Rubin, and R. H. Rubin. 1978. Efficacy of single-dose and conventional amoxicillin therapy in urinary tract infections localized by the antibody-coated bacteria technique. *N. Engl. J. Med.* **298**:413-416.
3. Goldberg, L. M., K. L. Vosti, and L. A. Rantz. 1965. Microflora of the urinary tract examined by voided and aspirated urine culture, p. 545-549. *In* E. H. Kass (ed.), *Progress in pyelonephritis*. F. A. Davis, Philadelphia.
4. Harding, G. K. M., T. J. Marrie, A. R. Ronald, S. Hoban, and P. Muir. 1978. Urinary-tract infection localization in women. *J. Am. Med. Assoc.* **240**:1147-1150.
5. Hawthorne, N. J., S. B. Kurtz, J. P. Anhalt, and J. W. Segura. 1978. Accuracy of antibody-coated bacteria test in recurrent urinary tract infections. *Mayo Clin. Proc.* **53**:651-654.
6. Jones, S. R., J. W. Smith, and J. P. Sanford. 1974. Localization of urinary-tract infections by detection of antibody-coated bacteria in urine sediment. *N. Engl. J. Med.* **290**:591-593.
7. Kohnle, W., E. Vanek, K. Federlin, and H. E. Franz. 1975. Lokalisation eines harnwegsinfektes durch nachweis von antikörperbesetzten bakterien im urin. *Dtsch. Med. Wochenschr.* **100**:2598-2602.
8. Riedasch, G., E. Ritz, K. Mohring, and H. E. Franz. 1978. Antibody coating of urinary bacteria: relation to site of infection and invasion of uroepithelium. *Clin. Nephrol.* **10**:239-244.
9. Smith, J. W., S. R. Jones, and B. Kaijser. 1977. Significance of antibody-coated bacteria in urinary sediment in experimental pyelonephritis. *J. Infect. Dis.* **135**:577-581.