Rapid Isolation and Presumptive Diagnosis of Uropathogens by Using Membrane Filtration and Differential Media

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Received 11 March 1991/Accepted 30 July 1991

Random urine samples from hospitalized patients (n = 550) and seeded sterile filtered urine samples (n = 730) were used to test a membrane filtration technique, Qualture (Future Medical Technologies International, Inc., West Palm Beach, Fla.), for the detection and identification of uropathogens. Results for each sample were compared with those obtained by the calibrated loop (0.01 ml) method to demonstrate the sensitivity of the method as a screening tool and the specificity of the presumptive diagnosis obtained from the pattern of growth on differential media. The medium was supplied as dehydrated nutrient pads (Sartorius AG, Goettingen, Germany) and was activated by rehydration by the addition of the liquid specimen. With a threshold of 10³ CFU/ml defining a positive culture, the sensitivity of the Qualture was 100%. At lower levels of bacteriuria, the Qualture was more sensitive than the calibrated loop method. Significant infections were presumptively diagnosed at 4 h by filtration rather than at 24 h on agar medium. The specificity of uropathogen identification ranged from 99% for Enterococcus spp. to 83% for Pseudomonas spp. Citrobacter spp. could not be differentiated from Escherichia coli and Providencia spp. could not be differentiated from Proteus spp., which does not create a therapeutic dilemma. Filtration, isolation, quantitation, and presumptive diagnosis are performed in one step, without subculture. Membrane filtration is a sensitive and rapid technique, with the advantage that it can be used as a collection and transport device without the use of growth inhibitors.

Each year, urinary tract infections presenting as acute dysuria affect large numbers of women (16, 25, 27). As many as 30% of these symptomatic patients that provide a clean-catch midstream urine sample have bacteriuria that does not meet the threshold set previously (13, 14, 26) and that is traditionally accepted by most physicians as a significant infection. Several studies have shown that a disparity exists between the diagnosis of cystitis or pyelonephritis in patients and the criteria used for the diagnosis, which is set at 100,000 bacteria per ml. Stamm et al. (25, 26) demonstrated that the most sensitive (0.95) and specific (0.85) guidelines to diagnose infections in symptomatic patients is the presence of bacteria at between 10² and 10⁴ CFU/ml. If therapy of patients with 100,000 bacteria per ml results in the suppression of bacteria by 90%, then low levels of colony counts may be responsible for the frequency of recurrent infections in about 20% of women (7, 16, 24). Symptoms of dysuria may be subjective, creating an asymptomatic pool of patients with low colony counts. Many variables play a role in the level of bacteriuria, such as antibiotics excreted in the urine, improper collection of the specimen, the state of hydration of the patient, or the pH and osmolarity of the urine. These conditions are most often unknown to the microbiologist who is called upon to interpret findings from the culture of body fluids. Screening of urine for bacteriuria therefore is limited by the method chosen and the variables concerning the levels of viable bacterial cells in the urine. Gram staining is inexpensive, but it is time-consuming and is neither quantitative nor diagnostic. Chemical strips or enzymatic methods are generally insensitive to low colony counts. Automated devices may require several hours for detection, and all require culture on an agar plate for the isolation of colonies for diagnosis (3, 21). Collection of urine in the outpatient setting is constrained by the time that has elapsed prior to culturing and the time that the bacteria are exposed to transport medium containing boric acid. Several studies have questioned the reliability of the transport medium (9, 19). Hubbard et al. (11) found that, in comparison with refrigerated specimens, transport medium inhibited the detection rates of bacteria exposed to boric acid for 24 h when the MS-2 system was used. Organisms that were most frequently missed were those that were most often found in patients with urinary tract infections. Geunther and Washington (9) are cautious about interpreting results from urine kept in preservatives for 24 h, since one-third of the specimens that contained bacteria at 10³ to 10⁵ CFU/ml yielded less than 10⁴ CFU/ml, increasing the reporting of negative cultures. It seems apparent that a method which can serve as a point-of-collection device, that is sensitive enough to detect those patients who are symptomatic with bacteria present at 10³ to 10⁴ CFU/ml, that acts as a rapid screening tool for significant infections, and that yields a presumptive diagnosis with one sample would be a valuable addition to the multiplicity of tools used in the laboratory. A membrane filter device that uses four differential media was studied and compared with the calibrated loop method. The purpose was to find a more sensitive and timely detection system that would resolve some of the limitations that exist.

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MATERIALS AND METHODS

Microorganisms. The microorganisms were prepared by using stock cultures of Escherichia coli, Citrobacter spp., Enterobacter cloaceae, Klebsiella pneumoniae, Proteus mirabilis, Providencia spp., Pseudomonas aeruginosa, Staphylococcus epidermidis, Staphylococcus aureus, Enterococcus faecalis, Streptococcus viridans, Candida albicans, and Serratia marcescens. These organisms were obtained from Scott Laboratories Inc. (Fiskville, R.I.) or were isolated from urine cultures and were preserved on the appropriate medium after subculturing. They were identified by Microscan (Baxter Systems Inc.) and Vitek (Vitek Systems Inc., Hazelwood, Mo.) and were chosen to represent uropathogens that are frequently isolated from bacteriuric patients.

Microorganisms were brought to the logarithmic growth phase in brain heart infusion broth (BBL, Cockeysville, Md.) after having been plated on 5% sheep blood or MacConkey agar. The isolates were diluted in broth to the equivalent of a 0.5 McFarland standard. Serial 10-fold dilutions in sterile normal saline were made to provide an inoculum of 3 ml with bacteria present at 10^2 to 10^5 CFU/ml. Each sample was plated onto the appropriate medium using a 0.01-ml calibrated loop, to determine a viable cell count. These organisms were used for evaluation of the differential media to provide a presumptive diagnosis. Serial dilutions were used to detect the time of appearance of colonies on the membrane and were compared with the quantitative results obtained by the calibrated loop method. Seeded cultures were observed every 2 h up to 12 h postinoculation (p.i.) for the development of visible colonies.

Urine cultures. Random urine samples that were received in the hospital laboratory were cultured immediately or were refrigerated for less than 24 h. A 0.01-ml calibrated loop was used to streak samples onto MacConkey and 5% sheep blood agar plates for quantitation and identification. In comparison, 3 ml of urine was loaded into a port atop a plastic culture device (Qualture; Future Medical Technologies, Inc., West Palm Beach, Fla.) by using a bulb pipette. Both cultures were incubated aerobically at 35 to 37°C, were examined every 4 h until 12 h p.i., and were then observed at 18 to 24 h. The colonies growing on the membrane were presumptively identified by their patterns of growth on the differential media. The appearance of colony growth morphology, color, and density of growth were recorded. Final identification was made by using the Microscan or the Vitek automated system.

Qualture. The Qualture is a plastic, self-contained, closed-membrane filtration culturing device with an entry port on the top. Microorganisms are trapped on a 0.45-μm-pore-size membrane. The filter membrane is supplied by four types of differential dehydrated media (Sartorius AG, Göttingen, Germany). The medium is rehydrated upon inoculation of 3 ml of broth, urine, or saline. When rehydrated, the medium diffuses through the pore system of the filter, supplying nutrients to the organisms trapped on the surface. The colonies that are formed on the membrane during incubation were counted and were related directly to the sample volume or to the time of appearance of confluent or heavy growth. Presumptive identification is based on the color of the colonies on these media (2, 4, 8, 12, 15). The media are arranged in quadrants juxtaposed to the underside of the membrane. Quadrant one contains Tergitol triphenyltetrazolium chloride (TTC), which detects and is differential for coliforms. Quadrant two contains cetrimide for the detection of Pseudomonas spp. Quadrant three contains membrane filtration for coliforms (m-FC), which is designed to detect fecal coliforms. Gram-positive organisms are inhibited on this medium. Quadrant four contains Chapman's medium for the isolation and detection of staphylococci. This medium may not inhibit growth of other gram-positive organisms. Colonies on the surface of the membrane were removed for additional studies and identification.

Presumptive identification. Qualture plates were inoculated with known reference organisms, and previously identified clinical isolates were resuspended in normal saline. The colors of the colonies and their morphologies were recorded for over 3,000 samples inoculated onto these media, from which a probability chart was developed (see Table 2). Biochemical spot tests were performed directly on the membrane or on filter paper to support the visual presumptive identification from the color change on the differential media (2, 4, 8, 12, 15).

Quantitation. Serial 10-fold dilutions of seeded sterile urine samples were inoculated into the Qualture. Observations at 2-h intervals for the first 12 h and then at 4-h intervals were made to determine visible colony formation. Colonies were defined as having sparse, moderate, heavy, or confluent growth. Each dilution was plated onto 5% sheep blood agar or MacConkey medium by using a 0.01-ml calibrated loop to obtain a viable cell count as a control.

RESULTS

Sensitivity of the procedure. Of the 550 random clinical specimens yielding greater than 10^3 CFU/ml, 22.7% were positive by the calibrated loop method and 23.8% were interpreted as having greater than 10^3 CFU by Qualture; this gave a sensitivity of 100%. At 4 h after inoculation, the positive predictive value for significant urinary tract infection was also 100%. Urine samples with 10^4 CFU/ml accounted for 13.8% of the total specimens by the calibrated loop method and 15.2% of the total specimens by the filtration technique. Twenty-nine agar plates streaked by the calibrated loop method had 10^5 CFU/ml or less, yielding 5.4% of the total positive cultures by that method (Table 1). By the Qualture technique, 61% of urine samples had growth, with 22% of the filtered urine samples demonstrating 10^3 CFU/ml or less, resulting in 91 more positive samples with low colony counts than were obtained by the agar plate method (Fig. 1). This would be expected, considering the limits of sensitivity of the calibrated loop method (Fig. 1).

Recovery of inoculum. Seeded sterile urine samples containing serial dilutions of stock cultures of Escherichia coli,
The sensitivity of the filter membrane method compared with that of Enterococcus faecalis. From $10^2$ to $10^5$ CFU/ml were observed for colony appearance at 2-h intervals p.i. until 12 h and then at 16 and 24 h (Fig. 2). Escherichia coli colonies were consistently visible at 4 h, while Staphylococcus epidermidis and Candida albicans and other gram-negative rods appeared at 6 h p.i. Presumptive identification was delayed until 8 h for Escherichia coli and until 10 h for Enterobacter spp. and Enterococcus faecalis. Staphylococcus epidermidis and Candida albicans were identified at 12 h. The last two microorganisms were more difficult to see because of their white color on a white background. Recovery was difficult to interpret from inocula greater than $10^3$ cells per ml if the membrane was read at 12 h or later because of heavy or confluent growth. At less than $10^3$ CFU/ml, recovery was 66 to 100%. Calculation of recovery from clinical specimens was not possible directly but correlated with the colony counts observed from the same specimens inoculated onto agar by the calibrated loop method. At 4 h p.i., urine samples containing greater than $10^5$ CFU/ml were confluent, while urine samples with $10^4$ CFU/ml became confluent or had heavy growth at 4 to 8 h. Most specimens reached a plateau within 8 to 10 h, after which no new colony formation was noted.

Presumptive diagnosis. Seeded stock cultures inoculated into Qualurate were observed for color and pattern development on the differential media (Table 2). The media are arranged in quadrants juxtaposed to the underside of the membrane. As noted from the data in Table 2, Citrobacter spp. could not be differentiated from Escherichia coli and Providencia spp. could not be differentiated from Proteus spp. on these media. All final identifications of bacteria were identified by the Microscan (Baxter Laboratories) and Vitek systems. The color of the colonies for each organism was tabulated into a probability chart for recognition of the most common uropathogens (Table 2). Enterococcus spp. was the most consistent in its appearance (a distinctive brick red color) and pattern of growth (pinpoint colonies) on Tergitol (quadrant one). Group B streptococci grew in a pattern similar to that of Enterococcus spp. and was indistinguishable without further analysis. All of the gram-positive organisms were inhibited on the m-FC (quadrant three). Klebsiella spp. and Proteus spp. were recognized by their pattern as frequently as Enterococcus spp. were. There was some overlap in color and development, which did not compromise identification because of significant morphological differences. Klebsiella spp. was very mucoid in appearance on the membrane and had colonies larger than those of Proteus spp. Escherichia coli and Enterobacter spp. were consistent in color on Tergitol (quadrant one) but were distinctive morphologically. The former was grey on m-FC 14% of the time. Candida albicans grew on all quadrants, displaying a creamy white raised colony that was easily recognized and that did not vary from its appearance on agar medium.

Staphylococcus aureus produced a typical yellow gold colony on Chapman's medium, (quadrant four), with a consistency of 94%, while coagulase-negative staphylococci appeared as a white or cream-colored colony. Catalase was positive on the membrane filter or on a glass slide by using colonies picked from the membrane. Serratia marcescens was crimson red on all quadrants, but there were a few nonpigmented strains. The percentages of these microorganisms recognized by the growth patterns are given in Table 2. Escherichia coli was the most prevalent organism isolated from urine samples, was typical in its appearance, and had a probability of 86%. Escherichia coli tested indole positive (1) on the membrane filter as well as on filter paper. Pseudomonas aeruginosa took longer to develop pyocyanins on cetrimide medium (12) and was positive when it was tested by the oxidase test (1).

![FIG. 1](image-url) Sensitivity of a 0.01 ml loop compared with that of filtration in detecting bacteriuria from 550 clinical urine samples. The sensitivity of the filter membrane method compared with that of the calibrated loop method was 100% for "significant" infections.

![FIG. 2](image-url) Range of time of appearance of visible colonies on the membrane filter (n = 3,000). Lower limit, colonies first observed; upper limit, no new colony formation noted. The bacterial isolates are those listed in Table 2.
TABLE 2. Appearance of bacterial and yeast colonies on differential media

<table>
<thead>
<tr>
<th>Colony</th>
<th>Colony appearance</th>
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<tbody>
<tr>
<td></td>
<td>Tergitol (one)</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>Yellow/orange</td>
</tr>
<tr>
<td>Citrobacter spp.</td>
<td>Yellow/orange</td>
</tr>
<tr>
<td>Enterobacter spp.</td>
<td>Yellow/orange</td>
</tr>
<tr>
<td>Klebsiella spp.</td>
<td>Green/red</td>
</tr>
<tr>
<td>Proteus spp.</td>
<td>Green/red</td>
</tr>
<tr>
<td>Providencia spp.</td>
<td>Green/red</td>
</tr>
<tr>
<td>Pseudomonas spp.</td>
<td>+</td>
</tr>
<tr>
<td>Staphylococcus coagulase-negative</td>
<td>±</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>±</td>
</tr>
<tr>
<td>Enterococcus spp.</td>
<td>Red</td>
</tr>
<tr>
<td>Group B streptococci</td>
<td>Red</td>
</tr>
<tr>
<td>Candida albicans (yeast)</td>
<td>White</td>
</tr>
<tr>
<td>Serratia spp.</td>
<td>Red</td>
</tr>
</tbody>
</table>

* A total of 3,000 samples were tested. Citrobacter spp. could not be differentiated from Escherichia coli on these media, and Providencia spp. could not be differentiated from Proteus spp. Acinetobacter spp. could not be differentiated from other gram-negative rods. Providencia, Proteus, and Acinetobacter spp. represent a small fraction of the isolates detected in clinical trials. Therapeutically, Citrobacter spp. falls under the same antibiotic regimens as Escherichia coli. Providencia spp. most often maintains the spectrum of antibiotic coverage of Proteus spp. Identification of these groups should be made by additional biochemical and susceptibility tests.

† Numbers in parentheses are quadrants. +, −, and ±, growth may or may not appear but is not differential for the presumptive diagnosis.

DISCUSSION

The microbiology laboratory has seen several recent innovations in urine screening techniques to reduce laboratory work loads and technician time. Most are automated with fair degrees of sensitivity and reliability for detecting urine samples containing significant (≥10⁸ CFU/ml) bacteriuria in asymptomatic patients (3, 21). Low colony counts (10² to 10⁴ CFU/ml) are not uncommon in clean-catch midstream urine samples from women with acute cystitis (16, 25, 26). This group of patients was described as having urethritis or acute dysuria and was reported as culture negative. In a study by Stamm et al. (24), approximately 30% of women presenting with urinary tract infections had levels of bacteriuria undetectable by screening methods with sensitivities of ≤8 × 10⁸ CFU/ml. Estimates of the incidence of urinary tract infections in the United States are in the millions, with 10 to 50% (24) of women having occult renal disease with serious therapeutic implications for the duration of therapy and prospects of long-term renal damage (23) because of either urea-splitting organisms that produce struvite stones or virulence factors contained by strains of Escherichia coli (6, 20, 22). The quantitation of bacteriuria and its causal relationship to clinical disease must take into account the status of the immune competence of the host and the susceptibility factors of the host’s uropathphone (22). Frequent recurrence of infection in some women raises the question of whether there was inadequate or inappropriate initial therapy or a low level of residual bacteriuria that was easily missed on repeat screening or culture with a 0.01-ml loop. Contributing to low colony counts and often overlooked in the commercial laboratory is the use of transport medium that has been shown to diminish the levels of bacteria in the urine. Autopsy data suggest that 80% of the cases of pyelonephritis go undiagnosed premortem, supporting the suspicion that a high percentage of women have occult renal disease (26).

In this combined study of clinical and seeded urine samples, we demonstrated the sensitivity of filtration for the recovery of bacteria from urine (Table 1). The technology is not new and has been used successfully in industrial and water-testing microbiology laboratories, where 1 CFU/100 ml is the threshold of sensitivity. Filtration has also been demonstrated in blood culture (10, 18) as a sensitive device that has the ability to shed inhibitors of bacterial growth commonly found in body fluids. The sensitivity in urine testing is evidenced by the percentage of clinical samples with growth determined by filtration techniques (61%) compared with the percentage of those with growth determined by streaking onto agar with a calibrated loop (42%). The differences are undoubtedly related to the sample size, in which one colony on an agar plate from a 0.01-ml calibrated loop is transposed into 100 colonies per ml and in which all of the aforementioned vagaries of urine culture are most apt to produce false-negative results. Mixed cultures were more evident in the filtration technique because of the sensitivity, but were no greater in terms of the number of positive samples in both groups. Mixed cultures cannot be ignored in symptomatic women, even in those exhibiting low colony counts (5).

Figure 2 demonstrates the time of appearance of colonies by the filtration method in relationship to the biological load. At 4 h p.i., urine samples with ≥10⁵ CFU/ml showed confluency on the membrane, with growth being distinguishable by the color (Table 2), pattern, and morphology, and had a positive predictive value of 100%. Reagent tests were useful in occasionally substantiating a diagnosis because of variation in the growth pattern that occurred, but this was usually only on one quadrant. Hospitalized patients with significant bacteriuria could be diagnosed in the same work shift. The absence of growth on the Qualtrac at 24 h had a negative predictive value consistent with growth on agar plates kept for several days. This provides a confidence level that bacterial infection is not causal to symptoms or pyuria, if it is present. Heavy growth was seen in 4 to 8 h when 10⁴ to 10⁵ CFU/ml was used. Colonies were detected in 8 to 12 h when 10⁴ CFU/ml was used. Infections in patients with spuriously low levels of bacteriuria as a result of antibiotic use or their state of hydration were detectable, limiting the number of false-negative results that were found, especially in elderly or chronically catheterized individuals. The issue of recurrent infections (7, 17) may be central to therapeutic success or failure, particularly since the efficacy of single-dose therapy has not been resolved. A sensitive in vivo test
that was suggested (6, 23) to increase the diagnostic acumen for incipient renal disease may now be available.

Four differential media which discriminate between gram-positive and gram-negative organisms and which allow the technician to distinguish members of the family Enterobacteriaceae and other gram-negative rods from each other were used. Rapid visible growth was related in part to the size of the specimen presented onto the membrane (Fig. 2). A consistent pattern of colony color developed, allowing for recognition of the genus of most common bacterial uropathogens (Table 2). Reagent tests, if needed, are rapid and inexpensive and can be performed in the usual manner or directly on the membrane. In mixed infections, discrete colonies can be removed to be tested for substantiation of the diagnosis. Media in the Qualture are in dehydrated pads, adding another dimension to this culture technique and thereby extending the shelf-life for at least 6 months to 1 year without refrigeration. The Qualture requires the addition of only 3 ml of urine, which is transferred by a bulb pipette into the port on top of the device. No special training is required, and nontechnical personnel in an office can easily perform the task. The implications of this are that the device can be used at the point of collection and, if necessary, sent to the hospital or commercial laboratory without the addition of boric acid to inhibit or suppress growth (9, 11, 19). Result reporting could be performed upon receipt in the laboratory, depending on the ambient temperature and the length of time transpired from collection to delivery.

REFERENCES