Automated Direct Antimicrobial Susceptibility Testing of Microscopically Screened Urine Cultures

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Two screening methods for urine microbiology are proposed: one in which the Gram-stained smear is used to detect significant bacteriuria, and another in which Autobac antibiotic susceptibility tests are performed directly on positive urine samples. Results on 1,350 specimens indicated that an average of 18 bacteria per oil immersion field were observed in the urine of patients with significant bacteriuria, and an average of <1 bacterium per oil immersion field was found in the urine of patients without significant bacteriuria. Direct susceptibility testing by Autobac proved to be rapid (3 h versus 24 h) and reliable (0.5 to 1.2% discrepancies).

The necessity for rapid and precise information on specimens for microbiological examination has been stated by many (3). Urine is the most numerous of the types of specimen submitted to the microbiology laboratory (1). A simple rapid screening test for significant bacteriuria is desirable, as is a rapid evaluation of the antimicrobial susceptibility of the alleged pathogens.

This report shows that microscopic evaluation of Gram-strained urine can accurately screen positive cultures and that direct Autobac antimicrobial susceptibility testing of the centrifuged urinary pathogens is indeed possible.

MATERIALS AND METHODS

Urine samples. Urine was received for culture from both inpatient and outpatient sources of the John Dempsey Hospital, University of Connecticut Health Center, and cultured within 1 h after collection. Urine samples were rejected if there was evidence of delayed transport (≥2 h).

Gram stain of urine. Ten microliters of urine was placed on a clean glass slide with a calibrated platinum loop (0.01 ml) and spread in approximately a 30-mm circle. The slides were air-dried and Gram-stained according to the procedure described in the second edition of the *Manual for Clinical Microbiology* (5).

Approximately 50 oil immersion fields (o.i.f.) were scanned, and the mean number of microorganisms per field was reported. If two or more morphological types of bacteria were present on a slide, the urine sample was not admitted to the study. All samples with ≥1 bacterium per o.i.f. were experimentally processed.

Routine urine culture. A 10-μl sample of urine was uniformly spread on the surface of a 10% sheep blood agar plate. A similar amount of urine was cultured on a MacConkey agar plate for optimal colony separation. Plates were incubated at 35°C for 18 h in an air incubator. Estimates of colony counts were made by visually examining the blood agar plates.

Urine cultures were routinely reported as "no growth," <1,000 colony-forming units (CFU) per ml, ≤10,000 CFU/ml, and actual colony count in 10,000-CFU/ml increments when the count was between 10,000 and 100,000 CFU/ml or greater. For the purposes of the study, three categories of urine colony counts were established: <10^4 CFU/ml, >10^4 but <10^5 CFU/ml, and ≥10^5 CFU/ml. Identification of organisms present in urine was according to methods outlined by Lennette et al. (5).

Routine antimicrobial susceptibility test. After overnight incubation of the blood agar plates and the MacConkey agar plates, the plates were examined, and individual colonies of representative bacteria were chosen for the Autobac antibiotic susceptibility test according to the procedure of Thornsberry et al. (9). Results of direct and pure culture susceptibility tests were compared, as were the results of the Gram stain on uncentrifuged urine and the semiquantitative colony count.

Experimental processing of urine. When the urine contained ≥1 bacterium per o.i.f., 12 ml of the sample was added to a sterile 15-ml conical centrifuge tube. Turbid urine was initially centrifuged at 500 × g for 10 min to remove cellular debris. The clarified sample was then centrifuged at 3,000 × g for 15 min, and the supernatant urine was removed.

The pellet was suspended in 5.0 ml of phosphate-buffered saline (Autobac Standardization Solution, Pfizer Diagnostics; pH 7.0). This suspension was used to adjust an Autobac inoculum standardization cuvette to the present machine value (1.5 × 10^7 to 3.0 × 10^7 CFU/ml). After inoculum standardization, the procedure for the Autobac antibiotic susceptibility test was followed as outlined by Thornsberry et al. (9). Autobac cuvettes were read at 3 h or when the growth index was ≥0.90. Susceptibility test results were carefully inspected for aberrant patterns due to a possible mixed bacterial inoculum.

Interpretation of discrepant Autobac results. "Minor discrepancies" were those in which there was a difference in interpretation as a function of method, e.g., between susceptible and indeterminate, resistant

and indeterminate, or vice versa. A "major discrepancy" was when the experimental Autobac procedure indicated resistance and the confirmatory method indicated susceptibility. A "very major discrepancy" occurred when the experimental method indicated susceptibility and the confirmatory method indicated resistance.

RESULTS

Table 1 shows the organisms isolated and the categories of colony counts observed in positive specimens. Since routine clinical specimens were used for this study, most of the significant isolates were *Escherichia coli* (63/84; 75%). The next most frequently isolated bacterium was *Klebsiella pneumoniae* (8/84; 9.5%). A positive result on which a susceptibility test was performed was defined as a pure culture of any microorganism, with the exception of *Staphylococcus epidermidis* or diphtheroid-like bacilli, in numbers >10,000 CFU/ml. (Although it is recognized that *S. epidermidis* and *Staphylococcus saprophyticus* may cause urinary tract infection, there are sufficient contraindications for Autobac susceptibility testing of these organisms not to include them in the study.) Of the 1,350 urine specimens evaluated, 84 (6.2%) were positive by our criteria. Ninety percent (76/84) of the positive specimens contained $\geq 10^5$ CFU/ml of urine.

A comparison of the numbers of specimens processed as a function of the average number of bacteria per o.i.f. can be seen in Fig. 1. There were 1,200 of 1,266 (94.7%) specimens in which no organisms were observed by Gram stain and in which $\leq 10^4$ CFU/ml were cultured. The majority of these specimens were either sterile or contained small numbers of normal urethral flora. There were 52 of 1,266 (4.1%) specimens containing $\leq 10^4$ CFU/ml in which 1 bacterium per o.i.f. was observed. Of 1,266 specimens, 14

(1.1%) revealed ≥ 2 bacteria per o.i.f.; the range was from 2 to 15 bacteria per o.i.f. with an average count of 5 bacteria per o.i.f. The overall average colony count for 1,266 specimens was <1 bacterium per o.i.f. In many instances in which bacteria were seen microscopically and the colony count proved insignificant, more than one morphological type was observed by Gram stain.

The distribution of microscopic counts among the 76 positive urine specimens ($\geq 10^5$ CFU/ml) can be seen in Fig. 1. Counts ranged from zero (3/76, 3.9%) to two specimens in which >50 bacteria were observed per o.i.f. The average count was 18. Eight urine specimens containing $>10^4$ but $<10^5$ CFU/ml, which were considered equivocal but still experimentally processed, revealed negative microscopic counts in two of eight specimens, 2 bacteria per o.i.f. in three of eight specimens, 5 bacteria per o.i.f. in one of eight specimens, and ≥ 10 bacteria per o.i.f. in two of eight specimens. The average microscopic count was 5. The difference between the microscopic counts of positive ($\geq 10^5$ CFU/ml) and negative specimens ($\leq 10^4$ CFU/ml) was statistically significant ($P < 0.001$). The eight specimens of equivocal significance were not analyzed statistically.

Table 2 shows the sensitivity, specificity, and predictive values of both positive and negative Gram-stain results. If a positive-negative threshold of ≥ 1 bacterium per o.i.f. is chosen, then the sensitivity and specificity of the method were 96.2% and 95%, respectively. Increasing the threshold to >5 bacteria per o.i.f. decreases sensitivity (90.5%) and increases specificity (99%). Predictive values of positive urine Gram stains were 53.5 and 99.7% for thresholds of ≥ 1 and ≥ 5 bacteria per o.i.f., respectively, and predictive values for negative urine Gram stains were 92.7 and 99.4% for the same two microscopic thresholds.

The data comparing direct versus pure culture antibiotic susceptibility test results by Autobac are presented in Table 3. Of the 75 specimens tested by both methods, no organism-antibiotic pair showed greater than 5.3% major or very major discrepancies. Minor discrepancies ranging from 1.3% to 6.0% were seen primarily with chloramphenicol. Of all discrepancies in the 1,200 individual tests performed, 0.5% (6) were minor (resistant or susceptible versus indeterminate), 1.2% (14) were major (resistant by Autobac instead of susceptible), and 0.5% (6) were very major (susceptible by Autobac instead of resistant).

With the exception of three urine samples containing *Pseudomonas aeruginosa*, which re-

TABLE 1. *Range of colony counts observed in 84 specimens of urine from patients with significant bacteriuria*

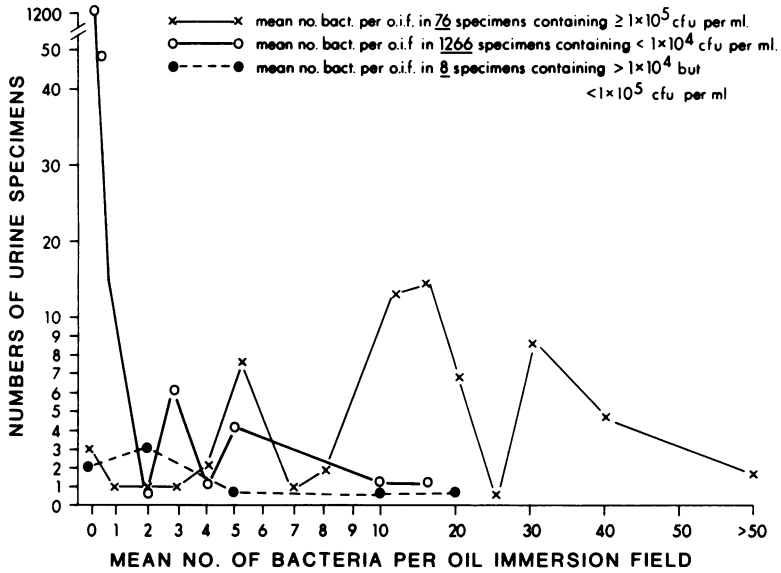| Isolate	No. of positive specimens	No. of specimens in range of colony counts:	
		$>10^4$–$<10^5$ CFU/ml	$\geq 10^5$ CFU/ml
Escherichia coli	63	6	57
Klebsiella pneumoniae	8	1	7
Enterobacter cloacae	1	0	1
Enterobacter aerogenes	1	0	1
Enterobacter agglomerans	1	0	1
Proteus mirabilis	3	1	2
Morganella morganii	1	0	1
Pseudomonas aeruginosa	3	0	3
Group B streptococci	1	0	1
Enterococci	2	0	2

FIG. 1. *Comparison of the numbers of bacteria per microscopic field with the colony count of urine samples.*

TABLE 2. *Sensitivity, specificity, and predictive value of urine screening by microscopy*

Avg bacterial count (per o.i.f.)	Sensitivity[a] (%)	Specificity[b] (%)	Predictive value of "positive"[c] (%)	Predictive value of "negative"[d] (%)
≥1	96.2	95	53.5	99.7
≥5	90.5	99	92.7	99.4

[a] True positive (TP)/[TP + false negative (FN)].
[b] True negative (TN)/[TN + false positive (FP)].
[c] TP/(TP + FP).
[d] TN/(TN + FN).

quired 4 to 5 h to reach a growth index of 0.90, all direct Autobac tests on urine samples attained a growth index of 0.90 in 3.0 to 3.5 h.

DISCUSSION

In this study it has been shown that urine can be screened microscopically and that an estimate can thus be made of the approximate colony count. Subsequently, the specimen, if positive, can be used as a source of bacteria for antimicrobial susceptibility testing. The screening procedure is rapid (a few minutes per specimen), and the ensuing antimicrobial test results are available within 3 to 4 h. Although such rapid results are not necessary for all patients, those hospitalized patients with urinary tract infection may benefit from early identification of a suitable antimicrobial agent. Persons with uncomplicated urinary tract infection may also benefit from the ability to detect significant bacteriuria in a few minutes.

TABLE 3. *Comparison of "rapid-direct" Autobac antibiotic susceptibility test results versus "pure culture" Autobac results[a]*

Antibiotic	% Discrepancies[b]		
	Minor	Major	Very major
Ampicillin	1.3	2.6	0.0
Cephalothin	2.6	5.3	1.3
Chloramphenicol	6.0	2.6	0.0
Colistin	0.0	2.6	2.6
Kanamycin	0.0	2.6	1.3
Tetracycline	1.3	0.0	2.6
Carbenicillin	0.0	1.3	0.0
Gentamicin	0.0	1.3	0.0

[a] Based on 75 pairs of determinations per antibiotic.
[b] For definitions of types of discrepancies, see the text.

Many methods have been proposed for rapid screening of urine for infection. They include detection of nitrite (4), disappearance of glucose (7), presence of catalase (4), tetrazolium reducing power (8), microbial adenosine 5'-triphosphate concentration (3), and pyuria. Most available methods suffer from imprecision, high cost, lack of ready availability, slowness of processing, and lack of practicality. On the other hand, investigators have suggested that the urine Gram stain is perhaps one of the most effective screening methods for bacteriuria currently available.

A report by Heinze et al. (2) indicates that the microscopic evaluation of urine is indeed a reliable screening device. Their work indicates that

urine containing $\geq 10^5$ CFU/ml usually will show >8 bacilli per o.i.f. and that specimens containing $\geq 10^3$ CFU/ml but less than 10^5 CFU/ml usually either are negative by microscopy or contain <1 bacterium per o.i.f. Although the present study is in general agreement with that of Heinze et al. (2), certain differences do exist. The idea that the presence of 1 to 2 bacteria per o.i.f. is indicative of a colony count of $\geq 10^5$ CFU/ml cannot be fully supported. The data in Fig. 1 show that 66 of 1,266 (5.2%) specimens whose colony count was $\leq 10^4$ CFU/ml revealed from 1 to 15 bacteria per o.i.f. (average count of 2), whereas those specimens (76/1,266; 6%) with culture-proven significant bacteriuria ($\geq 10^5$ CFU/ml) had microscopic counts significantly higher (average count of 18).

Those specimens termed equivocal (>10^4 to <10^5 CFU/ml) may often represent infection of the urinary tract, particularly when organisms are present in pure culture. There were eight specimens in this category; two were negative by microscopic screening, and six contained from 2 to 20 bacteria per o.i.f. (average count of 5).

It is difficult to determine a threshold microscopic count that predicts significant bacteriuria. If in this study an average count of 5 bacteria per o.i.f. was accepted, then 68 of 76 (90%) of the positives would have been predicted. However, 8 of 76 (10%) significant specimens would have been classified as negative. If, on the other hand, any number of bacteria per o.i.f. was accepted as a threshold, then 66 of 1,266 specimens (6%) would have been false-positive. With either threshold, three specimens which subsequently revealed $\geq 10^5$ CFU/ml and two specimens containing >10^4 to <10^5 CFU/ml would have been called negative as a result of no organisms observed by Gram stain.

Still another way to test the data on microscopic evaluation of urine specimens is to determine the sensitivity, specificity, and predictive values using certain threshold counts. Sensitivity can be defined as the probability of detecting a true-positive urine specimen by Gram stain, and specificity can be defined as the probability that a negative Gram stain is truly reflective of a negative urine specimen. Table 2 shows that reducing the threshold for a positive screening test from ≥ 5 to ≥ 1 bacteria per o.i.f. increases sensitivity (90.5 to 96.2%), decreases specificity (99 to 95%), markedly affects the predictive value of a positive (92.7 to 53.5%), and has essentially no effect on the predictive value of a negative. In practical terms a false-negative screening test has more dire results than a false-positive result. Thus, on the basis of the data in this report, a positive threshold of ≥ 5 bacteria per o.i.f. seems valid.

It must be emphasized that rejection of a specimen for culture based on a negative direct Gram stain cannot be recommended without reservation. All urine samples, collected and properly transported, should be processed. Whereas the microscopic evaluation of urine is not labor sparing, the ability to predict 90% of true positives based on a threshold count of ≥ 5 bacteria per o.i.f. would appear to be worthwhile. The false-negatives would be subsequently detected by routine culture.

The Gram stain then is used for two purposes: (i) to screen for true positives, and (ii) to trigger a rapid Autobac antibiotic susceptibility test. Direct antimicrobial susceptibility tests on urine suffer from the threat of multiple bacterial species being present. Although in all of the patients with two or more gram-negative rods in the urine, the rapid Autobac susceptibility results were sufficiently atypical as to be questioned, such cannot be guaranteed. Consequently, strict attention must be paid to microscopic morphology. Smears containing both gram-positive and gram-negative bacteria, or more than one morphological type regardless of Gram reaction, should not be rapidly processed.

The lack of precise correlation between the direct Gram stain and the colony count may be the result of a number of factors, including: (i) stained nonviable bacteria; (ii) the presence of substances in the urine inhibitory to bacterial growth; (iii) cellular components; and (iv) physical state of the urine (pH, temperature). The inability of the present study to corroborate that of Heinze et al. (2), particularly in those specimens containing only 1 to 2 bacteria per o.i.f., may reflect inherent interlaboratory variation, since the methods are similar.

The data indicate that most positive urine samples contain $\geq 10^5$ CFU/ml. In fact, Heinze et al. (2) reported that the majority of positive specimens in their study had colony counts of $\geq 10^8$ CFU/ml. This is a sufficient number of organisms upon which to perform a rapid Autobac susceptibility test. Since the advent of the standardized disk susceptibility test, direct analysis of specimens has not been encouraged due to the problems in inoculum standardization. The present study has attempted to circumvent this difficulty by using the Autobac nephelometer to standardize a suspension of bacteria removed from urine by centrifugation. It is clear that there are other cellular elements in urine besides bacteria, such as squamous epithelial cells, leukocytes, and erythrocytes. When the urine specimen was visibly turbid it was gently centrifuged (500 × g) for 10 min to remove cellular debris. The supernatant was then centrifuged, as previously indicated, for inoculum

preparation. Such a process usually removed most cellular debris that would have resulted in a spuriously low bacterial inoculum concentration.

The results of the rapid direct Autobac susceptibility test appear promising. The majority of very major discrepancies occurred with colistin and chloramphenicol, two antibiotics which would not normally be used for urinary tract infections. Overall, the results compare favorably with those of Heinze et al. (2): there were 0.5% very major discrepancies in our study, compared to 0.7% in theirs, and 1.2% major discrepancies in our study, compared to 3.0% in theirs. Similar procedures using direct Autobac susceptibility testing have been published for blood cultures (6). Both methods (urine and blood) have proven valuable in many situations where a 3.5- to 4-h delay was acceptable to obtain susceptibility results.

Lacking in this study was a susceptibility test for a sulfa derivative or sulfamethoxazole-trimethoprim. At the time, thymidine-free nutrient broth, required for sulfamethoxazole-trimethoprim analyses, was not widely available, and rapid tests for sulfa have not proved reliable. Recent results suggest that rapid sulfamethoxazole-trimethoprim tests are reliable.

A valuable adjunct to a simple microscopic screen and a rapid susceptibility test would be quick identification. Several products are available, one of which, Micro-ID (General Diagnostics), has shown promise for rapid identification of urine isolates within the same time frame as the direct Autobac susceptibility test.

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