Simplified Technique for Detection of Significant Bacteriuria by Microscopic Examination of Urine

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A comparative study of microscopic examination of 10 μ l (simplified loop technique) and 50 μ l (traditional drop technique) of uncentrifuged Gram-stained urine specimens for detection of significant bacteriuria was carried out. The results demonstrated that the 10- μ l loop technique can be used as an alternative to the 50- μ l drop technique for presumptive diagnosis of urinary-tract infection in bacteriological practice, with the advantages of greater rapidity and ease of performance.

Urinary-tract infections, including cystitis, pyelonephritis, asymptomatic bacteriuria, and acute urethral syndrome, constitute one of the most frequent causes of illness in humans (17, 18). Most such infections are caused by a few genera of bacteria, and the presence of these microorganisms in the urine is known as bacteriuria (6, 24). Quantitative urine culture is considered the standard procedure for adequate diagnosis of urinary-tract infections (25). Urine cultures represent 40 to 70% of the specimens sent for examination to clinical-microbiology laboratories (7, 14). Although the prevalence of urinary infections may vary in different patient populations, approximately 80% of urine cultures are negative (7, 14, 33). In an attempt to reduce the cost and time expended in examining these negative cultures, several rapid methods have been developed for characterizing bacteriuria, including microscopic examination, chemical tests, and automated systems (6, 7, 17).

Microscopic examination of an uncentrifuged Gram-stained urine drop constitutes one of the best diagnostic methods for detecting significant bacteriuria, i.e., the presence of 100,000 or more microorganisms per ml of urine (4, 6, 11, 26). Observation of one or more bacteria per oil immersion field correlates with 90% of cases of significant bacteriuria, thus indicating active urinary-tract infection (4, 20, 26, 30).

In Brazil, this method is not used in bacteriological practice, possibly because it is not widely known, because it is not standardized, or because of the prolonged drying time (probably due to the high concentration of salts and urea), about 3 to 4 h, of the urine drop, which is deposited, without spreading, on the slide. Use of a smaller volume of urine, leading to more rapid drying, might facilitate the use of this technique. However, no information regarding this subject was found in the literature, particularly in relation to the possibility of compromising the sensitivity or specificity of the method by using urine volumes less than 1 drop (50 μ l).

In order to better investigate this matter, we performed a comparative study of microscopic examination of an amount of urine applied with a calibrated loop (10 μ l) and a drop (50 μ l) of uncentrifuged Gram-stained urine, for detection of significant bacteriuria in patients with suspected urinary infections treated at the Maringá Regional University Hospital. As a

reference method, we performed parallel counts of bacterial colonies cultured from the 500 urine samples analyzed in the present study.

Samples. From October 1994 through July 1996, 500 urine samples were analyzed from patients treated at the Maringá Regional University Hospital of the State University of Maringá, in the city of Maringá, state of Paraná. Basic hygienic and aseptic precautions were taken in obtaining all urine specimens (4, 6). The time between collection and culture never exceeded 2 h. Urine samples not cultured immediately following collection were refrigerated at 4°C until processing (6).

Drop method. After homogenization of the urine sample, a hanging drop of approximately 50 μ l of the urine was deposited, by means of a 1- to 2-ml sterilized pipette, on the surface of a 25- by 75-mm microscope slide and was allowed to dry, without spreading, at ambient temperature (30).

Loop method. A 10- μ l volume of homogenized urine sample was applied, by means of a nickel-chrome loop calibrated to 10 μ l, to the surface of a 25- by 75-mm microscope slide and was allowed to dry, without spreading, at ambient temperature.

Gram staining. After air drying, the smears were fixed by passing the slides two or three times through the flame of a Bunsen burner, and then they were stained by the Gram method (5).

Microscopic examination. A preliminary inspection of the smears was performed by using a low-magnification $(10 \times to 20 \times)$ dry objective in order to locate the material on the slide. Next, with a $100 \times$ oil immersion objective, 50 fields were examined, and the shapes and number of microorganisms and cells per field were recorded. The microscopic reading was done systematically, beginning at the edge of the central region of the smear and continuing across its diameter. A positive microscopic examination was defined as the presence of ≥ 2 microorganisms uniformly distributed per oil immersion field, after observation of at least 20 fields, according to the criteria of Washington et al. (30).

Semiquantitative urine culture. Semiquantitative urine culture using the calibrated loop technique (6) and inoculation on cystine-lactose-electrolyte-deficient agar (Difco Laboratories, Detroit, Mich.) was employed as the reference method. Samples were considered positive if they contained $\geq 10^5$, or 10^4 to $<10^5$, CFU of the urinary pathogen/ml of pure culture. Also considered to indicate a urinary infection was isolation of two potentially pathogenic bacterial species, when the individual counts for the two species were $>10^5$ and $>10^5$, $>10^5$ and

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 $>10^4$, or $>10^4$ and $>10^4$ CFU/ml, or when the count for one organism was $>10^4$ CFU/ml and it was clearly predominant, i.e., at least 10-fold more than the other (6). Urine specimens containing $\geq 10^5$ or $< 10^5$ CFU of nonpathogenic bacteria (lactobacilli, diphtheroids, Staphylococcus epidermidis, or nongroup-D *Streptococcus* spp.)/ml or multiple (three or more) species of gram-negative bacteria, obtained from patients without clinical evidence of urinary infection, were considered contaminated and were excluded from the study. Isolated microorganisms were identified by standard biochemical procedures (4).

Statistical analysis. Sensitivity, specificity, and positive and negative predictive values were calculated by the method of Ransohoff and Feinstein (27), according to the following formulae: (i) sensitivity = TP/(TP + FN), the probability that the microscopic examination will be positive in patients with urinary infections (positive culture), (ii) specificity = TN/(TN + T)FP), the probability that the microscopic examination will be negative in patients without urinary infections (negative culture), (iii) positive predictive value = TP/(TP + FP), the probability that a urinary infection is present when the microscopic examination is positive, and (iv) negative predictive value = TN/(TN + FN), the probability that a urinary infection is not present when the microscopic examination is negative, where TP stands for true positive (microscopy and cultures both positive), FP for false positive (positive microscopy and negative culture), TN for true negative (microscopy and culture both negative), and FN for false negative (microscopy negative and culture positive). Comparison of the mean numbers of bacteria found in 10, 20, 30, 40, and 50 oil immersion fields of the microscopic Gram-stained preparations made with 10 μl (loop technique) and 50 µl (drop technique) of positive urine specimens (>10⁴ to \geq 10⁵ CFU/ml) was performed by using Student's t test for independent samples at a 5% significance level, with the program STATISTICA for Windows, release 4.3 (1993; StatSoft, Inc., Tulsa, Okla.).

Four hundred fifty-six of the 500 urine samples analyzed were collected by spontaneous urination (clean catch midstream), and 44 were collected with a plastic collecting bag. Most (80.42%) were from outpatients; the remainder were from inpatients. The ages of the patients ranged from 2 days to 87 years (mean, 32 years).

The incidence of positive cultures was 24.8% (124 of 500). The following groups of urinary pathogens (with the number of samples infected with each species in parentheses) were identified: (i) gram-negative bacilli, including Escherichia coli (74), Klebsiella pneumoniae (9), Pseudomonas aeruginosa (8), Klebsiella ozaenae (6), Enterobacter aerogenes (2), Citrobacter diversus (2), Proteus mirabilis (2), Enterobacter agglomerans (1), Enterobacter sp. (1), and Morganella morganii (1); (ii) grampositive cocci, including Staphylococcus saprophyticus (5), Enterococcus faecalis (3), S. epidermidis (3), Staphylococcus aureus (2), group-D Streptococcus sp. (2), and Enterococcus sp. (1); and (iii) fungi, i.e., yeast (1). In approximately 95% of the positive cultures (118 of 124), the etiological infective agent was isolated in pure culture, at a concentration of $\geq 10^5$ CFU/ ml.

The results of the two microscopy techniques showed 100% correlation. This was not at all surprising, considering that the 10- and 50-µl methods used the same urine with the same concentration of bacteria. The mean diameters of the smears made with the calibrated loop (10 μ l) and the drop (50 μ l) of urine were 6 and 11 mm, respectively. With regard to drying time, the smears made with the loop appeared dry in 10 to 15 min, as opposed to 3 to 5 h for those prepared with a urine drop.

TABLE 1. Correlation^a between microscopic examination and culture results of 500 urine specimens

Gram-stain result	No. (designation) of urine culture results		
	Positive ^b	Negative	Total
Positive ^c	119 (a)	3 (b)	122
Negative	5 (c)	373 (d)	378
Total	124	376	500

^{*a*} Sensitivity [a/(a + c)] = 119/124 (96.0%); specificity [d/(b + d)] = 373/376(99.2%); positive predictive value [a/(a + b)] = 119/122 (97.6%); negative predictive value [d/(d + c)] = 373/378 (98.7%). ^b Colony counts of >10⁴ to ≥10⁵ CFU/ml.

^c Observation of ≥ 2 bacteria per oil immersion field.

Table 1 shows the correlation of the microscopic examinations with the cultures of the 500 urine specimens studied. Disagreement was observed in eight samples, with five falsenegative results (negative microscopy and positive culture) and three false-positive results (positive microscopy and negative culture). In the latter there were pleomorphic, weakly stained gram-negative bacilli, suggestive of anaerobic bacteria. The microscopic examination, however, showed high sensitivity (96.0%) and specificity (99.2%) as a diagnostic method for significant bacteriuria (Table 1).

No statistically significant differences (P > 0.05) were found in relation to the mean numbers of bacteria observed in 10, 20, 30, 40, and 50 oil immersion fields of the stained microscopic preparations made with 10 μ l (loop technique) and 50 μ l (drop technique) of the positive urine samples.

In the positive microscopic urine examinations (122 of 500), the mean number of bacteria found in the 50 microscopic fields was 66.30 bacteria for the drop technique and 48.47 for the loop technique. On the other hand, in the 378 negative microscopic examinations, means of 0.16 and 0.18 bacteria, respectively, were found for the loop (10-µl) and drop (50-µl) techniques.

Microscopic examination of urine for detection of significant bacteriuria can be performed in bacteriological practice by four basic procedures: (i) examination of uncentrifuged fresh urine with a $40 \times$ dry objective, (ii) observation of fresh urinary centrifuged sediment with a $40 \times$ dry objective, (iii) examination with an oil immersion objective $(100 \times)$ of a Gram-stained smear of uncentrifuged urine, and (iv) observation of a Gramstained smear of centrifuged urine with an oil immersion objective (11). In the present investigation, we chose to use microscopic examination of uncentrifuged Gram-stained urine, because this is considered the most easily performed, the least expensive, and probably the most sensitive and reliable diagnostic method for identifying urine specimens containing more than 10^5 CFU/ml (4).

Although microscopic examination of an uncentrifuged Gram-stained urine drop is recognized as the conventional microscopic method for diagnosing urine specimens with counts of $\geq 10^5$ CFU/ml, being recommended as the routine procedure in bacteriological practice by several authors (4, 6, 7, 10, 11, 16, 17, 20, 25, 26, 30), no standardized technique exists for performing this procedure in the microbiological laboratory (11).

This lack of standardization is evidenced, for example, in 14 studies recorded in the literature, involving microscopic examination of approximately 46,200 urine specimens, in which the sensitivity of the method for detection of significant bacteriuria varied between 69 and 99%. In relation to the volume of urine used, in six of these studies (3, 7, 8, 15, 30, 31) the use of the

19, 23, 29, 32) the calibrated loop technique was used, with urine volumes varying from 5 to 10 μ l. The number of microscopic fields examined per urine sample was 5 (23, 31, 32), 10 (7–9), 20 (3, 30), or 50 (1, 29). Some authors used an observation parameter of 30 s (15), and others used an observation parameter of 3 min (9, 19). The different criteria for positivity for the microscopy included the presence of ≥ 1 (1, 12, 15, 23, 29, 32), ≥ 2 (3, 30, 31), ≥ 5 (8), or even any number of microorganisms per oil immersion field (7, 9, 10, 19).

The choice of criteria of positivity of the microscopic examination used in our investigation was based on a representative study in which the authors analyzed 32,076 urine specimens and obtained 94% sensitivity and 90% specificity in the detection of significant bacteriuria (30). In our study all the positive microscopic examinations (122 of 500) showed more than two bacteria per field, and in the 378 negative examinations we always observed fewer than two microorganisms per field. Nevertheless, it is worth mentioning that we found a mean of 1 to 1.5 bacteria per 20 fields in five negative urine specimens, which would give false-positive results if we were to employ a criterion of ≥ 1 microorganism per field.

Weinberg and Gan (31), on the basis of a study of microscopic examination of 1,019 urine specimens to diagnose urinary infection, emphasized that changing the criterion of positivity from ≥ 1 to ≥ 2 bacteria per oil immersion field improved the efficacy of the method, maintaining practically unchanged the 97.6% sensitivity but increasing the specificity from 87% to 94%. Our results were consistent with this observation.

Detection of significant bacteriuria by microscopic examination of 10 µl of uncentrifuged Gram-stained urine (loop technique) is described in three studies (1, 29, 32) in which the authors, using procedures similar to that employed in our investigation, found sensitivities of 94.1, 96.2, and 92.9%, respectively. These values were similar to the 96.0% sensitivity described in the present study. Perhaps the lower sensitivity obtained in one of the studies (32) was due to the criterion for positivity represented by the finding of ≥ 1 gram-negative bacillus in 5 fields examined, excluding the presence of grampositive bacteria, which, in our investigation, for example, represented 8.1% (10 of 124) of the isolates. In two of these studies (1, 29) a procedure slightly different from ours was used, in which the urine smear was spread with the 10-µl loop soon after application, over a 30-mm area of the slide. The authors used as a criterion of positivity the presence of ≥ 1 microorganism in 50 fields examined.

It is worth pointing out that in our study, spontaneous drying of the 10 μ l of urine applied on the slide led to accumulation of bacteria at the edge of the drop in all the positive urine specimens, facilitating the reading of the microscopic examination (Fig. 1). This was not observed in the negative urine samples.

The chief advantage of performing microscopic examination of uncentrifuged Gram-stained urine as part of the bacteriological routine of urine cultures is the presumptive rapid diagnosis of urinary infection and guidance for initial patient treatment based on the form and staining properties of the probable etiological infective agent; these can be made available while the clinic awaits the results of the urine culture and antibiotic sensitivity tests, which are generally available within 24 to 48 h (6, 11). Other advantages of this method include low cost and high specificity and sensitivity for detection of significant bacteriuria in urine specimens containing $\geq 10^5$ CFU/ml (4, 6, 11, 25, 28–30).

On the other hand, in the opinions of some authors (2, 6, 17), microscopic examination of stained urine preparations, besides being a lengthy, tedious process because of the large

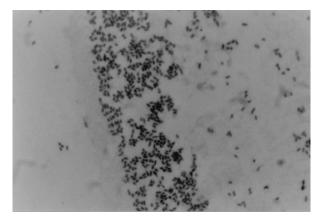


FIG. 1. Microscopic field (oil immersion objective) showing the accumulation of *E. coli* at the edge of the urine drop (to the left) with the Gram-stained preparation of 10 μ l of a positive urine specimen (\geq 10⁵ CFU/ml). A DIAPLAN microscope (Leitz, Wetzlar, Germany) and Tri-X pan ISO 400/27 film (Eastman Kodak Company, Rochester, N.Y.) were used.

number of negative urine cultures, has some limitations. There are the possibilities of false-negative results due to loss of bacteria in the case of inadequate fixation of the material on the slide and of false-positive results as a result of the presence of artifacts or the use of contaminated staining solutions (2). The low specificity and sensitivity of this method for detecting bacteriuria in urine specimens containing $<10^5$ CFU/ml, which may be significant at the 10^4 -CFU/ml level or in symptomatic patients with 10^2 to 10^4 CFU/ml, are well recognized (6, 26, 32).

Based on these considerations, a probable explanation for the false-negative results of the microscopic examination obtained in our study (5 of 124), is loss of the urine smear from the slide during the staining process because of inadequate fixation of the material. In relation to the false-positive results (3 of 376), the evidence indicates possible infections of the urinary tract caused by fastidious or anaerobic bacteria. In all three cases, there was a positive smear and the specimens failed to grow on aerobic culture. These patients were not being treated with antibiotics.

The major limitation of the microscopic method reported in this study is its decreased sensitivity for detecting bacteriuria in urine specimens containing $<10^5$ CFU/ml, a level that may be present in the acute dysuric syndrome in women, in infection in children, in infection in adult males, and in patients with urinary catheters (13, 21, 22, 26, 32). In addition, many clinical laboratories use 10^4 CFU/ml as a reportably and clinically significant result. This number is below the sensitivity of the microscopic method reported here. On the other hand, in urine specimens containing $\geq 10^5$ CFU/ml, usually associated with asymptomatic patients, patients with acute pyelonephritis, and patients with acute cystitis, a Gram-stained smear may be used as an accurate and inexpensive screening method (4, 6, 20-22, 26).

As evidenced in our study, microscopic examination of $10 \ \mu l$ of uncentrifuged Gram-stained urine showed no difference in the indices of efficiency for detection of significant bacteriuria compared to the conventional method of microscopic examination of a urine drop (50 μ l). The rapid drying time of the 10- μ l volume facilitates the use of this technique in bacteriological practice. Nevertheless, it is important to point out that for defining a positive microscopic examination, the microorganisms must be uniformly distributed over at least 10 oil immersion fields examined. The presence of many epithelial cells from desquamation, sometimes associated with the presence of different morphological and staining types of bacteria, indicates probable contamination of the urine specimen.

In summation, the results obtained in the present investigation demonstrated that the loop technique (10 μ l) can be utilized as an alternative to the conventional drop technique (50 μ l) for detecting significant bacteriuria, with the advantage of greater rapidity and simplicity of execution. As criteria for a positive microscopic examination, we recommend the reading of 10 oil immersion fields, with the presence of at least two microorganisms per field.

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