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To compare the efficacy of the conventional methods for detection of bacteriuria in married pregnant and non-preganant women

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Abstract
Calibrated platinum loop had come into general use in determining CFU in urine and found that it was comparable to the pour plate technique in determining the number of organisms present in urine. But later the it was considered only semiquantitative and not very reliable. Several other screening methods have been advocated for use in detecting bacteriuria like paper strips, nitrate reductase, TTC test, BACTEC® automated screening system and catalase test have been developed by rapid screening of urine for bacteriuria. However, scanty 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 50 µl. Hence, in this study, we carried out a comparative study of microscopic examination of un-centrifuged urine applied with a calibrated loop (10 µl) and stained with Grams staining, applied as a drop (50 µl) and examined as wet film, chemical tests of urine and counts of bacterial colonies cultured from urine samples. The study included the patients from Out-door and Indoor patients of Gynecology department which were recruited for bacteriologic evidence of asymptomatic bacteriuria by microscopy, culture and chemical examination. Patients included were randomly selected 3000 subjects who were married pregnant women reporting to ANC clinic of the Nair hospital of age group of 18 to 40 years. Collection, Transportation and Processing of urine like Microscopic examination (wet film and Gram staining), Chemical examination (Griess's nitrite test and Triphenyl tetrazolium chloride test) and Semiquantitative urine culture using standard loop technique was carried out by standard methods. Sensitivity of Nitrite test, TTC test, Wet mount, Gram's stain was 44.06%, 63.60%, 58.23% and 88.94% while specificity was 96.29%, 80.88%, 79.99% and 88.16% respectively, when compared to culture method. Total positive cultures was 8.26%. In approximately 100 % of the positive cultures, the etiological infective agent was isolated in pure culture, at a concentration of $\geq 10^5$ CFU/ml. Study results obtained in the present study demonstrated that the loop technique (10 µl) can be utilized as an alternative to the conventional drop technique (50 µl) for detecting significant bacteriuria. Also loop technique along with chemical test can be one of the better option with the advantage of greater rapidity and simplicity of execution. Still at the end culture method remains the gold standard for the detection of bacteriuria.

Keywords: Bacteriuria, calibrated platinum loop, triphenyl tetrazolium chloride test, wet mount married pregnant women

1. Introduction
Since 1960, the 0.001-ml calibrated platinum loop had come into general use in determining CFU in urine and was recommended by many workers [1]. Subsequently, in 1960, Hoeprich reported their use in determining CFU from urine cultures and found that the calibrated-loop technique was comparable to the pour plate technique in determining the number of organisms present in urine [2, 3]. Actually the use of calibrated loops for the quantitation of bacteria in a liquid suspension was first described in 1928 by Burri at the World's Dairy Congress [1]. But later the use of the calibrated loop by clinical microbiologists to determine colony counts from urine specimens was considered only semiquantitative and not very reliable as the reproducibility of the method was dependent on many uncontrolled variables [4]. In addition to microscopic method several other screening methods have been advocated for use in detecting bacteriuria which includes calorimetric filtration, bioluminescence, electric impedance, enzymatic methods, photometric detection of growth and enzyme immunoassay.

The most common and relatively simple screening methods in practice are paper strips, nitrate reductase, TTC test, BACTEC® automated screening system and catalase test have been developed by rapid screening of urine for bacteriuria [5].

Urine cultures represent 40 to 70% of the specimens sent for examination to clinical-microbiology laboratories [6, 7]. Urinary-tract infections, including cystitis, pyelonephritis, asymptomatic bacteriuria, and acute urethral syndrome, constitute one of the most frequent causes of illness in humans [8, 9]. Most such infections are caused by a few genera of bacteria, and the presence of these microorganisms in the urine is known as bacteriuria [10]. Quantitative urine culture is considered the standard procedure for adequate diagnosis of urinary-tract infections [11]. Urinary tract infections are frequently encountered medical complications of pregnancy. The majority of infections are asymptomatic; however even covert bacteriuria places the mother at risk for low birth weight and preterm birth. During pregnancy UTI are high potential risk for mother and child [12, 13]. Although the prevalence of urinary infections may vary in different patient populations, approximately 80% of urine cultures are negative [6]. 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].

The presence of bacteria on examination of an uncentrifuged drop of urine under the high-dry lens of the microscope is rapid, inexpensive and probably indicative of significant bacteriuria but is laborious and less effective. In addition, if these microscopic tests or Gram's stain of urine are performed their costs become prohibitive [14, 10]. 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 [10]. Observation of one or more bacteria per oil immersion field correlates with 90% of cases of significant bacteriuria, thus indicating active urinary-tract infection [15].

The chief advantage of performing microscopic examination of un-centrifuged 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 agents. These can be made available when the clinic awaits results of the urine culture and antibiotic sensitivity tests, which are generally available within 24 to 48 hours [16]. On the other hand, opinions of some other authors, microscopic examination of stained urine preparations, besides being a lengthy tedious process because of the large number of negative urine cultures has some limitations. Microscopic results have been found of questionable value for screening as well.

A number of chemical tests have been proposed as a means to establish significant bacteriuria. They are found on the principle of a colorimetric change based on reduction of a chemical substance by urinary bacteria present in sufficient numbers. These test has been found to be an insensitive means for identifying bacteriuria, but investigators have reported that it may be more useful when combined with other indicators, such as bacteria seen in uncentrifuged urine and pyuria [17].

However, non-culture methods are not generally, reliable for the identification of bacteriuria in asymptomatic populations, including pregnant women. Detection of significant bacteriuria by semi-quantitative loop culture technique is being commonly used by many researchers as most effective method [18]. Use of a smaller volume of urine, leading to more rapid drying, might facilitate the use of this technique.

However, scanty 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 50 µl. Hence, in this study, we carried out a comparative study of microscopic examination of un-centrifuged urine applied with a calibrated loop (10 µl) and stained with Grams staining, applied as a drop (50 µl) and examined as wet film, chemical tests of urine and counts of bacterial colonies cultured from the 3000 urine samples, for detection of significant bacteriuria in patients with suspected urinary infections treated at the Nair Hospital.

2. Material and methods

2.1 Place of work

This prospective longitudinal study was carried out over a period of two years, from January 2003 to December 2004 after taking the permission from Institutional Ethics committee of T. N. Medical College and B. Y. L. Nair Charitable Hospital, Mumbai. It was conducted in the Department of Microbiology in association with the Department of Obstetrics and Gynecology, of T. N. Medical College and B. Y. L. Nair Charitable Hospital.

2.2 Participants

The study included the patients from Out-door and Indoor patients of Gynecology department which were recruited for bacteriologic evidence of asymptomatic bacteriuria by microscopy, culture and chemical examination. Patients included were randomly selected 3000 subjects who were married pregnant women and 300 married non- pregnant women reporting to ANC clinic of the hospital of age group of 18 to 40 years. Counseling for enrollment procedure in the study was done. Detailed data from the patients were recorded in a specially formulated structured proforma.

2.3 Collection of urine

Before collection, all women were instructed to wash well and rinse periurethral area with water using front to back motion [19]. Saline soaked cotton was provided to wipe and clean periurethral area after washing. Patients were advised to pass urine, discarding the first part of the stream and collecting clean-catch "midstream" urine, in a graduated sterile wide-mouthed container covering around ¾ volume.

2.4 Transportation and Processing of urine

Urine samples were transported to the laboratory without delay. If a delay of more than 1-2 hrs was unavoidable, urine were stored in refrigerator at 4°C. 2ml of homogenized urine were centrifuged at 3000 r.p.m. for 4mins.

2.5 Microscopic examination of urine

a) Wet film: It was performed for both uncentrifuged and centrifuged urine to observe pus cells, casts, Renal tubular epithelial cells, RBC's (Red Blood corpuscles) crystals,

Trichomonas vaginalis, budding yeast and also pseudohyphae by observing under 40x magnification [20].

b) Gram staining using 10-µl volume Loop

10-µl volume of homogenized urine sample was applied, by means of a nickel-chrome loop calibrated to 10 µl, to the surface of a 25- by 75-mm microscope slide and was allowed to dry, without spreading, at ambient temperature. 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 with Hucker's modification. This method can detect the presence of both bacteria and pus cells in urine specimens [21].

c) Microscopic examination

A preliminary inspection of the smears was performed by using a low-magnification (10× to 20×) dry objective in order to locate the material on the slide. Next, with a 100× oil immersion objective, 50 fields were examined, and the shapes and number of microorganisms and cells per field was 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. [22]

2.6 Chemical examination

a) Griess's nitrite test: Many gram-negative bacilli reduce nitrate to nitrite. 2ml of urine is added to 2ml of 0.5% of sulphanilic acid to which 2ml of α- naphthylamine solution is added. In bacteria, presence of enzyme nitrate reductase formation of pink color indicates positive test [23, 24].

b) Triphenyl tetrazolium chloride (TTC) test: Reduction of triphenyl tetrazolium chloride test to red precipitate of triphenyl formazan takes place only at an alkaline pH. Such a pH in urine prevails only during a urinary tract infection [23].

2.7 Semiquantitative urine culture (using standard loop technique)

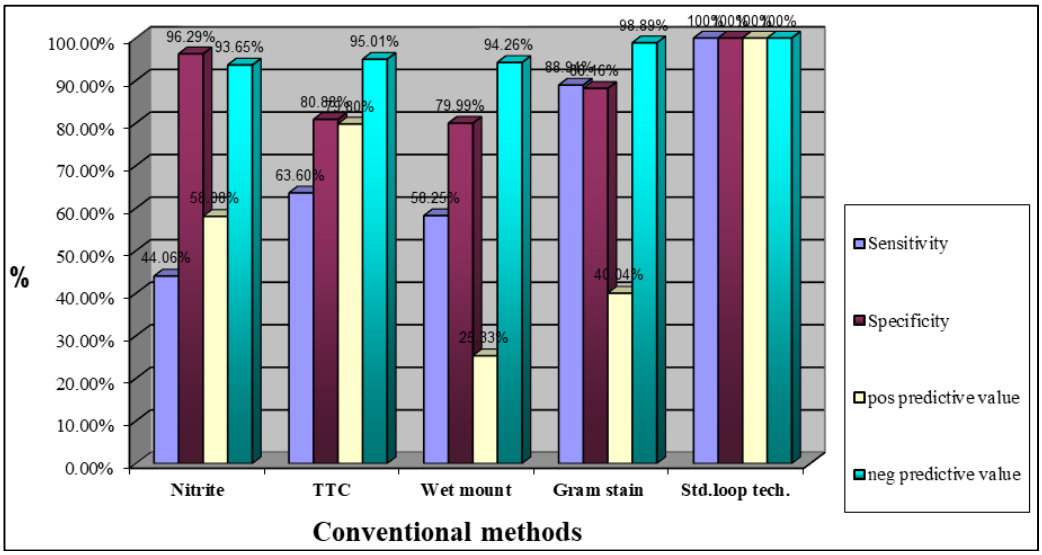
Culturing of un-centrifuged urine specimens was done using standard loop technique. 0.01ml of urine was surface streaked by calibrated loop on Nutrient agar [21]. If growth was seen, colonies were counted and recorded as colony forming units (CFU) /ml by multiplying count by 100. CFU more than or equal to 10^5 bacteria/ml of one or two organisms on two clean catch cultures in absence of symptoms were considered for significant bacteriuria.

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 $>10^4$, 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 [10]. Urine specimens containing $\geq 10^5$or $<10^5$ CFU of nonpathogenic bacteria /ml or multiple 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.

3. Results

Table 1: Comparison of Conventional methods for detection of bacteriuria

Method	Sensitivity	Specificity	Positive Predictive value	Negative Predictive value
1) Culture (using Standard loop technique)	100%	100%	100%	100%
2) Chemical examination				
a)Nitrite test	44.06%	96.29%	58.08%	93.65%
b)TTC test	63.60%	80.88%	79.80%	95.01%
3) Microscopic examination				
a)Wet mount	58.23%	79.99%	25.33%	94.26%
b)Gram's stain	88.94%	88.16%	40.04%	98.89%



Graph 1: Sensitivity, Specificity, +ve predictive value,-ve predictive value of different conventional methods compared with standard loop technique.

4. Discussion

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× dry objective, (ii) observation of fresh urinary centrifuged sediment with a 40× dry objective, (iii) examination with an oil immersion objective (100×) of a Gram-stained smear of uncentrifuged urine, and (iv) observation of a Gram-stained smear of centrifuged urine with an oil immersion objective [25]. In the present study, we used microscopic examination of uncentrifuged Gram-stained urine and centrifuged and un-centrifuged wet film of urine. These methods are easy to perform, cheap and probably the most sensitive and reliable diagnostic method for identifying urine specimens containing more than 10^5 CFU/ml [15].

Sensitivity, specificity, and positive and negative predictive values of the methods were calculated by the method of Ransohoff and Feinstein [26]. (i) sensitivity is (TP/(TP + FN), the probability that the microscopic and chemical examination will be positive in patients with urinary infections (positive culture), (ii) specificity is (TN/(TN + FP), the probability that the microscopic and chemical examination will be negative in patients without urinary infections (negative culture), (iii) positive predictive value is (TP/(TP + FP), the probability that a urinary infection is present when the microscopic and chemical examination is positive, and (iv) negative predictive value is (TN/(TN + FN), the probability that a urinary infection is not present when the microscopic and chemical examination is negative, where TP stands for true positive (chemical test, microscopy and cultures, all positive), FP for false positive (positive chemical test, positive microscopy and negative culture), TN for true negative (chemical test, microscopy and culture, all negative), and FN for false negative (chemical test negative, microscopy negative and culture positive).

Total 3000 urine samples were analyzed from pregnant women and the incidence of positive cultures was 8.26%, while 300 urine samples were analyzed from non-pregnant women and the incidence of positive cultures was 3.66%. Total 259 isolates were detected from these 3300 samples. The following groups of urinary pathogens were identified: (i) gram-negative bacilli, including *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Acinetobacter baumanii*, *Proteus mirabilis*, (ii) gram-positive cocci, including *Staphylococcus aureus*, *Enterococcus faecalis*, *Coagulase-ve Staphylococcus spp*), *Streptococcus agalactiae.* In approximately 100 % of the positive cultures, the etiological infective agent was isolated in pure culture, at a concentration of $\geq 10^5$ CFU/ml.

Table 1 Shows the sensitivity, specificity, positive predicative value and negative predictive value of chemical tests and microscopic test, compared with culture method. Specificity and negative predictive value of nitrite test was more than 90 %, but sensitivity and positive predictive value was lower than 50 %. Similar results were observed for TTC test and wet mount also. While Gram staining showed high value of sensitivity, specificity, negative predictive value (More than 80%) and but low value for positive value The results of the two microscopy techniques did not show 100% correlation in our study, while Some authors showed 100% correlation. This results may vary depending on the angle of tube while taking the urine sample, diameters of the smears made and drying time of smears [27].

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 [10, 15, 27]. No standardized technique exists for performing this procedure in the microbiological laboratory [25]. 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 [6, 15, 27] the use of the drop (50-µl) technique was described, and in eight [27, 28, 29] the calibrated loop technique was used, with urine volumes varying from 5 to 10 µl. The number of microscopic fields examined per urine sample was 5, 10, 20, or 50. Some authors used an observation parameter of 30 s, and others used an observation parameter of 3 min. The different criteria for positivity for the microscopy included the presence of ≥1, ≥2, ≥5 or even any number of microorganisms per oil immersion field [27, 28, 29]. *Bailey* [30] determined that microscopic detection of moderate numbers of bacteria and leukocytes in the urine had sensitivities of less than 75% and 85%. The specificity for combination of both tests was less than 85%. The positive predictive value of microscopic examinations for pyuria, bacteriuria or both has been shown to be as low as 33%.

The choice of criteria of positivity of the microscopic examination used in one 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 [22]. In same study all the positive microscopic examinations 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 one study found a mean of 1 to 1.5 bacteria per 20 fields in five negative urine specimens, which would give false-positive results if were to employ a criterion of ≥1 microorganism per field [27].

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%. Detection of significant bacteriuria by microscopic examination of 10 µl of uncentrifuged Gram-stained urine (loop technique) is described in studies [22, 28]. 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 very high to the 58 % sensitivity described in the present study. Perhaps the lower sensitivity obtained in the studies was due to the criterion for positivity represented by the finding of ≥1 gram-negative bacillus in 5 fields examined, excluding the presence of gram-positive bacteria. It is worth pointing out that in our study, spontaneous drying of the 10 µl 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. This was not observed in the negative urine samples. Same observations were made in some studies [27].

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 [10, 25]. 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 [15, 25].

On the other hand, in the opinions of some authors [10], microscopic examination of stained urine preparations, besides being a lengthy, tedious process because of the large 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. 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 [10].

Based on these considerations, a probable explanation for the false-negative results of the microscopic examination obtained in our study, 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, the evidence indicates possible infections of the urinary tract caused by fastidious or anaerobic bacteria [27].

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 [27]. 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 [27].

As evidenced in our study, microscopic examination of 10 µl 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 µl). The rapid drying time of the 10-µl 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.

When culture method and chemical testing results were compared, chemical test sensitivity, specificity and positive predictive value and negative predicitive values of the chemical tests are very poor in comparison of culture and 10 ul quantity urine gram stained smears. Chemical methods in combination with 10 ul quantity urine gram stained smears can provide appropriate results in laboratories, which are less costly and easy to perform.

5. Conclusion

Study results obtained in the present study demonstrated that the loop technique (10 µl) can be utilized as an alternative to the conventional drop technique (50 µl) for detecting significant bacteriuria. Also loop technique (10 µl) along with chemical test can be one of the better option with the advantage of greater rapidity and simplicity of execution. Still at the end culture method remains the gold standard for the detection of bacteriuria.

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