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Microscopic Detection of Urinary Tract Infection in Nepalese Patients

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Urinary tract infection (UTI) is one of the most common domiciliary and nosocomial bacterial infections prevalent in both males and females. UTI is diagnosed on the basis of clinical symptoms, microscopy and culture of urine. In order to evaluate the efficacy of microscopic detection for presumptive diagnosis of UTI we analyzed urine samples of Nepalese patients. We have conducted Gram staining and counting of pus cells, red blood cells (RBC) and epithelial cells. We observed that RBC and epithelial cell counts were not sensitive enough to be used for presumptive diagnosis of UTI. However, pus cell counts as well as Gram stain are sensitive and significant enough to presume UTI. When the Gram stain result was compared with the culture result, it was statistically significant. From this, we suggest that Gram stain of centrifuged urine is a very sensitive screening method to detect bacteriuria. In addition, we found that *E. coli* was the most predominant microorganism causing UTI and nitrofurantoin was the most effective antibiotic against the isolated urinary pathogens.

Key words: bacteriuria, urine, Gram stain, pus cells

UTI is the second commonest bacterial infection after respiratory diseases, prevalent in both males and females. The incidence of urinary tract infection is greatly influenced by age, sex and factors which impair the defense mechanisms that maintain the sterility of the urinary tract. Many predisposing factors have been described for the development of UTI including anatomical, pathological, infective, social and environmental factors (Leigh, 1990). Although UTI differs considerably in pathogenesis, natural history and management, it can be generally stated as a spectrum of diseases involving microbial invasion of any of the urinary tissues extending from the renal cortex to the urethral meatus (Singh, 1991). Urine secreted from kidneys is sterile unless any of the organs are infected. During infection, bacteria undergo multiplication in urine within the urinary tract causing a condition called bacteriuria (Leigh, 1990). Bacteriuria may lead to the infection of the male reproductive system, so the infection of the prostate, epididymes or testes are also included in the definition of UTI (Fowler, 1983).

Even though the prevalence of urinary infections may vary in different patient populations, approximately 80% of urine cultures are negative (Kolbeck *et al.*, 1985 and Wu *et al.*, 1985). 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 (Clarridge *et al.*, 1987). Among these non-cultural techniques, white blood cell (WBC) count and Gram stain have been proposed as sensitive and inexpensive methods (Baron and Finegold, 1994; Clarridge *et al.*, 1987; Pezzlo, 1988; Pezzlo, 1990 and Pollock, 1983). Quantitative urine culture with $\geq 10^5$ colony-forming units/ml (CFU/ml) remains the standard diagnostic method to diagnose UTI.

To assess the usefulness of Gram stain as a urine screening test in the clinical microbiology laboratory to eliminate culture negative specimens, we have analyzed one hundred and sixty urine samples obtained from Nepalese patients. When we compared the results of Gram stain of centrifuged urine to the results of culture, we found that Gram stain is a reliable and sensitive procedure with high sensitivity (94.0%) and specificity (96.0%) for the initial screening of urinary tract infection. In addition, observation of >5 pus cells per high power field added a subsequent value for the preliminary diagnosis.

Materials and Methods

Sample collection and preparation

Urine samples submitted to the clinical microbiology lab-

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oratory of Tribhuvan University Teaching Hospital, Kathmandu, Nepal by clinically suspected patients were collected and examined microscopically for epithelial cells, leukocytes, erythrocytes and microorganisms in both unstained and Gram-stained centrifuged urine. Urine samples were processed immediately, but in cases of delay they were refrigerated at 4°C until processing.

Ten milliliters of urine sample was taken in a clean sterile centrifuge tube, and centrifuged at 3000 rpm for 10 min. The supernatant was discarded aseptically and the sediment in the centrifuge tube was homogenized in 100 µl of supernatant. The sediment was then examined by wet preparation and Gram staining.

Gram staining of the sediment

A loopful of homogenized urine sediment was taken with a standard loop (4 mm diameter) on a new clean sterile glass slide and spread in an area of 1.5 cm × 1.5 cm. Gram staining of urine sediment was performed as described (Collee *et al.*, 1989) and the number of organisms was counted in each oil immersion field. One hundred fields were observed and an average was taken as the number of organisms per oil immersion field (OIF). A positive Gram stain was defined as the presence of >15 bacteria uniformly distributed per OIF after observation of at least 20 fields, according to the criteria described by Washington *et al.* (1981) and Cardoso *et al.* (1998).

Microscopic examination by wet preparation

Two loopfuls of homogenized urine sediment was placed on a clean dry glass slide which was then covered by a coverslip and observed on 40X dry objective. An average count of WBC, RBC or epithelial cells was taken per high power field (HPF) out of 50 fields examined.

Culture of urine specimens

Culture of each uncentrifuged urine specimen was done quantitatively on 5% Blood Agar and Mac Conkey agar (Oxoid, Unipath Ltd., Basingstoke, England) plates. An inoculating loop of standard dimension was used to take up approximately fixed and a known volume (0.001 ml) of mixed uncentrifuged urine. After incubating the plates aerobically at 37°C for 24 h, colonies were counted. Cultures were interpreted as positive, negative, non-significant or mixed for UTI according to the standard criteria endorsed by the American Society for Microbiology based on the colony count, the urinalysis findings and patient-specific clinical data as provided on the request slip (Isenberg, 1993).

Identification of significant isolates was done by using standard microbiological techniques as described which involves the morphological appearance of the colonies, staining reactions, biochemical properties and serotyping if required in specific cases (Murray *et al.*, 1995; Baron *et al.*, 1994; Collee *et al.*, 1989 and Cheesbrough, 1984).

Statistical analysis

Statistical analysis of Gram stain for sensitivity, specificity, and positive and negative predictive values was performed according to the formula described by Ransohoff and Feinstein (1978): (i) sensitivity = TP/(TP + FN), the probability that the Gram stain will be positive in a culture positive sample (indicating urinary infection), (ii) specificity = TN/(TN + FP), the probability that the Gram stain will be negative in a culture negative sample (indicating absence of urinary infection), (iii) positive predictive value = TP/(TP + FP), the probability that a positive Gram stain shows the presence of urinary infection, and (iv) negative predictive value = TN/(TN + FN), the probability that a negative Gram stain shows the absence of urinary infection. The abbreviations TP, TN, FP, and FN stand for true positive (both Gram stain and culture positive), true negative (both Gram stain and culture negative), false positive (Gram stain positive and culture negative), and false negative (Gram stain negative and culture positive), respectively.

Antibiotic sensitivity test

Antibiotic sensitivity testing is an *in vitro* method for estimating the activity of drugs against an infecting microorganism *in vivo*. The degree of sensitivity or resistivity of the isolated pathogens to an appropriate range of antibiotics was determined by the Kirby-Bauer method i.e. disc diffusion method as described (Collee *et al.*, 1989). The Kirby-Bauer method is based on the observation that the degree of inhibition of bacterial growth on agar medium surrounding an antimicrobial-containing disc correlates with susceptibility to the agent. Paper discs impregnated with standardized amounts of an antimicrobial agent and specifically certified for sensitivity testing were used. The test was carried out according to the method described by the National Committee on Clinical Laboratory Standards (NCCLS) (1999) guidelines. Briefly, four to five similar colonies of identified organism from pure culture plates were transferred into 5 ml nutrient broth and incubated at 37°C for 4 h. The prepared inoculum was compared with half the density of Mc Farland tube No. 1, which will give evenly spread semi-confluent growth. With a sterile swab the inoculum was spread on the entire surface of the dried Mueller-Hinton agar plate (Oxoid, Unipath Ltd., Basingstoke, England). The paper discs of selected antibiotics [nitrofurantoin, norfloxacin, trimethoprim-sulfamethoxazole (cotrimoxazole), nalidixic acid and ampicillin (Oxoid, Unipath Ltd., Basingstoke, England)] were gently pressed onto the organism-carpeted plate at a distance of 15 mm away from the edge and 24 mm apart from each other. After incubation at 37°C for 24 h the diameter of the zone of bacterial growth inhibition around each disc was measured and the susceptibility or resistance to the agent in each disc was determined according to the standardized table provided by the manufacturer.

Positive and negative controls were carried out using stock culture of standard organisms; *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853.

Results and Discussion

All the urine samples analyzed were collected by spontaneous urination (clean catch midstream). The majority of the samples (66%) were from out-patients and the remaining from hospitalized patients. About 49% of the specimens were received from males and the remainder (51%) from female patients. The ages of the patients ranged from 9 to 82 years with a mean age of 39 years. We found that a high number of male patients belonged to the age group older than 60 years, whereas for females the child-bearing age group was the highest. This pattern was consistent with the patient distribution of culture positive results. Similar to our results, Kosakasi *et al.* (1990) also observed a high incidence of bacteriuria in elderly men and women of child-bearing age.

A sample was considered as culture positive if it contained a pure culture of $\geq 10^5$ colony forming unit (CFU)/ml in asymptomatic cases where as in symptomatic conditions $\geq 10^4$ CFU/ml was considered significant. Based on this criterion, a total of 38 bacteria belonging to 8 different strains were isolated from 160 urine samples (Table 1). Among the isolated bacteria, Gram-negative bacteria were the most predominant uropathogens over Gram-positive bacteria. *Escherichia coli* was the most frequent isolate, followed by *Staphylococcus aureus, Klebsiella pneumoniae, K. oxytoca, Pseudomonas aeruginosa, Streptococcus faecalis, Staphylococcus epidermidis* and *Proteus vulgaris*. Leigh (1990) reported that infecting organisms are most commonly derived from the patient's own faecal flora. In women, they are usually present on the perineal skin before infection occurs. The majority of the organisms we isolated also belong to normal faecal flora suggesting that they might have entered the tract through the ascending route.

Antibiotic sensitivity tests of all 38 isolates were performed to determine the degree of sensitivity or resistivity to an appropriate range of commonly prescribed antimicrobial drugs. Antibiotic sensitivity profile (Fig. 1) showed that nitrofurantoin (F in Fig. 1) was the only effective antibiotic against both Gram-positive and Gram-negative isolates. Increased resistance was observed against other commonly used antibiotics, such as norfloxacin (NOR), trimethoprim-sulfamethoxazole [cotrimoxazole (SXT)], nalidixic acid (NA) and ampicillin (AMP) in this study and also in others (Obi *et al.*, 1996; Oh *et al.*, 2002). However, these antibiotics were reported as effective by Miano *et al.* (1990) and Schaeffer (1990). The increased resistance suggested that bacteria are acquiring resistance to commonly used antibiotics and also demonstrates a need for reevaluation of common antibiotics used to treat UTI.

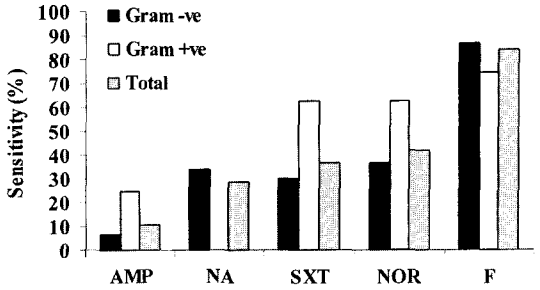

Fig. 1. Profile of antibiotic sensitivity. Antibiotic sensitivity pattern of Gram-negative, Gram-positive and total isolates. Nitofurantoin (F) was the most effective drug among those tested against both Gram-positive and Gram-negative pathogens. The abbreviations in the figure indicates AMP- Ampicillin, NA- Nalidixic Acid, SXT- Cotrimoxazole, NOR- Norfloxacin, and F- Nitrofurantoin

Culturing urine is the standard diagnostic method but on the basis of clinical symptoms, macroscopic and microscopic observation, initial presumptive diagnosis can be made. In this study we tried to evaluate these parameters for rapid diagnosis of UTI. First, macroscopically we focused on turbidity of urine and clinical symptoms (Table 2, first three rows). We observed that only about 46% of the samples that were turbid or cloudy were culture positive. Similarly, only 39% of the patients who developed symptoms were suffering from UTI (Table 2). Our result is consistent with the report of Leigh (1990)

Table 1. List of microbial isolates

Micro-organisms	Isolates (%)	Gram nature
Escherichia coli	21(55)	negative
Staphylococcus aureus	4(11)	positive
Pseudomonas aeruginosa	3(8)	negative
Klebsiella pneumoniae	3(8)	negative
Klebsiella oxytoca	2(5)	negative
Steptococcus faecalis	2(5)	positive
Staphylococcus epidermidis	2(5)	positive
Proteus vulgaris	1(3)	negative

Table 2. Correlation between some clinical parameters and culture

Parameters	Sample	Culture Positive (%)
Cloudiness of urine	82	38(46)
Symptoms*	71	28(39)
Cloudiness and symptoms	46	26(57)
Epithelial cells		
<2/HPF	67	24(36)
>2/HPF	18	4(22)
Erythrocytes (RBC)		
<3/HPF	12	2(17)
>3/HPF	18	13(46)

*Symptoms include either or a combination of dysuria, urgency, frequency, suprapublic pain, fever, rigors, flank pain, nausea or prostration.

who showed that diagnosis based on symptoms alone is highly inaccurate, with a more than 50% false negative rate. These results showed that cloudiness of urine and symptoms are insignificant in predicting possible infection but they can be useful together with microscopic results.

Next, we microscopically examined three different cell types; epithelial cells, red blood cells, and pus cells to correlate with the positive culture. First, epithelial cells were examined under the microscope. Epithelial cells appear in urine as a result of normal exfoliation along the urinary tract (Schumann and Schweizer, 1991). It is also reported that the finding of a large number of squamous epithelial cells or approximately 1-2/HPF, in the voided specimen is not uncommon if proper techniques for the collection of an uncontaminated specimen are not followed (Cheesbrough, 1984). To distinguish between contaminant and significant value, we evaluated a value of more than 2 epithelial cells per HPF as significant and analyzed accordingly. As shown in Table 2, a very low percentage (22%) of samples were culture positive in which we detected a significant number of epithelial cells (>2/HPF). From this finding we suggest that microscopy of epithelial cells has very poor significance for UTI prediction.

Second, we examined red blood cells under the microscope. The mechanism through which RBC enters urine is not known yet, but it is believed that increased numbers of erythrocytes are seen in renal disease, lower urinary tract disease, extrarenal disease, toxic reactions due to drugs and sometimes in physiologic causes including exercise. Schumann and Schweitzer (1991) suggested that the observation of 0 to 2 RBC per HPF on microscopic examination of the sediment is normal both in males and females. In another report, the finding of a red blood count greater than or equal to three per high power field was considered as abnormal (Wargotz *et al.*, 1987; Froom *et al.*, 1986 and Steward *et at* 1985). Based on this criterion we tried to investigate whether observation of greater than 3 RBC/HPF in urine deposits can be established as a potential predictor of significant bacteriuria. Among samples which had more than 3 erythrocytes per HPF, the majority of them (54%) were culture negative (Table 2). Our results revealed that microscopic observation of RBC is not sufficiently sensitive to be used as a screening test for the detection of UTI.

Third, we observed leucocytes under the microscope. The observation of leucocytes is suggestive of bacteriuria, but a substantial number of patients may excrete leucocytes in inflammatory disorders of the urinary tract. Increased numbers of leucocytes, principally neutrophils, are seen in almost all renal diseases and diseases of the urinary tract. Pyuria, the presence of WBC in urine, is considered significant if more than or equal to 5 white blood cells or pus cells are seen per high power field in the sediment (Steward *et al.*, 1985; Wargotz *et al.*, 1987; Pallares *et al.* 1988; Wenz and Lampasso, 1989; Ziloski and Smucker, 1989; Abyad 1991; Ouslander *et al.*, 1996 and Eisinger *et al.*, 1997). We observed that 8 samples out of 11 (about 73%) having 5-10 pus/HPF in microscopy were culture positive (Table 3). Thus, the presence of 5-10 pus cells per HPF can be a good marker of UTI.

Table 3. Correlation between pus cell count and culture result

Pus cells/HPF	Samples[1]	Growth Positive[2]	Growth Negative[3]	Not Significant[4]	Mixed Growth[5]
5	48	0	33	12	3
1-5	61	7	20	17	17
5-10	11	8	2	0	1
10-15	4	2	1	1	0
15-20	4	2	1	0	1
20-25	5	3	2	0	0
>25	27	16	5	0	6
Total(%)	160	38(24)	64(40)	35(19)	28(17)

[1]The number of cases observed.
[2]Colony counts of $\geq 10^4$ CFU/ml in symptomatic and $\geq 10^5$ CFU/ml in asymptomatic case.
[3]Samples which were sterile on culture.
[4]Colony counts of $<10^4$ CFU/ml in symptomatic and $<10^5$ CFU/ml in asymptomatic case.
[5]Growth of two or more types of bacteria, possibly due to contamination during sample collection by patients.

Finally, we Gram stained the urine samples and observed under microscope. The chief advantage of performing microscopic examination of Gram-stained urine 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 (Clarridge *et al.*, 1987 and Jenkins and Matsen, 1986). Although microscopic examination of an uncentrifuged Gram-stained urine drop is recognized as the conventional microscopic method for diagnosing urine specimens with counts of 10^5 CFU/ml (Baron and Finegold, 1994; Clarridge *et al.*, 1987; Hoeprich, 1960; Jenkins and Matsen, 1986; Pollock, 1983 and Washington *et al.*, 1981), it is a time consuming and tedious process looking for very few microorganisms (2 or less than 2) per field. Moreover, there is the possibility of false negative results due to loss of those small numbers of bacteria during the staining process because of inadequate fixation. To overcome this problem, we studied the centrifuged Gram stain smear and tried to set off a criterion that can distinguish between positive and negative cultures. During our study we discovered that finding bacteria within the microscopic field was very easy and less time-consuming compared to uncentrifuged urine having 1-2 bacteria per field (Weinberg and Gan, 1991). For example, Fig. 2 represents one oil immersion objective field which contained a significant number of bacteria, confirming that our method is

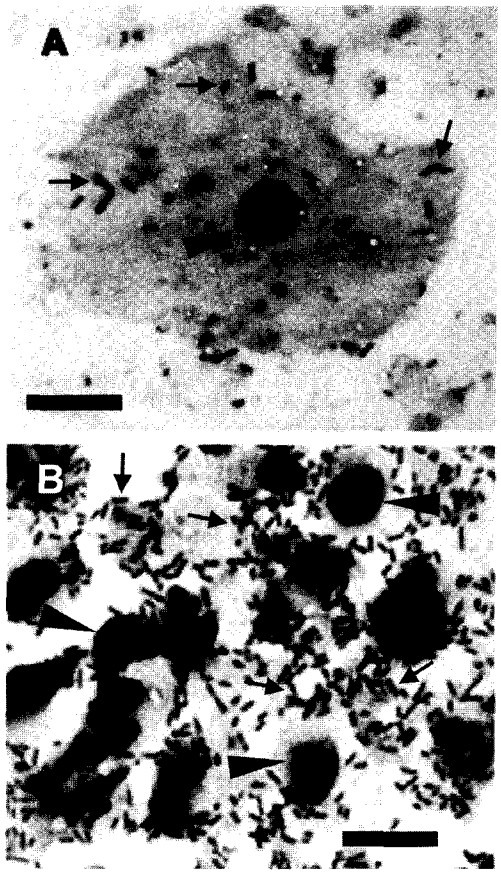

Fig. 2. Oil immersion objective fields (A) Gram stain of urine deposit showing *E. coli* in epithelial cell. Arrowhead indicates nucleus of epithelial cell and the arrows indicate *E. coli* (B) Gram stain of urine deposit showing plenty of *E. coli* and pus cells. Arrowheads indicate pus cells and the arrows indicate *E. coli*. Bars 10 μm.

Table 4. Correlation between Gram stain of urine sediment and culture

*Organisms/OIF[§]	Sample	Growth Positive	Growth Negative	Not Significant	Mixed Growth
0	88	0	69	15	4
1-5	11	1	3	4	3
5-10	6	1	1	2	2
10-15	2	0	0	1	1
15-20	9	7	0	2	0
20-50	17	16	0	1	0
>25	15	13	0	0	2
Mixed[@]	12	0	0	0	12

[§]One oil immersion objective field.
*Either Gram-positive cocci or Gram-negative rods only.
[@]Gram-positive cocci and Gram-negative rods mixed.

reliable to count bacteria.

The correlation of Gram stain of urine sediment with culture was examined and the results are summarized in Table 4. As shown in Table 4, with a cut-off value of ≥ 15 bacteria/OIF as positive, the microscopic examination of Gram-stained urine sediment showed significant correlation with growth positive culture. We further analyzed this data statistically and found it statistically significant (Table 5). It showed over 90% sensitivity [sensitivity (94.0%), specificity (96.0%), positive predictive value (88.0%), and negative predictive value (98.0%)] as a diagnostic method for the detection of significant bacteriuria. As shown in Tables 4 and 5, with our criteria of positive microscopy (≥ 15 bacteria/OIF) we observed a good correlation between culture and Gram stain except in 7 samples which showed false negative in two and false positive results in 5 samples. False positives were observed in the sample from patients who were under antibiotic treatment. Observation of Gram stain of urine deposits also helped us to predict the type of bacteria that will grow on culture and to evaluate whether the sample is contaminated, though it was very difficult to distinguish if bacteria of similar shape and Gram nature were present in the slide. In addition, fastidious and anaerobic bacteria that will not grow in routine culture media can be seen in the slide which will add a great value in the appropriate diagnosis of UTI.

Table 5. Statistical analysis of Gram stain and culture result

Gram stain result	Culture positive	Culture negative
≥ 15 bacteria/OIF	36(TP)	5(FP)
<15 bacteria/OIF	2(FN)	117(TN)
Total	38	122

Sensitivity [TP/(TP+FN)]=36/38 (94.0%)
Specificity [TN/(TN+FP)]=117/122 (96.0%)
Positive predictive value [TP/(TP+FP)]=36/41 (88.0%)
Negative predictive value [TN/(TN+FN)]=107/378 (98.0%)

It is worth pointing out here that despite the high sensitivity and specificity, the results of Gram stain might be misleading, showing false negative results (negative microscopy and positive culture) in symptomatic patients with a low CFU count. However, urine from asymptomatic patients, patients with acute pyelonephritis or patients with acute cystitis which can produce a CFU count of ≥ 10^5 per ml, Gram stain smear may be used as an accurate and cost effective screening method (Baron and Finegold, 1994; Clarridge *et al.*, 1987; Pezzlo, 1988; Pezzlo, 1990 and Pollock, 1983).

It has already been shown that pyuria accompanied by a lower or negligible bacterial count in uncentrifuged urine is a significant finding for UTI (Komaroff and Friedland, 1980 and Little *et al.*, 1980). The results obtained in the present study demonstrated that Gram stain of centrifuged urine can be used for detecting significant bacteriuria with criteria of ≥ 15 bacteria per oil immersion field and >5 pus cells per high power field.

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