Measurement of urine leukocytes by a second generation flow cytometer; application in the diagnosis of acute urinary tract infections in adult patients

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Introduction

The most important clinical manifestation of urinary tract infections (UTI) are fever, sovrapubic heaviness and pain, dysuria with frequent painful excretion of small amounts of turbid urine. Bacteriuria and pyuria, are the main Clinical Laboratory manifestations of UTI and a microbiological examination of an urine sample is usually necessary to establish the etiology of disease (1). For the Clinical and Laboratory diagnosis of UTI a definition of significant bacteriuria and pyuria is important. For bacteriuria, the criteria established by Kass (2,3) are now widely accepted and counts of more of 100,000 colony forming units (UFC) / mL are considered to be significant for UTI. For pyuria, the cut-off value is not well established because of the poor diffusion of routine quantitative microscopy methods (4,5,6). Different authors thus indicate different normal values and suggest various cut-off values for the diagnosis of UTI. Usually, in urine samples from healthy subjects there are only 1-2 white cells per high power field (HPF), and some authors suggest that more than 5 white cells for HPF might be considered significant for UTI (7) while others suggest higher cut-off value from 10 to 25 white cells / HPF (8,9)

In the Clinical Laboratory several screening tests have been devised for the rapid diagnosis of UTI because of several major considerations (10): firstly because of the great number of urinary samples sub-
mitted for microbiological examination; secondly because most urine specimens submitted for culture are negative or have bacterial counts below levels considered as significant; thirdly because UTI are often monomicrobial and small numbers of bacterial species are responsible for the great majority of UTI. Moreover, urine samples are usually available in large volumes without patients discomfort. Some screening test for rapid diagnosis of UTI considers together bacteriuria and pyuria. Microscopic examination of urine samples is a simple method for estimating bacteriuria and pyuria, but it is labor intensive and there are still open questions about standardization (11). The Catalase tube test to detect bacteriuria and pyuria has an unsatisfactory sensitivity (12,13). The most common approach to a non-culture screening for UTI is the use of reagent dipsticks for the presence of nitrites and leukocyte esterase (14,15). False negatives may result for group D Enterococci infection because these bacteria did not reduce nitrate (16). False positives result from the presence of ascorbic acid, drugs interference, or overgrows with nitrate producing bacteria (17).

The aim of this study is to evaluate the quantitative determination of pyuria obtained by using a second generation flow cytometer: Sysmex UF-100 (Toa Medical Electronics, Japan) versus the diagnosis of UTI by laboratory data from urine culture, microscopic examination, routine chemical analysis, and clinical data such as age, sex, symptoms, urinary tracts abnormalities, diagnostic suspect.

Materials and methods

Study Location: This study was performed in the Department of Clinical Pathology of ASL 14 “Chioggia”. The Department processes samples coming from two acute care facilities of 300 and 350 beds respectively and a chronic care facility of 120 beds. These facilities serve a 130,000 inhabitants South Venetian area (Italy).

Patients Selection: We considered 2010 consecutive patients, aged between 18 and 78 years (mean 56.4), whose recently collected urine samples were submitted, between January and August 1999, for diagnostic microbiological examination to our Laboratory. The samples were obtained from outpatients (1130, 496 males and 634 females) and inpatients (880, 374 males and 506 females): 123 from the department of Nephrology, 168 from Urology, 269 from Surgical Units, 47 from the Intensive Care Unit, 273 from Medical Units. (See Table I for description of the considered population). All the samples were obtained from patients over 18 years. The majority (1,812, 90.2%) of the samples were voided urine specimens collected by using the midstream technique (18), but 198 (9.8%) samples were collected through a bladder catheter. The samples were put in sterile containers and a 12-mL aliquot was transferred into test tubes and analyzed within one hour.

Chemical and Physical Examination: Dipstick analysis of urine samples was carried out before flow cytometer analysis by using URIFLET strips and a Super Auton automated reflectance photometer (Menarini, FI, Italy). The strips included reagent pads for semi-quantitative assessment of relative density, pH, leukocyte esterase, nitrite, protein, glucose, ketones, urobilinogen, bilirubin and hemoglobin (19).

Microscopic Examination: The classical microscopic examination was performed according to the NCCLS 1995 guidelines. After UF-100 analysis, each urine specimen (10 mL) was centrifuged at 400g for 5 minutes, and 9.5 mL of the supernatant was discharged. In each specimen at least 20 random microscopic fields were examined at 40x (HPF) from the same experienced technologist, and the mean number of cells/HPF were calculated with a cut-off at 10 WBC/HPF (20).

Culture of Urine Specimens: For microbiological examination, the samples were inoculated, within four hours, on agar plates by using a 0.001-mL calibrated loop. Both selective (McConkey agar (McC), and colistin-nalidixic acid blood agar (CNA) and non-selective (CLED agar) media were used. After 24 hours at 37°C the cultures were quantified, in CLED plate, as follows: 0-10 colonies (under 10,000 UFC/mL); 11 to 100 colonies (from 10,000 to 100,000 UFC/mL); over 100 colonies (over 100,000 UFC/mL). McC plates were adopted because thus allow a better demonstration of Enterobacteriaceae and CNA for a better demonstration of group D Enterococci (21). In each urine sample submitted for a microbiological examination we

<table>
<thead>
<tr>
<th>Patients</th>
<th>Number</th>
<th>Male</th>
<th>Female</th>
<th>Positives N∞</th>
<th>Positives %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outpatients</td>
<td>1,130</td>
<td>496</td>
<td>634</td>
<td>295</td>
<td>33.52</td>
</tr>
<tr>
<td>Inpatients</td>
<td>880</td>
<td>374</td>
<td>506</td>
<td>234</td>
<td>20.71*</td>
</tr>
<tr>
<td>Total</td>
<td>2,020</td>
<td>870</td>
<td>1,140</td>
<td>529</td>
<td>26.32</td>
</tr>
</tbody>
</table>

* The difference between the prevalence of urinary tract infections among outpatients and inpatients is statistically significant (p<0.05)
studied the Residual Antibacterial Activity (RAA) by using the Uro-Quick instrument. The urine was introduced in one of two culture vials containing a bacterium (Staphylococcus epidermidis ATCC 12228), and the resulting RAA was obtained from the analysis of the kinetic differential growth between the two culture vials (22). In cultures with a significant bacteriuria, an automated system DADE Microscan (Dade International Inc. Sacramento CA USA) (23, 24), allowed identification and evaluation of the sensitivity to antimicrobial drugs.

**UF-100 examination:** The Sysmex UF-100 is a second-generation automated analyzer that performs analysis of the formed urine elements by using a flow cytometer. The UF-100 analyzer aspirates 0.8-mL of uncentrifuged urine and performs the analysis (25). The sample is transferred to the reaction unit and diluted four times with a special buffer to dissolve the crystalline contents. A mixture of two dyes, carbocyanine and phenanthridine, is then added to the solution and the stained sample enters into the flow cell where urine conductivity is measured. After this step, the sample enters into a flow cell to form a sheath flow created by passing a sheath liquid irradiated by an argon laser beam. The fluorescence, the pulse intensity and pulse width of the forward-scattered light are measured (26). These measures together with the impedance data are converted by a microcomputer into three ranks of formed elements (scattergrams) (27).

Erythrocytes, leukocytes, epithelial cells and bacteria are recognized from the scattergrams of the forward-scattered light and the fluorescent light intensity. Epithelial cells are classified according the forward-scattered pulse width and fluorescence pulse width. For discrimination between casts and mucus it is important to consider the fluorescence pulse width. For erythrocytes, leukocytes, bacteria and epithelial cells the scattergrams and the counting results, expressed as number of cells / mL can be printed. Although white blood cells found in urine are mostly neutrophils they can form two characteristic scattergram populations one in a region of high intensity of scattered light and the other in a region of low intensity of scattered light. The region characterized by high intensity of scattered light typically contains amoebic white blood cells. On the other hand the region characterized by high intensity of scattered light typically contains glitter cells (28,29).

Statistical Analysis: In this study, the standard which the analytical performance of the screening for UTI obtained by using UF-100 was compared with , was the diagnosis of urinary tract infection. To assess the analytical performance of UF-100 screening for bacteriuria and pyuria in the diagnosis of UTI we considered the prevalence of the disease in the considered population (30). Statistical analysis include evaluation of Specificity (SP), Sensitivity (SE), Positive predictive value (PPV), Negative predictive value (NPV), Incidence of correctly classified (CCI) (31).

For comparison of means we adopted the Student’s t test, for comparison of proportion we adopted the Pearson’s Chi square test and a value for p<0.05 was considered significant (32). For comparison of results of the various tests we adopted the Spearman rank analysis (32) for an evaluation of the means of various white cells concentration in the urine samples we studied the cells distribution in healthy subjects and patients with UTI by means of the Relative Operating Characteristic curves (ROC) (32) with evaluation of the probability ratio L. (33). Moreover, after the establishment of an adequate cut-off for pyuria, the UF-100 results for WBC were compared with the results obtained by classical microscopic examination. Results were classified in groups i.e. group I (negative for WBC with both UF-100 and Microscope), group II (positive for UF-100 but negative for microscopy examination), group III (negative for UF-100 but positive for microscopic examination), group IV (positive for both the methods).

**Results**

Diagnosis of Urinary Tract Infections: Between the considered 2010 patients 529 (26.32%) were considered as affected by an UTI. As shown in Table I, the overall prevalence of UTI in the considered population was 26.32% with wide difference between the two groups: indeed the prevalence of subjects affected by UTI was 33.52% among in-patients versus 20.71% among out-patients, and the difference was statistically significant (p<0.05). Among the patients with UTI, 473 (89.41%) had a bacteriuria over 100,000 UFC/mL and so matched the classical microbiological criteria for diagnosis of UTI. In 22 (4.16%) patients the bacteriuria was between 10,000 and 100,000 UFC/mL, but these subjects were considered affected by UTI because of clinical considerations regarding presence of symptoms, underlying diseases, gender, results of the urine examination and species of bacteria. In 34 patients (6.43%) we did not observe a significant bacterial growth but the samples were positive for RAA and thus these subjects were considered affected by UTI too. These samples were obtained from patients with previously diagnosed UTI, a new urine sample of these patients was submitted to our Laboratory because of persistence of symptoms despite the antimicrobial therapy. Among the 1,491 subjects not considered affected by UTI, 1,014 had a bacteriuria under 10,000 UFC/mL, 432 between 10,000 and 100,000 UFC/mL and 35 a mixed bacteriuria with a microbial count over 100,000 UFC/mL. The latter 35 samples were not diagnosed for UTI because of contamination. In these samples we observed more than three bacterial strains, no one accounting for almost 80% of the colonies (33). These results are described in Table II. Among the patients with UTI we observed in 344 cases (65.03%) Gram negative bacteria, in 177 cases (33.46%) Gram
positive bacteria and in 8 cases (0.76%) a yeast. The data are shown in Table III.

Pyuria detection by Microscopic Examination: By using the classical microscopic observation at 400x of centrifuged unstained urine specimens and considering significant a pyuria over than 10 WBC/HPF, we observed 631 (31.39%) patients with significant pyuria, and of these 478 had an UTI, false positive results being 153 (7.61%), and false negative results 51 (2.54%). For this test the evaluation of analytical performance, as regard to diagnosis of UTI, gave the following results: SE 0.90, SP 0.90, PPV 0.76, NPV 0.96 and CCI 0.90.

Evaluation of Dipstick analysis: The analytical performance of a classic dipstick screening based on detection of nitrites and leukocyte esterase in the urine sample is relatively poor. The false negative results are 171 (8.51%) and the false positive results 184 (9.15%). For this screening we obtained SE 0.64, SP 0.88, PPV 0.63, NPV 0.89 and CCI 0.82.

Quantitative detection of Pyuria by UF-100: At first we evaluated the distribution of urine WBC in subjects with and without UTI. Figure 1 shows the distribution of pyuria values in out-patients and in-patients with and without UTI. Our data show than 70%, 80% and 90% of subjects without UTI had
less than 25, 50 and 100 WBC/mL respectively. On the other hand only 5%, 10% and 20% of patients with UTI had less than 25, 50 and 100 WBC/mL respectively. We then evaluated the relationship between urine WBC and UTI, and Figure 2 shows the relation between the number of urinary WBC/mL and the probability of an UTI in the patient. Both for in-patients and out-patients, male and female, if there are more than 25 WBC/mL in urine, there is more than 90% probability of an UTI. Finally we considered the results obtained by using the quantitative detection of pyuria to assess a cut-off value for diagnosis of UTI in adults. Figure 3 shows the ROC curves to assess the cut-off value for pyuria in the diagnosis of urinary tract infections. Both for in-patients and out-patients, male and female, if there are more than 25 WBC/mL in urine, there is more than 90% probability of an UTI. Finally we considered the results obtained by using the quantitative detection of pyuria to assess a cut-off value for diagnosis of UTI. Figure 3 shows the ROC curves to assess the cut-off value for pyuria in the diagnosis of urinary tract infections. Both for in-patients and out-patients, male and female, if there are more than 25 WBC/mL in urine, there is more than 90% probability of an UTI. Finally we considered the results obtained by using the quantitative detection of pyuria to assess a cut-off value for diagnosis of UTI.

Discussion

Usually, in clinical laboratory practice out of a great number of samples submitted for urinalysis and microbiological examination only few are obtained from patients for whom the clinical suspicion of UTI is confirmed by laboratory diagnosis. Indeed, in our experience, around 75% of urine samples submitted for a microbiological examination is not compatible with a diagnosis of UTI. The labor and resources expensive diagnostic protocol for urinalysis...
sis and microbiological examination gives negative results for around three quarters of the submitted samples. To save resources, many screening tests for rapid diagnosis of UTI were proposed in the past but few of them became widely used in the Clinical Laboratory. Among non cultural methods, the first screening test proposed was the direct microscopic examination (HPF) of Gram stained uncentrifuged urine. The presence of one WBC and one microorganism each HPF suggest a diagnosis of UTI with a sensitivity of 80% and a specificity of 60% (34). This test is labor expensive, needs an experienced observer, and has an unsatisfactory sensitivity and specificity because the diagnostic cut-off is equivalent to the threshold of microscopic detection (35).

In Italy, the microscopic examination of urine as screening for UTI, despite some supporters, has not great diffusion (36). The microscopic observation of centrifuged urine with or without staining, using a standard microscopic slide lacks in standardization (37,38). The use of classical quantitative microscopic examination performed on centrifuged stained urine samples by a count-chamber is too labor expensive for routine applications (39). The most widely used non cultural test for UTI is the dipstick screening for pyuria (which measures leukocyte esterase of neutrophil granules) and bacteriuria (which measures the presence of nitrite, reduced from nitrate by bacterial metabolic activity). This test shows a good correlation with diagnosis of UTI (SE 71%, SP 86%) only if in the sample there is a "large" number of microorganisms and pyuria is over 20 WBC/HPF (40). In the examination of urine samples with "moderate" bacteriuria or low pyuria the results obtained by using this screening test are absolutely unsatisfactory (41).

The only cultural method introduced in the clinical laboratory for rapid diagnosis of UTI is based upon the use of vials containing trypticase soy broth into which an aliquot of the urine sample to be tested is inoculated, with bacterial growth assessed by repeated photometry measurements. This test has a satisfactory sensitivity (90%) and specificity (98%) but requires some hours and it is quite expensive (42).

The aim of this study was an evaluation of the significance in UTI diagnosis of quantitative detection of urine WBC by the UF-100 cytometer. The authors at first evaluated the distribution of pyuria
among in-patients and out-patients with and without UTI, but no significant difference between the two groups was found. A significant difference was instead observed between UTI patients and patients without UTI. Indeed only 5% of the patients with UTI had less than 25 WBC/mL and less than 30% of the subjects without UTI had more than 25 WBC/mL, as it is shown in Figure 1. The analysis of the probability quotient “L” shows that for a pyuria over 15 WBC/mL the probability of an UTI is over 90% without difference between in-patients and outpatients, male or female, as shown in Figure 2. Figure 3 shows the ROC curves obtained for in-patients and out-patients, males and females. From our data it appears that by using the quantitative evaluation of pyuria by UF-100 the cut-off value for UTI diagnosis in adult patients is 25 WBC/mL, without significant variation between males and females, in-patients and out-patients. The correlation between the UF-100 and the dipstick data is satisfactory (Spearman r = 0.695, p<0.01). The correlation between the UF-100 data and the microscopic evaluation of pyuria is really very good (Spearman r=0.893, p>0.001).

The authors also studied the significance of the quantitative detection of pyuria by the UF-100 cytomter, with regard to the clinical diagnosis of UTI, by considering 2010 patients. For each of these patients, the clinical diagnosis of UTI was compared with the dipstick screening, the microscopic evaluation of pyuria, and the UF-100 results. The analytical performance of the three considered tests therefore is not obtained with reference to another laboratory test considered as a standard but with a real gold standard: the conclusive diagnosis of UTI thus emerges from the integration of clinical and laboratory data obtained in co-operation by Physicians, Microbiologists and Clinical Pathologists. The analytical performance of a classic dipstick screening based upon detection of nitrates and leukocyte esterase in the urine sample is relatively satisfactory. False negative results are 171 (8.51%) and false positive results are 184 (9.15%). In our experience, the dipstick screening gave SE 0.64, SP 0.88, PPV 0.63, NPV 0.89 and CCI 0.82, in good accord with the literature (43-46). By using the classical microscopic observation of centrifuged unstained urine with a cut-off at 10 WBC/HPF we observed 631 (31.39%) patients with significant pyuria, 478 of them with an UTI, while false positive results were 153 (7.61%), and false negative results were 51 (2.54%). In our laboratory, the microscopic evaluation of pyuria gave SE 0.90, SP 0.90, PPV 0.76, NPV 0.96 and CCI 0.90. The quantitative evaluation of pyuria performed by UF-100 with a cut-off at 25 WBC/mL shows 54 (2.69%) false negative results and 96 (4.78%) false positive results. For this test we obtained SE 0.90, SP 0.93, PPV 0.83, NPV 0.96 and CCI 0.93. Our results show that, the quantitative determination of pyuria performed by UF-100 has a better correlation with a diagnosis of UTI not only compared to the dipstick screening test, but also to the classical microscopic observation of urine WBC. This screening, in our study, demonstrates a negative predictive value of 0.96 and hence a subject without significant pyuria by UF-100 screening has 96% probability to be free from an UTI. This result is of great practical importance for a screening because a negative sample should be discharged without further tests. A little lack in specificity is less important because in each sample positive for UTI in the screening test, a culture should be performed to confirm the result, to identify the micro-organism and to determine antibiotic sensitivity (47-49). The examination of urine by UF-100 gives us more than the quantification of pyuria and bacterial count, i.e. an exhaustive evaluation of the urinary cells (50,51) and this information is useful in diagnosis or follow-up of UTI. For example UF-100 detects the presence of epithelial cells and large numbers of squamous epithelial cells in the sample may indicate that specimens contains more bacteria coming from vagina or perineum than from the urinary tract. The observation is useful if we observe bacteriuria (mixed or with prevalence of coagulase-negative Staphylococcus) without significant pyuria. These finding suggest a contamination better than an infection. Moreover, UF-100 gives us a quantification of hematuria and a distinction of the sit of bleeding with an evaluation of the morphology of RBC (52-54). In severe UTI a damage of the bladder’s epithelium with presence of hematuria is common and in this setting a differentiation between high (glomerular) hematuria and low hematuria (55,56) appears to be important. In acute uncomplicated UTI bacteria should be cleared from urine within 48 hours from the start of antibiotic therapy and the clearance of bacteriuria is concomitant with symptom remission. Persistent clinical signs of UTI indicate the persistence of the infection and the need of changing the antibiotic treatment or to search for another cause (i.e. yeast infection). In patients under antibiotic therapy the classical microbiological examination of urine is however very difficult because of the presence of RAA and the possibility that a bacterial strain gives negative results to the culture because the antibacterial drug inhibits the microbial growth in vivo but not in vitro. In these patients UF-100 give us important information in terms of a reliable quantification of bacteriuria and pyuria and morphologic data about urine WBC (57-60). In active UTI, the urinary WBC are young and form a population in a region of high intensity of scattered light but, after 24 hours of standing in the urine, the neutrophils shift to a region of low intensity of scattered light. Therefore UF-100 is able to give to the Laboratory information about the age of urinary WBC and this may be of great importance in the follow-up of some particular patients with UTI, such as patients in
critical conditions, with underlying disease (i.e. diabetes, obstruction, catheter).

The results presented here need obviously to be further confirmed by other authors with other populations and other pathological settings (i.e. children or chronic infections) (61,62). Moreover, the quantification of pyuria used in this study as a screening test for UTI, is not a new time and labor expensive test to be introduced in the Clinical Laboratory practice with further costs, since it is only a by-product of the examination of the corpusculated portion of urine by a flow cytometer (63, 64).

References

5. Gadeholt H. Quantitative estimation of urinary sediment to be introduced in the Clinical Laboratory practice with further costs, since it is only a by-product of the examination of the corpusculated portion of urine by a flow cytometer (63, 64).

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