Flow citometry for screening acute urinary tract infections and differentiation between Gram positive and Gram negative bacteria

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ABSTRACT

Urine culture is the most frequent test in microbiology laboratories. A screening tool, providing fast and reliable results to rule-out urinary tract infection (UTI), would be of great importance. We studied 1043 consecutive urine samples by Sysmex UF-1000i analyzer. Comparison was made by robotic urine culture on chromogenic agar with 1 μL loop, using 10^5 CFU/mL as a limit of positive growth. We evaluated bacteria quantification for rapid exclusion of UTI and bacteria forward scatter (B_FSC) in preliminary discrimination of UTI caused by Gram positive or Gram negative bacteria. For exclusion of UTI, the best cut-off value was 130 bacteria/μL. At this threshold, the sensitivity (SE) was 0.98 and the specificity (SP) 0.75. For exclusion of UTI sustained by Gram positive bacteria, the best cut-off value for B_FSC was 25ch. At this threshold, SE was 0.68 and SP was 0.89.

INTRODUCTION

Among transmissible diseases, urinary tract infections (UTI) are only second in frequency to the upper respiratory tract infections, but the request for microbiological examination of urine exceeds by far that for sputum; as a consequence, the urine culture is the most common bacteriological test in clinical laboratories (1).

The overall yield of positive results, even among patients with typical symptoms of UTI, is however low in spite of labour and time-consuming procedures. On the other hand, detection of microorganisms in a urine specimen is a necessary, but not sufficient criterion to establish a diagnosis, since contamination and/or colonization cannot be ruled out even when significant bacteriuria is present. Other criteria should be present in order to rule in a bacterial infection (2).

A concentration of $>10^5$ CFU/mL of a single microorganism in voided urine specimens is the generally accepted definition of significant bacteriuria (3), but lower limits have been suggested for children, men, patients with underlying diseases or when "fastidious" microorganisms are involved (4). Detection of leukocytes (LEU) in urine (pyuria) is a sensible clue for bladder infection, even though a universally accepted threshold for "significant" pyuria is still missing (5, 6). In the vast majority of patients, UTI are caused by Gram negative bacteria (*Escherichia coli* among *Enterobacteriaceae*, then non-fermenting Gram negative rods, such as *Pseudomonas species*), Gram positive bacteria (*Enterococcus species*, *Streptococcus species* and *Staphylococcus species*) being involved in about one third of UTI (7, 8). To get a reliable hint about the Gram characteristic of the germ involved in UTI certainly enhances the efficacy of the empirical therapy (9,10). Some evidence exists that the evaluation of "dimensional" parameters derived from the distribution histograms in the bacterial channel (bacteria forward scatter: B_FSC) of flow citometry-based analyzers may be useful for a rapid etiological differentiation (11).

By using flow citometry, this study aims to identify the screening threshold for bacteria and LEU providing the best performance in terms of diagnostic sensitivity and negative predictive value with respect to the reference culture method and to establish a presumptive morphological distinction between UTI caused by Gram positive and Gram negative bacteria on the basis of B_FSC and bacteria fluorescent light scatter (B_FLH) characteristics.

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MATERIALS AND METHODS

Sample selection

We considered 1043 consecutive mid-stream urine samples from adult outpatients, with age between 15 and 91 years (median, 63.2) [688 females (66%)], submitted to our institution from December 2012 to January 2013 for suspected UTI. Patients and their general practitioners were advised both verbally and by written instructions on the correct method for urine culture collection. Urines were collected in a sterile container (100 mL), fully equipped for sampling by vacuum tubes (Vacutest Kima). Two separate tubes with no additive were immediately sampled, one for microbiological examination and one for Sysmex UF-1000i examination and kept refrigerated until analysis. Plate inoculation and Sismex UF-1000i analysis were performed within 4 h from sample collection (12, 13).

Microbiological analysis

Quantitative urine culture was performed by using the Copan walk-away specimen processor (WASP) (Copan Diagnostics Inc.). This robotic platform is able to plant and streak samples using whole plates with 1, 2, 3 or 4 streaked zones, and bi-plates using horizontal or vertical patterns. Actual plating is done by three metal loops incorporated in a three-cornered loop inoculation tool (14). For the purpose of this study, we selected a 1 μL inoculation loop. Urine samples were routinely cultured for pathogens using the commercial chromogenic agar medium CPS ID3 (Biomerieux). Culture plates were aerobically incubated at 35 °C for 24 h. Quantification in CFU/mL was obtained multiplying by the dilution factor the colonies numbered on the agar plate. The culture was labelled as positive if containing $\geq 10^5$ CFU/mL (15, 16). Standard biochemical identification and susceptibility tests to antimicrobic drugs were performed by using Vitek 2 analyzer (Biomerieux) (17).

UF-1000i analysis

All samples were processed on a Sysmex UF-1000i Analyser (Dasit). Briefly, this is a flow cytometer that counts, analyzes and separates microscopic particles suspended in a stream of fluid. It can also perform simultaneous multiparameter analysis of physic-chemical properties of single cells flowing through a detection system and hence classify urinary particles. The measured parameters are converted into electric signals and the analysis of these electrical signals enables each particle to be classified accordingly and analyzed quantitatively. All the measurements are presented by the software as a scatter gram. Particles include erythrocytes, LEU, epithelial cells, casts, bacteria, crystals and yeasts. UF-1000i has a separate analytical channel for bacteria, where the urine specimen is mixed at 42 °C with diluents that increase cell wall permeability and enable specific staining of bacteria nucleic acids with a dedicated polyethinic fluorescent dye. The particles are classified and quantified by considering their size (impedance) and staining characteristics using the forward scatter and the intensity of fluorescent light. B_FSC and B_FLH are reported in arbitrary units (analytical channel – ch) and provide information about the size (B_FSC) and the nucleic acid contents (B_FLH).

Statistical analysis

Statistical tests were performed using a dedicated software (Analyse-it© version 2.03). A Kruskal-Wallis test was performed for comparison of data. The following parameters were considered: median (ME), with 95% confidence interval (CI), and the interquartile range (IQR). ROC curves, drawn by plotting sensitivity versus 1-specificity, were used to assess the best cut-off values and areas under curve (AUC) were also estimated. Specificity (SP), sensitivity (SE), positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy (DA) were calculated.

RESULTS

Microbiological analysis

A total of 287 out of 1043 samples (27.5%) met the prescribed criterion and were considered positive. A mono-microbial infection was found in 240 samples (193 Gram negative and 47 Gram positive), two bacterial strains were isolated in 11 samples (4 had two Gram negative bacteria; 7 a Gram positive plus a Gram negative), 31 patients showed a polymicrobial flora and were dropped out as contaminated and in 5 samples an infection sustained by *Candida species* was observed. In 256 positive samples, 267 bacterial strains were found: 208 Gram negative, 54 Gram positive bacteria, plus 5 *Candida species* (Table 1).

Bacteriuria quantification by UF-1000i

The following values for counting bacteria: 19/μL, 130/μL 187/μL and 345/μL corresponded to SE equal to 100%, 98%, 95% and 90%, respectively, and the value of 515/μL corresponded to the best cut-off suggested by the ROC curve to find the best compromise between SE and SP in our patient series. The AUC was 0.96.

By using a cut-off of 130/μL, we observed 281 true positive, 567 true negative, 6 false negative and 189 false positive results, with SE of 0.98, SP 0.85, PPV of 0.81 and NPV of 0.99 (Table 2). In the 6 false negative results, a bacterial growth of 105 CFU/mL was observed (*Escherichia coli* in two samples and Gram positive bacteria in four samples).

For *Candida species*, a cut-off of 200 yeast/μL was set. Under this setting, a full identification (5/5) of *Candida* infection was obtained.

LEU quantification by UF-1000i

The following LEU values: 20/μL, 40/μL, 100/μL and 150/μL corresponded to SE equal to 100%, 99%, 98% and 96%, respectively, and the value of 200/μL corresponded to the best cut-off suggested by the ROC

curve. The AUC was 0.84.

By using a cut-off value for LEU of 40/μL, we observed 204 true positive, 618 true negative, 83 false negative and 138 false positive results, with SE of 0.71, SP 0.81, PPV of 0.59 and NPV of 0.88 (Table 2).

By considering in association bacteria and LEU quantification, in comparison with results obtained for bacteria alone, we observed an increase in false positive results without any significant reduction in false negative results. So, we adopted an algorithm in which, alternatively or in combination, with the cut-off value for bacteria at 130/μL, different cut-offs for LEU were considered. Corresponding data are reported in Table 3.

Table 1
Strains isolated from positive urine samples

Microorganism	No. of strains
Candida species	5
Citrobacter species	2
Enterobacter species	5
Enterococcus species	30
Escherichia coli	155
Klebsiella species	29
Morganella species	1
Proteus species	8
Pseudomonas species	8
Staphylococcus species	5
Streptococcus species	19

Presumptive differentiation between Gram positive and Gram negative bacteria by UF-1000i

240 urine samples with a mono-microbial positive culture were considered (193 Gram negative and 47 Gram positive). Gram negative bacteria gave a B_FSC median value of 20ch (IQR: 14-33) and Gram positive bacteria had a B_FSC median value of 41ch (IQR: 28-60) (Figure 1). A statistically significant difference (P <0.001) in B_FSC between Gram positive and Gram negative was obtained.

In Gram negative bacteria B_FLH median value was 98.1ch (IQR: 87.1-115.6), while in Gram positive bacteria the B_FLH median value was 85.6ch (IQR: 83.3-112.3), with no statistically significant difference (P=0.63).

By using ROC curves, the best cut-off value for B_FSC was 25ch (AUC 0.82, SE 0.68, SP 0.89, NPV 0.91, PPV 0.38 and DA 0.71); for B-FLH the best cut-off value obtained was 90ch (AUC 0.61, SE 0.57, SP 0.65, NPV 0.87, PPV 0.24 and DA 0.54) (Table 4).

Table 2
Performance of screening assays by flow citometry in comparison with urine culture

	SE	SP	PPV	NPV	DA
BACT	0.98 (0.96-0.99)	0.85 (0.82-0.88)	0.81 (0.78-0.85)	0.99 (0.95-1.00)	0.96 (0.91-0.99)
LEU	0.71 (0.65-0.76)	0.81 (0.75-0.84)	0.59 (0.51-0.67)	0.88 (0.83-0.92)	0.80 (0.75-0.85)

Numbers in parentheses are 95% confidence intervals.
SE, sensitivity; SP, specificity; PPV, positive predictive value; NPV, negative predictive value; DA, diagnostic accuracy; BACT, bacteria; LEU, leukocytes.

Table 3
Evaluation of the UF-1000i performance at different cut-off values for bacteria (BACT) and leukocytes (LEU)

BACT[a]	LEU[a]	SE	SP	NPV	PPV	DA
19	-	1.00	0.37	1.00	0.37	0.54
130	-	0.98	0.75	0.99	0.60	0.81
187	-	0.95	0.79	0.99	0.64	0.84
345	-	0.90	0.85	0.98	0.70	0.87
515	-	0.89	0.88	0.96	0.74	0.88
130	20	1.00	0.60	1.00	0.49	0.71
130	40	0.99	0.67	1.00	0.53	0.76
130	100	0.98	0.71	1.00	0.56	0.78
130	150	0.96	0.72	0.99	0.57	0.79
130	200	0.95	0.72	0.99	0.57	0.80

[a]*Results are reported /μL.*
SE, sensitivity; SP, specificity; NPV, negative predictive value; PPV, positive predictive value; DA, diagnostic accuracy.

DISCUSSION

Many reports evaluating rapid methods for UTI diagnosis have been published in the past years, using microscopic observation of untreated or stained samples, enzymatic methods using catalase, glucose-oxidase, nitrate reductase, leukocyte esterase, filtration-based colorimetric methods, bioluminescence assays, photometric growth detection and, more recently, flow cytometry (18-23).

Using the UF-1000i analyzer, we established a threshold of 130 bacteria/μL for rapid UTI detection. Our data are in good accordance with those previously reported in literature (22, 23). The effectiveness of the screening by UF1000i would, therefore, be high even without considering LEU.

The experimental cut-off value reliable as "significant" pyuria (40/μL) was similar to those previously reported, both for UF-1000i and UF-100 analyzers (5, 6, 21, 22). In our experience, despite a small gain in SE, the association between bacteria and LEU counts determined a significant decrease in SP, making controversial its clinical use. Similar observations were already present in the literature (21, 22). This could be explained by considering a preanalytical phase still inadequate. In samples collected without a clean catch midstream technique, a contamination by LEU from the genital mucosa may be present. On the other hand, the presence of LEU in urine is of significance in differentiating contamination from infection. We have, therefore, decided to maintain LEU among parameters to be considered in the rapid diagnosis of UTI using Symex UF-1000i, but rather using a cut-off at 200/μL. In this way, important information about the presence of infections caused by slow growth or annoying bacteria should not be lost in spite of a slight decrease in SP.

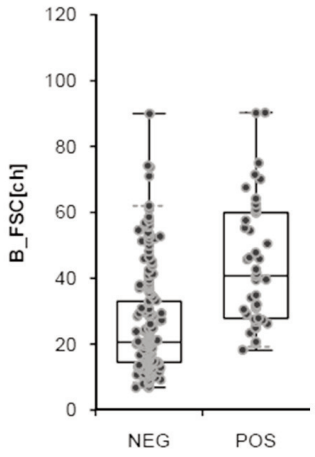

Figure 1
Bacteria forward scatter (B_FSC) in urinary tract infections sustained by Gram positive (POS) and Gram negative (NEG) bacteria .

In this study we observed six false negative results in samples with bacterial count >10^5 CFU/mL, in four of these samples Gram positive bacteria being observed. Limits in UF-1000i ability for detection of UTI sustained by Gram positive bacteria were reported in literature and usually ascribed to bacteria particles aggregation (15, 21, 22).

When setting an empirical treatment for UTI, a reliable indication of Gram properties of bacteria is valuable in selecting the most effective agent (15, 21, 22). UF-1000i has a dedicated analytical flow channel named "BACT channel", where specific reagents and algorithms provide data for bacteria detection and counting. Furthermore, a number of additional parameters are available, among which B_FSC is potentially useful in differentiating Gram positive and Gram negative bacteria (11, 24). Usually, Gram negative bacteria do not aggregate to form clusters or chains and tend to remain in suspension as single cells. On the contrary, Gram positive bacteria tend to form chains (*Streptococcus* and *Enterococcus species*) or clusters of different sizes (*Staphylococcus species*). The B_FSC measured in the bacteria channel of Sysmex UF-1000i analyzer can effectively detect this difference and the parameter correlates with the linear dimensions of examined particles (11, 24). When aggregated in clusters or chains, Gram positive bacteria show larger sizes compared to Gram negative. De Rosa et al. (11) suggested to set a cut-off value <30ch when using B_FSC in identifying Gram positives strains. In the present study, we found that a lower figure, namely <25ch, was slightly better in terms of SE and SP (0.68 and 0.89, respectively) compared to the value of <30ch (SE 0.73, SP 0.71).

In conclusion, we aimed to check the clinical performance of UF-1000i as a fast and reliable screening tool in the diagnosis of UTI. Our results strengthen the positive conclusions of other studies in presenting flow cytometry as a cost-effective and safe device in the diagnostic work up of this frequent condition. The

Table 4
Performance of flow citometry in discrimination between urinary tract infections sustained by Gram positive and Gram negative bacteria

	SE	SP	PPV	NPV	DA
B_FSC	0.68 (0.65-0.71)	0.89 (0.76-0.96)	0.38 (0.32-0.44)	0.91 (0.88-0.94)	0.71 (0.66-0.76)
B_FLH	0.57 (0.49-0.64)	0.65 (0.49-0.78)	0.24 (0.18-0.31)	0.87 (0.81-0.93)	0.54 (0.49-0.59)

Numbers in parentheses are 95% confidence intervals.
SE, sensitivity; SP, specificity; PPV, positive predictive value; NPV, negative predictive value; DA, diagnostic accuracy; B_FSC, bacteria forward scatter; B_FLH, bacteria fluorescent light scatter.

application of this technology holds promises in terms of cost savings, antibiotic stewardship and, hence, of better overall healthcare outcomes.

CONFLICTS OF INTEREST

None.

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