Estimation of the Causative Bacterial Group from Bacterial Scattergrams of the Fully Automated Urine Particle Analyzer UF-1000*i*

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We analyzed bacterial scattergrams generated by a Sysmex UF-1000i for estimating whether the causative micro-organisms were bacilli, cocci, or both.

We classified the distribution patterns of the scattergrams of 81 urine samples found positive in bacterial culture, among those submitted for flow cytometric urinalysis to the Kawasaki Hospital clinical laboratory. The separation line between "High Angle pattern" and "Low Angle pattern" had an angle of 30° from x-axis. Some cases had dots distributed widely with no relationship with the 30° line. This was defined as "Wide pattern". The incidence of the Low Angle pattern, High Angle pattern and Wide pattern was 57%, 20% and 23%, respectively. Most samples having the Low Angle pattern showed bacilli in culture (89%), and those with the High Angle pattern generally showed growth of cocci (69%). Multi-drug resistant bacteria (e.g., methicillin resistant staphylococcus and extended spectrum β-lactamase producing bacteria) were found at high incidence (32%) in the Wide pattern samples. Diagnosis of urinary tract infection (UTI) is primarily done by microbial culture, which takes a few days to give results. The possibility of estimating the group of the causative bacteria from the distribution angle of the bacterial scattergram could enable prompt diagnosis of complicated UTI and early discontinuation of antibiotic agents in patients with uncomplicated UTI.

Key Words Urinary Tract Infection, Causative Organisms, UF-1000*i*, Antibiotic Agents

INTRODUCTION

Urinary tract infection (UTI) is a nonspecific inflammation that develops in a kidney, ureter, urinary bladder or urethra, which in a majority of cases is caused by infection by intestinal bacteria. Often, the bacteria enter through the external urethral orifice, ascend the urinary tract, and cause infection in the bladder or kidney[1)]. UTI are classified into uncomplicated UTI, which have no underlying disease, and complicated UTI, which are accompanied by a systemic or localized underlying disease that makes the patient more susceptible to the infection. Uncomplicated and complicated UTI are handled differently, as they differ in the disease type, causative organisms, symptoms, treatment policy, and the patients' response to treatment. At the point of care, the urine is tested at the time of the initial outpatient examination. Urine bacterial culture and antimicrobial susceptibility tests of the bacteria are ordered while diagnosing UTI. As it takes a few days to get the results of urine bacterial culture, resorting to empiric treatment with antibiotic agents right from the patient's first visit is unavoidable. But if the patient has complicated UTI, such initial empiric antibiotic therapy is often ineffective, and currently, physicians can identify the primary disease only when the results of urine culture become available, a few days after the initial consultation.

Urine particle analysis of non-centrifuged samples by flow cytometry is covered by the National Health Insurance Scheme of Japan (Category D002-2, 30 points), independently from urine sediment microscopy (Category D002, 25 points) after the April 2006 revision of medical fees. Urine particle analyzers are being increasingly used in ordinary hospitals. The UF series urine particle analyzers (Sysmex Corporation; Sysmex) are systems that can quantitatively measure RBC, WBC, epithelial cells, casts, and bacteria in non-centrifuged urine in 72 seconds, using flow cytometry. The fully automated urine particle analyzer UF-1000*i* (hereinafter UF-1000*i*; Sysmex) uses a red semiconductor laser (wavelength λ = 635nm), and is smaller and consumes less power than earlier models. In this analyzer, a fluorescent polymethine dye specifically stains nucleic

Note: This article is translated and republished from Sysmex J.34 (Suppl.1): 19-26, 2011.

acids of bacteria, and carries out highly accurate quantitative analysis of bacteria using the dedicated bacteria channel (BACT channel). At the same time, the results of the analysis can be graphically displayed as scattergrams[2].

The fact that the bacterial group can be roughly estimated from the distribution angle of dots with respect to the x-axis in the bacterial scattergrams generated by UF-1000*i* has been reported in some academic conferences and scientific papers. Muratani et al.[3] and Nakagawa et al.[4] have reported that gram-negative *bacilli* were detected more often in urine samples with distribution angle <30°, and that *cocci* were detected more often when the angle was ≥30°. However, there have been no reports on the possibility of estimating the bacterial group from the angle that the distribution of the dots makes with the x-axis in bacterial scattergram, based on studies that used only urine samples of actual outpatients of a urology department.

In the present study, we examined the possibility of broadly estimating the bacterial group (whether it is a *bacillus* or a *coccus*) from the UF-1000*i* bacterial scattergram pattern of patients' urine samples, to gather data for use in the selection of suitable antibiotics at the first visit to the hospital.

MATERIALS AND METHODS

Urine samples from a total of 81 subjects (23 males and 58 females, mean age 59.6, and age range 17 to 89) selected from among persons who were examined in the Department of Urology, Kawasaki Hospital, Kawasaki Medical School during February to June 2010 were used in the study. The subjects comprised 78 persons whose urine had ≥10 WBC/μL as analyzed by UF-1000*i* and the bacterial species present in whose urine were identified by urine bacteria culture, and 3 persons who were positive for Neisseria gonorrhoeae when tested by transcription-mediated RNA amplification. The breakdown of the subjects is shown in ***Fig. 1***. Age group-wise, 33% of the subjects, the largest group, were in their 70s. Next were those in their 60s and those aged ≥80, each of these groups accounting for 18%. Although the proportion of patients generally decreased with decrease in age, there were more patients in their 20s than in their 30s, which matched with the peak incidence of uncomplicated cystitis in young people. Among underlying diseases, acute cystitis was the most common (48%). This was followed, in decreasing order, by prostatitis, enlarged prostate, and infection during intermittent catheterization. The bacteria detected by urine culture are shown in ***Fig. 2***. *Bacilli* were found in a

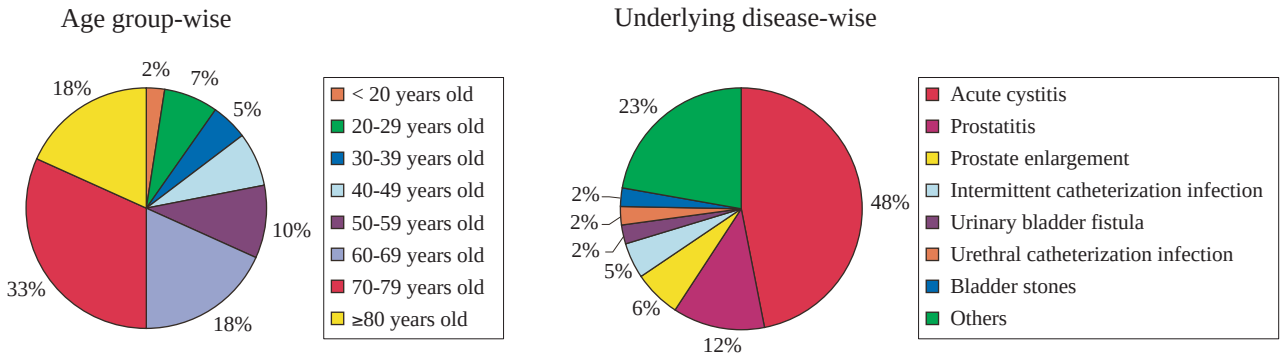


Fig. 1 *Distribution of cases*

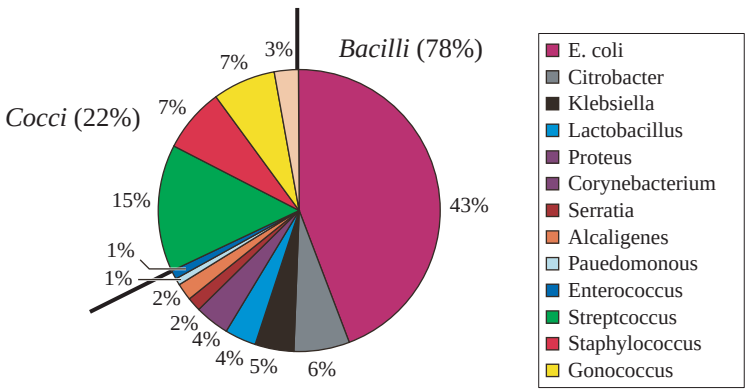


Fig. 2 *Bacteria detected in urine culture*

majority (78%) of the subjects and 22% had *cocci*. 43% of all subjects had Escherichia coli.

We examined the pattern of dot distribution in the scattergrams generated by the UF-1000*i* BACT channel. ***Fig. 3*** is a scattergram of the sediment channel which shows the overall pattern of distribution of dots representing urine sediment components. Both the sediment channel and the BACT channel use combinations of forward scattered light intensity (FSC) and fluorescent light intensity (FL) to create 2-dimensional scattergram. The FSC on the y-axis reflects the size of the particle, bacterial cluster, etc, while FL on the x-axis reflects the depth of staining of the cell nuclei in such particles and clusters. WBC, epithelial cells and spermatozoa are far larger than bacteria, and only RBC will be distributed close to the y-axis, around the area where the bacterial clusters are distributed.

The the BACT channel, on the other hand, gives an enlarged view of the area near the intersection of the x and y axes of the sediment channel scattergram, where relatively small particles like bacteria are distributed. The staining fluid UF II SEARCH-BAC used in the BACT channel contains a polymethine dye that specifically stains the nucleic acid of bacteria, and it does not stain microfragments of non-bacterial cells in the urine, which are of about the same size as bacteria. The diluent UF II PACK-BAC contains a cationic surfactant that lyses blood cell components. The use of the dye and diluent creates a mechanism wherein the nonbacterial cell components and microfragments of such cells are not stained.

The UF-1000*i* analysis screen (of a 22-year-old female with acute uncomplicated cystitis) is shown in ***Fig. 4***. The parameters are listed on the left side of the screen. We can see that large numbers of WBC and bacteria are present. The BACT channel output is shown as a 2-dimensional scattergram at the bottom right of the screen. In this patient, the distribution angle, from the x-axis, of the dots that represent bacteria is clearly less than 30°.

In the analysis of the present study, the distribution patterns of dots that represented bacteria in the BACT channel were classified into 3 types, namely, a "Low Angle Pattern" that passes through the origin with the dots distributed clearly below the angle of 30° from the x-axis, a "High Angle Pattern" with the dots distributed around the 30° line or above it, and a "Wide Pattern" with the dots distributed over a wide range from low to high angles.

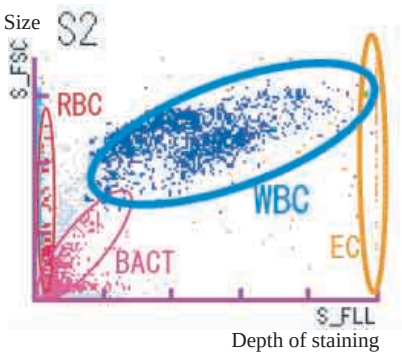
StatMate III (Atoms Co.) was used for testing statistical significance. Wilcoxon test, Mann-Whitney U test and chi-square test were used for testing the differences between groups. In all these tests, $p<0.05$ was taken as significant difference.

RESULTS

Fig. 5-A shows a BACT channel scattergram with the Low Angle Pattern. 46 (57% of the total) subjects had this pattern. Among these, 32 (70%) had *E. coli*, 4 (9%) had the genus *Citrobacter*, 3 (7%) had the genus *Klebsiella*, and another 3 (7%) had the genus *Gonococcus*. 41 (89%) of the subjects with the Low Angle Pattern had gram-negative *bacilli*.

Fig. 5-B shows a BACT channel scattergram with the High Angle Pattern. 16 (20% of the total) subjects had this pattern. The breakdown of bacteria found in them was, in decreasing order, genus *Enterococcus* in 5 (31%), genus *Staphylococcus* in 3 (19%), genus *Streptococcus* in 2 (13%), and E. coli in 2 (13%). 11 (69%) the subjects of this group had gram-positive *cocci*.

Fig. 5-C shows a BACT channel scattergram with the Wide Pattern. 19 (23% of the total) subjects showed this pattern. 11 (79%) of the 14 subjects who had polymicrobial infection, i.e., 2 or more bacteria identified by urine culture, showed the Wide Pattern. The bacterial species identified in individual samples of this category are listed in ***Table 1***. In most of the cases of



- Sediment channel
- RBC are shown as red dots
- WBC are blue dots
- Epithelial cells are orange dots
- In the BACT channel, the area close to the intersection of the two axes is magnified, and the RBC components are hemolyzed with a surfactant.

Fig. 3 *An example of scattergram displayed (sediment channel)*

S_FSC: Forward scattered light intensity (size)
S_FLL: Fluorescent light intensity (low) (depth of staining)
RBC: Red blood cells (red)
WBC: White blood cells (blue)
BACT: Bacteria (pink)
EC: Epithelial cells (orange)

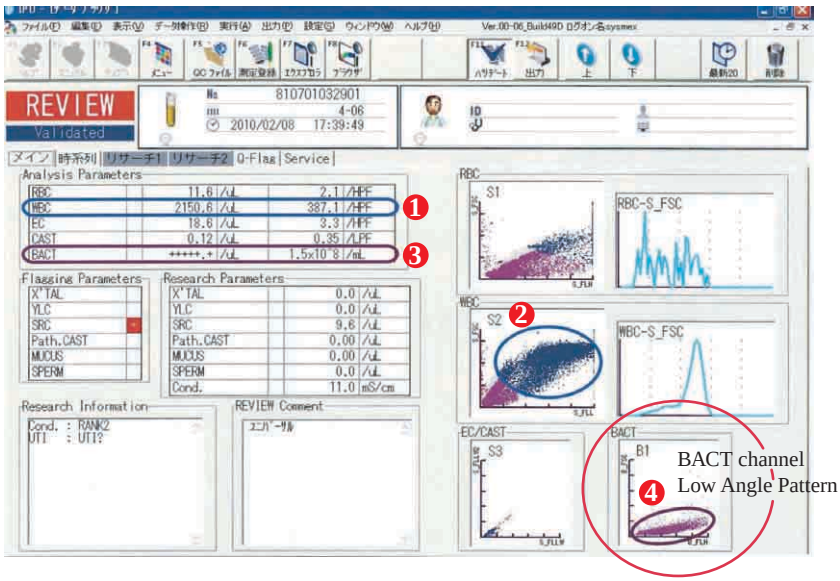


Fig. 4 *UF-1000i analysis screen (acute uncomplicated cystitis)*

This method uses the BACT channel scattergram, shown at bottom right.
The dots representing bacteria are distributed in the zone of less than 30° angle from the x-axis (Low Angle Pattern).

❶ Table at the third row from bottom, on the left side of the screen: The WBC count in the urine was elevated at 387.1/HPF.

❷ Enhanced image of WBC distribution in the sediment channel (graph at second column from the right, second row from the bottom) shows a large number of blue dots.

❸ Table at the third row from bottom, on the left side of the screen: The bacterial count in the urine was elevated at BACT = 1.5×10^8/mL.

❹ BACT channel

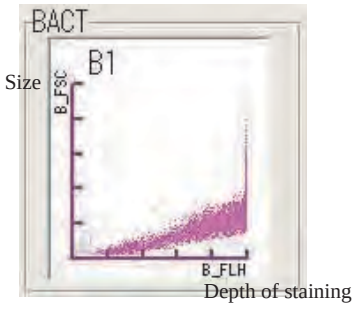


Fig. 5-A. *Low Angle Pattern*

Dots representing bacterial clusters are distributed in a zone below the line with 30° angle.

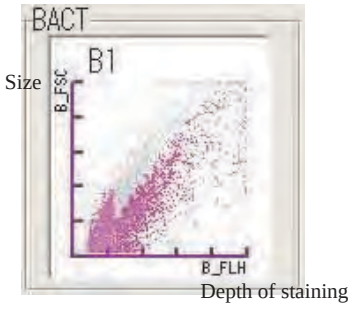


Fig. 5-B. *High Angle Pattern*

Dots representing bacteria are distributed above the 30° line.

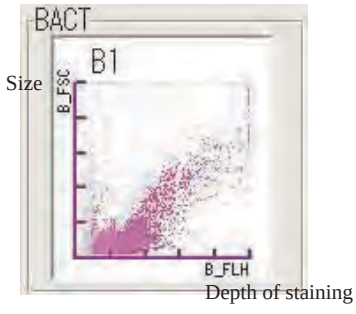


Fig. 5-C. *Wide Pattern*

The dots are distributed in the areas both above and below the 30° line.

Table 1 *Bacterial species detected by urine culture of each case that showed the Wide Pattern, listed in the order of decreasing counts from left to right.*

86 F	E. faecalis		
84 F	E. coli	S. haemolytics MRS*	
82 F	E. coli ESBL*		
81 M	K. pneumniae	E. faecalis	
79 F	P. mirabilis	E. faecalis	
79 F	MRSA*		
75 F	K. pneumoniae	E. aerogenes	S. haemolyticus MRS*
74 M	K. pneumoniae	E. faecalis	
73 F	E. coli	S. agalactiae	
72 F	E. coli ESBL*	S. salivarius	
72 M	E. coli		
72 M	E. coli		
70 F	Streptococcus spp.		
66 F	E. faecalis	Lactbacillu spp.	
44 F	S. epidermidis		
39 M	M. morganii	Streptcoccus spp.	
36 M	E. coli	Alcaligene spp.	E. faecalis
23 F	E. coli		
22 F	E. coli	S. epidermidis MRS*	

* Multidrug resistant bacteria

polymicrobial infection, one or more gram-negative *bacilli* and gram-positive *cocci* were found together. Multidrug resistant bacteria such as methicillin resistant *coccus* (MRS) and extended spectrum β-lactamase (ESBL)-producing E. coli were detected at a significantly ($p = 0.02$) higher frequency in 6 (32%) of the 19 subjects who had the Wide Pattern compared to only in 3 (5%) of the 62 subjects who did not have this pattern.

DISCUSSION

UTI patients are diagnosed by testing their urine at the initial examination as outpatients. If the causative organism can be differentiated into coccus or bacillus at that time, the possibility of complicated UTI can be estimated at an early stage, particularly in patients infected with a gram-positive coccus, and the QOL can be improved. Moreover, patients infected with gram-negative *bacilli* can be treated by short-term administration of a suitable antibiotic agent. In urine bacteria culture tests, a certain amount of urine is applied with a quantitative platinum loop on an agar medium used for colony development, and cultured for 24h or even longer to obtain the results. Thus the results of the culture test are not available at the first visit, and currently a few days are required to arrive at definite diagnosis. In this respect, gram-negative/positive differentiation of the bacteria by gram staining is a useful method. But there is a possibility of the result being affected by the level of expertise of the technologist, or the bacterial cells being missed sometimes[5]. Contrary to such testing, UF-1000*i* can display the RBC count, WBC count, and bacterial count in the urine through its basic cell analysis function in a very short time and can thus contribute to diagnosis of UTI[6-8]. Furthermore, it can output a bacterial scattergram without any additional treatment of the urine sample[2]. In the present study, by measuring the angle of the distribution of dots in the bacterial scattergram, *cocci* can be broadly differentiated from *bacilli* in actual clinical samples rapidly, in about a minute. This is considered a very useful method as the causative organism can be rapidly differentiated into coccus or bacillus without any additional processing, unlike in conventional testing.

Muratani et al. measured the distribution angle of the dots from the x-axis in the UF-1000*i* bacterial scattergrams of suspensions of known bacterial species. They reported that gram-positive *cocci* showed a distribution angle of about 40°, *Corynebacterium*, a gram-positive bacillus, had 43°, gram-negative *bacilli* had about 20°, and that Neisseria *gonorrhoeae*, a gram-negative coccus, had 11°. In short, their results showed that the distribution angle of the scattergram was primarily determined by whether the bacterium was positive or negative in gram staining, rather than their morphology, i.e., whether they were *bacilli* or *cocci*[3]. Our results also point to a similar conclusion. This suggests that in flow cytometric analysis with UF-1000*i*, not all the individual dots in the scattergrams represent individual bacterial cells. Some represent certain clusters of bacteria, without differentiating between *cocci* and *bacilli*. For example, gram-positive *cocci* and the genus *Corynebacterium* (gram-positive bacillus) both multiply by dividing in 2 or 3 dimensions to form of grape-like irregular clusters. Therefore, they appear on the scattergrams as relatively large dots with strong staining intensity. On the other hand, gram-negative *bacilli* and bacteria of Neisseria *gonorrhoeae*, a gram-negative diplicoccus, divide after reaching a certain size, scatter, and exist as individual cells, the depth of staining is almost constant for all the bacterial cells, which appear as

small dots with weaker staining of the bacterial nuclei by flow cytometry than in the clusters of gram-positive *cocci*, and the distribution is more convergent[4]. On the basis of these facts, we can say that the difference in the distribution angle of the dots in the scattergram is the result of the reflection in two dimensions of the differences in the pattern of bacterial cell multiplication. This suggests the possibility of estimating the bacterial group from the scattergrams.

Muratani et al. had grouped the distribution patterns of the BACT scattergrams into those of ≥30° and <30° distribution angle[3]. In our study, however, apart from those in the High Angle Pattern (≥30°) and Low Angle pattern (<30°), there were also more than a few cases that showed the Wide Pattern (dots distributed widely both below and above the 30° line). This was probably because the subjects of our study included all patients who had abnormal WBC counts (≥10/μL) in the urine and were positive in urine culture for bacteria, among those who visited the Department of Urology for examination. In particular, 79% of the samples which were found to have polymicrobial infection in the urine culture test showed the Wide Pattern, and there was a high incidence of mixed infection involving both *bacilli* and *cocci*. Furthermore, the Wide Pattern group had significantly higher incidence of involvement of multidrug resistant bacteria such as methicillin resistant staphylo*cocci* (MRS) and ESBL-producing *E. coli*. According to some reports, gram-negative *bacilli* showing filamentation due to prior exposure to antibiotic agents have a High Angle distribution of the dots. Therefore, Wide Pattern scattergrams could arise from complicated UTI that could not be fully cured by earlier antibiotic treatment.

How to apply scattergram-based diagnosis of UTI in therapy is an important issue. It is generally held that the causative organism is predominantly a gram-negative bacillus in uncomplicated UTI with no prior exposure to antibiotic agents. Therefore, we may say that Low Angle Pattern suggests uncomplicated UTI without prior exposure to antibiotics, except in cases of male urethritis with *N. gonorrhoeae* infection. In other words, a typical Low Angle Pattern means that the patient can be fully cured by short-term oral administration of ordinary wide spectrum antibiotics. The High Angle Pattern and the Wide Pattern suggest the possibility of complicated UTI. With such patients, it is important to avoid empiric antibiotic therapy, except in cases that require urgent intervention because of high fever or septicemia detected at the time of the first visit itself, and give priority to identification of the underlying condition that led to the urinary tract disease.

We feel that future investigations should cover the incidence of different distribution patterns separately in uncomplicated and complicated UTI patients, whether all patients showing the Low Angle distribution can be cured by short-term administration of wide spectrum antibiotics, how the dot distribution pattern varies in relation to prior exposure to antibiotics, etc.

So far we have been discussing the usefulness at the point of care of classification of dot distribution patterns of UF-1000*i* bacterial scattergrams. Although it is needless to state it, the results of microscopy of urine sediments and gram staining, apart from blood cell counts in urine, must be given a predominant role in the diagnosis of UTI. Gram staining can give results in about 10 minutes, and thus rapid reporting is possible. Moreover, it provides many types of other information, such as the possible causative organism, morphological changes in the bacterium caused by administration of antimicrobial agents, phagocytosis of bacteria by neutrophils, etc However, when only a small number of bacteria are present, their presence cannot at times be confirmed by microscopy, and currently many medical institutions are forced to depend on urine culture tests as they lack facilities for real time gram staining and microscopy. UF-1000*i* can output the WBC and bacterial counts in a short time through the use of flow cytometry, and the reliability of the results has been sufficiently validated by past reports[2,8]. The present study of ours, which analyzed the distribution pattern in the BACT channel scattergram, automatically displayed on the computer screen of the analyzer, shows that cases with a Low Angle Pattern (distribution of dots below the 30° line) had a high incidence of infection by a *bacilli* and the majority of cases with the High Angle Pattern had infection by cocci, whereas the majority of cases with the Wide Pattern had complicated UTI involving more than one bacterium. The method described here enables a broad judgment as to whether the UTI is caused by a bacillus, coccus, or more than one bacterium, within about one minute on the day of the consultation itself without having to wait for the results of urine culture, and therefore can be used as a guide in the selection of antimicrobial agents for treating UTI patients.

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