The Possibility of the Bacterial Class Estimate Using Urine from Patients with the Urinary Tract Infection by the Fully Automated Urine Particle Analyzer UF-1000*i*

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We investigated bacterial counts and bacterial discrimination in urine samples from patients with urinary tract infection, and compared Sysmex UF-1000i with standard culture method. This study investigated 208 urine samples from different patients. Regarding bacterial counts in urine, these two methods had good correlation, however, the results of the UF-1000i generally showed 10 times higher than culture results method generally. Five of 15 samples that showed result of no growth by culture method and more than 10^4cfu/mL by UF-1000i were positive for bacteria by microscopy. This outcome may be attributed to the use of antibiotics or existing bacteria that were unable to grow on blood agar or Drigalski agar.
Scattergram in bacterial channel of UF-1000i was a graph drawn at Y-axis front airlight width, X-axis front airlight strength, and I pulled a straight line centrally and measured the angle from this X-axis by a protractor.
In the study using bacterial suspensions of identified organisms, the angle of the gram-positive cocci and rods were distributed around 40 degrees, and that of the gram-negative cocci and rods were distributed around 20 degrees. Therefore these results suggest the possibility that one can distinguish gram-negative bacteria from gram-positive bacteria by an angle with the X-axis of scattergram of UF-1000i.
In results using clinical specimens, when the angle of the scattergram was less than 30 degrees, the probability that gram-negative bacterium were present was 89.2%. In contrast, when the angle of the scattergram was more than 30 degrees, the probability gram-positive bacterium was 55.8%. Considering that samples with a scattergram angle of more than 30 degrees accounted for 83.3% when isolating only gram-positive bacteria, it is considered that scattergram analysis of UF-1000i might be useful in choosing appropriate antibiotic therapy.

Key Words Urinary Tract Infection, Causative Organism, Gram-stain, UF-1000*i*

INTRODUCTION

In general, urinary tract infection (UTI) usually refers to pyelonephritis and cystitis among non-specific infections of the urinary tract. UTIs are caused by various organisms. Almost all of the causative organisms of UTIs originate from faecal material or the periurethral environment. The organisms ascend the urinary tract from the urethral opening or from the insertion site of catheters. UTIs are classified into uncomplicated and complicated infections depending on underlying diseases. Uncomplicated UTIs, without underlying diseases occur commonly with an acute onset. They include acute uncomplicated cystitis and acute uncomplicated pyelonephritis. Complicated infections are also subdivided as chronic complicated cystitis and chronic complicated pyelonephritis occurring in conjunction with underlying conditions in the urinary tract. Acute exacerbation can occur in chronic complicated UTIs with acute and sometimes severe symptoms similar to that in acute uncomplicated UTIs. Significant underlying conditions and indwelling urinary catheters are quite important underlying conditions in inducing complicated UTIs. The causative organisms are *Escherichia coli* in about 3/4th of the cases, followed by *Staphylococcus* spp., especially *S. saprophyticus*, and *P. mirabilis* as shown in ***Fig. 1*** [1]. On the other hand, in complicated UTI *Enterococcus faecalis*, *E. coli*, and *Pseudomonas*

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aeruginosa are the main causative organisms and they along with *Enterococcus faecium* and *Staphylococcus* spp. account for about 2/3rd of the cases. Apart from these, many other types of bacteria, including *Enterobacteriaceae* species such as *Citrobacter* spp., *Enterobacter* spp. and *Serratia marcescens*, and the generally weakly virulent glucose nonfermentable gram-negative rods like *Acinetobacter* spp. are also involved. Uncomplicated and complicated UTI not only differ in their causative organisms but also in the resistance of the causative organisms to antibiotics. Thus it becomes necessary to adopt completely different strategies in selecting the appropriate antibiotics. Clinical symptoms and urine leukocyte counts are the important diagnostic parameters. Definitive diagnosis can be made if the causative bacterium is detected by microscopically examining smear samples. In the early stages of uncomplicated UTI in particular, the bacterial counts in urine are low, and negative results accounted for about 10% not only in microscopy but also in culture. Treatment with antibiotics should not be considered if patients of complicated UTI do not show clinical symptoms in spite of being positive for certain bacteria and having high urine leukocyte counts. However, if the patients show symptoms attributable to cystitis, like frequent urination and pain in the lower abdomen, they are considered to be cases of acute exacerbation, which requires administration of antibiotics. Causative organisms of complicated UTI are fairly varied, and could be gram-negative as well as gram-positive bacteria. The antibiotics are therefore selected by making an educated guess about the identity of the causative organisms through microscopic examination of gram-stained samples, etc.

The UF-1000*i*, a fully automated urine particle analyzer from Sysmex Corporation (Sysmex) is based on fluorescence flow cytometry technology, which can quantitatively analyze red blood cells, white blood cells, epithelial cells, and casts and bacteria. Furthermore, the fully automated urine particle analyzer UF-1000*i* (hereinafter, UF-1000*i*) is capable of quantitatively analyzing bacteria. It is provided with an exclusive dedicated bacteria (BACT) channel where a polymethine fluorescent dye specifically stains bacterial nucleic acids. UF-1000*i* can measure the urine leukocyte count and urine bacterial count in a short time after urine sampling, and thus it is would be useful in the diagnosis of UTI. If such data is to be used in the selection of antibiotics, however, it becomes important to decide whether the bacterium is gram-positive or gram-negative. There have been some reports that suggested that the UF-1000*i* could differentiate bacterial species to some extent[2]. Although a sufficient amount of data on urine leukocyte counts has been reported [3,4], most of the studies on bacterial counts and the possibility of identifying bacteria have so far used cultured bacterial strains and not non-clinical samples. We therefore examined these aspects using clinical samples.

MATERIALS AND METHODS

Two-hundred and eight urine samples received for quantitative bacterial culture tests during July and August 2008 were studied. Bacteria counting and analysis of bacterial scattergrams using a UF-1000*i*; microscopic examination of gram-stained urine sediments; quantitative culturing on Drigalski agar medium, blood agar medium and chocolate agar medium; and bacterial identification were carried out.

The scattergrams of the BACT channel of UF-1000*i* are plots of dots with fluorescent light intensity on the x-axis and forward scattered light intensity on the y-axis. A straight line that passed through the origin was drawn

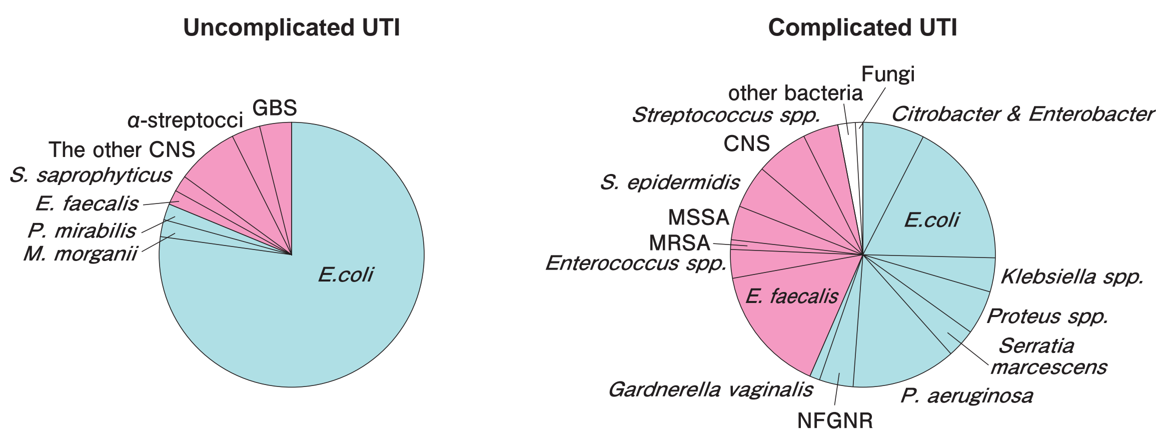


Fig. 1 *Causative organisms of uncomplicated and complicated urinary tract infections1)*

E. coli is responsible for 70-80% of the cases of uncomplicated urinary tract infections (UTI). However, apart from E. coli, various bacteria such as Pseudomonas aeruginosa, Enterococcus spp., and Staphylococcus spp. cause complicated UTI, which have underlying diseases that affect urine flow.
: Gram-negative bacteria, : Gram-positive bacteria

centrally through the collection of dots, and the angle that this line made with the x-axis was measured with a protractor. In addition, piperacillin was allowed to act on *E. coli* ATCC25922. The bacteria were observed microscopically, and the scattergrams prepared using UF-1000*i* were analyzed.

RESULTS

1. Bacterial count

Fig. 2 shows the BACT channel scattergrams of the urine samples from 3 patients with different inoculum size of bacteria. The difference in bacterial count is obvious by visual comparison of the scattergrams. The bacterial counts output by UF-1000*i* tended to be about 10 times higher than those obtained by culture method.

Fig. 3 shows the correlation between the results of quantitative culture on Drigalski agar or blood agar and the bacterial counts measured by UF-1000*i*. The correlation was fairly good but there was a trend of UF-1000*i* reporting counts that were 10 times higher of those obtained by culture method. Moreover, 5 among the 16 samples that had bacterial count ≥ 10^4 cfu/mL by UF-1000*i* and were negative for bacterial growth in quantitative culture, were positive in microscopy after gram-staining. Among the 144 samples that showed bacterial count ≥ 10^4 cfu/mL in quantitative culture, 143 cases gave similar results in UF-1000*i* analysis as well. The remaining 1 sample had 4.6×10^3 cfu/mL.

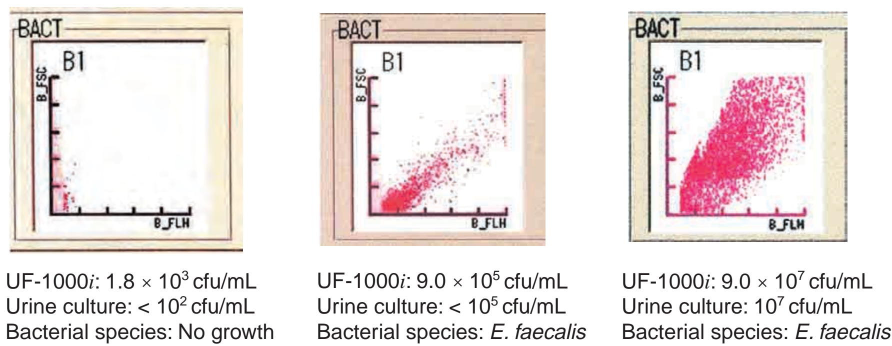


Fig. 2 *Scattergrams of samples with different bacterial counts*

X-axis: Fluorescent light intensity
Y-axis: Forward scattered light intensity
All the samples were of patients' urine. Bacterial counts are shown in units of cfu/mL.

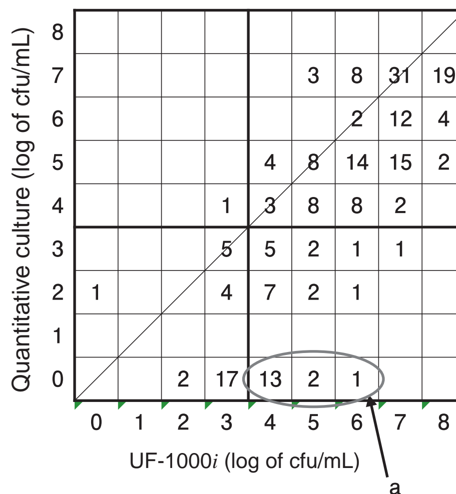


Fig. 3 *Correlation between bacterial counts determined by quantitative culturing and those measured using UF-1000i*

a: Smears of 5 of the 16 samples were positive

2. Relationship between the bacterial species identified and the distribution angle of the BACT channel scattergram generated by UF-1000*i*.

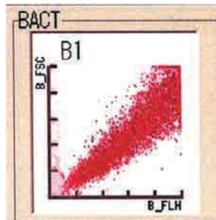
Typical examples of scattergrams are shown in ***Fig. 4***. Dots representing gram-positive cocci are distributed along an angle of about 40° and gram-negative rods along an angle of about 20°. The distribution of *Corynebacterium* sp., a gram-positive rod, formed an angle of 43°, and that of *Neisseria gonorrhoeae*, a gram-negative cocci, formed an angle of 11°. Thus, while there was little difference in the distribution angle between rods and cocci, there was difference between gram-positive and gram-negative bacteria (***Fig. 4-a*** and ***b***).

Fig. 4-c shows some of the scattergrams with the dots distributed along two directions, among samples from which both gram-positive and gram-negative bacteria were isolated. The discrimination is not always easy with such patterns, but these are distinctly different from the patterns shown in ***Fig. 4-a*** and ***b***.

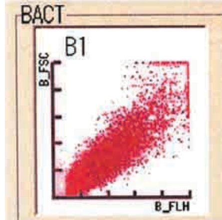
The angle could not be determined in 40 samples because of insufficient number of dots. Among these, the bacterial counts from UF-1000*i* were $< 10^3$ cfu/mL in 4 samples, $\geq 10^3$ cfu/mL but $< 10^4$ cfu/mL in 25 samples, and 1.0-3.9 $\times$ 10^4 cfu/mL in 11 samples. The results of culturing these 40 samples were bacterial count $< 10^3$ cfu/mL in 31 samples, $\geq 10^3$ cfu/mL but $< 10^4$ cfu/mL in 6 samples, 10^4 cfu/mL in 2 samples, and 10^5 cfu/mL in 1 sample.

a. Gram-positive bacteria

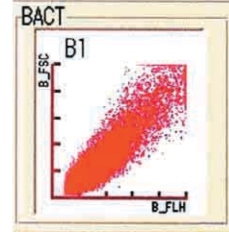
Staphylococcus sp. (cocci)



α-streptococci (cocci)

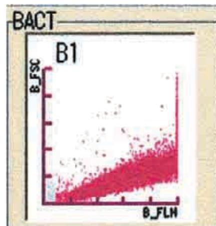


Corynebacterium sp. (rods)

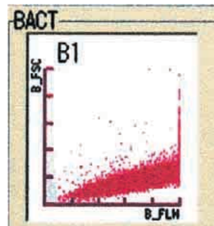


b. Gram-negative bacteria

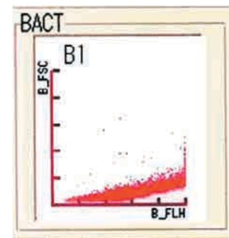
E. coli (rods)



K. pneumoniae (rods)

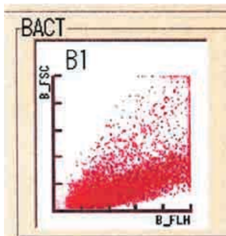


Neisseria gonorrhoeae (cocci)



c. Gram-positive and gram-negative bacteria

E. faecalis
C. koseri, A. faecalis



E. faecalis
K. oxytoca

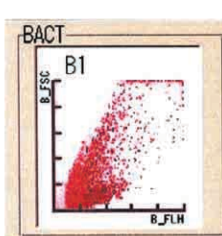


Fig. 4 *Scattergrams generated by the BACT channel of UF-1000i, and the bacterial species isolated*

The Corynebacterium sp. and Neisseria gonorrhoeae samples were of bacteria grown in liquid culture and the others were urine samples of patients.

With the 140 samples that showed unidirectional distribution of dots on the scattergram, the relationship of the angle of the distribution with the x-axis and the bacterial class identified through isolation culture is shown in ***Table 1-a*** and ***b***. The bacteria were classified as follows: family *Enterobacteriaceae* (EC, gram-negative), glucose nonfermentable gram-negative rods (NFGNR, gram-negative), genus *Staphylococcus* (SA, gram-positive), genus *Enterococcus* (EF, gram-positive), genus *Streptococcus* (St, gram-positive), and genus *Corynebacterium* (Cor, gram-positive). Among the samples from which only species of EC were isolated, 82.8% (53/64) had a distribution angle < 30° in the scattergram. Among samples from which species of more than one group including EC were isolated, 75.0% (21/28) had a distribution angle of < 30°. Four samples from which NFGNR alone were isolated had angles of 10-18°. 84.3% (59/70) of the samples from which only gram-negative rods (including NFGNR) were isolated had an angle of < 30°, and 82.8% (24/29) of the samples from which only gram-positive bacteria were isolated had an angle of ≥ 30°. Only 30.0% (9/30) of samples from which both gram-positive and negative bacteria were isolated had an angle of ≥ 30°.

Table 1 *Relationship between the class of bacteria isolated and identified and the angle of the distribution with the x-axis.*

a. The results of specimen with one way distribution in the bacterial channel scattergram anlyzed by UF-1000*i*

	Isolated bacterium							The angle of the distribution with the X-axis								No. of specimen with angle < 30° The ratio of gram-negative bacteria	
	EC	NFGNR	SA	EF	St	Cor	Not analyzable	<10°	10°-<20°	20°-<30°	30°-<35°	35°-<40°	40°-<50°	50°≦	Total		
Monomicrobial group	+						4	3	40	10			5	6	64	53	82.8%
		+						1	3						4	4	100%
			+				2			2		1	5	3	11	2	18.2%
				+			1		2		3	2	3	3	13	2	15.4%
					+				1		1	1			3	1	33.3%
						+							1		1		
Polymicrobial group	+	+							2						2	2	100%
	+		+						2		1				3	2	66.7%
	+			+					4	2	1	2		2	11	6	54.5%
	+				+		1		4	3					7	7	100%
	+					+			1						1	1	100%
	+		+	+			1		1	1			1		3	2	66.7%
	+		+		+				1						1	1	100%
			+							1					1	1	100%
		+		+							1				1		
		+			+					1					1	1	100%
		+	+		+						1				1		
		+	+		+								1		1		
No growth							31		3	2	1		3	2	11	6	54.5%
							Total 40								Total 140		

b. Summary of results

	Gram-negative bacteria		Gram-positive bacteria	Angle of the Scattergram※1		Total	
	Intestinal bacteria	NFGNR		30° >	30°≦		
Monomicrobial group			+	5	23	28	A
	+			53	11	64	B
		+		4		4	C
Polymicrobial group	+	+		2		2	D
	+		+	19	7	26	E
		+	+	2	2	4	F
			+		1	1	G
No growth				5	6	11	
Total				90	50	140	

※1 The angle of the distribution with X-axis in the bacterial channel scattergram anlyzed by UF-1000*i*

Gram-positive bacteria alone; 29 (A+G)
Gram-negative bacteria alone; 70 (B+C+D)
Gram-positive and gram-negative bacteria; 30 (E+F)
Multi bacteria+; 28 (D+E)

Of the 28 samples that showed bidirectional distribution of dots on the scattergram, 18 from which only gram-negative or only gram-positive bacteria were isolated are shown in ***Fig. 5-a*** and the remaining 10 samples from which both gram-negative and gram-positive bacteria were isolated are shown in ***Fig. 5-b***. The 18 samples of ***Fig. 5-a*** all showed bidirectional distribution with one angle of < 30° and the other angle of ≥ 40°. Out of the 10 samples shown in ***Fig. 5-b***, 9 had bidirectional distributions with one angle of < 30° and the other angle of ≥ 30°. The sample from which 10^7 cfu/mL *E. coli* and 10^4 cfu/mL *E. faecalis* was isolated, had the distribution angles of 18° and 28° respectively.

When we pool the results of samples with unidirectional and bidirectional distributions, gram-negative bacteria were isolated from 89.2% (81/91) of the samples that had a distribution angle of < 30° and gram-positive bacteria were isolated from 55.8% (43/77) of the samples with distribution angle ≥ 30°.

a. The results of specimen that isolated either gram-negative bacteria or gram-positivebacteria (n = 18)

	<10	<20	<30	40≦
≧50		8 (①①①① ①①②③)	5 (①①① ①④)	
<50		3 (①①③)	2 (①③)	
<40				
<30				

① *Enterobacteriaceae* alone: 13
② NFGNR alone: 1
③ *Enterobacteriaceae* and NFGNR: 3
④ *Enterococcus faecalis* alone: 1

b. The results of specimen that isolated both gram-negative bacteria and gram-positive bacteria (n = 10)

	<10	<20	<30	40≦
≧50		2 (②⑤)	2 (③⑤)	
<50		4 (①①④⑥)		
<40		1 (②)		
<30		1 (①)		

① *Enterobacteriaceae* and enterococci: 3
② *Enterobacteriaceae* and staphylococci: 2
③ *Enterobacteriaceae*, enterococci, and staphylococci: 1
④ *Enterobacteriaceae* and streptococci: 1
⑤ NFGNR and streptococci: 2
⑥ *Haemophilus parainfluenzae* and streptococci: 1

Fig. 5 *Distribution angles of specimen that showed bidirectional distribution of dots in the bacteria channel scattergrams*

3. The effect of piperacillin treatment on UF-1000*i* scattergrams of *E. coli* ATCC25922

Fig. 6 shows the UF-1000*i* scattergrams and images of gram-stained *E. coli* ATCC25922 cultured in Mueller Hinton broth and treated for 3 hours with piperacillin at different concentrations. The distribution angle of the scattergram was 17° when no drug or 1/2 of the minimum inhibitory concentration (MIC) of piperacilline was used. The patterns obtained with 1-8 MIC were almost identical, with the dots shifted to the region of high forward scattered light intensity, i.e., a large distribution angle. Microscopic examination of these treated samples at a magnification of 1,000 times revealed that the bacteria had retained their normal bacillus morphology up to a drug concentration of 1/2 MIC. At 1 MIC, the rods showed signs of filament formation, and at higher concentrations, filamentous bacterial cells were seen all over the field.

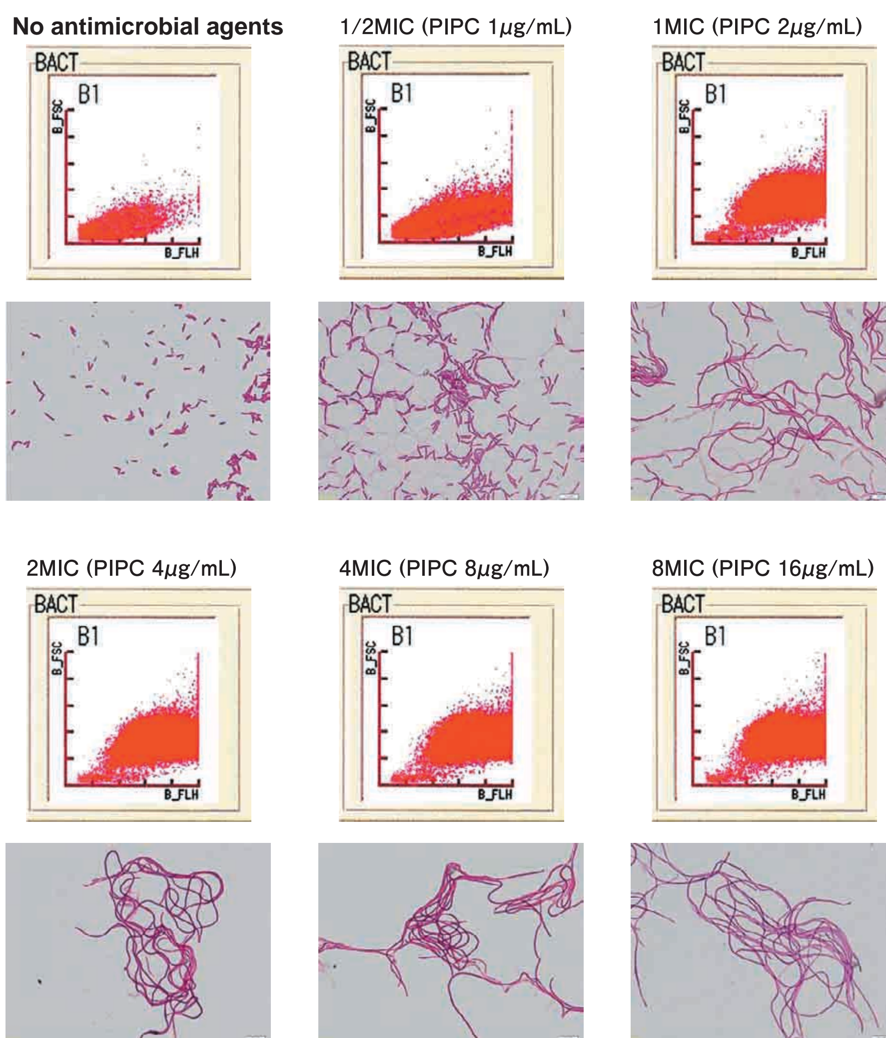


Fig. 6 *Scattergrams generated by the BACT channel after adding piperacillin (PIPC) to Escherichia coli ATCC25922*

The bacteria were cultured in Mueller Hinton broth. Three hours after adding the piperacillin they were gram-stained and also analyzed by UF-1000i.

DISCUSSION

In the present study, we came across some urine samples that were positive for the bacteria in UF-1000*i* analysis and microscopic observations on gram-stained samples, but were negative in culture tests. It is possible that these were obligate anaerobes or Gardnerella vaginalis and the like, which do not grow in the culture media in this study. It could also be the effect of administered antibiotics, etc because in the present study we did not check the clinical background, i.e., whether antibiotics had been administered to the patients, or whether there was any underlying disease. A similar reason could probably be behind the bacterial count being higher from UF-1000*i* than in the culture tests, but we cannot rule out factors like inadequate adjustment of the analyzer.

There have been reports in academic meetings, etc on the relationship between bacterial class and the angle of the distribution in the scattergram. Our present study showed that both gram-positive cocci and gram-positive rods tended to have high forward scattered light intensity and large angle of distribution, and gram-negative rods and gram-negative cocci tended to have low forward scatter and low angle of distribution. The theoretical reason for this difference is something to be found out in the future. However, the results suggest that a cut off value of 30° appears to be appropriate. There were 5 samples with a distribution angle of < 30° from which only gram-positive bacteria were isolated. These comprised one sample each with *Streptococcus agalactiae* (18°), *Enterococcus faecalis* (19°), *E. faecium* (20°), *S. aureus* (24°), and coagulase-negative staphylococci (28°). Most strains of E. faecalis cultured on brain heart infusion broth also showed distribution angles of < 30° (data not shown). Thus, the 30° cut off angle may not be applicable in all cases.

There were 11 samples with the distribution angle of ≥ 30° with only gram-negative bacteria isolated from them. Their angles were in the high range of 43-54°. The bacterial species isolated were *E. coli* alone from 6 samples, *E. coli* and *Citrobacter farmeri* from 1 sample, and *C. koseri, Klebsiella pneumoniae* and *K. oxytoca* from 1 sample each, and *C. koseri* and *Providencia rettgeri* from 1 sample. Besides these, there were 17 samples (***Fig. 5-a*** (1) to (3)) from which only gram-negative bacteria were isolated and which had a bidirectional distribution in their scattergrams with one distribution having an angle of < 30° and another ≥ 40°. From these results, the possible reasons for the scattergrams having large angles of distribution despite only gram-negative bacteria being isolated are the use of anti-gram-positive bacteria drugs because of which gram-positive bacteria could not be detected in the culture test although they were present in the samples, and the use of β-lactam, which has some antibacterial action, leading to filament formation in gram-negative rods.

We believe that further investigations with patients' urine samples are necessary with regard to the bacterial counts generated by UF-1000*i*. Nevertheless the UF-1000*i* data were at least correlated with the results of isolation culturing. Therefore, the former can be used as a guide in the diagnosis of UTI. As for the differentiation of gram-positive and gram-negative bacteria, with scattergram distribution angle cutoff of < 30°, the probability that the bacterium was gram-negative was as high as 89.2%. Among samples with an angle of ≥ 30°, gram-positive bacteria were isolated from only 55.8% of the samples. If we consider the fact that among the samples (with unidirectional or bidirectional distribution) from which only gram-positive bacteria were isolated, 83.3% (25/30) had the distribution angle of ≥ 30°, we can see that such UF-1000*i* scattergrams have the potential to be used as a rough guide for selecting antibiotics. The results of the present investigation suggest that gram-negative bacilli which formed filaments have a high distribution angle in the scattergrams. We might be able to do a more detailed evaluation through further investigations that the information on the administration of antibiotics such as those effective against gram-negative or gram-positive bacteria, etc. are taken into account.

Apart from the urine leukocyte count, gram-staining and microscopy of urine sediments should of course be the standard in the diagnosis of UTI. Gram-staining and microscopy is the basic technique for estimating the identity of bacterial species. However, we believe that there are a large number of medical facilities where gram-staining and microscopy cannot be done in real time. UF-1000*i*, which can provide fast and accurate urine leukocyte counts and bacterial counts in a short time and potentially differentiating the bacterial species, would be useful in such facilities. We look forward to further investigations along these lines.

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