The Basic Performance of Bacteria Counting for Diagnosis of Urinary Tract Infection Using the Fully Automated Urine Particle Analyzer UF-1000*i*

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Improvements in the quality and the rapidity of laboratory testing are getting more and more significant in accordance with laboratory automation in urinalysis. In 2005, the "Guidelines for clinical laboratory tests" were published by the Japanese Society of Laboratory Medicine.

The new instrument UF-1000i does not only provide automation of standard testing, but also assists with diagnosis in accordance with the above guidelines. We have evaluated the basic performance of bacteria counting and/or white blood cell counting especially for diagnosis of urinary tract infection (UTI). We conclude that the UF-1000i was useful for clinical diagnosis of UTI.

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Key Words UF-1000*i*, Flow Cytometry (FCM), Pyuria, Bacteriuria, Urinary Tract Infection (UTI)

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INTRODUCTION

Urinary tract infection (UTI) is a disease frequently experienced in clinical practice. Since the disease is painful and uncomfortable, rapid diagnosis and treatment are desirable. Recently, the concept of comprehensive medicine, aimed at standardization of tests and reduction of medical costs, has been introduced, and the "Guidelines for laboratory tests" to promote proper testing were issued. In these guidelines, the diagnosis of UTI is based upon clinical observations such as micturition pain and frequent urination, and the existence of significant amounts of pus in urine. Specifically, a white blood cell count (WBC) $\geq$ 10/μL by the chamber method using uncentrifuged urine or WBC $\geq$ 5 /400 $\times$ field by urinary sediment analysis and a bacterial count $\geq 10^4$CFU/mL by urinary culture should be demonstrated in urine samples.[1)]
To count bacteria in urine, a certain amount of urine is spread onto an agar medium plate for colony counting with a quantitative platinum loop and cultured overnight. Thus rapid diagnosis is currently impossible.[2)] Gram staining to demonstrate the existence of bacteria under a microscope is useful since it can omit culturing procedures and rapidly provide results about the presence or absence of pathogens, However, it has other problems such as low sensitivity in the case of a small number of bacteria in the sample.[2,3)] We had the opportunity to evaluate the performance of the fully automated urine particle analyzer UF-1000*i*, newly-developed by Sysmex Corporation (Kobe, Japan). The UF-1000*i* is a flow cytometer that stains cell components with a fluorescent dye and rapidly measures cells in urine such as erythrocytes, leukocytes and bacteria. It is notable that the channel exclusively used for bacterial detection of the UF-1000*i* allows accurate detection and counting of minute amounts of bacteria. Sysmex's unique fluorescent dye that binds to nucleic acid in bacteria is used as a staining solution. The reagent composition of the diluent was improved to suppress the effects of contaminants, including minute cell fragments and/or mucus components in urine that were difficult to be discriminated from minute bacteria in the past.
To evaluate the clinical usefulness of the UF-1000*i* in the rapid diagnosis of urinary tract infection, we conducted a basic study mainly for the evaluation of measuring performance with samples obtained from patients of the Urology Department of our hospital, and report the results.

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MEASUREMENT PRINCIPLE

As a basic principle of the UF-1000*i*, flow cytometry is used in the same manner as for the existing UF-100, UF-100*i* and UF-50.
The UF-100, so far the representative instrument of the UF-series uses an argon ion laser (wavelength λ=488nm) while the UF-1000*i* utilizes a red semiconductor laser (wavelength λ=635nm) to further reduce the size of the laser and to save electric power (***Fig. 1***). The UF-1000*i* has two sample modes, analyzing 1 and 6μL respectively, which can be used depending on the individual requirements on accuracy, reagent consumption and sample throughput. The urine aspirated through a sample probe is automatically mixed with the diluent and the staining solution at a specific ratio. The sample is then delivered to a flow cell using a sheath flow technique to ensure that a single-object stream passes through the flow cell. The laser beam is aimed in a direction perpendicular to the flow of the sample passing through the flow cell, and scattered light and fluorescence of individual formed elements are detected by light detectors. The scattered light is detected at two different positions (forward scattered light (FSC) and side scattered light (SSC)) by a photo diode and converted into electric signals. The fluorescence is likewise converted into an electric signal (FL). Waveform processing analysis is conducted on these electric signals by a dedicated signal processing circuit, and the characteristic parameters such as pulse-height and pulse-width of each formed element are stored internally. A two-dimensional scatter diagram of the combination of these parameters is automatically created.
Finally the scattergram is automatically analyzed using specific algorithms to classify and quantify the different particles of the urine.

MATERIALS AND METHODS

Materials

Reproducibility and linearity

(1) The following ATCC strains were used to demonstrate the performance of the detection of bacteria.
Reproducibility: 3 strains
- *Escherichia coli* (ATCC11775)
- *Staphylococcus aureus* (ATCC29213)
- *Enterococcus faecalis* (ATCC29212)

Linearity: 2 strains
- *E. coli* (ATCC11775)
- *S. aureus* (ATCC29213)

(2) To confirm the linearity of the leukocyte count, purulent urine was selected from urine samples obtained from patients of the Urology Department of Teikyo University Hospital to be used for evaluation.

Correlation

(1) 81 urine samples obtained from patients of the Urology Department were used to evaluate the correlation between the bacterial count by the UF-1000*i* and that by the quantitative culture.
7 samples, positive with Urotest (Merck Ltd., Tokyo, Japan), a test system for the detection of antimicrobial agents in urine, and negative in agar culture, were excluded from this evaluation. An accurate result of the culture method could not be expected in the presence of these substances.[4)]

(2) 128 urine samples obtained from patients of the Urology Department were used to evaluate the correlation between the leukocyte count by the UF-1000*i* and that by microscopy as a control method.

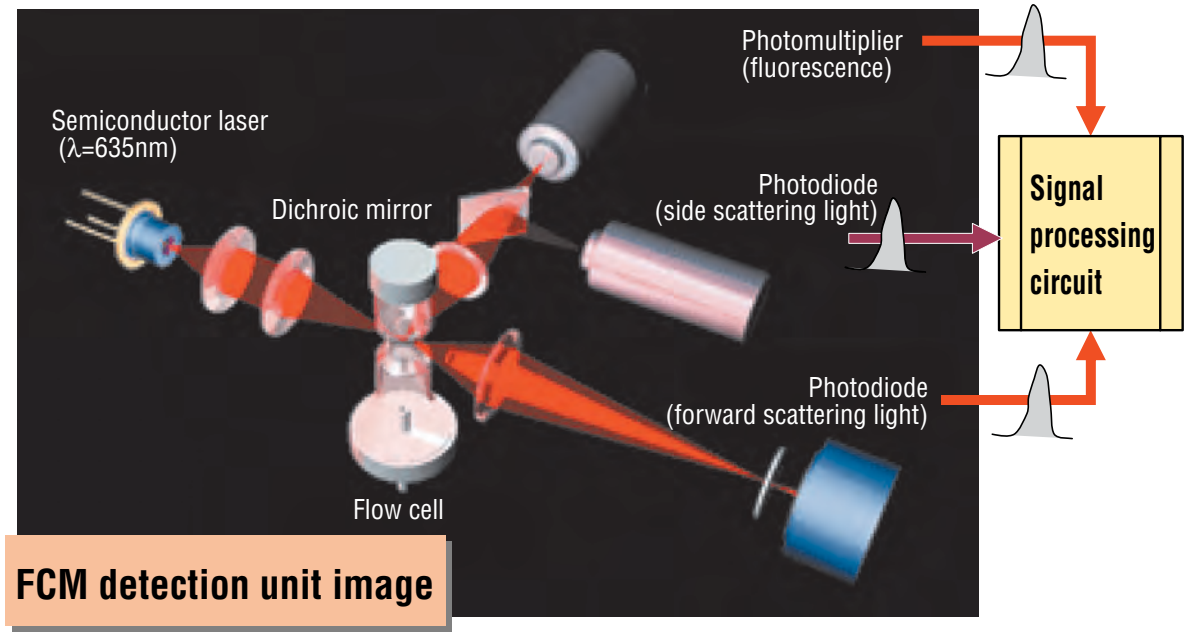


Fig. 1 *Flow cytometry technique using red semiconductor*

Methods

Comparison of within-run reproducibility of the UF-1000i and quantitative culture using ATCC strains

The ATCC strain was initially pre-cultured (35˚C, static culture) in heart infusion liquid medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) to be used as a concentrated bacterial stock solution (10^7 CFU/mL and above). Samples were prepared by diluting this stock solution with saline (0.9w/v% sodium chloride) (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) to the predetermined concentration (10^4-10^5 CFU/mL). The samples were measured 10 times, and mean value and standard deviation were obtained from the measurement results to calculate the CV (%).

The quantitative platinum loop method, a routinely-used quantitative cultural method, was employed as a comparison method. The bacterial suspension prepared as described above was preliminarily diluted with saline to a concentration that allows colony counting, and the diluted bacterial suspension was spread onto 10 different CLED agar medium plates (Nissui Pharmaceutical Co., Ltd. Tokyo, Japan) using a 1μL quantitative platinum loop, followed by overnight (about 18-24 hours, 35˚C) static culture. An effort was made to keep the spreading to the agar medium plates concurrent with the measurement by the UF-1000*i* in the 6μL analysis mode. On the following day, the colonies seen on the CLED medium were counted visually. The number of colonies and the volume of bacterial suspension used were converted into the actual bacterial count, and the mean value and standard deviation were obtained from the computed values of the 10 plates to calculate the CV (%).

Linearity of the bacteria count using ATCC strains and linearity of the leukocyte count using urine samples obtained from patients of the Urology Department

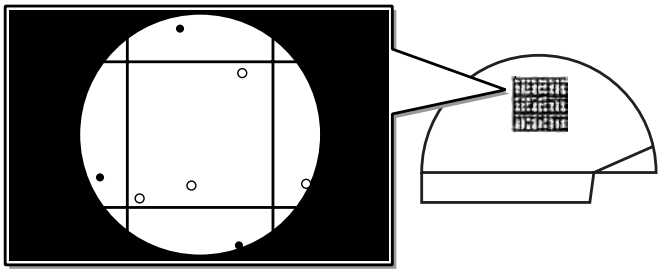
E. coli and *S. aureus* strains, pre-cultured in heart infusion liquid medium, were used as bacterial stock solutions (10^7-10^8 CFU/mL). These solutions were used to prepare a 6-stage series of 10-fold dilutions with saline (up to 10^5-fold dilution). The mean value of two measurements was used. The measurement result closest to the upper limit of the linearity range of the instrument (10^7/mL) was used as the reference point for the true value of the bacterial count.

To assess the linearity of the leukocyte count, purulent urine (approx. 2,000μL of leukocyte concentration) was selected from clinical samples to prepare a 2-fold dilution series with saline. The mean value of two measurements was used as a measurement result.

Correlation between the control method and the UF-1000i using urine samples

(1) Quantitative bacterial cultural method
CLED agar medium containing a migration inhibitor was used for quantitative culture. Dilution series were prepared in advance with saline using a micropipette (Eppendorf Co., Ltd. Hamburg, Germany) to make the colony count after sample spreading to be about 100-1,000. 100μL of each dilution was uniformly spread onto agar medium. Depending on the measurement results by the UF-1000*i*, two different concentrations of samples were spread. The culture conditions were 35˚C and 18-24 hours under aerobic conditions. The number of colonies on the agar plates was counted visually. The number of colonies obtained and the dilution ratio were converted to the bacterial count in the original urine sample.

(2) Visual measurement of the leukocyte count (KOVA direct method: chamber method using uncentrifuged urine)
The leukocyte count in uncentrifuged urine on a KOVA slide (Bayer Medical Ltd.) was used as the control method. As seen in ***Fig. 2***, chambers and compartments for estimation (grids) are incised on the KOVA slide. The volume of all these compartments for measurement is equal to 0.9μL. The number of particles counted and the number of the compartments, observed in a low or high power field of a microscope, can be converted to the number of particles per 1μL urine.[5] The values measured using this KOVA slide were correlated to the UF1000*i*. Microscopic observation was conducted without staining using a phase-contrast microscope (OLYMPUS BX-51; Olympus Corporation, Tokyo, Japan).



Chamber volume : 6.6μL
Area of large compartment : 3mm×3mm
Volume of large compartment : 0.9μL
Area of small compartment : 0.33mm×0.33mm

Fig. 2 *Large and small compartments of a KOVA slide*

(3) Measurement of blood cells and bacterial count by the UF-1000*i*

The UF-1000*i* is provided with special staining reagents for two analytical sub-systems, responsible for measurement of bacteria in urine and measurement of other particles such as erythrocytes or leukocytes. Using these special reagents improves the accuracy of leukocyte and bacteria detection in urine compared to conventional instruments. Measurement of blood cells and measurement of bacteria in urine are conducted by a single aspiration of the urine sample.

First, for the measurement of blood cells, about 1 mL of urine sample is aspirated through a probe and 435µL of diluent and 15µL of stain solution are automatically added to and mixed with 150µL of urine in a reaction chamber whose temperature is maintained at about 35˚C to stain the cells in the urine with a fluorescent dye. In the flow cytometry chamber, the particles are exposed to a laser beam. The scattered light signals and fluorescent signals obtained at that time are used as parameters to reveal the characteristics of each particle for classification and counting.

Next, in the measurement of bacteria in urine, 62.5µL of urine sample, aspirated as described above, are mixed with 425µL of diluent in another reaction chamber whose temperature is maintained at about 42˚C. Subsequently 12.5µL of staining solution is automatically added to and mixed with this. The special diluent increases the permeability of the bacterial cell membrane and thus facilitates the specific staining of the nucleic acids in the bacteria with a fluorescent dye (***Fig. 3***). The stained sample is delivered to the detection unit to detect scattered light signals and fluorescent signals by flow cytometry, followed by counting of the bacteria using special algorithms.

RESULTS

Reproducibility

Tests for three different bacterial strains were conducted: One each for *S.aureus* and *E.coli* and two independent experiments for *E.faecalis*. Results in the 6µL analysis mode were compared to the 1µL quantitative platinum loop method. CVs for the UF-1000*i* ranged from 7.6% (*E.coli*) to 11.2% (*S.aureus*). CVs for the comparison method proved much more variable, even using the same strain, with results for *E.faecalis* of 27.8% in one experiment and 62.6% in the other. Results for the other strains could be found between those values (***Table 1***). The 1µL analysis mode was evaluated for *S.aureus* and in one experiment using *E.faecalis*, with results (25% and 32% respectively) inferior to the 6µL mode, but superior to the comparison method in the respective experiment.

Linearity

The linearity of bacteria counts within the range of 10^3-10^7/mL could be confirmed using both *E.coli* and *S.aureus* (***Table 2***, ***Fig. 4-1, 4-2***). For leukocyte counts, linearity in the range of 3-2,500/µL was demonstrated (***Fig. 4-3, 4-4***).

Correlation

Using 74 urine samples the concordance of the bacterial count with the UF-1000*i* and the quantitative bacterial cultural method was investigated by a concordance table. Using a cutoff value of 104 CFU/mL, the sensitivity was 96.4% (27/28), specificity 89.1% (41/46), concordance rate 91.9% (68/74), PPV 84.4% (27/32) and NPV 97.6% (41/42) (***Table 3***). The correlation diagram between the quantitative cultural method and the UF-1000*i* is shown in ***Fig. 5-1***.

The correlation equation against the comparison method for the leukocyte count (KOVA direct method) was y=1.01x+8.88 with r=0.9592 at n=128 (***Fig. 5-2***).

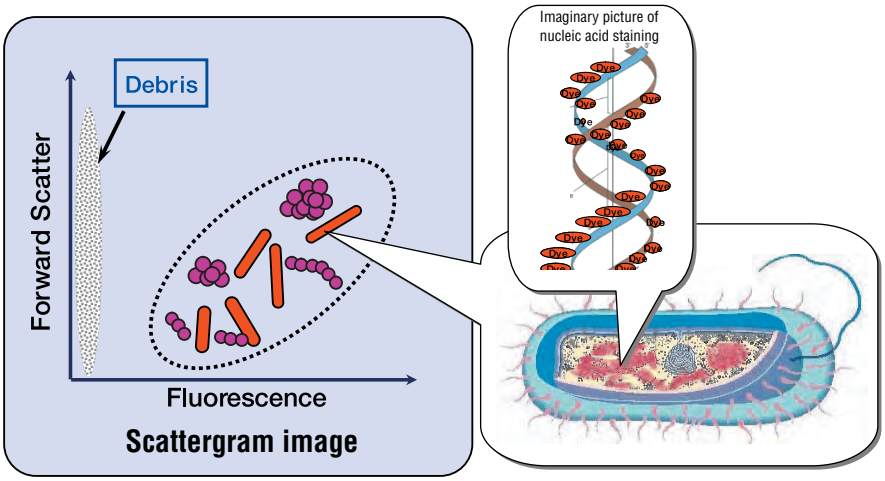


Fig. 3 *Bacterial stain image and scattergram by UF-1000i*

Table 1 *Within-run reproducibility of the control method (cultural method) and UF-1000i in bacterial measurement*

	E.coli **(ATCC11775)**		***S.aureus*** **(ATCC29213)**			***E.faecalis*** **(ATCC29212)(1)**			***E.faecalis*** **(ATCC29212)(2)**	
Strain	Culture 1μL Loop	UF-1000*i* 6μL analysis mode	Culture 1μL Loop	UF-1000*i* 1μL analysis mode	UF-1000*i* 6μ L analysis mode	Culture 1μL Loop	UF-1000*i* 1μL analysis mode	UF-1000*i* 6μL analysis mode	Culture 1μL Loop	UF-1000*i* 6μL analysis mode
AVE.(/mL)	1.2×10^4	1.6×10^4	1.2×10^5	4.8×10^4	5.2×10^4	6.3×10^3	1.4×10^4	1.4×10^4	2.5×10^4	1.2×10^4
SD (/mL)	6.0×10^3	1.2×10^3	3.7×10^4	1.2×10^4	5.8×10^3	3.9×10^3	4.5×10^3	1.2×10^3	6.9×10^3	1.0×10^3
CV(%)	50.8%	7.6%	30.4%	25.3%	11.2%	62.6%	32.1%	8.4%	27.8%	8.9%

Table 2 *Linearity of bacterial measurement*

E.coli **(ATCC11775) [/mL]**			***S.aureus*** **(ATCC29213) [/mL]**		
Theoretical value	6μL analysis	1μL analysis	Theoretical value	6μL analysis	1μL analysis
6.9×10^2	1.3×10^3	2.5×10^3	1.1×10^3	1.4×10^3	1.0×10^3
6.9×10^3	5.5×10^3	6.0×10^3	1.1×10^4	1.1×10^4	1.5×10^4
6.9×10^4	6.7×10^4	6.2×10^4	1.1×10^5	1.0×10^5	0.9×10^5
6.9×10^5	6.5×10^5	6.4×10^5	1.1×10^6	1.1×10^6	1.0×10^6
6.9×10^6	6.9×10^6	6.7×10^6	1.1×10^7	1.1×10^7	1.1×10^7
6.9×10^7	5.7×10^7	5.5×10^7	1.1×10^8	7.8×10^7	7.6×10^7

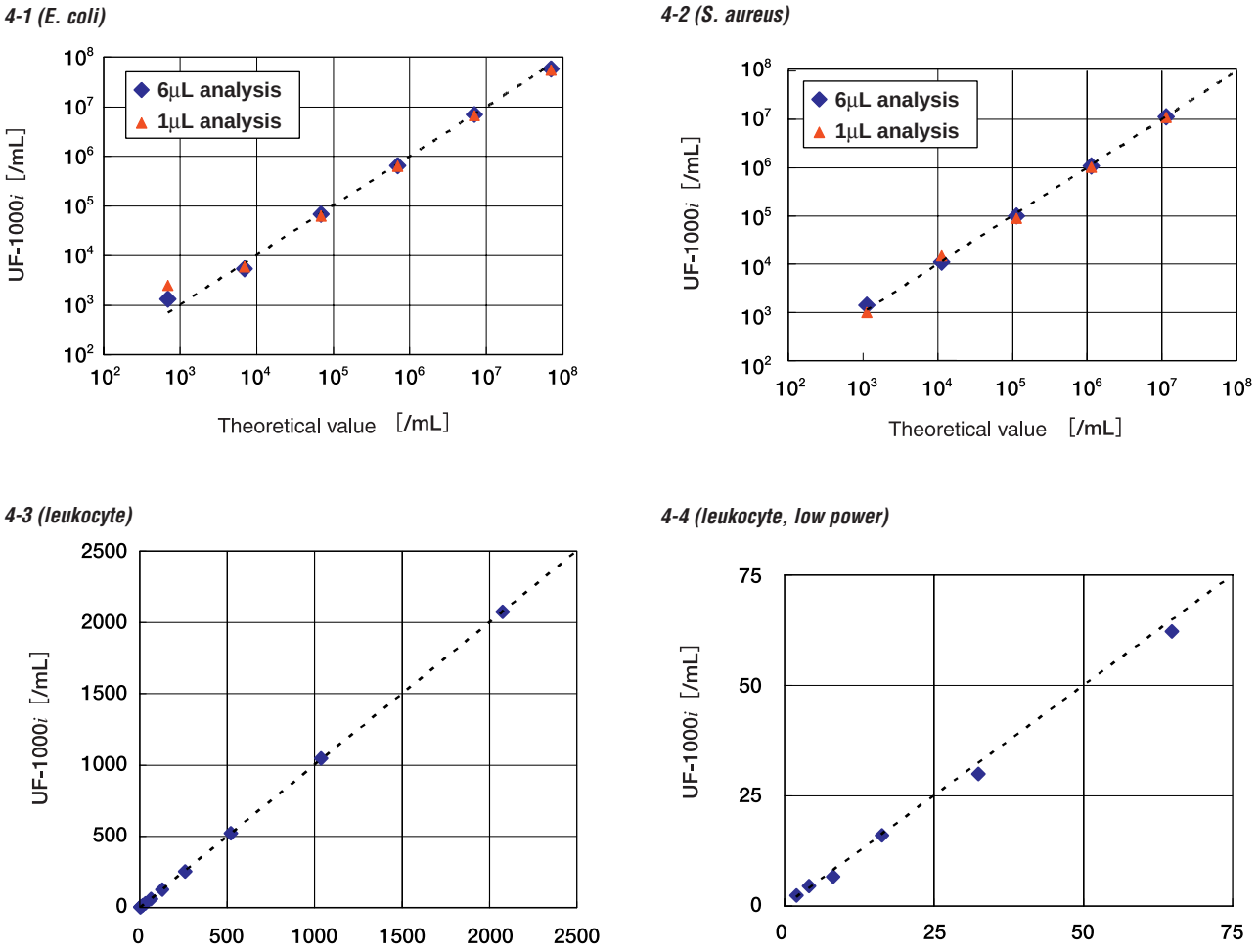


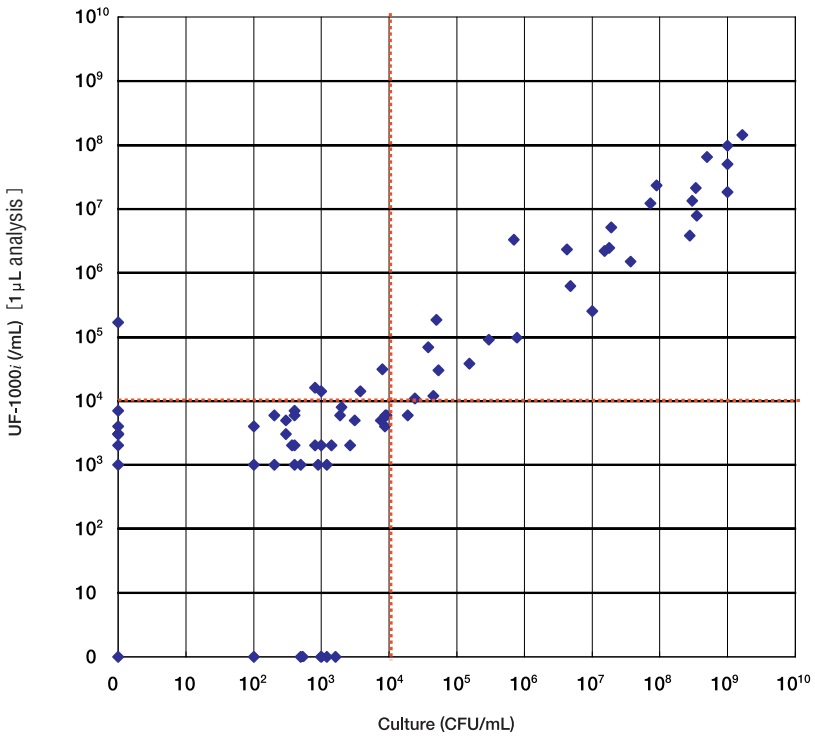
Fig. 4 *Linearity*

Table 3 *Evaluation results of bacterial detection by 2 x 2 Table*

		Reference culture		
		Positive	Negative	Total
UF-1000*i*	Positive	27	5	32
	Negative	1	41	42
	Total	28	46	74

Criteria for positive judgment Reference culture : 10^4CFU/mL, UF-1000*i* : 10^4/mL

5-1 (bacteria)



5-2 (leukocyte)

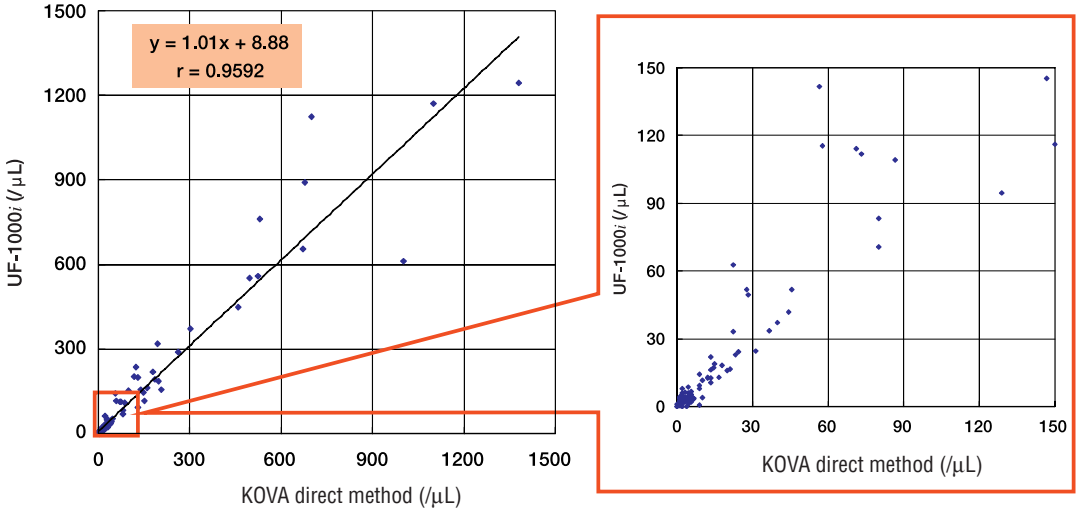


Fig. 5 *Correlation diagram*

DISCUSSION

In the study on reproducibility, the quantitative platinum loop method was considered to have several confounding factors. The first potential factor is variation in sample volumes when samples are collected with a quantitative platinum loop, the second possible factor is handling variations in sample spreading on the agar medium. Care must thus be taken to exclude these factors as much as possible especially for detection of low concentrations of bacteria. In the measurement system used by the UF-1000*i*, all the operations from aspiration to dilution, staining and measurement of samples are automated and thus human error factors can be largely excluded. In addition, the use of an optional sampler can automate even the sample feed.

As it was the intent of this study to focus on the performance of the bacterial count, the full evaluation of the reproducibility of leukocyte counts is excluded here. However, the within-run reproducibility of the UF-1000*i* on a sample in which a low concentration of leukocytes (about 10/μL) appeared indicated a favorable result (CV=about 15%), demonstrating precise measurement against the comparison method with KOVA slides.[6)]

The results of the correlation study showed that the UF-1000*i* could accurately detect bacteria even at low concentrations (in the 10^3 CFU/mL range). However, there was a sample group (n=9) in which the presence of bacteria was indicated by the UF-1000*i* among cases in which no bacterial growth was detected by the quantitative cultural method. Both the UF-1000*i* and the comparison method are considered to be responsible for the divergence: The first factor for divergent results is detection and counting of viable and dead bacteria. Due to the characteristic of the reagent, the UF-1000*i* counts all the bacteria included in urine independent of their viability. However, only viable cells form colonies on agar medium in the comparison method. The second confounding factor may be so-called VBNC (viable but non-culturable) conditions under which the bacteria used in the study cannot grow on agar.[7)] The actual cause of the divergence of some samples in this study remains open. In future, in order to characterize samples with a divergence towards the comparison method in the bacterial count, other procedures to confirm the presence of bacteria, such as gram staining, will have to be used in addition. Furthermore, it will be essential to critically assess the samples used both in future evaluations and actual clinical use concerning viability of bacteria. Thus, for patient samples, clinical information such as treatment history is important to recognize bias due to bacteria already having succumbed to antibiotic treatment.

CONCLUSIONS

The results of the basic study showed that the UF-1000*i* had a higher reproducibility than visual microscopy and quantitative culture. Bacterial count performance was satisfactory in the range of 10^3-10^7/mL. In addition, the leukocyte count had a better performance than visual microscopy. For the bacterial count, problems such as the degree of reproducibility of the cultural method itself were identified, but generally good correlation with the cultural method was obtained in the performance of overall bacterial counting in urine.

Rapid counting of leukocytes and bacteria by the UF-1000*i* is useful for diagnosis of UTI and may prove suitable for monitoring of antibiotic treatment.

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