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Original Article

Flow Cytometric Analysis of Viable Bacteria in Urine Samples of Febrile Patients at the Emergency Department

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Background: Fast and reliable diagnostics are important in febrile patients admitted to the emergency department. Current urine diagnostics are fast but moderately reliable or reliable but time consuming. Flow cytometry (FC) is a new promising technique in the diagnostics of complicated urinary tract infections by counting bacteria in urine samples. The aim of this study is to improve the FC method by counting only viable bacteria.

Methods: Urine was obtained from 135 consecutive febrile patients at the emergency department. According to protocol regular diagnostic urine tests were performed. In addition, FC counting of viable and non-viable bacteria was executed after staining with thiazole orange and propidium iodide. All test results were compared to the results of urine culture ($\geq 10^5$ colony forming units/mL).

Results: At a cut-off value of 2.01×10^5 viable bacteria/mL the sensitivity was 100% and specificity was 78.4% (AUC-value 0.955 on ROC-curve). Spearman correlation test exhibited a higher correlation for flow cytometric counting of only viable bacteria than counting of all bacteria (0.59 vs. 0.37). Using ROC-curves, the AUC-values for FC counting of all bacteria, only viable bacteria and Gram staining were respectively 0.935, 0.955, and 0.968 ($P > 0.05$).

Conclusion: FC counting of only viable bacteria can predict quickly and reliably positive and negative urine cultures in febrile patients admitted to the emergency department. It can help to improve the speed and accuracy of the diagnostic procedure at the emergency department. © 2017 Clinical Cytometry Society

Key terms: flow cytometry; viability; bacteria; fever; urinary tract infection

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INTRODUCTION

Urinary tract infection (UTI) is one of the most common bacterial infections and a frequent cause for hospitalization in febrile or complicated cases (1). Symptoms of urinary tract infections can present in variable and nonspecific ways, especially in the elderly. Apart from fever, characteristic symptoms of complicated urinary tract infection such as dysuria and flank pain are not always present. Early goal-directed treatment of septic febrile patients requires fast and accurate urine analysis. If complicated urinary infection has been demonstrated, appropriate empirical antibiotic treatment can be started

Abbreviations: CFU, colony forming unit; CRP, C-reactive protein; ED, emergency department; FC, flow cytometry; NPV, negative predictive value; PI, propidium iodide; PPV, positive predictive value; ROC, receiver operating curve; SG, Sybr Green; SIRS, systemic inflammatory response syndrome; TO, thiazole orange; UTI, urinary tract infection

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quickly after admission to the emergency department (ED) which can be life saving (2).

Different techniques exist to predict the presence or absence of urinary tract infection. Current diagnostic methods are fast but moderately reliable, or reliable but time consuming. The dipstick test is fast and has a high specificity; it is able to rule in UTIs. However, due to a limited sensitivity, the dipstick test is less suitable to rule out UTIs. Microscopic examination of the urine sediment is only moderately reliable. For executing urine sediments, analysts have to be trained but the results have a high inter-individual variation (3). The Gram staining is a more reliable test but time consuming and also labor-intensive (4–9). The golden standard for urinary tract infection remains the urine culture but the results of this test are only available after at least 24 to 48 h.

FC is a new promising and fast diagnostic method in determining urinary tract infections (10,11). Previous research showed that counting bacteria in urine samples by FC is more reliable than counting leukocytes or bacteria in the urine sediment to predict positive urine cultures and showed a high sensitivity of 81–97% and a specificity of 78–94% (12–15). In our previous study, FC is able to predict negative and positive urine cultures at different cut-off levels with a high predictive value (10). However, the total number of bacteria includes both non-viable and viable bacteria. Today, distinction between non-viable and viable bacteria can be made using thiazole orange and propidium iodide during the FC analysis (16–18). Because only viable bacteria are able to grow and to form colonies in urine culture, we hypothesized that counting viable bacteria by FC is more reliable than counting the total number of bacteria. Therefore, we investigated the reliability of counting viable bacteria by FC to predict the outcome of urinary culture. We investigated this in a specific population of febrile patients admitted to the ED because fast and accurate diagnostic procedures are important in this patient category.

MATERIAL AND METHODS

Patients and Samples

This prospective cohort research was performed at Isala hospital Zwolle, one of the largest nonacademic hospitals in the Netherlands (1,116 clinical beds). Hundred-thirty-five consecutive adult patients admitted to the ED with a temperature $\geq$38.0°C in the last 24 h measured at home or at the emergency department, or$\geq$2 SIRS (systemic inflammatory response syndrome)-criteria and suspected infection, were included. SIRS was defined by the presence of at least 2 of the following symptoms: body temperature >38.5°C, heart rate > 90 beats/minute, respiratory rate > 20 breaths/minute, an arterial partial pressure or carbon dioxide < 4.3 kPa or white blood cell count $> 12 \times 10^9$ cells/L. Patients with a urinary catheter were excluded. The inclusion period started at 1 December 2014 and ended on 25 February 2015 (12 weeks). The medical ethics committee of our hospital gave approval for the execution of this study.

Patient characteristics were obtained from electronic patient files. Gender, age, and presence of immune comprised status, chemotherapy <3 months, diabetes mellitus, use of antibiotics, signs and symptoms, temperature, heart rate, frequency of breathing, blood pressure, and hemoglobin, leucocytes and C-reactive protein measurements in blood, were noted. ED nurses collected urine of each patient and divided it in three samples: one sample for the dipstick test and urine sediment, one sample for the Gram staining and urine culture, and one sample for flow cytometric analysis.

The dipstick test was performed with the Aution Max (AX-4280 Menarini Diagnostics ARKRAY Benelux, Valkenswaard). For the urine sediment, 10 mL urine was centrifuged at 425 rpm during 3 minutes and supernatant was removed and the sediment was microscopic investigated by an enlargement of 40x10 (Nikon Eclipse E400) after addition of a droplet Kova Stain. The Gram staining was performed by fixating 10 μL of urine using methanol, adding crystal violet and fuchsia, and analyzing microscopically with an enlargement of 10x100 (Axioskop, Zeiss, Germany). Gram staining results are expressed semi-quantitatively (as +/++/+++) as observed by trained analysts based on numbers per field of view. Urine cultures were performed using standard laboratory protocols. Ten μl urine was placed on two different Agars (a chromogenic agar and a sheep blood agar) and growth was determined after overnight incubation at 37°C by semi quantitatively counting of CFU/mL per isolated organism. A specimen that grew $\geq 10^5$ CFU/mL of one or two uropathogens was defined as a positive urine culture, the internationally accepted golden standard for the presence of urinary tract infection.

All urine samples were also analyzed by the flowcytometer Accuri C6 (BD Biosciences, USA). The instrument was validated and maintained daily according to the user manual. The Accuri C6 is equipped with two excitation lasers: a blue solid state (488 nm) and a diode red (640 nm) providing up to six simultaneous detection parameters, including 4 fluorescent colors plus forward scatter (FSC) and side scatter (SSC). The accuracy of the bacterial counting by the Accuri C6 was validated described earlier (10). Ten mL staining buffer was added to 5 mL urine and centrifuged at 5,000 rpm for 5 minutes. Staining buffer consisted of a solution of Phosphate Buffered Saline (PBS), 0.01% Tween-20 and 1 mmol EDTA. The residue was resuspended with 5 mL of staining buffer at room temperature. For counting of the total number of bacteria 500 μL 100× diluted urine solution (5 μL urine + 495 μL staining buffer) was added to 500 μL of “prediluted” (30.000×) Sybr Green (SG, Life Technologies, Invitrogen) and incubated at 37°C during 10 minutes. SG has an excitation/emission maxima at 494 and 521 nm and binds to DNA of bacteria and can therefore be used to count bacteria. Next, a

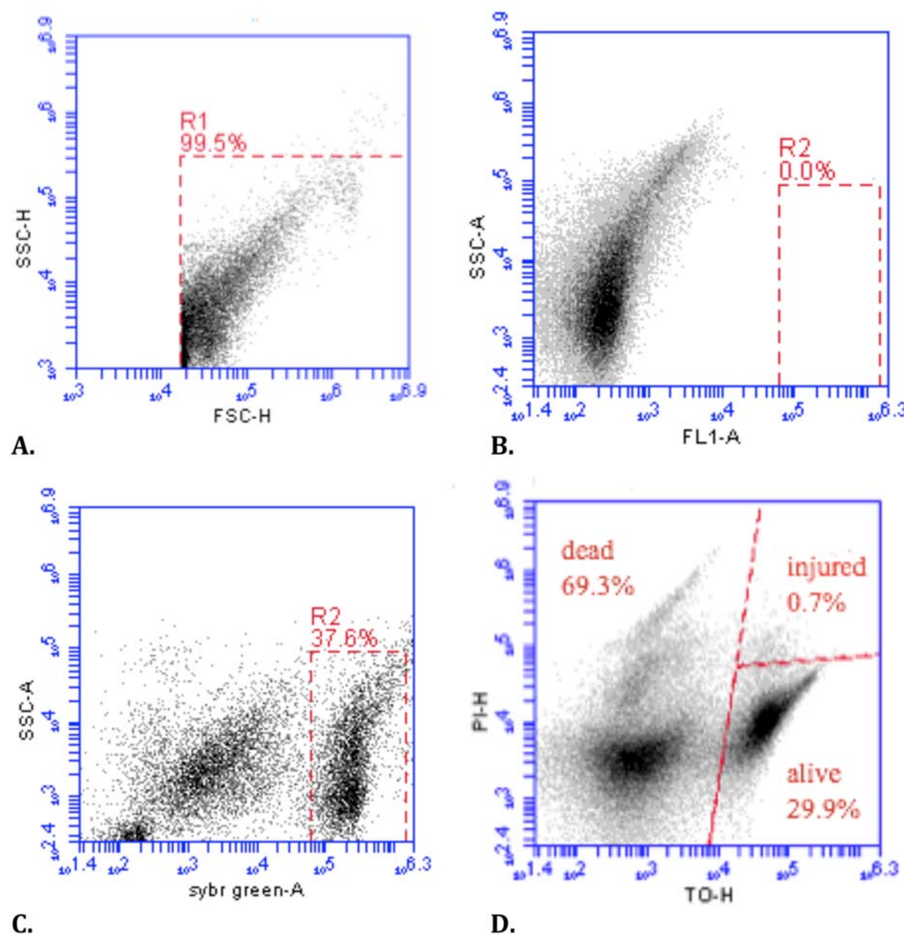

FIG. 1. Representative example of FC bacterial counting. A-C: Representative example of FC bacterial counting using SG. (A) FSC vs.SSC dotplot of bacteria detected in a urine sample as descripted in methods (gate R1). (B) FL1 vs.SSC dotplot of an unstained urine sample (negative control) for SG staining. Gate R2 shows the background staining. (C) SG vs.SSC dotplot of a SG-stained urine sample. Gate R2 shows the SG stained bacteria in this sample. (D) Representative FL1 vs.FL3 dotplot of bacteria stained with TO and PI to detect the viable bacteria. Regions set around different bacteria populations: live (TO+/PI-, 29.9%) and dead (TO-/PI±, 69.3%) bacteria. The TO and PI dye combination provides significant resolution between live and dead cell populations. An intermediate or injured population (TO+/PI+, 0.7%) can often be observed between the live and dead population. [Color figure can be viewed at wileyonlinelibrary.com]

fixed volume (50 μL) of the urine SG-stained bacterial cells was analyzed on the flow cytometer during 5 minutes using low sample rate and a selected threshold setting on FSC and SSC to discriminate bacterial cells from relatively large particles. A representative example is shown in Figure 1A–C. The instrument threshold defines the minimum scatter needed to trigger an event that will be processed by the system software. It allows not only to reduce the electronic background noise, but also to get rid of the unwanted nontarget particles. Particles with low fluorescence and low SSC have a greater potential to interfere with the actual determination of the bacterial density, but these can easily be separated in the SG fluorescence plot as they do not stain with SG.

For the determination of viable and non-viable bacteria 5 μL thiazole orange (TO, 42 μmol/L, BD Biosciences) and 5 μL propidium iodide (PI, 4.3 mmol/L, BD Biosciences) were added to 500 μL diluted urine solution and incubated at room temperature during 5 minutes. Live cells have intact membranes and are impermeable to dyes such as PI, which only leaks into cells with compromised membranes and binds the DNA. TO is a permeable dye and enters all cells, viable and dead, to varying degrees. TO and PI have an excitation/emission maxima at 510/530 and 536/617 nm respectively and can be detected in FL1 and FL3 fluorescence channels respectively. Thus a combination of these two dyes provides a rapid and reliable method for discriminating live and dead bacteria (19).

Next, a fixed volume (50 μL) of the urine TO/PI-stained bacterial cells was analyzed on the flow cytometer during 5 minutes using low sample rate and a selected threshold setting on FSC and SSC to detect the number of viable bacteria. A representative example is shown in Figure 1D. Considering the dilution steps in the process absolute numbers of bacteria in both methods were calculated. Before every measurement with

Table 1
Descriptive Statistics of the Study Population, Binominal Variables

Characteristic	Number (*n*)	Percentage (%)
Gender:		
Male	69	51.1
Female	66	48.9
Immunocompromised	49	36.3
Chemotherapy < 3 months	19	14.1
Diabetes mellitus	34	25.2
Use of antibiotics	38	28.1
Dysuria	28	20.7
Hematuria	7	5.2
Fever at emergency department	104	77.0
SIRS	94	69.6
Fever and SIRS	82	60.7
Fever within 24 h before admission to emergency department	19	14.1

Immunocompromised: patients with cancer and use of immunosuppressive drugs; Use of antibiotics in the last 3 days; Fever: body temperature of >38.5°C; SIRS: systemic inflammatory response syndrome.

the flow cytometer, samples were vibrated for an equal distribution of bacteria in the fluid. After each measurement the aspiration needle was flushed to prevent contamination.

Statistics

The statistical analysis was performed using SPSS version 22.0 for Windows (IBM SPSS Statistics, IBM Corporation, Armonk, NY). All variables were nonparametric distributed and presented by a median and a 25- and 75-percentile. Based on 2 × 2 cross tabs, sensitivity, specificity, positive and negative predictive values (PPV and NPV), were calculated using urine culture ($\geq 10^5$ CFU/mL) as golden standard. Receiver operating curves (ROC-curves) were performed with the related "area under the curve" values (AUC-values). The optimal cut-off point was determined using the Youden-index. The Spearman correlation test was used to correlate the results of the total number of bacteria and the number of viable bacteria to the results of the urine culture. A significant test was defined as a P values <0.05.

RESULTS

Patient Characteristics

At the emergency department 166 patients were considered for eligibility. Due to lack of fever or absence of SIRS-criteria, an indwelling catheter or failure to perform a urine culture, 31 patients were not analyzed. The statistical analysis was performed on 135 patients. The demographic data are shown in Tables 1 and 2.

Slightly more males (51.1%) than females were included with a median age of 69 years. Eleven patients complained of dysuria (8.1%), 6 patients of flank pain (4.4%) and 10 patients of frequent urination (7.4%). Seventy-seven per cent of patients had fever at the emergency department with a median temperature of 38.6°C (IQR 38.1–39.2°C). The median CRP was 60 mg/L (IQR 24–140 mg/L).

Nineteen patients had a positive urine culture. *Escherichia coli* was the most frequent dominant cultured species (10 cultures, 52.6%). Identical bacteria were seen in 7 blood and urine cultures.

Thirty-eight patients (28.1%) were using antibiotics at presentation at the emergency department. Thirty-three out of these 38 patients had negative urine cultures. Of 5 out of 38 patients with positive urine cultures, 2 patients were treated with antibiotics which the bacteria was resistant for, 2 patients were treated with antibiotics suitable for cystitis and not for complicated urinary tract infections, 1 patient started a suitable antibiotic treatment only a few hours before visiting the emergency department.

Evaluation of Diagnostic Tests

The nitrite in the dipstick test was performed in all patients and had a sensitivity of 21.1% (4 out of 19) and specificity of 99.1% (115 out of 116; Table 3). Urine sediment was performed in 125 of 135 patients. In 10 patients the urine sediment was lacking due to logistic reasons or an insufficient quantity of urine. Urine sediment had a sensitivity of 100% (11 out of 11) with a NPV of 100% (10 out of 10) at a cut-off value of ≥1+ bacteria, and a specificity of 100% (63 out of 63) with a PPV of 100% (4 out of 4) at a cut-off value of ≥3+ bacteria. The Gram staining was performed in all patients. Bacteria in Gram staining were found in 64 patients with a sensitivity of 100% (19 out of 19) at a cut-off value ≥ 1+ bacteria and a specificity of 98.3% (113 out of 115) at a cut-off value of ≥ 3+ bacteria. In 26 urine samples Gram-positive bacteria were found, in 18 samples Gram-negative bacteria and in 20 samples both Gram-negative and -positive bacteria were found.

Results of Flow Cytometry

FC analysis was performed in all patients. The number of total bacteria varied between 1.2×10^4 and 4.2×10^8 bacteria/mL, the number of only viable bacteria varied between 2.2×10^2 and 1.8×10^8 bacteria/mL. At

Table 2
Descriptive Statistics of the Study Population, Nonparametric Variables

Characteristic	Median	25th–75th percentile
Age (years)	69	59–78
Temperature (°C)	38.6	38.1–39.2
Heart rate (bpm)	100	87–113
Respiratory rate (per minute)	20	17–25
Systolic blood pressure (mmHg)	127	111–140
Diastolic blood pressure (mmHg)	72	63–80
Hemoglobin (mmol/L)	8,1	7.3–8.9
Leucocytes ($\times 10^9$/L)	10.3	6.6–14.4
CRP (mg/L)	60	24–140

CRP: C-reactive protein.

Table 3
Sensitivity, Specificity, Positive and Negative Predictive Value with Their Fractions are Shown of Dipstick Nitrite, Urine Sediment, and Gram Staining Compared to the Results of Urine Culture (≥ 10^5 CFU/mL)

Test with cut-off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Dipstick nitrite	21,1 (4/19)	99,1 (115/116)	80,0 (4/5)	88,5 (115/130)
Urine sediment Bacteria ≥ 1+	100 (11/11)	15,9 (10/63)	17,2 (11/64)	100 (10/10)
Urine sediment Bacteria ≥ 2+	81,8 (9/11)	85,7 (54/63)	50,0 (9/18)	96,4 (54/56)
Urine sediment Bacteria ≥ 3+	36,4 (4/11)	100 (63/63)	100 (4/4)	90,0 (63/70)
Gram staining Bacteria ≥ trace	100 (19/19)	60,9 (70/115)	29,7 (19/64)	100 (70/70)
Gram staining Bacteria ≥ 1+	100 (19/19)	86,1 (99/115)	54,3 (19/35)	100 (99/99)
Gram staining Bacteria ≥ 2+	78,9 (15/19)	93,0 (107/115)	65,2 (15/23)	96,4 (107/111)
Gram staining Bacteria ≥ 3+	68,4 (13/19)	98,3 (113/115)	86,7 (13/15)	95,0 (113/119)

PPV: Positive Predictive Value; NPV: Negative Predictive Value.
The result for urine sediment and Gram staining were sorted for different cut-off values.

cut-off value 1.0×10^4 total bacteria/mL and at cut-off value 1.0×10^5 viable bacteria/mL we found both a NPV of 100% (respectively 84 out of 84; 50 out of 50). At cut-off value 1.0×10^8 total bacteria/mL and cut-off value 1.0×10^8 viable bacteria/mL the PPV was both 100% (3 out of 3).

Using the Spearman correlation test the results of urine culture were compared to the results of FC counting of bacteria and the counting of only viable bacteria. FC counting of only viable bacteria showed a higher correlation coefficient (0.59) than FC counting of all bacteria (0.37).

The diagnostic value was analyzed with a ROC-curve and optimal cut-off values with highest sensitivity and specificity were calculated based on the golden standard. Counting only viable bacteria by FC showed an AUC-value of 0.955 with a sensitivity of 100% (19 out of 19) and specificity of 78.4% (91 out of 116) at an optimal cut-off value of 2.01×10^5/mL. Counting of total (viable and non-viable) bacteria by FC had an AUC-value of 0,935 and the optimal cut-off value was set on 4.79×10^6 bacteria/mL with a sensitivity of 89.5% (17 out of 19) and a specificity of 83.6% (97 out of 116) (Fig. 2 and Table 4). Semi quantitative counting of bacteria of Gram stained urine samples showed an AUC-value of 0.968. However, the 95%-confidence intervals of AUC values of abovementioned tests were overlapping and showing the results were not significantly different (Fig. 2). Also the 95%-confidence intervals of the ROC-curves specified for use of antibiotics are overlapping, so use of antibiotics did not have a significant impact on the FC method in our study population (Table 4).

DISCUSSION

Fast and reliable diagnostic procedures are required at the ED in febrile patients. In this study the diagnostic value of FC counting of viable bacteria in the demonstration of complicated urinary tract infections was investigated and compared to the other current diagnostic techniques. Our results showed that FC counting of only viable bacteria could predict quickly and reliably positive and negative urine cultures in febrile patients admitted to the emergency department. This can be of clinically importance for the speed and accuracy of the diagnostic process at the ED.

Although this study was done in a small study population, the results of dipstick, urine sediment and Gram staining were in accordance to the literature (3,7,20–22). In literature FC showed a sensitivity of 73.0–97.0% and specificity of 61.8–94% to predict positive urine cultures (5,12–15), our results were in accordance (sensitivity 89.5%, specificity 81.9%).

This study was performed to show that FC counting could be of relevance in the diagnostics of febrile patients to diagnose urinary tract infections. The study results seem to be similar to the results in literature.

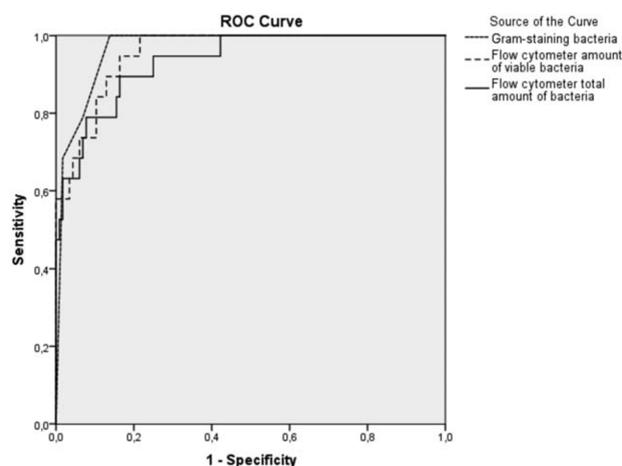

Fig. 2. ROC-curve of Gram staining and FC analysis of total number of bacteria and number of viable bacteria using urine culture (≥ 10^5 CFU/mL) as golden standard ($n = 135$). The curves are overlapping which indicates no superiority of one particular test ($P > 0.05$).

Table 4
The "Area Under the Curve"-Values are shown for the Total Number of Bacteria by FC, Number of Viable Bacteria by FC (Quantitative) and Number of Bacteria after Gram Staining (Semi-Quantitative), and all Values are also specified for Use of Antibiotics

Diagnostic test	AUC	95%-confidence interval	AUC—use of antibiotics	95%-confidence interval—use of antibiotics	AUC—no use of antibiotics	95%-confidence interval—no use of antibiotics
FC total amount of bacteria	0.935	0.881–0.988	0.933	0.847–1.000	0.940	0.874–1.000
FC amount of viable bacteria	0.955	0.920–0.991	0.952	0.878–1.000	0.954	0.910–0.998
Gram staining bacteria	0.968	0.941–0.995	0.961	0.901–1.000	0.969	0.936–1.000

AUC: Area under the curve. P values < 0.05.

Therefore, we concluded that this study was done in a relevant and representative study population.

Of 135 urine cultures only 19 cultures (14.4%) were positive in this study. In comparison to literature (49–64.5%) (5,9,14) this is a relatively low number, probably due to the fact that our inclusion period was in winter and a lot of patients with fever had respiratory problems such as pneumonia.

Counting only viable bacteria using FC was a reliable test method (sensitivity 100% and specificity 78.4%) and had a higher correlation to the results of urine cultures than counting all bacteria by flow cytometry (Spearman correlation coefficient 0.59 versus 0.37).

FC measurement takes manually about 5 minutes for counting of total bacteria with additional 5 minutes for the viability measurement. It can become standardized and automated with accompanying shortening in time that is necessary for the measurements. As expected the Gram staining has also an excellent diagnostic potential, but it takes much more time to gain the result (3,22). Therefore, we concluded that FC is a fast and accurate method in predicting both positive or negative urine culture, which is necessary for an optimal treatment in the clinical patient care.

Although this study was executed carefully, limitations are present. The Accuri flow cytometer has not been automated yet and all tests were done manually, which stimulated measurement errors. Also the use of antibiotics might influence the results. Because pretreatment with antibiotics induces negative urine cultures and almost no viable bacteria in FC analysis will be detected, the use of antibiotics did not influence our results (Table 4). This is not surprising because both the viable flow FC test as well as the culture results depend on the viability of the bacteria and will be affected by previous antibiotic treatment to the same extent. Viability testing with TO and PI suggests a strict distinction in viable ("alive") and non-viable ("dead") bacteria. Although it is uncertain whether this distinction can be made so strictly (23,24), in our study the number of viable bacteria showed to be a good predictor of positive urine culture.

In future research the diagnostic value of FC should be further investigated. A larger cohort of patients, different patient populations and cut-off values can be used. Also, the extensive applicability's of FC can be explored. Using (viability) testing of bacteria the antibiotic resistance of the bacteria can be investigated (25,26). Also, Gram positive and negative bacteria can be distinguished by applying the technique of Wada (27) or Gessoni (28) in the FC analysis.

CONCLUSION

FC is a fast, accurate and promising technique in the diagnostics of complicated urinary tract infections. FC counting of only viable bacteria has a higher correlation to the results of urine culture than FC counting of all bacteria. Fast and accurate diagnostics can improve the early appropriate therapy in this ill patient category.

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