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Use of Sysmex UF-5000 flow cytometry in rapid diagnosis of urinary tract infection and the importance of validating carryover rates against bacterial count cut-off

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Abstract

Introduction. Urinary tract infections are common bacterial infections worldwide. Urine culture is the gold standard method to identify and quantify the presence or absence of bacteria in urine. Flow cytometry, which can differentiate and quantify multiple particles (including bacteria) in the urine, presents an alternative method for rapid screening to rule out bacteriuria.

Hypothesis. Adding flow cytometry to identify urine samples without bacteriuria could substantially reduce the number of urine samples that need to be cultured as well as the response time for negative results. However, the level of instrument rinsing between samples could affect sample-to-sample carryover rate, a concept given little attention in previous studies.

Aim. We aimed to evaluate urine flow cytometry as a rapid screening method to identify urine samples without significant bacterial growth, including analyses of cross-contamination and sample-to-sample carryover rate.

Methodology. We analysed 3919 urine samples by quantitative urine culture and flow cytometry screening (Sysmex UF-5000). Receiver operator characteristic (ROC) curve analyses were used to test method agreement to identify: (a) positive vs. negative culture and (b) mixed vs. pure culture. In addition, we performed carryover and cross-contamination studies.

Results. ROC curve analyses identified bacterial count (BACT ml^{-1}) and leucocyte count (WBC μl^{-1}) as possible predictors of bacterial growth in the total material and subpopulations, except pregnant women (n=451). This subgroup was excluded from further analyses, leaving a final 3468 urine samples. Area under the ROC curve was 0.94 (95% CI 0.93–0.95) and 0.81 (95% CI 0.79–0.82) for bacterial and leucocyte count, respectively. A bacterial count cut-off of 30 BACT ml^{-1} resulted in 95.2% sensitivity and 91.2% negative predictive value, resulting in approximately 30% of urine samples that could be reported as negative without culture. Use of high-level rinse modes was necessary to ensure carryover rates <0.05%.

Conclusion. Flow cytometry is a suitable and rapid method to rule out urine samples without significant bacterial growth. Rinses between samples should be adjusted, depending on the cut-off used, to prevent sample-to-sample carryover, whereas cross-contamination can be eliminated by the use of separate urine aliquots for flow cytometry analysis and urine culturing respectively.

BACKGROUND

Urinary tract infections (UTIs) are among the most common bacterial infections worldwide, affecting approximately 150 million people annually [1]. Generally, women are at a higher risk and the prevalence of UTIs increases with increasing age [2, 3]. Urine culture is the gold standard method to identify and quantify the presence or absence of

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Keywords: urine flow cytometry; urinary tract infection; urine screening; sample-to-sample carryover.

Abbreviations: AUC, area under the ROC curve; BACT, bacteria; c.f.u., colony forming unit; CI, confidence interval; EC, epithelial cells; h, hour; NPV, negative predictive value; PPV, positive predictive value; ROC, receiver operating characteristic; ROC, receiver-operating characteristics; SquaEC, squamous epithelial cells; UFC, urine flow cytometry; UTI, urinary tract infection; WBC, White blood cells, leucocytes.

†These authors contributed equally to this work

Table 1. Characteristics of the study population (*n*=3919)

Characteristics of study population	*n*=3919
Age, mean (range), years	59 (0–95)
Sex	
Female, *n* (%)	2565 (65)
Male, *n* (%)	1354 (35)
Patient category	
Hospitalized, *n* (%)	1865 (48)
Outpatients, *n* (%)	2054 (52)
Immunocompromised, *n* (%)	245 (6)
Pregnant women, *n* (%)	451 (12)
Sample collection method	
Midstream, *n* (%)	3091 (79)
Indwelling urethral catheter, *n* (%)	516 (13)
Single-use urethral catheter, *n* (%)	213 (5)
Other*, *n* (%)	99 (3)
Classification of cases:†	
UTI symptoms reported, *n* (%)	3743 (96)
Complicated UTI, *n* (%)	3198 (82)
Non-complicated UTI, *n* (%)	545 (14)
Asymptomatic/pre-operative screening, *n* (%)	176 (4)

*Urine samples with no information on specimen collection provided or samples collected from paediatric urine collection bag, external catheter, uro-/nephrostomy or cystoscopy (one sample).
†Based on information provided in the microbiology request form.

bacteria in urine. However, it is a time-consuming and tedious method with a turn-around time of up to 48 h, and generally a large number of specimens yield no bacterial growth (i.e. culture-negative) [4–6]. Empirical treatment of clinically suspected UTIs normally starts before microbiological analysis is completed. This might lead to unnecessary antibiotic prescriptions and increased rates of bacterial antibiotic resistance.

In recent years, several European laboratories have implemented urine flow cytometry (UFC) as part of their urine routine diagnostics [4–9]. By use of flow cytometry, urine samples with no or insignificant concentration of bacteria could quickly be reported as negative to the clinician, up to 48 h earlier than when conventional culture is used. This could benefit both the patient and healthcare system, as it allows for a more rapid clarification of the patient's condition, possibly reducing the number of hospital bed-days per patient, as well as the use of antibiotics. Flow cytometry is reported to be a rapid, accurate and robust screening method that can substantially reduce the number of excessive cultivations in the microbiology laboratory [4–9]. However, if the instrument cut-off regarding bacterial counts is set low, according to defined thresholds for positive urine cultures [2, 10], there is a risk that the count measured by the flow cytometer represents carryover from a previous, rather than the current sample if rinsing of the instrument between samples is insufficient. Sample-to-sample carryover can be defined as increased erroneous counts in one sample due to contamination from the preceding sample within the instrument [11]. Thus, it is important to ensure that measured bacterial counts in the flow cytometry instrument do not represent carryover contamination from a previous sample. This can be avoided by adjusting the level of rinsing between samples in the instrument [12].

In this study, we evaluated urine flow cytometry for rapid screening of urine samples in our patient population, with the main aim to identify culture-negative samples. In addition, we investigated whether it could be possible to identify urine samples resulting in mixed bacterial growth. Carryover and cross-contamination studies were performed to assess optimum rinse modes during urine flow cytometry analysis.

METHODS

Urine sample collection

We included all 3919 urine samples from both hospitalized patients and outpatients routinely sent to our laboratory at the Department of Medical Microbiology, St. Olavs Hospital, Trondheim, Norway, for routine testing for bacteriuria/UTI from 4 June to 2 August 2019. Of the included samples, 3091 were midstream urine and 729 were catheterized urine samples (Table 1). Urine samples from the majority of patients were collected in sterile Vacuette Urine tubes without preservatives (Greiner Bio-One), and were sent to the laboratory shortly after specimen collection. A subset of the outpatient samples from a geographical area in mid-Norway with transportation time longer than 4 h were collected in sterile Vacuette Urine CCM tubes with added boric acid (Greiner Bio-One). Due to the longer transportation time, these samples could not be analysed within the recommended 4 h, and were therefore processed upon arrival. Urine samples that were not transported immediately after sampling were stored on site at 4–8 °C, followed by transportation at room temperature. At the laboratory, all samples were stored at 4–8 °C if not processed immediately and discarded after analysis, according to standard operating procedure.

Patient data collection

From the microbiology request form, we registered data from all participants regarding age (in years), sex, hospitalized or outpatient status, and tentative clinical diagnosis [i.e. clinical suspicion of UTI (complicated or uncomplicated) or no clinical suspicion of UTI (asymptomatic or pre-operative screening), pregnancy and immunosuppression], alongside culture and UF results. Events classified as 'uncomplicated UTI' were female patients with no information of pregnancy and reported symptoms consistent with uncomplicated cystitis, according to guidelines [2]. All other UTI events

Table 2. Number of rinses, and corresponding rinse modes in the Sysmex UF-5000

Rinsing was programmed to be performed automatically after flow cytometry analysis of samples with predefined bacterial counts, as given in the table. The different rinse modes were used to investigate carryover in the UF-5000 instrument.

Bacterial count (BACT ml^{-1})	Rinse mode – no. of rinses								
	0-0-1-2-3	0-0-1-3-3	0-0-1-3-4	0-0-1-3-5	0-0-1-4-5	0-0-1-5-5	0-0-1-6-6	0-0-1-6-7	0-0-1-7-7
1×10^4–9.9×10^4	0	0	0	0	0	0	0	0	0
1×10^5–9.9×10^5	0	0	0	0	0	0	0	0	0
1×10^6–9.9×10^6	1	1	1	1	1	1	1	1	1
1×10^7–9.9×10^7	2	3	3	3	4	5	6	6	7
$\geq10^8$	3	3	4	5	5	5	6	7	7

which were not defined as 'uncomplicated' were categorized as 'complicated'.

Standard microbiological analyses

According to our standard procedure for cultivation of urine specimens, urine samples were inoculated at 10 µl onto blood agar and at 1 µl onto Brilliance UTI clarity agar (Oxoid) using a WASP DT: Walk-Away Specimen Processor (COPAN Diagnostics) when received at the laboratory. Samples obtained by cystoscopy were manually inoculated onto the same plates using the same volumes as mentioned above. These samples were also manually inoculated at 100 µl onto Neisseria-GC Agar (Bio-Rad) and 100 µl onto Anaerobe Basal Agar (Oxoid). For urine samples where fungal cultures were requested, manual inoculation was done with 500 µl on Sabouraud dextrose agar (Mast Group) and 500 µl on CandiSelect (Bio-Rad). All agars were incubated for 24–48 h for subsequent species identification and antimicrobial susceptibility testing. Bacterial colonies were counted, and for diagnostic purposes, significant bacteriuria was defined as growth on agar plates at concentrations ranging between $\geq10^2$ c.f.u. ml^{-1} (for e.g. bladder puncture) and $\geq10^5$ c.f.u. ml^{-1} (for the majority of our classifications) [10]. For the purpose of this study, due to the need to decide on a general cut-off to best cover all scenarios, we defined cultures with two or fewer bacterial species each at concentrations $\geq10^4$ c.f.u. ml^{-1} as significant bacteriuria, in concordance with previous urine flow cytometry studies [9]. Mixed cultures were defined by the growth of three or fewer microorganisms (any) at total concentrations $\geq10^4$ c.f.u. ml^{-1}, whereas samples with non-significant bacteriuria were defined by microbial growth (any) $<10^4$ c.f.u. ml^{-1}.

Flow cytometry analysis with Sysmex UF-5000

Following inoculation, we analysed the urine samples for all parameters available in the Sysmex UF-5000 instrument (Sysmex), which is the latest urine and body fluid analyser from Sysmex. In the instrument, cells are counted and classified by analysing forward scattered light, side scattered light, side fluorescent light and depolarized side scattered light (Sysmex).

Sysmex UF-5000 has a theoretical maximum throughput of 105 urine samples per hour, assuming no rinsing between samples (Sysmex). Automated rinsing between samples can be programmed to reduce the risk of sample-to-sample carryover from samples with a high bacterial count to samples with a lower bacterial count [11]. In this study we programmed rinsing according to the manufacturer's instructions with rinse mode 0-0-1-3-5 as follows: no rinsing after samples with $\leq9.9\times10^5$ BACT ml^{-1}, one rinse after samples with 1×10^6–9.9×10^6 BACT ml^{-1}, three rinses after samples with 1×10^7–9.9×10^7 BACT ml^{-1}, and five rinses after samples with $\geq10^8$ BACT ml^{-1} (Table 2). Sample throughput will therefore depend of the number of rinses, which again depends on the concentration of bacteria in the samples.

Sample-to-sample carryover analysis

We evaluated sample-to-sample carryover in the flow cytometer by measuring in triplicate a selected clinical urine sample with a high bacterial load ($>10^5$ c.f.u. ml^{-1}), as defined by urine culture, followed by triplicate measurements of sterile filtered saline (blank). Subsequent instrument measurement of bacteria (any) in the sterile filtered saline was defined as carryover contamination of the tube system in the instrument from the previous sample. The triplicate measurements of the urine sample with high bacterial load were denoted high 1, 2 and 3, while the triplicate measurements of the blank were denoted blank 1, 2 and 3, and were used to calculate the carryover rates according to the following formula: $Carryover = \frac{(blank\ 1 - blank\ 3)}{(high\ 3 - blank\ 3)} * 100\ \%$ [4, 11].

In this study, evaluation of carryover was done using nine different automatic rinse modes, 0-0-1-2-3 to 0-0-1-7-7 (Tables 2 and 3). For rinse modes 0-0-1-2-3 to 0-0-1-6-6, we measured urine samples with different Gram-negative rods (either *Escherichia coli*, *Klebsiella* species, *Proteus mirabilis* or *Pseudomonas aeruginosa*). For the highest level rinse modes (0-0-1-6-7 and 0-0-1-7-7), we spiked sterile saline to ensure a bacterial count as close as possible to 10^8 BACT ml^{-1} (100 000 BACT µl^{-1}), as a bacterial concentration this high was hard to find in urine samples. This concentration corresponded to a McFarland standard of approximately 3.

Table 3. Results from carryover analyses at nine different rinse modes in the Sysmex UF-5000 flow cytometer

Rinsing in the instrument was programmed to initiate automatically after samples with predefined bacterial counts in flow cytometry analysis as given in Table 2.

			Investigation of carryover (BACT ml^{-1})			
Rinse mode	**Patient number**	**Sample type (bacterial isolate, c.f.u. ml^{-1})**	**Sysmex run 1**	**Sysmex run 2**	**Sysmex run 3**	**Carryover rate (%)**
0-0-1-2-3	1	Urine (*Escherichia coli*, >10^5)	6.55×10^7	5.87×10^7	5.47×10^7	0.21
		Sterile saline	1.40×10^5	3.86×10^4	2.47×10^4	
	2	Urine (*Klebsiella* spp., >10^5)	3.55×10^7	3.06×10^7	2.83×10^7	0.04
		Sterile saline	1.16×10^4	1.10×10^3	1.10×10^3	
	3	Urine (*P. mirabilis*, >10^5)	6.67×10^7	6.52×10^7	6.45×10^7	0.20
		Sterile saline	1.35×10^5	9.92×10^4	5.60×10^3	
0-0-1-3-3	4	Urine (*E. coli*, >10^5)	1.46×10^7	1.64×10^7	1.79×10^7	0.01
		Sterile saline	2.30×10^3	2.30×10^3	0	
	5	Urine (*Klebsiella* species, >10^5)	2.31×10^6	2.52×10^6	3.14×10^6	0.00
		Sterile saline	0	0	0	
	6	Urine (*P. mirabilis*, >10^5)	1.61×10^7	1.93×10^7	2.06×10^7	0.08
		Sterile saline	1.88×10^4	4.60×10^3	2.00×10^3	
0-0-1-3-4	7	Urine (*E. coli*, >10^5)	3.71×10^7	3.42×10^7	3.59×10^7	0.06
		Sterile saline	2.47×10^4	7.00×10^3	2.30×10^3	
	8	Urine (*Klebsiella* spcies, >10^5)	3.18×10^7	4.00×10^7	3.66×10^7	0.01
		Sterile saline	7.00×10^3	3.50×10^3	2.30×10^3	
	9	Urine (*P. aeruginosa*, >10^5)	1.69×10^6	1.52×10^6	1.68×10^6	0.84
		Sterile saline	1.41×10^4	1.10×10^3	0	
0-0-1-3-5	1	Urine (*E. coli*, >10^5)	5.97×10^7	6.32×10^7	6.33×10^7	0.02
		Sterile saline	2.36×10^4	7.00×10^3	8.00×10^3	
	2	Urine (*K. pneumoniae*, >10^5)	3.35×10^7	3.03×10^7	3.06×10^7	0.02
		Sterile saline	7.00×10^3	5.80×10^3	2.20×10^3	
	3	Urine (*P. mirabilis*, >10^5)	7.75×10^7	8.31×10^7	8.01×10^7	0.15
		Sterile saline	2.15×10^5	1.45×10^5	9.30×10^4	
0-0-1-4-5	1	Urine (*E. coli*, >10^5)	5.76×10^7	6.87×10^7	7.03×10^7	0.08
		Sterile saline	6.22×10^4	1.98×10^4	5.60×10^3	
	2	Urine (*Klebsiella* species, >10^5)	3.02×10^7	3.24×10^7	3.23×10^7	0.01
		Sterile saline	3.50×10^3	1.10×10^3	0	
	3	Urine (*P. mirabilis*, >10^5)	6.62×10^7	6.64×10^7	6.63×10^7	0.09
		Sterile saline	5.78×10^4	1.04×10^4	1.10×10^3	

Continued

Table 3. Continued

			Investigation of carryover (BACT ml^{-1})			
Rinse mode	**Patient number**	**Sample type (bacterial isolate, c.f.u. ml^{-1})**	**Sysmex run 1**	**Sysmex run 2**	**Sysmex run 3**	**Carryover rate (%)**
0-0-1-5-5	1	Urine (*E. coli*, >10^5)	7.01×10^7	6.60×10^7	7.23×10^7	0.06
		Sterile saline	4.47×10^4	9.30×10^3	0	
	10	Urine (*E. coli*, >10^5)	2.99×10^7	2.37×10^7	2.95×10^7	0.04
		Sterile saline	1.05×10^4	7.00×10^3	0	
	3	Urine (*P. mirabilis*, >10^5)	6.34×10^7	5.62×10^7	5.62×10^7	0.03
		Sterile saline	4.96×10^4	6.83×10^4	3.05×10^4	
0-0-1-6-6	10	Urine (*E. coli*, >10^5)	3.21×10^7	2.93×10^7	2.67×10^7	0.13
		Sterile saline	4.37×10^4	1.89×10^4	9.30×10^3	
	10	Urine (*E. coli*, >10^5)	2.99×10^7	3.05×10^7	2.96×10^7	0.02
		Sterile saline	2.95×10^4	3.19×10^4	2.48×10^4	
	11	Urine (*P. aeruginosa*, >10^5)	1.45×10^6	1.03×10^6	1.01×10^6	1.29
		Sterile saline	1.53×10^4	5.90×10^3	2.30×10^3	
0-0-1-6-7	NA[1]	Spiked saline (*E. coli* CCUG17620, McFarland 3)	1.00×10^8	9.02×10^7	8.68×10^7	0.01
		Sterile saline	9.40×10^3	5.60×10^3	2.20×10^3	
	NA[1]	Spiked saline (*K. pneumoniae* ATCC700603, McFarland 3)	5.31×10^7	4.79×10^7	5.19×10^7	0.01
		Sterile saline	5.80×10^3	4.60×10^3	0	
	NA[1]	Spiked saline (*P. aeruginosa* CCUG17619, McFarland 3)	9.02×10^7	7.69×10^7	7.55×10^7	0.00
		Sterile saline	3.50×10^3	5.90×10^3	5.90×10^3	
	12	Urine (*E. coli*, >10^5)	1.69×10^7	1.44×10^7	1.31×10^7	0.04
		Sterile saline	5.80×10^3	0	0	
	13	Urine (*E. coli*, >10^5)	5.72×10^7	5.22×10^7	5.21×10^7	−0.01
		Sterile saline	1.10×10^3	3.50×10^3	4.60×10^3	
0-0-1-7-7	NA[1]	Spiked saline (*E. coli* CCUG17620, McFarland 3)	9.27×10^7	9.99×10^7	1.00×10^8	0.01
		Sterile saline	9.30×10^3	7.00×10^3	1.10×10^3	
	NA[1]	Spiked saline (*K. pneumoniae* ATCC 700603, McFarland 3)	4.02×10^7	3.98×10^7	5.13×10^7	0.00
		Sterile saline	2.30×10^3	0.00×100	1.10×10^3	
	NA[1]	Spiked saline (*P. aeruginosa* CCUG17619, McFarland 3)	7.80×10^7	8.64×10^7	9.14×10^7	0.00
		Sterile saline	4.70×10^3	3.50×10^3	1.10×10^3	
	13	Urine (*E. coli*, >10^5)	5.13×10^7	5.36×10^7	5.66×10^7	0.00
		Sterile saline	6.90×10^3	6.90×10^3	4.70×10^3	

NA, Not applicable.

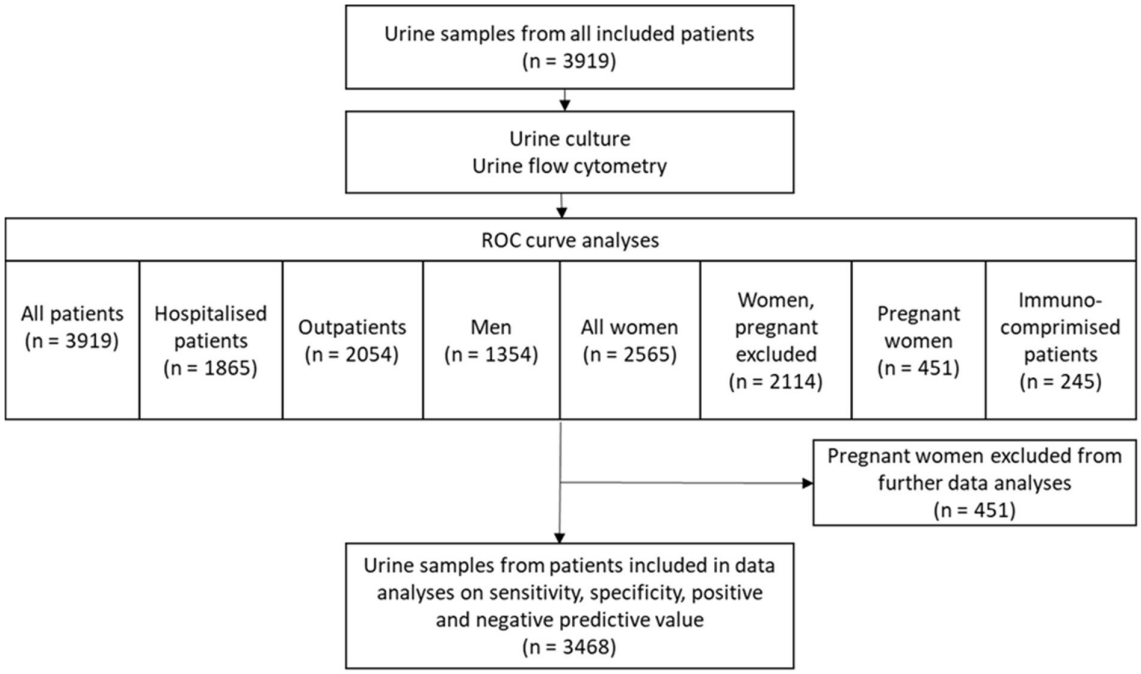

Fig. 1. Flowchart of urine samples from patients included in the study. Final data analyses were based on urine samples from 3468 patients, where urine samples from pregnant women (*n*=451) were excluded.

Here, we used three different reference strains: *Escherichia coli* CCUG 17620, *Klebsiella pneumoniae* ATCC 700603 and *Pseudomonas aeruginosa* CCUG 17619. For comparison, we also included two different urine samples identified as *E. coli* with growth $>10^5$ c.f.u. ml^{-1}. As described above, we measured all samples in pairs of technical triplicates (i.e. triplicates of urine or spiked saline, followed by triplicates of sterile saline.

According to the manufacturer's recommendation (Sysmex), the carryover rate for urine samples with a bacterial count in the instrument of 10^6 BACT ml^{-1} should be <0.05%.

Cross-contamination analysis

In the Sysmex UF-5000 instrument, a reusable probe is used to aspirate urine from sample vials. According to the manufacturer (Sysmex), cross-contamination, i.e. transfer of cells or particles from one sample tube to the next, might occur, although the probe is washed between succeeding samples. The level of probe washing is constant and independent of the rinsing of the tube system in the instrument, as described above regarding sample-to-sample carryover. Sample-to-sample cross-contamination was evaluated with flow cytometry to analyse a urine sample with a high bacterial load ($>10^5$ c.f.u. ml^{-1}), followed by three samples of sterile filtered saline. In total, 12 clinical urine samples followed by triplicates of sterile saline were analysed. All samples were cultured on agar plates after having been exposed to the flow cytometer sample probe. Subsequent bacterial growth (any) on the agar plate was regarded as cross-contamination.

Data analysis

To compare results from urine culture at the threshold $\geq 10^4$ c.f.u. ml^{-1} with parameters from urine flow cytometry analyses, we used receiver-operating characteristics (ROC) curve analyses. ROC curve analysis was applied for the total population, and subsequently for the following subpopulations: inpatients, outpatients, men, women (including and excluding pregnant women, respectively), pregnant women and immunocompromised patients (Fig. 1). The diagnostic accuracy of the urine flow cytometry parameters was defined by the area under the ROC curve (AUC). The Youden index, defined as the sum of sensitivity and specificity minus one (J=sensitivity + specificity – 1) was calculated and used to guide selection of cut-off values for urine flow cytometry parameters. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated at selected cut-offs. IBM SPSS Statistics 27 was used for all statistical analyses.

Ethical considerations

The Regional Committee for Medical and Health Research Ethics Mid-Norway (REK Midt) reviewed the study and considered it a quality control project. It was therefore deemed exempt from their approval. Urine samples were used without consent from the patients from whom samples were collected based on the Norwegian infection control legislation (§ 3.7 in 'Lov om vern mot smittsomme sykdommer') which allows laboratories to use patient samples for method evaluation without consent from the patients.

Table 4. Microorganisms identified in the 1320/3919 (34%) urine samples yielding significant bacterial growth on agar plates (i.e. two or fewer bacterial species at concentrations ≥10^4 c.f.u. ml^{-1})

	No. of samples	
Microorganism	***n*=1320**	**%**
Escherichia coli	721	54.6
Klebsiella species	131	9.9
Enterococcus species	124	9.4
Streptococcus agalactiae	47	3.6
Proteus mirabilis	46	3.5
Staphylococcus epidermidis	28	2.1
Staphylococcus saprophyticus	25	1.9
Pseudomonas aeruginosa	20	1.5
Staphylococcus aureus	24	1.8
Aerococcus urinae	23	1.7
Enterobacter cloacae complex	19	1.4
Other Gram-positive bacteria	62	4.7
Other Gram-negative bacteria	28	2.1
Yeast	22	1.7

RESULTS

Diagnostic performance of UFC in comparison to urine culture

Of the 3919 urine samples initially included in the study, 2565 (65%) were from women and 1354 (35%) from men, and included both hospitalized patients (*n*=1865, 48%) and outpatients (*n*=2054, 52%) (Table 1). Of the 1320 (34%) culture-positive samples (Table 4), we observed a single species in 1150 (29%) cultures and two species in 170 (4%) cultures.

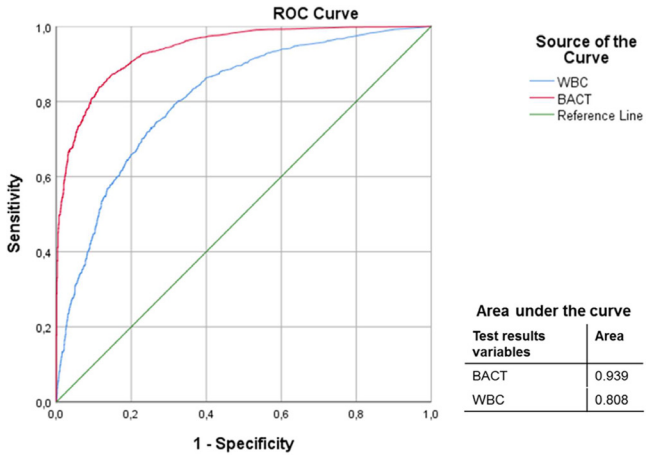

Fig. 2. ROC curve for urine flow cytometry bacterial count (BACT ml^{-1}, red line) and leucocyte count (WBC μl^{-1}, blue line) versus culture results (c.f.u. ≥10^4) in 3468 urine samples (pregnant women excluded).

We observed negative culture from 1662 (42%) samples and mixed cultures from 937 (24%) samples. *E. coli* (*n*=721, 55%), *Klebsiella* species (*n*=131, 10%) and *Enterococcus* species (124, 9%) were the most frequently isolated bacterial species in positive cultures (Table 4).

ROC curve analyses of data from the total population (*n*=3919) identified bacterial (BACT μl^{-1}) and leucocyte counts (WBC μl^{-1}) as possible predictors of bacterial growth. By analysing subpopulations, we found poor agreement between results from culture and urine flow cytometry in pregnant women (*n*=451), and samples from this subpopulation were therefore excluded from further analyses (Fig. 1). The discriminatory ability of bacterial and leucocyte counts, respectively, in the remaining subpopulations was comparable to that of the total population (data not shown). Further analyses were therefore done on the remaining 3468 urine samples (Fig. 1), of which 2114 (61%) were from women and 1354 (39%) from men. In this population, the AUC for bacterial count was 0.939 [95% confidence interval (CI) 0.931–0.946]. For leucocyte count, the AUC was 0.808 (95% CI 0.794–0.823) (Fig. 2).

Using calculated Youden indexes as initial guidance, we examined different cut-off values for the flow cytometry parameters bacterial and leucocyte count, separately and in combination, to discriminate samples with significant bacteriuria from those with non-significant bacteriuria based on urine culture (data not shown). Evaluation of results showed that using a cut-off of 30 BACT μl^{-1} (3.0×10^4 BACT ml^{-1}) provided the best results, obtaining 95.2% sensitivity, 67.8% specificity, 91.2% NPV and 80.1% PPV (Table 5). In the female subpopulation, specificity and NPV were slightly lower, whereas sensitivity and PPV remained unchanged. In the male subpopulation, however, specificity and NPV were slightly higher, whereas sensitivity and PPV remained largely unchanged. We observed no improved diagnostic accuracy using differentiated cut-offs in male and female subpopulations, in contrast to some other studies [4, 6]. The discriminatory ability of leucocyte count was lower than that of bacterial count (Fig. 2, Table 5). Combining leucocyte and bacterial count did not improve overall diagnostic performance. Although both sensitivity and NPV slightly improved when bacterial and leucocyte count were included, the number of false positive samples increased from 473 to 654, whereas the number of false negatives was reduced by only 28 (Table 5). Our data demonstrate that using a cut-off of 30 BACT μl^{-1} (3.0×10^4 BACT ml^{-1}), 2375 vs. 3468 cultures need to be cultured, giving a reduction in need for culturing of 32% (Table 5).

At a bacterial cut-off of 30 BACT μl^{-1} (3.0×10^4 BACT ml^{-1}), 96 samples were initially classified as false negative (negative by flow cytometry while growth ≥10^4 c.f.u. ml^{-1} by culture) (Table 5). Of these, however, 69 samples were reported with three or more microbial species (bacteria/yeast) or as mixed genital or urethral flora, consistent with true negative observations (data not shown). Hence, the remaining 27 samples were indeed false negative (two or fewer microbial species), in which the predominant findings

Table 5. Performance of the Sysmex UF-5000 in urine samples from the total study population (*n*=3468), the female study population (*n*=2114) and the male study population (*n*=1354) at a bacterial count cut-off of 30 BACT ml^{-1} and leucocyte count cut-off of 30 WBC μl^{-1}, alone or in combination, compared to results from urine culture (c.f.u. ml^{-1} $\geq 10^4$)

		Cut-off for UF-5000									
Samples	***n***	**BACT μl^{-1}**	**WBC μl^{-1}**	**Sensitivity (%)**	**Specificity (%)**	**NPV (%)**	**PPV (%)**	**True positive, *n***	**False positive, *n***	**True negative, *n***	**False negative, *n***
All*	3468	30	NI†	95.2	67.8	91.2	80.1	1902	473	997	96‡
All*	3468	NI†	30	78.0	69.5	69.9	77.6	1558	449	1021	440
All*	3468	30	30	96.6	55.5	92.3	74.7	1930	654	816	68
Women*	2114	30	NI†	95.9	52.4	86.4	80.3	1356	333	367	58
Women*	2114	NI†	30	75.4	68.0	57.8	82.6	1066	224	476	348
Women*	2114	30	30	97.2	44.7	88.7	78.0	1374	387	313	40
Men	1354	30	NI†	93.5	81.8	94.3	79.6	546	140	630	38
Men	1354	NI†	30	84.2	70.8	85.6	68.6	492	225	545	92
Men	1354	30	30	95.2	65.3	94.7	67.5	556	267	503	28

*Urine samples from pregnant women are excluded.
†NI, not included.
‡Initially, 96 false negative samples were identified when using only the bacterial count parameter. The majority (69/96, 72%) yielded three or more microorganisms by culture, suggesting contamination. Consequently, only 27/3468 (0.77%) urine samples were identified as actual false negative (i.e. growth of two or fewer bacterial species at concentrations $\geq 10^4$ c.f.u. ml^{-1} on an agar plate, while negative by flow cytometry).

Table 6. Overview of microorganisms isolated by culture from the 27 urine samples classified as true false negative* in a total of 3468† samples, using a cut-off of 30 BACT ml^{-1} in the Sysmex UF-5000 instrument

	Growth of one microbial species (*n*=24)		**Growth of two microbial species (*n*=3)**	
	Hospitalized patients	**Outpatients**	**Hospitalized patients**	**Outpatients**
Enterococcus faecalis	3	0	0	2
Streptococcus agalactiae	1	1	0	1
Streptococcus dysgalaciae	1	0	0	0
Staphylococcus simulans	1	0	0	0
Proteus mirabilis	0	0	0	1
Klebsiella pneumoniae	0	1	0	0
Serratia marcescens	0	1	0	0
Streptococcus mitis group	0	1	0	0
Gram-positive cocci unspecified	4	4	0	1
Gram-negative rods unspecified	0	0	0	1
Diphteroid rods	0	1	0	0
Yeast	5	0	0	0

*Samples with a negative result by flow cytometry but which showed growth of two or fewer microorganisms at concentrations $\geq 10^4$ c.f.u. ml^{-1} by urine culture were regarded as true false negative
†Urine samples from pregnant women were excluded.

Table 7. Results of cross-contamination analyses using 12 different urine samples, showing growth in triplicate sterile saline samples after each urine sample was tested in a Sysmex UF-5000 flow cytometer; bacterial growth (any) on an agar plate, after inoculation of the saline sample, was regarded as cross-contamination

	Growth in triplicate saline samples (c.f.u. ml^{-1})		
Bacterial species of urine sample, c.f.u. ml^{-1}	**Sample 1**	**Sample 2**	**Sample 3**
E. coli, >10^5	1000	1000	100
E. coli, >10^5	100	0	100
E. coli, >10^5	1000	100	100
E. coli. >10^5	0	one colony	one colony
Klebsiella species, >10^5	0	0	0
Klebsiella species, >10^5	1000	1000	1000
Klebsiella species, >10^5	100	100	100
K. pneumoniae , >10^5	0	0	0
P. mirabilis, >10^5	1000	100	100
P. mirabilis, >10^5	1000	100	1000
P. mirabilis. >10^5	100	100	100
P. aeruginosa, >10^5	0	0	100

were *Enterococcus faecalis* (five samples), *Streptococcus agalactiae* (three samples), unspecified Gram-positive cocci (nine samples) and yeast (five samples) (Table 6).

ROC curve analyses comparing various urine flow cytometry parameters, including squamous epithelial cells (SquaEC µl^{-1}) and epithelial cells (EC µl^{-1}), with culture results, showed that none of the urine flow cytometry parameters had any diagnostic value in predicting mixed culture (data not shown).

Carryover and cross-contamination analyses

For each of the rinse modes 0-0-1-2-3 to 0-0-1-6-6, the carryover rates were >0.05% for at least one of the high and low sample pairs that were analysed (Table 3). In addition, the bacterial counts measured in numerous sterile saline samples were above the cut-off of 30 BACT µl^{-1} (3.0×10^4 BACT ml^{-1}). For the highest level rinse modes, 0-0-1-6-7 and 0-0-1-7-7, carryover rates were <0.05% and bacterial counts were below the cut-off in all sterile saline samples (Table 3).

Cross-contamination analyses showed bacterial growth of up to 1000 c.f.u. ml^{-1} for several samples (Table 7).

DISCUSSION

The main aim of this study was to evaluate UFC as a rapid screening method to rule out culture-negative urine samples preceding urine culture in a population of both in- and outpatients with suspicion of bacteriuria. We analysed and compared results from urine culture and UFC analyses using the Sysmex UF-5000 in a large collection of nearly 3500 urine samples, which to our knowledge is the largest UFC study so far reported.

Diagnostic cut-offs applied by different laboratories vary greatly, probably owing to different patient populations and different definitions used to classify significant bacteriuria [4, 9, 10, 13–15]. By using ≥10^4 c.f.u. ml^{-1} as cut-off for significant urine culture in our population, we observed the highest sensitivity and NPV at a cut-off of 30 BACT µl^{-1} (3.0×10^4 BACT ml^{-1}), for both bacteria and leucocyte counts. The latter demonstrated lower discriminatory ability, compared to bacterial count, in predicting bacterial growth in culture. This could be due to the fact that not all observations of bacteriuria were associated with UTI, but rather contamination of the specimen by commensal bacteria. The leucocyte count in urine samples is known to decrease during longer transportation and storage time [16, 17]. However, results from ROC curve analyses of leucocytes did not indicate any difference between samples from hospitalized patients and outpatients in our study (data not shown).

Our analyses showed poor agreement between UFC results and culture in pregnant women, leading to the exclusion of this subgroup. Here, UFC analyses often reported high bacterial counts, which were not reflected in corresponding cultivation results. It could be that urine samples from pregnant women contain strict anaerobic and slow-growing bacteria not cultivated by routine procedures, or that sampling according to the instructions is not easy to obtain in pregnant women. Recent studies of female urinary microbiota has challenged the paradigm of 'sterile urine' specimens, following the detection of uncultivated bacteria by deep 16S rRNA gene sequencing of urine samples from both asymptomatic and symptomatic women [18]. The viability of these bacteria was further investigated and

confirmed by expanded quantitative urine culture using a modified culture protocol with larger volumes of urine and prolonged incubation times [19]. It is possible that expanded quantitative urine culture could obtain better concordance with positive Sysmex screening results, but this was beyond the aim of our study. Another reason for disagreement could be due to the possible counting of non-viable bacteria by the Sysmex UF-5000. Although technical solutions to separate non-viable from viable bacteria using special dyes in UFC exist in certain instruments [20, 21], this is not included as an option in the Sysmex UF-5000. Thus, urine samples from pregnant women should always be cultured, for the detection of uropathogens as well as group B streptococci [22]. Furthermore, samples obtained by cystoscopy and suprapubic bladder puncture should always be cultured manually.

Culture results provide important information to clinicians, both with respect to differential diagnostics and choice of antimicrobial treatment. We identified 997/3468 (29%) true negative urine samples using flow cytometry. This number of samples is indeed manageable by urine culture in our laboratory. Yet, it would take up to 48 h before negative results were reported to the clinician. By implementing flow cytometry as daily routine for urine screening, incoming urine samples can be analysed successively on the flow cytometer as they arrive in the laboratory and negative screening results can be reported to the clinician within the same day of sample collection. Because reporting by rapid screening would be up to 48 h earlier than by regular culture methods, the initiation of possibly unnecessary antibiotic treatment could be avoided in nearly 30% of the patients. Thus, the patients will benefit from implementing flow cytometry analysis of urine samples by a shorter time from sample collection to reporting of negative results.

According to the literature, both Gram-positive and Gram-negative bacteria are reported among false negative samples [4, 14, 23, 24]. We initially identified 96 false negative samples in this study, when using only the bacterial count parameter (Table 5). When combining the bacterial and leucocyte counts, the number of false negative samples was reduced to 68 (Table 5). However, the number of false positive samples increased by nearly 200 samples, from 473 to 654 samples. These samples would need to be cultured, generating unnecessary work in the laboratory. Thus, based on the results in our material, the bacteria-only analysis gave the best outcome. Also, among the 96 samples initially identified as false negative, the majority (69/96, 72%) yielded three or more microorganisms by culture, suggesting contamination. Consequently, only 27/3468 (0.77%) urine samples were identified as actual false negatives (i.e. growth of two or fewer bacterial species at concentrations $\geq 10^4$ c.f.u. ml^{-1} on an agar plate, while negative by flow cytometry), a result which we consider clinically acceptable. Also of note is that in these 27 false negative samples the great majority showed growth of either one or two Gram-positive bacteria or yeasts. The clinical relevance of these findings is in many cases dubious [10].

Mixed cultures are frequently observed in urine samples due to inappropriate sampling. We evaluated whether any of the UFC parameters could predict mixed culture, with the intention of also ruling these out by screening. We hypothesized that the UFC parameters squamous epithelial cells (SquaEC μl^{-1}) or epithelial cells (EC μl^{-1}), alone or in combination with BACT μl^{-1}, could serve as possible predictors of mixed culture. Unfortunately, however, our results did not support this hypothesis. None of the UFC parameters, alone or in combination with bacterial count, were able to predict mixed culture, in accordance with other reports [5, 9]. Thus, these samples still need to be cultured, since in many cases they have bacterial counts above the diagnostic cut-off.

Sample-to-sample carryover within the instrument can lead to erroneous high measurements in a sample with an originally low bacterial count if it is analysed directly after a sample with a high bacterial count. In contrast to several other studies, we find that carryover may be a considerable problem when the local cut-off is set to a low bacterial count, in line with a recently published report [12]. In contrast, in our study carryover was found at all rinse modes 0-0-1-2-3 to 0-0-1-6-6 for bacterial counts $>10^6$ BACT ml^{-1}. Moreover, and of high importance, several measurements exceeded our cut-off value of 30 BACT μl^{-1} (3.0×10^4 BACT ml^{-1}). This could erroneously classify samples as positive for bacteriuria by UFC screening. Both issues, however, were resolved when the number of rinses was increased substantively (Table 3). During the main part of this study, we therefore used the rinse mode 0-0-1-3-5. Carryover could have contributed to some of the 473 false positive samples observed, yet this remains unknown. Based on the results from the carryover analysis we decided to use the rinse setting 0-0-1-7-7 (Tables 2 and 3). This setting should prevent carryover between succeeding urine samples when using 30 BACT μl^{-1} (3.0×10^4 BACT ml^{-1}) as cut-off in flow cytometry analysis, as we do in our laboratory . The number of rinses between samples should be evaluated and set according to the locally selected cut-off and quality recommendations. A higher number of rinses will, however, reduce the instrument throughput of samples per hour.

We also observed cross-contamination when urine was cultured after UFC analysis in experiments (Table 7). Because it was previously known from the manufacturer that cross-contamination could occur, we inoculated all included urine samples on agar before UFC analysis during the study period. Based on these results we now ask for two separate urine sample vials, with aliquots from the same urine sample: one for flow cytometry analysis and one for urine culture of UFC screening positive samples.

Our study has some limitations. All urine samples were collected over a period of 3 months in the summer, which could potentially contribute to a slightly different cohort than the all-year average population. According to the manufacturer's protocol, urine samples analysed on the Sysmex UF-5000 should be without preservatives. Ideally, they should also be analysed within 4 h after collection. Our hospital

serves a large geographical area, with longer transportation time than recommended for some of the urine samples from outpatients. These urine samples are collected in sample tubes with boric acid. Because for practical reasons it was impossible to mark or sort out these samples, they were included in the study. When analysing subgroups in our material using ROC curve analysis, however, we did not find any differences between outpatients and hospitalized patients. Thus, based on these results it is unlikely that the addition of boric acid affected the results of the UFC analysis in this study.

In conclusion, we demonstrate in this study that UFC results are in high agreement with urine culture results, and that UFC as a rapid screening method for ruling out urine culture-negative samples is indeed eligible in our population. Also, our results show that with the cut-off for bacterial count used in this study, in addition to quality recommendations, the rinse mode 0-0-1-7-7 should be used to avoid carryover between consecutive urine samples.

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Author contributions
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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The Regional Committee for Medical and Health Research Ethics Mid-Norway (REK Midt) reviewed the study, and considered it a quality control project. It was therefore deemed exempt from their approval.

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