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Coherent fluctuation nephelometry as a promising method for diagnosis of bacteriuria

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ABSTRACT

Objectives: Specialized analyzers are used to automate the diagnosis of bacteriuria in laboratory practice. They are based on analysis of microorganisms concentration in urine samples or recording the growth of urine microflora. Coherent fluctuation nephelometry (CFN) has high sensitivity and allows analyzing both parameters simultaneously. The aim of the study is to compare the effectiveness of CFN-based and flow cytometry based analyzers.

Design and methods: Total 117 urine samples from children were studied in parallel using the CFN-analyzer and UF-1000i (Sysmex), the results were confirmed by conventional microbiological methods.

Results: In 21 urine samples (18%), significant bacteriuria was determined (≥10^4 CFU/ml). The best diagnostic indicators were obtained while testing urine samples using the CFN-analyzer. The most efficient bacteriuria diagnosis is achieved by simultaneous analyses of microorganisms concentration in urine and growth of urine microflora (sensitivity – 95.2%, specificity – 96.9%, positive predictive value – 87%, negative predictive value – 98.9%, diagnostic odds ratio – 81.7, positive likelihood ratio – 30.5, negative likelihood ratio– 0.049, area under curve in ROC-analysis – 0.987). The CFN-analyzer allows the preliminary selection of negative urine samples, which do not require further analysis by conventional microbiological methods, thereby decreasing the number of cultures by 80.3%.

Conclusions: This study suggests that the CFN-analyzer is the effective tool for bacteriuria screening in children.

Introduction

Urinary tract infections (UTI) belong to the most common infectious diseases, making 20–49% of all nosocomial infections [1,2]. For correct UTI diagnosis, the knowledge about the presence of microorganisms in urine is needed. Such diagnosis is traditionally carried out by culture methods using solid nutrient media. New technologies have been introduced to laboratory practice for acceleration of diagnosis recently. Since most urine samples received for testing turn out to be negative, preliminary selection of

Abbreviations: AUC, area under curve; B, bacteriuria; CFN, coherent fluctuating nephelometry; CFU, colony forming units; G, growth time; T, turbidity; TN, traditional nephelometry; T & G, function of turbidity and growth time; UTI, urinary tract infections

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incoming samples allows to optimize the work process and to reduce the costs. A simple and rapid point-of-care dipstick bacteriuria screening test by determination of nitrites and leucocyte esterase levels in urine is developed [3], however its specificity and sensitivity are insufficient for use in laboratories. In veterinary practice, immunochromatographic test based on the detection of widespread bacterial pathogens in urine using specific antibodies begins to be used. Although the test cannot determine fungi and rare bacterial strains, it is promising for application in laboratory diagnosis [4], further multicenter studies are required to prove its diagnostic significance.

Flow cytometry based analyzers are used for bacteriuria screening in laboratory practice, for example UF-1000i (Sysmex). It combines cytofluorimetry and conductometry and can analyze the composition of urine sediment and also the concentration of microorganisms in urine. In case of significant bacteriuria, the concentration of microorganisms in urine sample is higher than in sterile samples and some of contaminated samples. That allows to define cut off level and to separate positive and negative samples prior to culturing to reduce the workload of the laboratory and to obtain negative results on the day of urine collection. UF-1000i was shown to be a useful tool for preliminary selection of urine sample for further bacteriuria testing [5]. Despite of high sensitivity, flow cytometry does not provide any information about viability of the detected microorganisms, which has negative influence on the specificity of the method.

Since 1980s nephelometry based systems, which detect scattered light intensity changes in time due to microorganisms division, are used for direct determination of viable microorganisms in urine samples [6]. Uro-Quick (Alifax) and BacterioScan 216Dx (BacterioScan Inc.) systems allow not only to perform bacteriuria screening, but also to test bacterial cultures for antibiotic susceptibility [7–10]. For bacteriuria screening, the analyzed parameter is growth delay time after start of incubation: in the case of significant bacteriuria, fast growth is observed, in the case of contamination, growth is delayed for a few hours. In nephelometry, high sensitivity is achieved only if cuvettes of high optical quality are used and complicated devices for stray light reduction are designed. It makes the used analyzers more complicated, while their sensitivity is still insufficient for turbidity measurement of urine samples in addition to recording growth curves. The data about urine turbidity would allow estimating the initial concentration of microorganisms, thus increasing diagnostic reliability of negative and positive urine sample determination.

Coherent fluctuation nephelometry (CFN) is a variant of nephelometry. In traditional nephelometry (TN), the mean intensity of scattered light in time is measured, while in CFN, the intensity fluctuations of scattered light are recorded. In TN, the stray light from optical parts of the device (first of all from the cuvette) and the light scattered by microorganisms are mixed and fundamentally cannot be separated in the resulting signal. In CFN, only particles moving in the cuvette make contributions to fluctuations of scattered light intensity, and the stray light from immovable parts of the device (cuvette, diaphragm, etc.) are subtracted and almost do not contribute to the resulting signal. Asymmetrical heating of the cuvette is used to provide the convection of the contained liquid, which makes the particles under investigation move with enough velocities. Furthermore, due to technological simplicity, in CFN light scattered to small angles (5–7°) can be detected easily, while in TN, stray light have the most impact at small angles. In turn, microorganisms also scatter light mostly to small angles, therefore recording intensity fluctuations of light scattered at low angles allows achieving the sensitivity limit of 600 CFU/ml in CFN, while the sensitivity of the best traditional one-angled nephelometers is limited by 25,000 CFU/ml [11].

High sensitivity of CFN allows not only to record growth curves of microorganisms at low concentrations, but also to estimate the concentration of microorganisms in urine samples by its turbidity. CFN-analyzers were used for bacteriuria screening by growth curve and by concentration of microorganisms separately [12] and also for antibiotic susceptibility testing [13].

The aim of the work is evaluation of diagnostic effectiveness of CFN-analyzer for bacteriuria screening.

Materials and methods

Total 117 urine samples were collected for routine microbiological testing in hospital and outpatient departments of the Federal State Autonomous Institution "Scientific Center of Children's Health" of the Ministry of Health of the Russian Federation (Moscow). Samples were stored in sterile containers without preservative agents at room temperature and analyzed within 2 h after collection. Every urine sample was divided into 3 parts. One part was used for culturing on *Uriselect* chromogenic agar (Bio-Rad, France) with the Ø4 mm calibrated loop for 24 or 48 h. The second part was tested using the CFN-analyzer, the third part was analyzed using UF-1000i.

The turbidity of the whole urine sample is analyzed using the CFN-analyzer, but not single cells flowing along the capillary, as in flow cytometry. For that reason, urine samples were centrifuged for 60 s at 3000 rpm (1700 g) to sediment large impurities (such as cells, salts, mucus). During such centrifugation, microorganisms do not sediment onto the bottom of the test tube and stay in the volume of the liquid. Then 0.5 ml of the supernatant was mixed with 0.5 ml sugar broth, placed into disposable 1 ml semi-micro cuvettes, and closed with disposable stoppers (LP ITALIANA SPA, Italy). The cuvettes were placed into CFN-P-12 analyzer (Medtechnopark Ltd., Russian Federation, Moscow) and incubated for 8 h. Muller-Hinton broth (Bio-Rad, France) with addition of 0.5% glucose was used in this work.

The results obtained using UF-1000i and CFN-analyzer were compared with those of culturing on solid media, which is considered the gold standard. The result of analysis of initial turbidity and growth curves using the CFN-analyzer were interpreted both separately and together. The objective of use of both analyzers is detecting and excluding from further testing maximal percentage of negative samples, and saving maximal percentage of positive samples for further testing by culturing on solid media.

Samples with positive cultures (N_+) tested with the analyzer can be either positive (true positive – N_{TP}) or negative (false negative – N_{FN}), $N_+ = N_{TP} + N_{FN}$. Samples with negative cultures (N_-) tested with the analyzer can be either positive (false positive – N_{FP}) or negative (true negative – N_{TN}), $N_- = N_{TN} + N_{FP}$. To estimate the diagnostic informative value of the test, the following indicators were used (Eqs. 1–9): sensitivity (the percentage of detected positive samples); specificity (the percentage of detected negative

samples); PPV – positive predictive value (the probability that the positive sample tested with the analyzer will be positive in culturing); NPV – negative predictive value (the probability that the negative sample tested with the analyzer will be negative in culturing); LR^+/LR^- – positive/negative likelihood ratio (showing how more often a positive/negative sample by the analyzer results is positive than negative by the culture results); DOR – diagnostic odds ratio (the ratio of the odds of the test being positive if the sample is positive relative to the odds of the test being positive if the sample is negative). Clinically useful tests usually show $LR^+ > 10$ or $LR^- < 0.1$, $DOR > 20$ [14,15].

$$N_+ = N_{TP} + N_{FN}; \tag{1}$$

$$N_- = N_{TN} + N_{FP}; \tag{2}$$

$$Sensitivity = N_{TP}/N_+; \tag{3}$$

$$Specificity = N_{TN}/N_-; \tag{4}$$

$$PPV = N_{TP}/(N_{TP} + N_{FP}); \tag{5}$$

$$NPV = N_{TN}/(N_{TN} + N_{FN}); \tag{6}$$

$$LR^+ = \frac{N_{TP}}{N_+}/\frac{N_{FP}}{N_-} = Sensitivity/(1-Specificity); \tag{7}$$

$$LR^- = \frac{N_{FN}}{N_+}/\frac{N_{TN}}{N_-} = (1-Sensitivity)/Specificity; \tag{8}$$

$$DOR = \frac{N_{TP}}{N_{FP}}/\frac{N_{FN}}{N_{TN}} = \frac{PPV}{(1-PPV)}/\frac{(1-NPV)}{NPV} = LR^+/LR^-. \tag{9}$$

ROC analysis was used to estimate the diagnostic characteristics of the analyzers and to choose cut-off levels. ROC curve shows the dependence of *sensitivity* on (*1 – specificity*), while area under curve (AUC) shows the analytical precision of the test. AUC can be from 0 to 1, useful tests have AUC > 0.9. Mann-Whitney *U*-test was used to determine the statistical significance of the differences between the samplings. Statistical processing of the results was performed using Excel 2016 and IBM SPSS Statistics 20.

Results and discussion

The results obtained by culturing were interpreted in the following way:

- Negative samples with no growth – 34 (29%);
- Negative samples with non-significant bacteriuria: one uropathogen at low concentration < 10^4 CFU/ml – 6 (5%);
- Negative samples with contaminating mixed flora at low concentration ≤ 10^4 CFU/ml – 56 (45%);
- Negative samples with contaminating mixed flora at high concentration ≥ 10^5 CFU/ml – 3 (3%);
- Positive samples with significant bacteriuria: one or two uropathogens in concentration ≥ 10^4 CFU/ml – 21 (18%).

In samples with significant bacteriuria *E. coli* (*N* = 8; 38%), *Enterococcus* spp. (n = 6; 27%) and *Klebsiella* spp. (*N* = 4; 18%) were most common. The data about the microorganisms isolated from urine samples are shown in Table 1.

Seventeen of total 117 urine samples were tested using the UF-1000i analyzer both before and after centrifugation to confirm the insignificant influence of centrifugation on microorganism concentration. In average, the concentration of microorganisms was reduced insignificantly (by 3%) after centrifugation (Fig. 1).

Further, the values determined for positive and negative urine samples using UF-1000i and CFN-analyzers are shown.

According to the results of the measurements with UF-1000i, bacteria concentration in urine samples (B in CFU/ml) was 6.7×10^3 (95% CI: 4.1×10^3 to 11×10^3) for negative samples and 5200×10^3 (95% CI: 2000×10^3 to 7000×10^3) for positive ones, the

Table 1
Data on the microorganisms isolated from the positive urine samples.

Microorganisms species	N	%
E. coli	8	36
Enterococcus faecalis	4	18
Klebsiella pneumoniae	3	14
Klebsiella oxytoca	1	5
Enterococcus faecium	2	9
Citrobacter freundii	2	9
Staphylococcus simulans	1	5
Acinetobacter pittii	1	5
Total	**22**	**100**

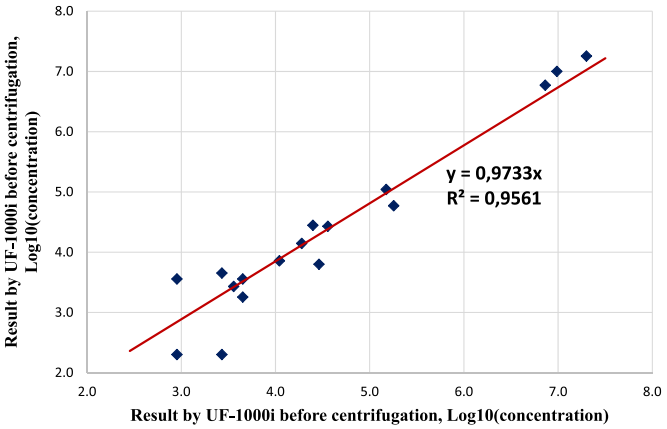

Fig. 1. The results of urine samples testing by analyzer UF-1000i before and after centrifugation.

differences between the groups are significant ($p = 0.0001$).

According to the results of the measurements with the CFN-analyzer, the turbidity of urine samples (T in arbitrary units) was 2.6×10^3 (95% CI: 1.9×10^3 to 3.5×10^3) for negative samples and 170×10^3 (95% CI: 70×10^3 to 410×10^3) for positive ones, the differences between the groups are significant ($p = 0.004$).

According to the results of the measurements with the CFN-analyzer, the time needed for microflora of urine samples to show growth (G in hours) was 5 (95% CI: 4.5–5.4) for negative samples and 0 (95% CI: 0–0.8) for positive ones, the differences between the groups are significant ($p < 0.00001$).

To record the results of turbidity and growth measurements using the CFN-analyzer together, we chose the simplest linear combination of Log(T) (decimal logarithm of turbidity) and G (growth detention time) parameters, called T & G. It is defined as $T\&G = c + k \times Log(T) - G$, where k – coefficient of order of unity, c – constant of order of unity. Linear combination T & G is measured in arbitrary points, the chosen coefficients are $k = 1.7$, $c = 4.6$ (coefficient k is chosen according to Fig. 8, since it defines the inclination of the cut-off line, and constant c is chosen so that the minimal T & G value is 0).

According to the results of the measurements of both turbidity and growth together using the CFN-analyzer (T & G) negative urine samples scored 6 (95% CI 5.5 – 6.5), while positive ones scored 13.5 (95% CI 12.6–14.4), the differences between the groups are significant (p = 0.0002).

The measured values of B, T, G and T & G for samples with different culture results are shown in Table 2 and Figs. 2–5 (the columns on the diagrams represent inter-quartile range of 25–75 percentiles, that is central 50% samplings; the lines inside the columns show the medians; upper and lower ends cover the whole range of values; the dots represent statistical outliers – the values which 3 times exceed the inter-quartile range). A group of samples with contaminating flora was divided into two subgroups (N = 3 and N = 53) with high ($\geq 10^5$ CFU/ml) and low ($\leq 10^4$ CFU/ml) bacteria concentrations.

ROC analysis was used to choose the cut-off levels for the investigated tests. ROC curves and corresponding AUC values are shown in Fig. 6.

To achieve optimal balance between sensitivity and specificity, the following cut-off levels were chosen: B $\geq 87 \times 10^3$ CFU/ml (UF-1000i), T $\geq 11.5 \times 10^3$ (turbidity by CFN), G ≤ 2.1 h (growth time by CFN), T & G ≥ 8.7 points (simultaneous turbidity and growth analyses using the CFN). In Fig. 7, all 117 tested urine samples are shown as dots, and their colour corresponds to the result of urine culture. Green lines illustrate the choice of cut-off levels for measurements of bacteriuria using UF-1000i and turbidity using the CFN-analyzer.

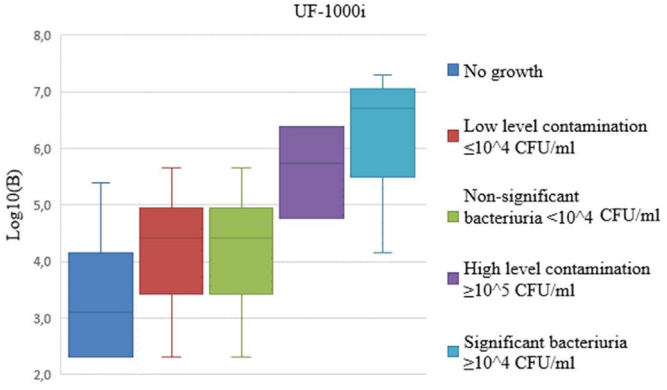

Fig. 2. Distribution of bacteriuria levels measured by UF-1000i (B) according to urine culture results.

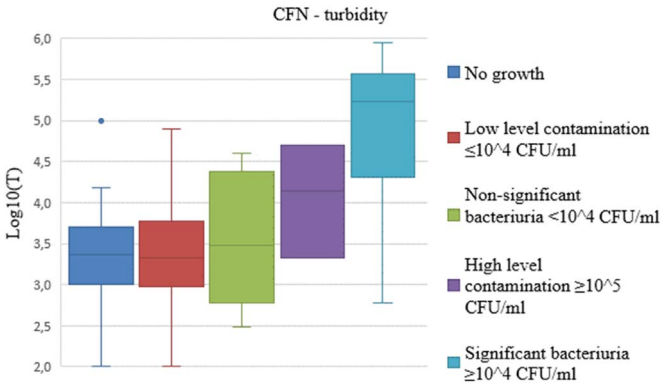

Fig. 3. Distribution of turbidity levels measured by CFN-analyzer (T) according to urine culture results.

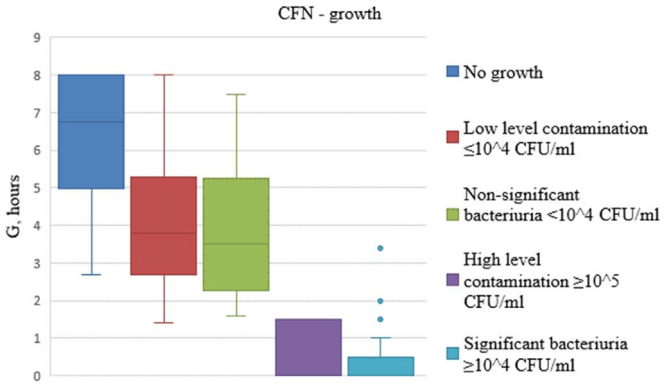

Fig. 4. Distribution of growth times measured by CFN-analyzer (G) according to urine culture results.

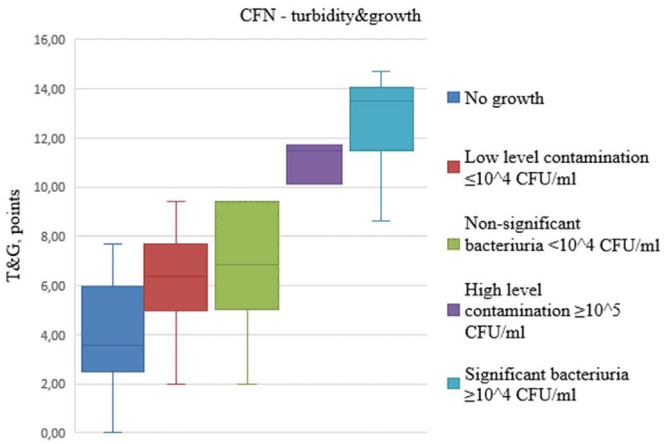

Fig. 5. Distribution of turbidity and growth time linear combination values measured by CFN-analyzer (T & G) according to urine culture results.

Fig. 8 illustrates the simultaneous analyses of turbidity and growth time using the CFN-analyzer, all 117 tested urine samples are shown as dots, and their colour corresponds to the result of urine culture. Green line illustrates the choice of cut-off level, its inclination is defined by coefficient k. Value $k = 1.7$ is chosen based on dot distribution on the diagram.

According to the chosen cut-off levels for all methods, 19–20 positive urine samples were determined correctly (true positive, N_{TP}), 1–2 positive samples were determined incorrectly as negative (false negative, N_{FN}), 79–93 negative samples were determined correctly (true negative, N_{TN}) and 3–17 negative samples were determined incorrectly as positive (false positive, N_{FP}). The contingency table is shown in Table 3.

Based on Table 3, diagnostic indicators for the investigated methods were calculated using formulas 1–9, sensitivity was 90.5–95.2%, specificity: 82.3–96.9%, PPV: 54.1–87%, NPV: 97.6–98.9%, LR^+: 3.4 – 30.5; LR^-: 0.049 – 0.058; DOR: 25.2 – 81.7. The percentage of incoming samples, determined as negative and excluded from further analyses, was 68.4 – 80.3%. Detailed information is summarized in Table 4.

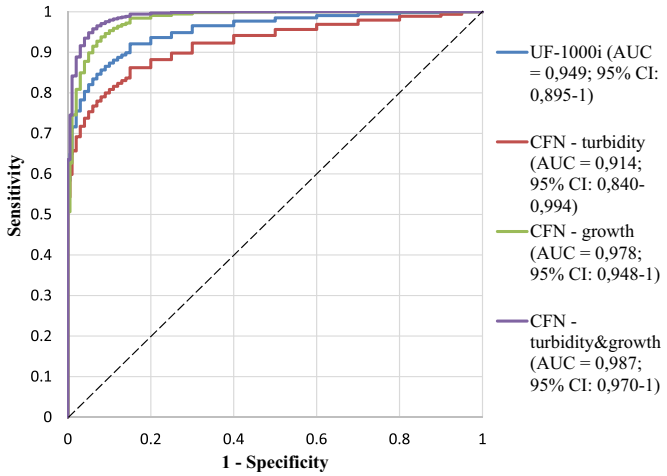

Fig. 6. ROC-curves and AUC values for bacteriuria measurements by UF-1000i, turbidity, growth time and their linear combination measured by CFN-analyzer.

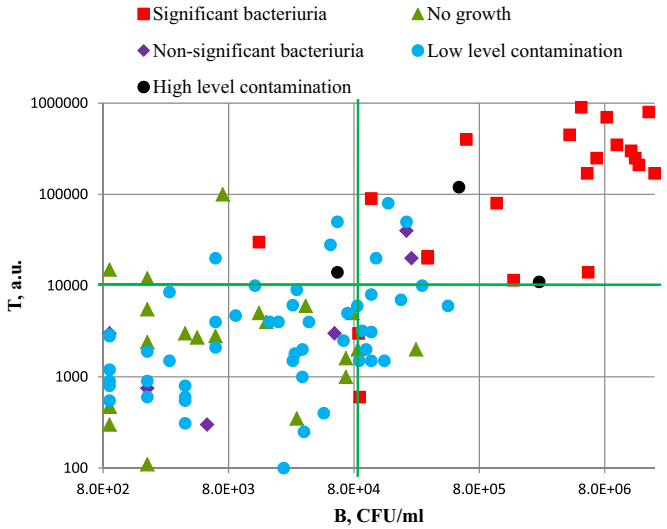

Fig. 7. The results of bacteriuria measurements by UF-1000i (B) and turbidity measurements by CFN-analyzer (T) according to urine culture results, green lines correspond to chosen cut-off levels (87×10^3 CFU/ml and 11.5×10^3 a.u. accordingly).

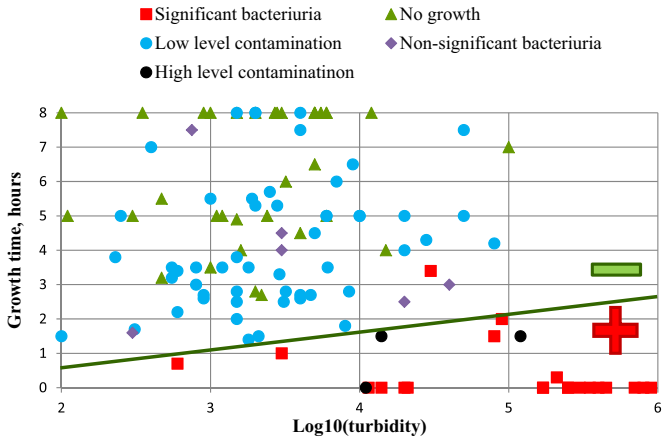

Fig. 8. Result of turbidity (T) and growth time (G) measurements by CFN-analyzer according to the urine culture results, green line corresponds to chosen cut-off level cut-off (8.7 points) for linear combination T & G.

Table 2
Comparison of the results of urine culture and bacteriuria (B) measurement using UF-1000i, turbidity and growth (T, G, T & G) measurements using the CFN-analyzer.

Urine culture result		No growth	Contamination and non-significant bacteriuria < 10^4 CFU/ml	Significant bacteriuria ≥ 10^4 CFU/ml
	N (%)	34 (29%)	62 (53%)	21 (18%)
UF-1000i: B (×10^3 CFU/ml)	Median	1.3	26	5200
	95% CI	0.5–3.4	15–46	2000–7000
	Min–Max	0.2–250	0.2–2400	14 – 20,000
CFN: T (×10^3 a.u.)	Median	2.3	2.8	170
	95% CI	1.3–4.1	1.9–4.2	70–410
	Min–Max	0.1–100	0.1–120	0.6–900
CFN: G (hours)	Median	6	3.5	0
	95% CI	6.1– 7.4	3–4	0–0.8
	Min–Max	2.7–8	0–8	0 – 3.4
CFN: T & G (points)	Median	3.6	6.5	13.5
	95% CI	2.9–4.3	6–7.1	12.6–14.4
	Min–Max	0–7.7	2–11.7	8.6–14.7

Table 3
Contingency table for distribution of the results of urine culture and bacteriuria testing using UF-1000i, turbidity and growth analysis using the CFN-analyzer.

Test results		Significant bacteriuria by culture	
		Yes	No
Total		N_+ = 21	N_- = 96
Bacteriuria, UF–1000iB ≥ 87 × 10^3 CFU/ml	Yes	20	17
	No	1	79
Turbidity, CFN-analyzerT ≥ 11.5 × 10^3 a.u.	Yes	19	13
	No	2	83
Growth time, CFN-analyzerG ≤ 2.1 h	Yes	20	10
	No	1	86
Turbidity & growth, CFN-analyzerT & G ≥ 8.7 points	Yes	20	3
	No	1	93

Table 4
Indicators of diagnostic informative value for bacteriuria determination using UF-1000i, turbidity and growth analysis using the CFN-analyzer.

Analyzer	UF-1000i	CFN-analyzer		
Test	B ≥ 87 × 10^3 CFU/ml	T ≥ 11.5 × 10^3 a.u.	G ≤ 2.1 h	T & G ≥ 8.7 points
Sensitivity, %	**95.2**	**90.5**	**95.2**	**95.2**
95% CI	51.6–100	52.7–100	51.6–100	51.6–100
Specificity, %	**82.3**	**86.5**	**89.6**	**96.9**
95% CI	61.1–100	64.6–100	67–100	72.8–100
AUC	**0.949**	**0.914**	**0.978**	**0.987**
95% CI	0.895–1	0.84–0.994	0.948–1	0.97–1
PPV, %	**54.1**	**59.4**	**66.7**	**87**
95% CI	28.3–100	29.3–100	31.2–100	25.8–100
NPV, %	**98.8**	**97.6**	**98.9**	**98.9**
95% CI	72.4–100	72.2–100	73.4–100	74.3–100
LR^+	**5.4**	**3.4**	**9.1**	**30.5**
95% CI	2.4–12	1.5–7.5	3.7–22.4	8.3–112
LR^-	**0.058**	**0.056**	**0.053**	**0.049**
95% CI	0.013–0.24	0.008–0.44	0.007– 0.4	0.006– 0.37
DOR	**43.2**	**25.2**	**58**	**81.7**
95% CI	5.6–334	5.6–114	7.5–460	10.4– 641
The percentage of samples excluded from further analyses, %	**68.4**	**72.6**	**74.4**	**80.3**
95% CI	51.5–90.9	54–97.7	56.3–98.1	61.2–100

All tested methods demonstrated diagnostic usefulness for preliminary selection of urine samples for the microbiological culture for bacteriuria. The samples without growth, with non-significant bacteriuria and contaminating flora in average have lower concentration of microorganisms, and the growth in such samples is observed later than in those with significant bacteriuria. At the same time, the samples with high concentration of contaminating flora (≥ 10^5 CFU/ml, N = 3) had high initial concentration of

microorganisms and showed fast growth. This suggests the special significance of pre-analytical stage for bacteriuria screening.

The values of bacteriuria measured with UF-1000i and of initial turbidity measured with the CFN-analyzer correspond to the same measured parameter, namely initial concentration of microorganisms in urine samples, but their viability is not analyzed. The samples with higher amount of non-viable microorganisms increase the percentage of false-positive results, therefore the separation of positive and negative samples is not maximally efficient. Analysis of growth curves allows detecting the viable microorganisms, while urine can contain growth inhibiting agents which can cause false negative results, while rapidly growing contaminating bacteria can cause false positive results. The simultaneous analysis of initial concentration of microorganisms represented by the initial turbidity of the centrifuged sample and growth curves allows to separate preliminary urine samples for further culturing with maximal effectiveness, which is proved by the diagnostic indicators of the simultaneous analysis of turbidity and growth delay time using the CFN-analyzer (Table 4).

Simultaneous analysis of turbidity and growth curves of urine samples using the CFN-analyzer allows detecting maximum number of negative samples and preserving almost all positive samples for further testing by conventional methods. It allows to decrease the costs for urine testing and to obtain negative results on the day of urine sampling.

This study was performed using prototypes of CFN-analyzers. The future serial devices will be provided with corresponding reagents kits, composed of disposable cuvettes already containing nutrient broth. Hence, the preparation for urine testing will take only 15 min, including fast centrifugation during one minute and inserting the samples into the cuvettes. Together with analytical procedure it will take only 3–3.5 h to test the samples, allowing to obtain negative result on the day of urine collection and to reduce the number of samples to be cultured by 80%. At the same time, 3-h delay in positive samples culturing will not influence the result obtaining the next morning significantly.

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Conflict of interests

Authors declare no conflict of interests

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