Original paper

The development of an analysis protocol based on flow cytometry for rapid detection of uropathogenic E. coli in artificially contaminated urine samples

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Abstract

Inappropriate empiric treatment for urinary tract infections (UTI) has led to a significant increase in the prevalence of resistant pathogens and is associated with a rise in hospital mortality. Novel diagnostic platforms, including flow cytometry (FC) devices, are needed for timely microbial identification and rapid antimicrobial susceptibility testing (AST). The aim of the present study was the design and optimization of a rapid assay, based on FC, for direct detection of resistant E. coli directly in artificially urine samples. A total of thirty Escherichia coli strains, isolated from UTI, grown in artificial urine were investigated and four antibiotics were tested. FC analysis investigated the bacterial viability based on the assessment of increased membrane permeability for propidium iodide. The results obtained by FC tests were compared with standard AST cultivation methods (VITEK-2 automated system). The developed FC based assay was demonstrated to detect antibiotic resistant E. coli isolates, in less than four hours analysis time, directly in artificial urine samples. The developed analysis method could improve the accuracy of emergency antimicrobial therapy in UTIs.

Keywords

Urinary tract infections (UTI), E. coli, Flow cytometry (FC), non-viable:viable bacteria.

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Introduction

Urinary tract infections (UTIs) are among the most common bacterial infections and a significant cause of morbidity and mortality (Blango and Mulvey, 2010). Studies are indicating an increase in resistance, particularly the Gram-negative bacilli, to multiple antimicrobial agents, such as β-lactams and fluoroquinolones (Falagas and Karageorgopoulos, 2008; Mladin, 2009). Also, several studies showed a rise of the prevalence of carbapenem resistant Enterobacteriaceae among patients with serious infections, including UTIs (Zilberberg et al., 2017).

Recently, fosfomycin has been introduced for the treatment of infections with MDR (multidrug-resistant) uropathogens for which other treatment options are limited (Kalal and Nagaraj, 2016). Therefore there is an urgent need for the availability of rapid AST methods in order to optimize initial antibiotic therapy.

Severe UTIs are often treated empirically with broad-spectrum antibiotics. Preliminary data on antibiotic susceptibility could improve the initial therapy of UTIs and reduce the use of unnecessary drugs. Additionally, patients carrying resistant microorganisms could be rapidly isolated, preventing the spread of MDR organisms within hospital settings and other institutions. Thus, the availability of a rapid method for the direct detection of resistant bacteria in the clinical sample, before the standard susceptibility testing results, would improve the empirical therapy. Standard phenotypic methods used for AST are based on the growth of bacteria therefore requiring an analysis time of at least 18 hours. Additionally, these methods are dependent on the identification of pathogens, hence the susceptibility results are available after two days or more.

Flow cytometry (FC) is a quantitatively analysis tool that enables the detection and enumeration of thousands of cells or particles in a few seconds. Light scattering and fluorescence emissions are used for the measurement of structural and functional characteristics of individual cells or particles interrogated by the laser beam. Multiparameter acquisition results in large data that is further analyzed using statistical software. Several studies have demonstrated that FC technology could be employed for the simultaneous detection, identification and susceptibility testing of bacteria directly in clinical specimens (Gauthier and Villemur, 2002; Nuding and Zabel, 2013). The studies indicated a good agreement with standard cultivation-based methods, i.e. the reference disc diffusion method and the reference microdilution method. Despite its advantages over conventional susceptibility assays, the application of this technique in microbiological laboratories is infrequent. The aim of the present study was the development of an assay based on FC for direct detection of antibiotic resistant E. coli directly in artificially urine samples. Detection of resistant bacterial isolates was performed by measuring the alteration of bacterial membrane integrity using the intercalating dye propidium iodide (PI). Ciprofloxacin, fosfomycin, nitrofurantoin, cotrimoxazole were tested against uropathogenic E. coli strains grown in artificial urine samples.

Materials and Methods

Uropathogenic bacterial isolates

The bacterial strains used in this study were isolated from urine specimens, collected from 30 patients (27 females and 3 males, mean age 50 years) with UTIs (Private Medical Laboratory, Bucharest, in March-April 2015). The identification and AST of the clinical E. coli isolates were performed using the automated system Vitek 2 (bioMerieux), following the recommendations of the international standard CLSI (2018).

Artificial urine medium formula

We used an adapted artificial urine medium (AUM) (Chutipongtanate and Thongboonkerd, 2010) with the following composition: 200 mM urea, 1 mM uric acid, 5 mM Na₂C₃H₆O₇, 54 mM NaCl, 30 mM KCl, 15 mMNH₄Cl, 3 mM CaCl₂, 2 mM MgSO₄, 2 mM NaHCO₃, 0.1 mM Na₂C₃O₇, 9 mM Na₂SO₄, 3.6 mMNaH₂PO₄, 0.4 mM Na₃HPO₄. The physical parameters of the AUM were: pH 6.2, specific gravity 1.008, 446 mOsm/kg osmolality.

Bacterial cultivation in AUM

Different ratios of AUM and Luria Bertani (LB) medium, i.e. 2:1, 7:5, 5:7 and AUM and distilled water, i.e. 2:1 and 5:7 were investigated to establish theoptimal formula and incubation period necessary for bacterial growth. Suspensions of E. coli ATCC 21925, prepared in sterile saline, from 16-18 h solid culture, and adjusted to 1.5 X 10⁸ CFU/mL, was added (v) to the different AUM: LB medium or AUM: distilled ratios to a final volume of 10% (v/v). The bacterial growth was evaluated by spectrophotometric analysis after 4, 5 and 6 h incubation, at 37°C in aerobic conditions.

Detection of viable and nonviable bacteria by FC analysis

FC analysis was performed using a Becton-Dickinson FACS Calibur instrument equipped with a 488 nm argon laser. For all experiments, approximately 10000 events were collected per sample. The SSC-FSC dot plot referring to cell size and granularity was employed for bacterial population gating. Red fluorescence was detected in the FL3 channel (670 nm long pass filter).
Suspensions of *E. coli* ATCC 21925 were prepared in physiological saline from 18 h solid culture and adjusted to 1.5 X 10^8 CFU/mL. Serial dilutions were carried out in saline from 10^3-10^6 CFU/mL. Different ratios of heat inactivated bacterial cells and viable cells (100°C – 30 min) were analysed: 9:1, 4:1, 7:3, 3:2, 1:1, 2:3, 3:7, 1:4, 1:9. A volume of 100 µL of the each mix inoculum was added to 900 µL of AUM: LB (7:5) followed by four h incubation at 37°C. Thereafter the samples were stained at room temperature in dark for 10-15 minutes with PI (final concentration of 1 µg/mL). Samples fluorescence was measured by FC. The sample preparation and analysis is presented in Figure 1.

![Figure 1. Sample preparation and analysis by FC method.](image)

**Evaluation of uropathogenic *E. coli* antibiotic susceptibility directly in artificially urine samples using FC**

For FC AST, bacterial suspensions prepared in physiological saline from solid overnight cultures were inoculated in AUM: LB (7:5) containing different antibiotics to obtain final cellular density of 10^8 CFU/mL. The following antibiotics (Oxoid) and concentrations were tested: ciprofloxacin (5 µg/mL), fosfomycin (50 µg/mL), cotrimoxazol (25 µg/mL), and nitrofurantoin (50 µg/mL). After 4 hours incubation the antibiotic discs were removed, the bacterial cells were pelleted at room temperature at 7000 rpm for 3 minutes, and resuspended in 500 µl filtered phosphate buffered saline (PBS). The controls were represented by: non-treated bacterial cells, heat inactivated bacterial cells *E. coli* ATCC 21925. At the end of incubation all the samples and controls were PI labelled (1 µg/mL PI final concentration) and after 10 minutes at room temperature, in dark, the FC measurements were performed. The FC AST results were compared to those obtained from the Vitek®2 (bioMérieux) analysis.

**Results**

**Evaluation of bacterial growth in the AUM**

The bacterial growth in AUM was measured by spectrophotometric readings after 4, 5, and 6 h of incubation at 37°C in aerobic conditions (Figure 2).

The results indicated an increase in the absorbance values after 4 h of incubation for the AUM: LB medium mixtures, the trend being maintained up to 6 h. In case of AUM diluted with water after an increase of absorbance values at 4 h, a decrease was observed at 6 h.

**Discrimination between viable and non-viable bacteria**

FC analysis of different ratios of viable and non-viable bacteria cells was carried out to determine the median fluorescence intensity (MFI) of the gated bacterial population. Histograms corresponding to viable and non-viable bacteria are showed in figure 3. Samples from the M1-M5 range exhibited a profile superposed with the corresponding control, represented by heat inactivated cells (data shown for the M1/ heat inactivated cells). Also, the sample M9 and the viable cells control revealed similar profiles on the histograms.

The results showed that the red fluorescence could be easily detected for high densities of non-viable bacteria (9:1 to 1:1 viable: non-viable bacteria ratio). A decrease of red fluorescence was detected for 2:3, 3:7, 1:4, 1:9 viable: non-viable bacteria ratios (figure 3).

**Detection by FC of antibiotic sensitive and resistance *E. coli* directly in artificially contaminated urine samples**

The antibiotic susceptibility profiles of the uropathogenic *E. coli* isolated from patients with cystitis...
and pyelonephritis were assessed using the automated Vitek system. The results are presented in Table 1. Multi-drug-resistance was detected for 24 strains (56%) and the ESBL (extended spectrum β-lactamases) phenotype for 19 strains (44%). High resistance rates were observed for ampicillin (100%), followed by fluoroquinolones (ciprofloxacin, levofloxacin) (96.66%). The tested strains were susceptible to all carbapenems, i.e. imipenem, meropenem and ertapenem (Table 1).
The development of an analysis protocol based on flow cytometry for rapid detection of uropathogenic E. coli

Table 1. Antibiotic resistance phenotypes of the tested E. coli strains.

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Regarding the FC AST method, for fosfomycin, all the E. coli tested susceptible to this antibiotic. After 4 h of incubation in presence of this antibiotic no bacterial growth was detected by the FC protocol. These results were in accordance with the standard method.

The 29 ciprofloxacin resistant E. coli formed a distinct population on the FSC-SSC dot plot after 4 h of incubation in AUM. The measurement of red fluorescence on the gated bacterial population allowed the determination of MFI that indicated a low red fluorescence corresponding to viable bacteria. In case of the ciprofloxacin susceptible E. coli strain, the FSC-SSC dot plot did not revealed any bacteria in the gate corresponding to the bacterial population suggesting that the antibiotic was efficient in inhibiting the bacterial growth. Concerning the cotrimoxazole results, the FC AST method detected a total of 26 strains exhibiting resistance and 4 sensitive to this antibiotic. In case of nitrofurantoin, the 26 E. coli strains were assessed as sensitive, and 4 were as resistant by FC AST.

Comparative analysis of the AST results for the four antibiotics: fosfomycin, ciprofloxacin, cotrimoxazole and nitrofurantoin, obtained by standard method and by the
novel protocol based on FC showed 100% correlation for fosfomycin, ciprofloxacin, cotrimoxazole and 86.67% for nitrofurantoin.

After 4 hours of incubation, in case of sensitive *E. coli* strains the FC AST analysis did not detect bacterial population on the controls’ gated regions of the FSC-SSC dot plot. For resistant *E. coli* strains the FC AST analysis allowed the detection of bacteria on the FSC-SSC dot plot and the subsequently measurement of the MFI corresponding to the red fluorescence. The resistant and sensitive phenotypes revealed by the FC histograms (figure 4) are presented below.

![Overlay histograms corresponding to the red fluorescence](image)

**Figure 4. Overlay histograms corresponding to the red fluorescence.** A. Black line corresponds to the viable control. The red line corresponds to the strain *E. coli* 4 exposed to the antibiotics (cotrimoxazole 100 μg/mL, nitrofurantoin 150 μg/mL, ciprofloxacin 4 μg/mL and fosfomycin 4 μg/mL). B. Black line corresponds to the viable control. The red line corresponds to the strain *E. coli* 5 exposed to the antibiotics (cotrimoxazole 100 μg/mL, nitrofurantoin 150 μg/mL, ciprofloxacin 4 μg/mL and fosfomycin 4 μg/mL). If the histogram sample is superposed with the viable control, the strain is resistant.

![Graphic representation of the MFI of the PI labelled *E. coli*](image)

**Figure 5.** Graphic representation of the MFI of the PI labelled *E. coli* after 4 h of incubation in contact with ciprofloxacin (5 μg/mL).

The MFI values corresponding to the red fluorescence of the *E. coli* strains exposed to ciprofloxacin for 4 h are presented in Figure 5, showing the standard deviation for three independent experiments. No statistical significance for the differences between the three experiments has been observed. Z score calculation for each strain tested revealed values between +2 and -2 σ.
The development of an analysis protocol based on flow cytometry for rapid detection of uropathogenic E. coli

Discussions

Empirical treatment regimens and increased incidence of resistant multi-drug resistant strains strongly indicates the requirement for the development of rapid identification and susceptibility methods.

The analysis of resistance profiles indicated that all the 30 UPEC strains were susceptible to fosfomycin, confirming that for uncomplicated cystitis fosfomycin remains a first line oral therapy. Resistance to fosfomycin most commonly arises by chromosomal mutations which do not spread easily (Ballestero-Telles et al, 2015). Fosfomycin demonstrates a favorable safety profile, and clinical trials have demonstrated that its efficacy is comparable to that of ciprofloxacin, nitrofurantoin, and trimethoprim-sulfamethoxazole (Zhanel et al, 2016). The analyzed UPEC strains revealed high resistance rates (97%) to ciprofloxacin that could be explained by the extensive use of this antibiotic in our country.

Currently, FC instruments are used for urine analysis with the purpose of detection of bacteriuria, leukocyturia, and erythrocyturia (Okada et al., 2001; Okada et al., 2006). Studies are suggesting that the FC analysis could allow identification of uropathogens prior to culture (Monsen and Ryden, 2015). The FC instruments could predict diagnosis of UTIs by ‘ruling out’ bacteriuria, leading to a substantial reduction of culture and ‘ruling in’ bacteriuria, confirming the UTI (Moshaver et al, 2016).

The comparative analysis of the standard AST method represented by the automated Vitek system and the FC assay developed for rapid detection of antibiotic resistance directly in urine samples has shown good correlations regarding the four tested antibiotics, i.e.: 100% for fosfomycin, cotrimoxazole ciprofloxacin and nitrofurantoin, and 86.67% for nitrofurantoin. The mismatches results could be explained by the fact that the nitrofurantoin disk is colored and thus interfered with the fluorescence analysis.

The overall results are suggesting that the FC assay developed for direct detection antibiotic resistance directly in artificially contaminated urine samples could represent a valuable alternative to classical screening approach of urine samples. Further tests are needed to validate the method on clinical specimens (i.e., urine samples). Also, the use of two or more fluorescent dyes could allow the detection of microbial metabolic changes induced by antibiotics could further improve the reliability of the FC AST method (Saint-Ruf et al., 2016).

Conclusion

Life-threatening infections require prompt antimicrobial therapy and therefore need rapid and accurate diagnostic tests. The FC-based protocol developed in this study could detect susceptibility of uropathogenic E. coli in artificially contaminated urine samples to four antibiotics (fosfomycin, cotrimoxazole, ciprofloxacin and nitrofurantoin), after 4 h of incubation. The results indicated good correlation of the FC-based assay results with those obtained with the standard AST method. Further tests are needed to validate the FC-based protocol on clinical specimens.

Acronyms and abbreviations

AST - antimicrobial susceptibility test
AUM - artificial urine medium
FC - flow cytometry
LB - Luria Bertani medium
MDR - multi-drug resistance
MFI - median fluorescence intensity
PI - propidium iodide
UTI - urinary tract infections

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References

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