A novel flow cytometric assay for rapid detection of extended-spectrum beta-lactamases

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Abstract

The rapid detection of extended-spectrum beta-lactamases (ESBLs) is a challenge for most clinical microbiology laboratories because inaccurate identification of ESBL producers has important clinical implications for both antibiotic treatment and infection control. The aim of our study was to develop a rapid detection assay of ESBL producers based upon flow cytometric analysis. Antimicrobial susceptibility testing followed by molecular characterization of bla_{TEM}, bla_{SHV} or $bla_{CTX\text{-}M}$ genes was performed on clinical isolates (41 ESBL positive and 20 ESBL negative) and isolates expressing well-characterized beta-lactamases, including ESBLs (n = 13), plasmid AmpCs (n = 3), oxacillinases (n = 5) and carbapenemases (n = 3). Additionally, two ATCC strains recommended by CLSI for susceptibility testing were used as controls. The flow cytometry analysis protocol involved an incubation of bacterial cells with different concentrations of ceftazidime (1, 2 and 4 mg/L) and cefotaxime (4, 8 and 16 mg/L) for 1 and 2 hours, in the presence and absence of clavulanic acid; subsequently, cells were stained with the fluorescent dye Bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$], a lipophilic anion able to diffuse across depolarized membranes. Additionally, CFU counts were performed. Susceptible isolates displayed increased fluorescence after 1 hour of incubation; conversely, the increase of the depolarized population was only observed after incubation with clavulanic acid associated with ceftazidime or cefotaxime in ESBL producers. An excellent correlation was obtained between the number of non-depolarized bacteria quantified by flow cytometry and by conventional CFU assays. A novel, accurate and fast flow cytometric assay is available to detect the presence of ESBLs.

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Introduction

Extended-spectrum beta-lactamases (ESBLs) are a large and rapidly evolving group of plasmid-mediated enzymes that confer resistance to oxyimino-cephalosporins and monobactams. These drugs are widely used for the treatment of serious invasive infections by Gram-negative bacteria [1]. ESBL recognition has an important clinical impact as inappropriate treatment can lead to therapeutic failures and consequently to adverse clinical outcomes [2]. Furthermore, failure to rapidly and reliably detect ESBL-producing isolates may impair appropriate infection control measures, which further contributes to its diffusion. Rapid detection of ESBL producers may not be easy and readily available during routine susceptibility testing because confirmation methods are often necessary. The semi-automated systems used by most clinical laboratories vary considerably regarding the detection capacity of ESBLs [1–3]. This detection has been invariably based upon growth assessment in the presence of cephalosporins with and without CLA.

On the other hand, although molecular methods brought speed and accuracy, several drawbacks prevent their full use

for ESBL diagnosis. DNA microarrays and multiplex PCR assays aiming to detect the most prevalent ESBL genes in the clinical setting, namely TEM, SHV and CTX-M, have been developed and are commercially available [4–6]. However, the genes encoding ESBL enzymes are highly diverse due to a variable number of amino acid substitutions as a result of continuous mutation. This contributes to the high cost of molecular assays. Moreover, although they provide valuable information for ESBL epidemiological investigation, phenotype tests could have a wider spectrum, making them more adequate for clinical use.

In order to cope with ESBL diversity, a comprehensive set of molecularly well-characterized isolates were hereby studied by classic methods and by flow cytometry (FC) assay. FC is a rapid, powerful high-throughput technology, which allows the analysis of several thousand cells per second, providing quantitative and statistically significant data [7]. Our group has developed several FC microbiological applications, namely to characterize and distinguish different physiological states of microorganisms, which could be addressed in relation to antibiotic susceptibility [8–11]. In the present study, an FC assay for the detection of ESBL producers is proposed. This method represents progress when comparing it with the conventional procedures.

Material and Methods

Bacterial strains

A total of 61 clinical isolates (64% from intensive care units) were tested; 20 ESBL-negative and 41 ESBL-positive isolates. All ESBL producers were resistant to cefotaxime and/or ceftazidime. Additionally, isolates expressing well-characterized beta-lactamases were also included in order to determine the true specificity of the cytometric assay (Table 1). Two strains from the American Type Culture Collection (ATCC), (*Klebsiella pneumoniae* ATCC 700603 and *Escherichia coli* ATCC 25922) recommended by the CLSI for susceptibility testing were used as controls [12].

Antimicrobial drugs and ESBL phenotypic detection

Ceftazidime (CAZ), cefotaxime (CTX) and CLA were purchased from Sigma Chemical Company (St Louis, MO, USA). Stock solutions (10 mg/ml) were prepared in sterile distilled water. Bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$], a fluorescent probe that binds to membranes and to intracellular proteins of depolarized cells, was purchased from Sigma Chemical Company; a stock solution (1 mg/ml) was prepared in dimethyl sulphoxide (DMSO).

The evaluation of the susceptibility profile to common cephalosporins and a screening for ESBL producers was conducted with VITEK 2 System panel AST-151 (BioMérieux, Paris, France). The disc diffusion method, using CAZ (30 μg) and CTX (30 μg) with and without CLA (10 μg), was used as a confirmatory test in isolates classified by the automated system as ESBL producers. The CLSI M2-A10 protocol was followed [13].

Multiplex PCR assay for ESBL characterization

A multiplex PCR protocol was used for the most common ESBL genes (TEM, SHV and CTX-M) according to Espinar and colleagues [14].

Flow cytometric analysis

The bacterial strains were incubated at 35 ± 1°C with shaking in Müeller-Hinton broth (Difco™) until the log phase was reached (about 1 h and 15 mins). Subsequently, a suspension containing 5×10^6 cells/ml in fresh medium was prepared and the bacterial cells were exposed either to 1, 2 and 4 mg/L of CTX, or 4, 8 and 16 mg/L of CAZ, alone or with 4 mg/L of CLA, for 60 and 120 min. In parallel, similar incubations with antimicrobials were performed after preparing bacterial suspensions directly from 24-h colonies. After incubation, the cells were washed and stained with 1 mg/L $DiBAC_4(3)$ for 30 min, at room temperature, protected from light. For each strain, non-treated and non-stained cells were also analysed in order to evaluate the native cell autofluorescence.

The fluorescence of bacterial suspensions was measured on a FACSCalibur Cytometer (BD Biosciences, Sydney, Australia). The data generated by FC were analyzed by CELL QUEST PRO software (version 4.0.2, BD Biosciences). The acquisition settings were defined using non-treated and non-stained cells (autofluorescence) and after adjusting the PMTs voltage to the first logarithmic (log) decade. The fluorescence intensity at 530/30 nm (FL1) was registered after incubation with antimicrobials and staining with 1 mg/L $DiBAC_4(3)$; the percentage of depolarized cells (cells with high intensity of green fluorescence) was quantified based upon the gated population and the area under the obtained curve, which is automatically normalized by the CYTOMETER software.

Cytometric susceptibility phenotype to cephalosporins and CLA Index

According to the results of the percentage of depolarized cells of each isolate after incubation with the cephalosporin, a cut-off value was defined in order to classify them as susceptible or resistant. In order to evaluate the CLA effect on resistant strains, a CLA index was calculated as the ratio of