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Short Communication

Evaluation of rapid colistin susceptibility directly from positive blood cultures using a flow cytometry assay

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ABSTRACT

Accurate assessment of colistin susceptibility is crucial with the increasing number of multidrug-resistant Gram-negative bacteria and simultaneously increasing colistin resistance. Both EUCAST and CLSI recommend broth microdilution (BMD) to determine colistin susceptibility, however it is cumbersome and growth-dependent. In this study, a rapid flow cytometry method (FASTinov®) to determine colistin susceptibility directly from positive blood cultures (BCs) was evaluated. BCs were spiked with 204 Gram-negative bacilli (137 Enterobacterales, 35 *Pseudomonas* spp. and 32 *Acinetobacter baumannii*) at a concentration of 2×10^3 cells/bottle, inoculated with human donor blood and incubated until flagged positive. As quality control strains, two susceptible (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) and two resistant (colistin-resistant *mcr-1*-positive *E. coli* NCTC 13846 and *Serratia marcescens* ATCC 14756) were used. Bacteria were extracted according to assay instructions and were incubated for 1 h at 37 °C with 2 and 4 mg/L colistin and a fluorescent dye, previously optimised. Cells were analysed on CytoFLEX (Beckman Coulter) and Accuri™ C6 Plus (BD Biosciences) flow cytometers. Colistin susceptibility results were automatically provided by BioFAST software (FASTinov®) and compared with those obtained with standard BMD. Overall categorical agreement between this new flow cytometry method and BMD was 99.0%. No very major errors were detected as well as no discrepancies between both flow cytometers. Here we describe a rapid and accurate assay for colistin susceptibility directly from positive BCs with a turnaround time of 2 h versus 48 h required for BMD. This method represents an accurate alternative to standard BMD.

1. Introduction

Blood culture (BC) represents the gold standard for identification of micro-organisms causing bloodstream infections or septicaemia [1]. For antimicrobial susceptibility testing (AST) of the micro-organisms involved, the current reference procedure requires positive BCs to be subcultured on solid media, thus usually requiring 48–72 h from BC positivity to the availability of AST results to clinicians. Empirical broad-spectrum therapy is commonly initiated to circumvent the reporting delay, representing a costly approach that often fails to effectively target the correct microbe to achieve therapeutic success.

FASTinov® (Porto, Portugal) has developed a disruptive flow cytometry method to evaluate several phenotypic lesions induced by antimicrobial drugs over bacterial cell populations [2,3]. This novel assay allows the evaluation of AST in a turnaround time of 1–2 h. It can be used directly from colonies, saving almost 1 day, but also directly from positive BCs, thus saving ≥2 days in result reporting time [4,5]. This technology provides the potential for real-time antibiotic decision-making, thus ensuring more rapid initiation of targeted therapy.

Colistin is currently considered one of the few 'last-line' therapies for multidrug-resistant Gram-negative bacilli. It is a membrane-active drug with several human toxicity side effects [6]. The reference methodology for colistin AST is minimum inhibitory concentration (MIC) determination by standard broth microdilution

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(BMD) [7]. However, colistin BMD has several technical issues, thus limiting it use in most routine clinical laboratories. Recently, a joint Clinical and Laboratory Standards Institute and European Committee on Antimicrobial Susceptibility Testing (CLSI-EUCAST) working group investigated colistin BMD and gave specific recommendations [8]. Nevertheless, this method remains cumbersome, time consuming and difficult to perform in routine daily practice, requiring ≥48 h from the moment that the BC is flagged positive. Consequently, different methods to determine colistin susceptibility have been proposed, including colorimetric and mass spectrometry assays [9–11]. Here we describe a rapid susceptibility test for colistin clinical categorisation based on flow cytometry methodology that takes <2 h to perform from the time the BC turns positive.

2. Methods

2.1. Blood cultures samples

BC bottles (Becton Dickinson, USA) were spiked with 204 Gram-negative bacilli, comprising: 137 Enterobacterales, including 12 intrinsically resistant isolates presenting colistin MICs > 32 mg/L (8 *Proteus* spp., 1 *Serratia marcescens*, 2 *Morganella morganii* and 1 *Providencia rettgeri*) and 11 isolates with acquired colistin resistance with colistin MICs in the range 4–8 mg/L (10 *Klebsiella pneumoniae* and 1 *mcr-1*-positive *Escherichia coli*); 35 *Pseudomonas* spp., including 5 resistant isolates with colistin MICs of 8 mg/L ($n = 4$) or 4 mg/L ($n = 1$); and 32 *Acinetobacter baumannii*, including 1 resistant to colistin with an MIC of >64 mg/L. BCs were inoculated with non-treated human blood from donors and were incubated until obtaining a positive flag [12]. As quality control strains, two colistin-susceptible (*E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) and two colistin-resistant (*mcr-1*-positive *E. coli* NCTC 13846 with a colistin MIC of >32 mg/L and *S. marcescens* ATCC 14756 with a colistin MIC of >16 mg/L) were used.

2.2. Extraction of micro-organisms and incubation with colistin and a fluorescent probe

A gel tube (BD Vacutainer® Barricor LH Plasma; Becton Dickinson) was filled (≈5 mL) with the positive BC and the bacteria were extracted after two centrifugation steps (1500 × *g* for 5 min and 1 min, respectively). The pellet was resuspended in brain–heart infusion broth (Liofilchem, Roseto degli Abruzzi, Italy) and a 0.5 MacFarland suspension was prepared and diluted 10-fold. The cells were then incubated for 1 h at 37 °C with 2 mg/L and 4 mg/L colistin according CLSI and EUCAST protocols for AST and with a fluorescent probe that only labels bacteria with cell membrane damage.

2.3. Flow cytometry analysis

Subsequently, cells were analysed in parallel both in a CytoFLEX (Beckman Coulter, USA) and an Accuri™ C6 Plus (BD Biosciences, USA) flow cytometer. Data analysis of the cytometric files was automatically performed by BioFAST software, a dedicated software developed by FASTinov®.

2.4. Colistin broth microdilution routine procedure and antimicrobial susceptibility testing flow cytometry evaluation

Spiked positive BCs were subcultured on blood agar plates for 24 h [7]. BMD AST was performed as recommended and the results were interpreted according both to EUCAST and CLSI guidelines. For the EUCAST protocol, the clinical breakpoints are susceptible ≤2 mg/L and resistant >2 mg/L for Enterobacterales, *Pseudomonas* spp. and *Acinetobacter* spp., and an Area of Technical Uncertainty (ATU) at 4 mg/L for *Pseudomonas* spp. [13]. For the CLSI protocol, breakpoints are susceptible ≤2 mg/L and resistant ≥4 mg/L for *Pseudomonas* spp. and *Acinetobacter* spp. No breakpoints are yet established for Enterobacterales and only epidemiological cut-off values suggested that wild-type isolates have colistin MICs ≤ 2 mg/L and non-wild-type isolates have colistin MICs ≥ 4 mg/L [14].

2.5. Data analysis and comparison between methods

Categorical agreement was calculated according to ISO standard 20776-2:2016, as well as very major errors (VME) and major errors (ME). VME rates were calculated using the total number of resistant isolates as the denominator, whereas ME rates were calculated using the total number of susceptible isolates as the denominator [15]. Minor errors were not calculated as no intermediate category has been established. Results obtained from CytoFLEX and Accuri™ C6 Plus were compared using Cohen's kappa (κ).

3. Results

Fig. 1 illustrates the distribution of fluorescence obtained with three control strains as an example of two susceptible strains (one Enterobacterales and one *Pseudomonas*) and one colistin-resistant strain, treated or not with the two colistin concentrations (2 mg/L and 4 mg/L) and stained with a membrane potential fluorescence marker after 1 h of incubation. In the case of the colistin-resistant control strain (*mcr-1*-positive *E. coli* NCTC 13846), there were no differences between the fluorescence displayed by cells exposed to colistin and the internal control (stained bacteria unexposed to colistin) (Fig. 1B). Regarding colistin-susceptible strains, there was an increase in fluorescence intensity compared with the internal control (Fig. 1A,C). The increase in fluorescence indicates cell membrane damage (shift to the right). Thus, differences in fluorescence intensity allow the discrimination of resistant from susceptible strains.

The overall categorical agreement between this new rapid flow cytometric method and the standard BMD reference method was 100% for Enterobacterales and *A. baumannii* and 94.3% for *Pseudomonas* spp. with one ME (1/30) with a BMD MIC of 2 mg/L and one ME in a strain with a BMD MIC of 4 mg/L, an ATU according to EUCAST criteria but resistant for CLSI. Regarding the strain classified as ATU by the reference method, it was categorised as resistant by flow cytometry. Moreover, there were no differences between susceptibility results obtained from CytoFLEX and Accuri™ C6 Plus ($\kappa = 1.00$).

4. Discussion

In this study, we validate the performance of a rapid flow cytometric assay for the study of colistin susceptibility directly from positive BCs. Indeed, it was possible to discriminate between clinically susceptible and resistant isolates by quantitative assessment of fluorescence intensity differences in bacterial cells exposed to different concentrations of colistin. Among colistin-susceptible isolates, there was an increase in fluorescence of treated cells compared with the control, whilst in colistin-resistant isolates there were no differences between treated bacteria and the control. The increase in fluorescence intensity signifies that the cell membrane was damaged as a result of colistin exposure according to the mechanism of action of the drug. Colistin acts on the cell membrane of Gram-negative bacteria, leading to disruption of the outer membrane with consequent leakage of intracellular contents and subsequent bacterial death [6]. Accordingly, susceptible bacteria treated with colistin exhibit a measurable higher fluorescence.

Different probes have been proposed to test AST using flow cytometry [16]. According to the colistin mechanisms of action, a

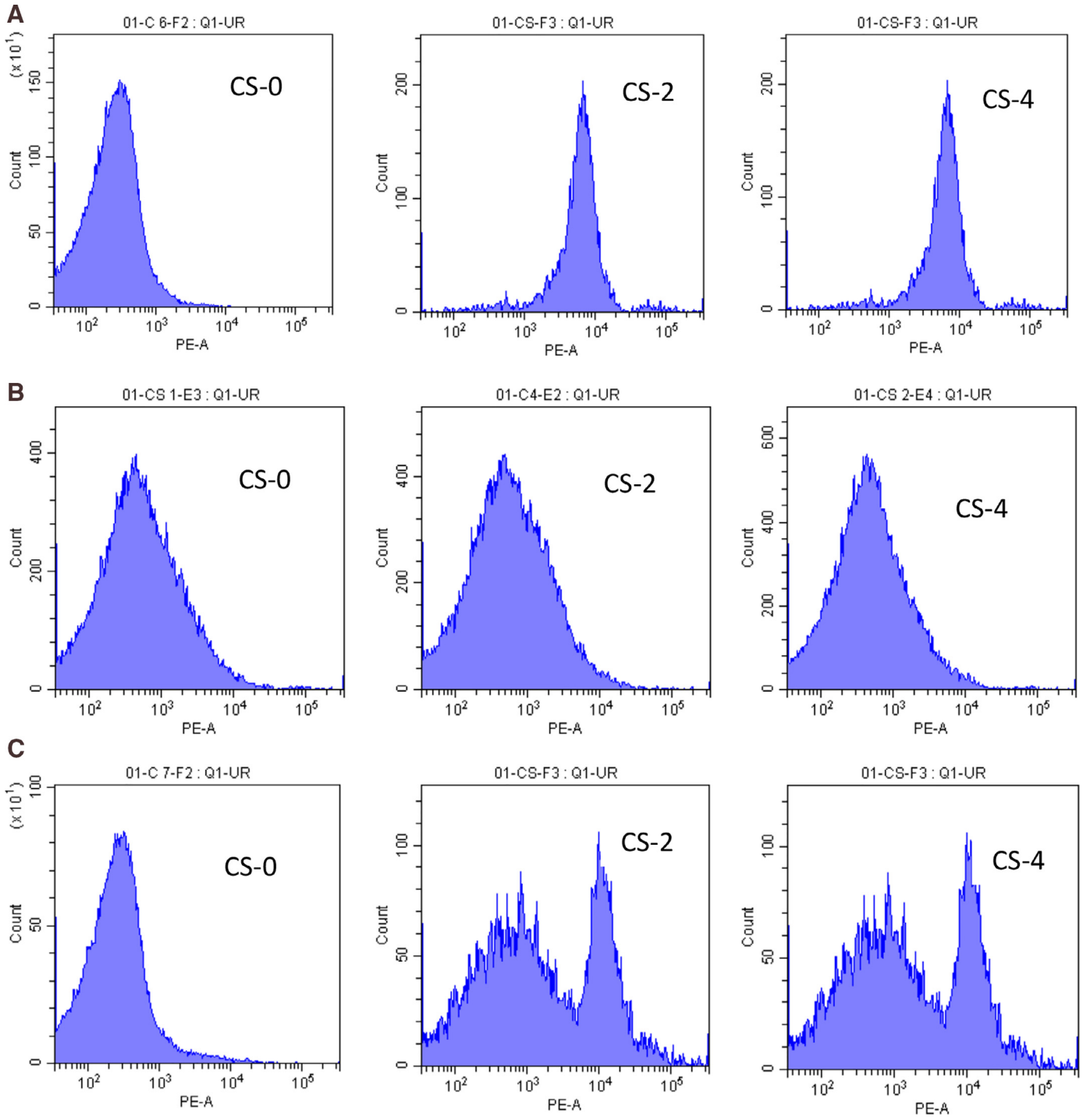

Fig. 1. Flow cytometry histograms of (A) *Escherichia coli* ATCC 35218 (colistin-susceptible), (B) *E. coli* NCTC 13846 (colistin-resistant strain *mcr-1*-positive) and (C) *Pseudomonas aeruginosa* ATCC 27853 (colistin-susceptible). Fluorescence displayed by control cells non-exposed to colistin (CS-0) and stained with fluorescent dye; cells exposed to 2 mg/L colistin and stained (CS-2); and cells exposed to 4 mg/L colistin and stained (CS-4).

probe that only penetrates when the cell membrane is permeable appears to be the most appropriate to study susceptibility to this particular drug. This approach allows much earlier detection of response compared with growth-dependent assays for AST. Notably, it should be stressed that the described flow cytometric test evaluates the antibiotic-dependent bacterial cell membrane lesion, thus being a functional assay, with the advantage of not being growth-dependent, contrary to other existing methodologies [17]. In addition, flow cytometric assay is very rapid and accurate method, requiring <2 h after the BC becomes positive, with excellent correlation with the current reference method for colistin AST.

In conclusion, we propose a novel rapid diagnostic assay for colistin susceptibility directly from positive BCs. We believe that its implementation in clinical microbiology laboratories should impact the patient management of bacteraemia in this era of an increasing number of multidrug-resistant Gram-negative bacteria and simultaneously increasing colistin resistance. This methodology represents a valid alternative to the current cumbersome BMD refer-

ence method, which is difficult to perform in most routine clinical laboratories. It was developed to be easy to perform, needing no special abilities. The results are provided by the developed software without the need for great expertise in flow cytometry. Identification by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) directly from BC coupled with the FASTinov® flow cytometry assay would help to effectively decrease reporting time in septic patients.

Declarations

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Competing Interests: None.

Ethical Approval: Not required.

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