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# Detection, Identification and Susceptibility Testing of Bacteria by Flow Cytometry

**Sabine Nuding[1]* and Lutz T Zabel[2]**

[1]Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology Stuttgart and University of Tuebingen, Germany
[2]Institute of Laboratory Medicine, ALB FILS KLINIKEN GmbH Göppingen, Germany

## Summary

Flow cytometry permits the simultaneous analysis of numerous parameters of eukaryotic cells as well as microorganisms making it a powerful tool for differentiation and functional analysis. However, application of flow cytometry in clinical microbiology is still infrequent. Here, we report on the development of flow cytometric assays to detect and identify bacteria, and determine antibiotic susceptibility. Susceptibility testing of a wide spectrum of bacterial culture isolates was based on the oxonol $DiBAC_4(3)$, and further modified to allow susceptibility testing using specimens such as blood cultures or swabs. Additionally an extended assay was developed for the rapid detection of methicillin resistant *Staphylococcus aureus* (MRSA) within 6 hours from swabs. The results of 402 flow cytometric susceptibility tests were confirmed by Bauer-Kirby agar disk diffusion or automated systems with an average agreement of 94%. This assay brought essential time saving of one day particularly concerning extended beta lactamase producing bacteria (ESBL). Susceptibility testing performed directly from positive blood cultures or from swabs increased the rapidness furthermore. Susceptibility results of bacterial strains tested directly from blood culture achieved an accordance of 89% compared to Vitek® 2. Investigating 140 screening swabs of patients colonized with MRSA and of their contact persons 22 swabs were identified correctly to contain MRSA, 2 MRSA samples were not identified due to a lack of protein A expression of the MRSA-strains. False classifications as MRSA-positive swabs did not occur. Flow cytometry offers a suitable technique for the rapid detection, identification and susceptibility testing of bacteria with great potential.

**Keywords:** Flow cytometry; Microbiology; Bacterial identification; Susceptibility testing; Membrane potential; Extended spectrum beta lactamases; Methicillin resistant *Staphylococcus aureus* (MRSA)

## Introduction

With the multiparametric measurement of individual cells at a rate of several thousand per second, flow cytometry offers a rapid method for the detailed analysis of microorganisms. This technique provides quantitative data for cell size or granularity through light scattering signals and via fluorescence signals quantitative information on the expression of surface antigens, or intracellular components such as nucleic acids, proteins or lipids, on membrane potential, and ion fluxes.

First applications of flow cytometry in microbiology appeared in the late 1970's. In these studies Hutter and Eipel [1] as well as Paau et al.[2] determined DNA content of bacteria with propidium iodide and total cell protein of microorganisms with fluorescein isothiocyanate[3]. Allmann et al. [4] monitored changes in light scattering signals as indicator for changes in bacterial cell size. Improvements of the instrumentation provided better differentiation between bacteria and background noise and led to further applications in microbiology as cell cycle kinetics [5], differentiation between Gram-positive and Gram-negative cell wall [6,7], and viability determination. The latter was then further modified for antimicrobial susceptibility testing based on metabolic activity or membrane integrity [8]. Currently, flow cytometry is used in numerous studies about bacterial viability in research and for the detection of contaminants in food products [9,10], the monitoring of fermentation processes [8,11] or in environmental research [12]. But although in clinical laboratories the application of the flow cytometric technique is well established in haematology and immunology, the use of this method in routine clinical microbiology is even rare. Indeed, in recent years automated platforms for identification of bacteria and susceptibility testing found their way in microbiological routine laboratories, but the potential of flow cytometry in this field is still underutilized. This is despite flow cytometric susceptibility testing can offer advantages over conventional susceptibility assays. For example, the commonly used agar diffusion or turbidimetric assays such as broth microdilution depend on cell growth of culturable cells and give an endpoint for the whole bacterial population. Although it is possible to identify individual colonies among a population coming up within a zone of inhibition in conventional assays, flow cytometry has the advantage to look at the single cell level and is able to identify heterogeneous subpopulations during susceptibility testing. Additionally, current standard susceptibility assays do not provide information on neither morphological changes of individual bacterial cells after antibiotic treatment nor changes in surface protein expression.

Although some flow cytometric methods for susceptibility testing have been published [13-16], none of these provided an insight into the range of possibilities using flow cytometric methods in a microbiological laboratory comprising detection, identification and susceptibility testing of bacteria. We developed a susceptibility testing assay based on measuring the breakdown of the membrane potential with the membrane potential sensitive probe oxonol $DiBAC_4(3)$ and employed it for a wide range of bacterial species and extensively utilized it for susceptibility testing [17,18]. Modifications of the assay were used to detect extended spectrum beta lactamase producing bacteria (ESBL).

***Corresponding author:** Sabine Nuding, Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology and University of Tuebingen Auerbachstr. 112, 70376 Stuttgart, Germany,Tel: (49) 711-8101-3736; Fax: (49) 711-85 92 95; E-mail: Sabine.Nuding@ikp-stuttgart.de

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Rapidness was achieved, performing susceptibility tests from primary material as blood cultures or swabs without previous isolation or overnight cultivation of the bacteria on agar plates. Furthermore for the detection of methicillin resistant *Staphylococcus aureus* identification of bacteria by the species specific detection of staphylococcal surface protein A was combined with susceptibility testing.

## Material and methods

### Bacterial strains and culture

The bacterial strains analysed with the flow cytometric assay included ATCC reference strains and clinical isolates from the Institute of Laboratory Medicine at the Klinik am Eichert, Göppingen, Germany. In the flow cytometric susceptibility test, 67 bacterial strains were investigated, comprising 10 strains *Escherichia coli*, 10 *Klebsiella pneumoniae or Klebsiella oxytoca*, 4 *Pseudomonas aeruginosa*, 10 *Staphylococcus aureus*, 10 *S. epidermidis*, 2 *S. lugdunensis*, 1 *S. capitis*, 1 *S. hominis*, 1 *S. haemolyticus*, 1 *Streptococcus pyogenes*, 1 *S. agalactiae*, 4 *Enterococcus faecalis*, 1 *E. faecium*, 3 *Acinetobacter baumannii*, 2 *Proteus mirabilis*, 1 *P. vulgaris*, 1 *Enterobacter aerogenes*, 1 *E. agglomerans*, 1 *Citrobacter freundii*, 1 *Serratiam arcescens* and 1 *Morganella morganii*.

Testing for extended spectrum beta lactamases was performed for further 24 clinical strains of *E. coli* and *K. pneumoniae*. An *E. coli* with CTX-M-type resistance was kindly provided by Prof. H. K. Geiss, formerly University of Heidelberg.

For susceptibility testing, isolated colonies from Columbia blood agar with 5% sheep blood (BD, Sparks, USA) were inoculated into Schaedler broth (BD, Sparks, USA) and cultured to the early logarithmic growth phase at 37°C, anaerobic species were grown in an anaerobic jar using Anaerogen$^{tm}$ compact (Oxoid, Wesel, Germany).

### Susceptibility testing

Several dyes were tested for viability determination: the intercalating dye propidium iodide, the esterase substrate fluoresceine diacetate and the membrane potential sensitive dyes $DiOC_5(3)$ and $DiBAC_4(3)$.

The detection of antibiotic susceptibility was performed with the membrane potential sensitive dye $DiBAC_4(3)$ [bis-(1,3-dibutylbarbituric acid) trimethineoxonol] (Invitrogen/Life technologies, Carlsbad, USA). $DiBAC_4(3)$ has an emission maximum of 516 nm and the green fluorescence can be detected in the channel of fluorescence 1 [6]. When bacteria reached the early logarithmic growth phase the culture was adjusted to 0.5 McFarland in prewarmed Schaedler broth and further incubated with antibiotics for 120 minutes at 37°C. Initially, different concentrations of antibiotics were tested to determine a breakpoint comparable to the minimal inhibitory concentration. For final susceptibility testing the following antibiotics were added to 1 ml bacteria suspension: mezlocillin (Bayer Vital GmbH, Leverkusen, Germany) or oxacillin (Bayer Vital GmbH, Leverkusen, Germany), piperacillin/tazobactam (Lederle, Wolfratshausen, Germany), cefuroxime (Eli Lilly, Indianapolis, USA), cefazolin (Eli Lilly, Indianapolis, USA), ciprofloxacin (Bayer Vital GmbH, Leverkusen, Germany) or gentamicin (Merck, Darmstadt, Germany) in concentrations as listed in table 1. Bacteria without antibiotic treatment served as controls. The dye $DiBAC_4(3)$ was added in a concentration of 0.5 µg/ml. After 120 minutes the suspensions were centrifuged for 10 minutes at room temperature at 420×g and the bacterial pellets were resuspended in PBS and analysed by flow cytometry. The results were compared to those of the Bauer-Kirby agar disk diffusion test and the MicroScan WalkAway-96® (Dade-Behring, Marburg, Germany) or the Vitek®2 (bioMérieux, Marcy-l'Etoile, France).

For testing of extended spectrum beta lactamases 24 strains suggestive of ESBL-production were incubated with serial dilutions from a stock solution of 128 µg/ml using following antimicrobials: ceftazidim (GlaxoSmithKline, London, United Kingdom), cefpodoxime (Sanofi-Aventis, Frankfurt, Germany), cefotaxime (Sanofi-Aventis, Frankfurt, Germany). Each antimicrobial was tested alone and with 4 µg/ml clavulanic acid (GlaxoSmithKline, London, United Kingdom) as additive. The procedure was done as described above.

### Blood culture

Susceptibility testing was performed with 41 bacterial strains from positive blood cultures identified with the BACTEC®9240 system (BD, Sparks, USA). A sample of 1 ml was removed and erythrocytes were lysed with 0.1% Saponin (Merck, Darmstadt, Germany). After centrifugation the pellet was washed with PBS, resuspended and inoculated into 8 ml Schaedler broth, and further incubated at 37°C. Bacteria in exponential growth phase, measured by optical density determination, were applied to the following tests: Initially, a classification as Gram-negative or Gram-positive was done to choose the appropriate antimicrobials. Therefore, the bacteria were stained with SYTO® 9 and hexidium iodide (LIVE BacLight™ Bacterial Gram Stain Kit, Invitrogen/Life Technologies, Carlsbad, USA). The SYTO® 9 stain labels all bacteria, whereas hexidium iodide is excluded from Gram-negative bacteria by the lipopolysaccharide layer and replaces SYTO® 9 only in intact Gram-positive bacteria. Thus, Gram-negative bacteria appear green fluorescent while Gram-positive are yellow-red fluorescent. Subsequently, the samples were applied to susceptibility testing as described above.

### Identification of methicillin-resistant *Staphylococcus aureus*

The assay to detect methicillin-resistant (oxacillin-resistant) *S. aureus* required preliminary experiments. Firstly the time needed for bacterial growth from swabs was determined by culturing low concentrations of *S. aureus* in Schaedler broth, which was controlled in intervals of 1 hour in the flow cytometer for a well-defined bacterial population in the forward scatter – side scatter dot plot.

Secondly, to investigate the suitability of the protein A expression as marker for *S. aureus*, 200 *S. aureus*-isolates, including 155 MSSA and 45 MRSA, and 100 coagulase negative staphylococci including *S. lugdunensis, S. capitis, S. cohnii, S. chromogenes, S. haemolyticus, S. hominis, S. simulans, S. warneri, S. xylosus* and 30 Gram-negative strains such as Enterobacteriaceae, *Pseudomonas aeruginosa* or *Neisseria spp*. were tested with a biotinylated monoclonal anti protein A goat antibody (Biotrend, Cologne, Germany). 100 µl bacteria suspension were incubated 10 minutes with 1 µl of the 1:100 diluted anti protein A antibody and washed with PBS. The pellet was resuspended in 300 µl PBS and 2 µg/ml streptavidin-phycoerythrin (BD, Sparks, USA) was added. After 10 minutes incubation and an additional washing step fluorescence 2 was measured by flow cytometry. For screening of *Staphylococcus aureus* in swabs we cultured the bacteria with a specific medium of Mueller-Hinton II (BD, Sparks, USA) enriched with 15% Schaedler to detect protein A on the bacterial surface. High levels of glucose decreased the expression of protein A, therefore, original Schaedler broth was not feasible for culture.

The detection of staphylococci from primary material was performed in 140 swabs from throat, nose or wound infections. They were submerged in a combination of Mueller Hinton Broth (BD, Sparks, USA) with 15% Schaedler broth and cultured at 37°C in a shaking water

bath for 3 hours. The broth was divided into a sample with oxacillin (1 µg/ml, Bayer Leverkusen, Germany), and a control without oxacillin. $DiBAC_4(3)$ (0.5 µg/ml) was added to the bacteria in the exponential log phase. After 120 minutes incubation at 37°C, the suspension was centrifuged for 10 minutes at room temperature at 420×g and the bacterial pellets were washed once with PBS. Samples with oxacillin and control were divided in oxacillin-1 and oxacillin-2 and in control-1 and control-2, respectively. Control-1 and oxacillin-1 were incubated for 10 minutes with the protein A antibody, diluted 1:100; to control-2 and oxacillin-2 no antibody was added. After washing step with PBS, streptavidin-phycoerythrin (BD, Sparks, USA) was added to each sample in a concentration of 2 µg/ml. The samples were incubated for 10 minutes and washed with PBS. Subsequently, the pellet was resuspended in 300 µl PBS. Fluorescence 1 ($DiBAC_4(3)$, susceptibility) and fluorescence 2 (Streptavidin-PE, protein A) were analysed by flow cytometry (German Patent 19945553; 1999). A test was valid, when no fluorescence occurred in samples oxacillin-2 and in both controls.

To detect protein A expression, the marker for fluorescence 2 was set at the highest fluorescence of the control-2 for each bacterial strain. To differentiate between viable and depolarized bacteria, the marker was set at the highest fluorescence 1 of the untreated control-2. The results of the flow cytometric test have been compared to routine methods, which were agar diffusion, growth on oxacillin screening agar with 6 µg oxacillin, the detection of the *mecAgene* by PCR and susceptibility testing by the automated systems Vitek®2 or WalkAway-96®.

## Flow cytometry

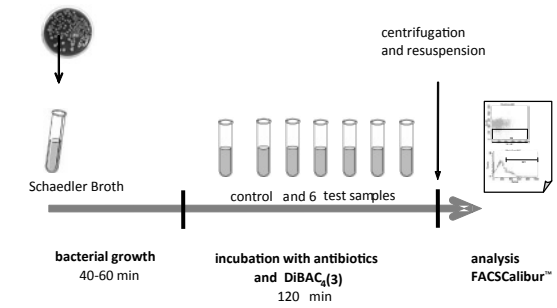
The flow cytometric assay was performed on a FACSCalibur flow cytometer (BD, Sparks, USA) equipped with an argon laser with an excitation wavelength of 490 nm and a red diode laser. 10,000–30,000 events of each sample were measured with the Software Cell Quest. The bacterial population was gated in the FSC-SSC-Dot Plot referring to cell size and granularity. Fluorescence 1 was measured for susceptibility testing ($DiBAC_4(3)$) or for Gram-determination (SYTO®9). For detection of protein A (Streptavidin-PE) and for Gram-determination (Hexidium iodide) fluorescence 2 was measured.

## Results

### Susceptibility testing of clinical isolates

In the last decades the prevalence of multiresistant bacteria has increased and it has become more and more important to shorten the time of susceptibility analyses. Therefore, the aim of this study was to establish a rapid flow cytometric susceptibility assay (Figure 1).

First, viable unstained bacteria were differentiated by a side scatter threshold from electronic noise, medium components and cellular debris. The susceptibility test was performed with the oxonol $DiBAC_4(3)$, which accumulates in the cytoplasm of depolarized bacteria by a Nernst equilibrium dependent uptake from the extracellular solution [19]. The dye binds to intracellular proteins or membranes and thus results in an enhanced green fluorescence. Bacterial cells of susceptible strains showed with respect to the untreated control an enhanced fluorescence 1 after treatment with antimicrobials which was dependent on the concentration of the antibiotic tested (Figures 2A-2D). Cells of resistant strains were not stained by $DiBAC_4(3)$. Consequently, their fluorescence intensity was similar to that of bacteria not treated with antibiotics in the control.




| Parameter | Measuring principle | Interpretation |
|---|---|---|
| FSC | scattered light | size |
| SSC | scattered light | granularity, internal complexity |
| Fl1 (green) | membrane potential | susceptibility |

**Figure 1: Scheme of the flow cytometric susceptibility test**. Process of flow cytometric susceptibility test-assay. Inoculation from agar, cultivation in broth, incubation with 6 different antimicrobials and the membrane potential sensitive dye $DiBAC_4(3)$ and flow cytometric analysis.

The concentrations of the different antibiotics, which led to membrane depolarisation, were determined by serial dilutions based on the concentrations recommended for testing by the Clinical and Laboratory Standards Institute (CLSI) (formerly NCCLS). A definite depolarisation was generally achieved with concentrations of the 1-2 fold of the minimal inhibitory concentrations (MIC) determined by E-Test or the automated systems. For the validation of the method 67 Gram-negative and Gram-positive isolates were treated with 6 antimicrobials of different classes comprising penicillins such as mezlocillin, piperacillin combined with tazobactam and oxacillin, the cephalosporins cefazolin and cefuroxime, the fluoroquinolone ciprofloxacin and the aminoglycoside gentamicin.

The oxonol $DiBAC_4(3)$ was suitable as indicator for bacterial viability for a broad range of bacteria species including mucoid species as *Pseudomonas spp*. and encapsulated species as *Klebsiella spp*. 95% of the flow cytometric susceptibility results were concordant with results derived by the agar diffusion method and 94% with the results obtained with the WalkAway-96®. Compared to the results of the agar disk diffusion, very major errors reporting susceptibility according to flow cytometry and resistance according to reference methods occurred in 4 of 402 susceptibility tests. When compared to the WalkAway-96® very major discrepancies increased to 6 of 402 susceptibility tests. Major errors reporting resistance in flow cytometry and susceptibility in reference methods were found in 16 cases compared to agar disk diffusion and in 17 cases compared to the WalkAway-96®. Most very major deviations were seen with the cephalosporin cefazolin, whereas cefuroxime, which possess the same mode of action, showed rare discrepancies.

However, each antimicrobial, whether it acts directly at the cell wall as the penicillins mezlocillin, oxacillin and piperacillin as well as the cephalosporins cefazolin and cefuroxime or on other targets as the quinolone ciprofloxacin or the aminoglycoside gentamicin, caused membrane depolarisation to sensitive bacteria and resulted in increased fluorescence 1. Figure 3 shows a flow cytometric susceptibility test (Figure 3A), the corresponding agar diffusion test (Figure 3B) and the results of the WalkAway-96®(Figure 3C). *E. coli* treated with mezlocillin

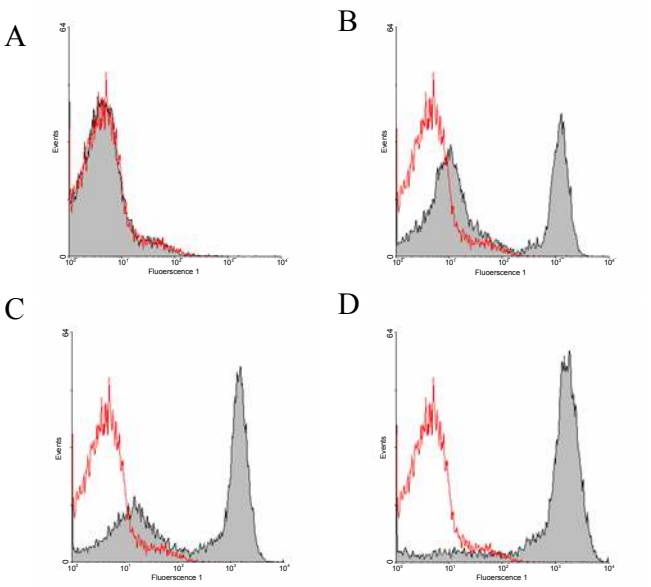



**Figure 2: Effect of increasing concentrations of ciprofloxacin to the membrane potential of *S. pneumoniae*.** Toxicity of ciprofloxacin resulted in depolarisation of membrane potential followed by increased binding of $DiBAC_4(3)$ indicated by increased fluorescence intensity (grey filled histogram). Red line: Bacteria not treated with ciprofloxacin. Ciprofloxacin concentrations were A) 0.015 µg/ml B) 0.125 µg/ml C) 1 µg/ml D) 4 µg/ml.

displayed a reproducible change in the light scattering to a higher side scatter signal corresponding to an altered granularity, and an increased forward scatter signal referring to larger cell size (Figures 4 A and 4 B). This could be confirmed by fluorescence microscopy. *E. coli* treated with mezlocillin exhibited a morphological change to more filamentous bacterial cells (Figures 4C and 4D).

Applying the flow cytometric test it was also possible to identify extended-spectrum β-lactamase (ESBL)–producing bacteria. Using an antimicrobial assortment, recommended by CLSI 24 strains suggestive of ESBL-production were tested. No depolarization of bacterial membranes was observed applying the antimicrobials alone, but in combination with clavulanic acid, all strains depolarized at minimal antimicrobial concentrations (Figure 5). A confirmation test for all strains was performed using the double diffusion test and double disk test. AmpC-producers were not significantly inhibited by the combination of antimicrobials with clavulanic acid, whereas all ESBL-producers did. Each ESBL-producer was sensitive to cefoxitin. Compared to the double diffusion test and double disk test, the concordance was 100%.

### Susceptibility testing directly from blood cultures

From 41 positive blood cultures Gram determination was carried out (Figure 6 A) and subsequently the flow cytometric susceptibility test was performed (Figure 6 B). Monomicrobial bloodstream infection was detected in 40 cases; in one case an infection with 2 different staphylococcal species was determined after culturing the bacteria. 22 bacterial strains were correctly classified as Gram-positive, 15 correctly as Gram-negative. Four strains of *Enterococcus faecalis* were misclassified as Gram-negative. Two of these Enterococcus strains underwent cell death of nearly 50% of the bacteria in the untreated controls. Therefore, it was not possible to differentiate spontaneous cell death from cell death caused by antimicrobial action and the susceptibility test of these two strains could not be evaluated.

234 susceptibility tests were performed by flow cytometry from isolates of the blood cultures. In 7 cases the isolate was determined false susceptible in the flow cytometric analysis and in 18 analyses false resistant compared to the results in the Vitek®2. Thus the preliminary flow cytometric results were confirmed in 89.3% after culturing by the Vitek® 2. The concordance was higher when the strains had been grown on solid media before susceptibility testing.

### Detection of bacteria from swabs

In cultures from swabs with origin from throat, nose, groin and wounds bacteria could be well distinguished from debris on the basis of forward and side scatter signals after an incubation of the culture of 3 hours. A pronounced incubation time of 5 hours led to a well-defined bacterial population (Figures 7 A and 7 B).

### Identification of methicillin-resistant *Staphylococcus aureus*

To investigate whether protein A expression is a reliable parameter for *S. aureus* identification 200 *S. aureus*-isolates, including 155 MSSA and 45 MRSA, additionally 100 coagulase negative staphylococci and 30 Gram-negative strains were investigated for protein A expression. The flow cytometric detection of protein A resulted in a specificity of 96.1% and a sensitivity of 97.5%.

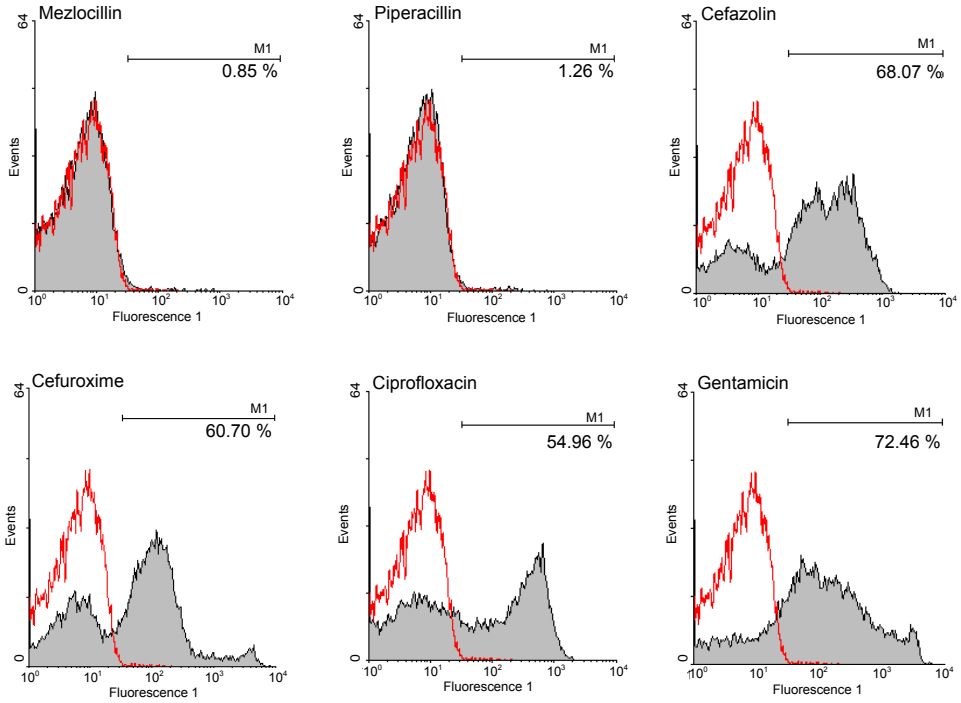
In mixed cultures the detection of MRSA subpopulations was not affected by high concentrations of MSSA in the same suspension. Using a ratio of 1:5 in mixed cultures of MRSA and MSSA, the MRSA strain could be clearly identified after 180 minutes of incubation with 1 µg oxacillin/ml. After this time on average only 7% of the bacteria were MSSA, due to growth inhibition and killing of the sensitive staphylococci. With other species than *S. aureus* no unspecific binding of the antibody was observed, therefore, *S. aureus* could be well detected in suspensions from swabs, even in mixed cultures (Figures 8 A-8 E).

The results of the flow cytometric test and the routine culture methods of the clinical laboratory were concordant in 138 of 140 swabs analysed. Without previous isolation of the staphylococci it was possible to detect all but 2 of 24 MRSA within 6 hours. The identification of 2 strains failed because of a negative result for protein A detection that was confirmed by a negative latex agglutination test for protein A and clumping factor. The oxacillin susceptibility test of all *S.aureus* strains was in concordance to the routine results. No false resistant results occurred, thus no strain was misclassified as MRSA. The sensitivity of the flow cytometric MRSA detection was 91.7% and the specificity was 100%.

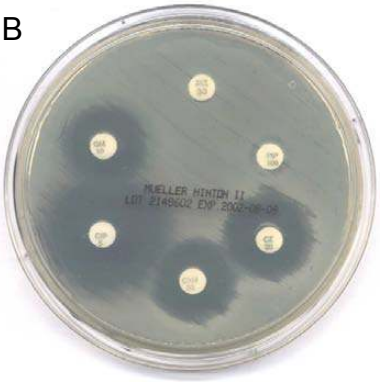
## Discussion

One of the most done analyses in clinical microbiology is susceptibility testing as basis for a specific therapy of bacterial infections. We established a fast and reliable flow cytometric assay that was feasible for all bacteria species tested. Depolarisation of cell membranes could be observed generally with $DiBAC_4(3)$, independent of the killing mechanism of the antimicrobials. With slight modifications the assay was used for the detection of extended spectrum β-lactamase producing bacteria (ESBL). To gain useful time the susceptibility test was performed from primary material as blood cultures. Furthermore, *Staphylococcus aureus* could be identified by the species specific detection of staphylococcal surface protein A. Simultaneous analysis of the oxacillin susceptibility allowed the detection of methicillin resistant *S. aureus* (MRSA).

A




B



| antibiotics | zone of inhibition | interpretation |
|---|---|---|
| mezlocillin | - | R |
| piperacillin | - | R |
| cefazolin | 20 mm | S |
| cefuroxime | 21 mm | S |
| ciprofloxacin | 28 mm | S |
| gentamicin | 19 mm | S |

C

00000247 Not Specif **/** ****;** **/**/** **;** FINAL

| TESTED | PANEL TYPE | ORGANISM | BIOTYPE | WARD OF ISOLATION | NOTE (S) |
|---|---|---|---|---|---|
| | Neg BP Combo 10 | E. coli | 7711501-0 | Not specified | |

| An | Pi | Mz | Cf | T/S | Cfz | P/T | A/S | Aug | Gm | To | Tsp | C | ra | Cax | Caz |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| >16 | >64 | >64 | <8 | <2 | <8 | <16 | <16 | <8 | <4 | <4 | <4 | <4 | | <8 | <8 |
| R | R | R | S | S | S | S | I | S | S | S | S | S | | S | S |

| Cp | Of1 | Te |
|---|---|---|
| <1 | <2 | > 8 |
| S | S | R |

**Figure 3: Antimicrobial susceptibility testing of *E. coli* isolate 247**. Flow cytometric test (A), agar diffusion test (B) and result of the automated system WalkAway-96® (C). The red line shows the untreated control, the grey filled histograms bacteria incubated with different antibiotics. The part of fluorescent depolarized bacteria in the suspension is shown in percent. In accordance with the reference methods, this strain was resistant to mezlocillin, piperacillin and sensitive towards cefazolin, cefuroxime, ciprofloxacin and gentamicin.

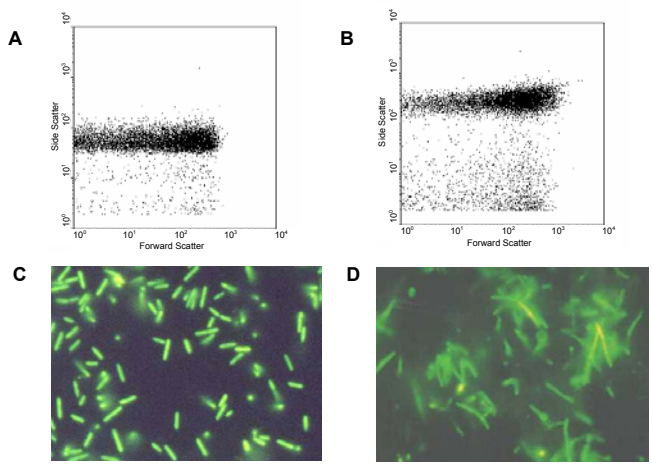



**Figure 4: Morphological changes of *E. coli* after treatment with mezlocillin** FSC-SSC-Dot plot of *E. coli* isolate 247 after 120 min incubation with cefazolin (A) or mezlocillin (B). Fluorescence microscopy of *E. coli* isolate 247 with cefazolin (C) or mezlocillin (D), colored with $DiBAC_4(3)$ (100er optical enlargement, spot, light exposure 3 s). Cefazolin, in contrast to mezlocillin, lead to filamentous elongation of bacterial cells.

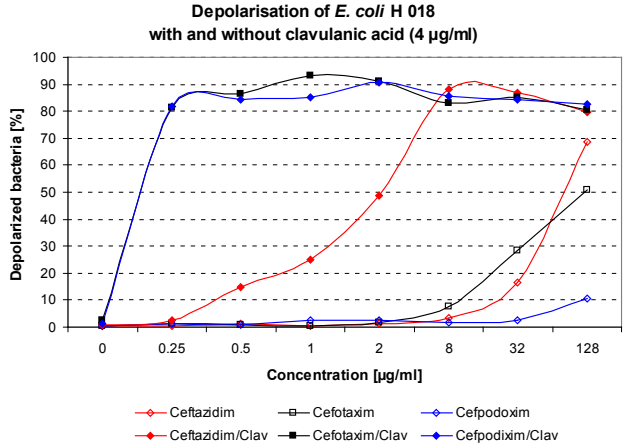



**Figure 5: Susceptibility test with antimicrobial arrangement proposed by the CLSI to detect extended spectrum beta lactamases.** The antimicrobials were added each alone or in combination with clavulanic acid (4 µg/ml) at each concentration to the suspension, antimicrobial combination with clavulanic acid showed significant depolarisation in the samples at minimal concentrations, whereas the samples with antimicrobials alone showed no or marginal depolarisation. The strain H 018 was an *E. coli* with CTX-M-type resistance.

The concordances of the flow cytometric susceptibility results compared to those of the agar diffusion assay and of the WalkAway-96®, respectively, are within the range seen in studies, which compared agar diffusion testing with automated systems [20,21].

For detection of ESBL the principle of common disk testing, double-disk synergy testing, and Etest, was transferred to the FACS assay, which overcomes the disadvantage of incubating cultures for 18 to 24 hours. The presence of KPCs or hyperproduction of K1 penicillinases in combination with high-level AmpC β-lactamase production can result in false-positive and -negative results, respectively [22]. For most TEM- and SHV-derived ESBLs cefpodoxime is a reliable substrate for the detection of most ESBLs. Nevertheless, usually it is preferred to utilize cefpodoxime in combination with ceftazidime and cefotaxim.

| Gram-negative bacteria | Gram-positive bacteria |
|---|---|
| Sample 1: control without antibiotic | Sample 1: control without antibiotic |
| Sample 2: mezlocillin 128 µg/ml | Sample 2: oxacillin 0.25 µg/ml |
| Sample 3: piperacillin 128 µg/ml | Sample 3: piperacillin 1 µg/ml+ tazobactam 0.1 µg/ml |
| Sample 4: cefazolin 8 µg/ml | Sample 4: cefazolin 1 µg/ml |
| Sample 5: cefuroxime 8 µg/ml | Sample 5: cefuroxime 1 µg/ml |
| Sample 6: ciprofloxacin 2 µg/ml | Sample 6: ciprofloxacin 1 µg/ml |
| Sample 7: gentamicin 6 µg/ml | Sample 7: gentamicin 2 µg/ml |

**Table 1:** Samples for the flow cytometric susceptibility test of Gram-negative and Gram-positive bacteria. Depending on Gram classification different penicillins and different concentrations of the other antibiotics were used.

The latter combination allows reliable detection of CTX-M producers and those with ceftazidimase-type TEM variants [23]. Faria-Ramos et al. [24] recently reported a flow cytometric assay performed with slight differences from that used in this study. Their susceptibility test was followed by molecular characterization of the $bla_{TEM}$, $bla_{SHV}$ or blasgenes, genes were found to distinguish reliably between ESBL-producers and non-producers. Moreover, they defined the CLA index that was calculated as the ratio between the percentage of depolarised cells after treatment with 4 mg/L of cefotaxim or with 16 mg/L of ceftazidime in the presence versus the absence of clavulanic acid. An index value higher than 1.5 discriminated all ESBL-positive clinical strains from ESBL-negative strains.

The flow cytometric susceptibility test in this form did not distinguish between sensitive and intermediate. Further experiments may allow even a classification of intermediate, because it could be well differentiated between strains that were killed in high percentages and strains which were killed only to 10-20% even by higher antibiotic concentrations.

To perform routine susceptibility testing positive blood cultures need to be subcultured on solid media. This procedure is time consuming. Using flow cytometry for susceptibility testing directly from blood cultures, results could be obtained within 4 hours. Compared to the Vitek® 2 system a concordance of 89% was achieved. Hansen et al.[25] determined identification and susceptibility testing in the Vitek® 2 by inoculating the Vitek® panels with Gram-negative bacteria directly from positive blood cultures. They found an agreement in 85% of the results compared to conventional susceptibility testing with strains grown on solid media after one day of incubation. This corresponds well to the observation with the flow cytometric test. However, the flow cytometric susceptibility test should be designed as a rapid preliminary test and should be confirmed with standard tests recommended by the CLSI. The suitability of the flow cytometric Gram-determination is limited to viable bacteria, because damaged cells stain variably with the dyes SYTO® 9 and hexidium iodide. Cell death led to a misclassification of 4 *Enterococcus faecalis* strains.

The combination of susceptibility testing and identification with antibodies was used successfully to distinguish MRSA from other bacteria in mixed cultures [26]. *Staphylocccus aureus* is a widespread nosocomial pathogen and due to the increasing resistance to antimicrobials, a serious global public health problem. Detection of methicillin resistant *Staphylococcus aureus*, based on the flow cytometric oxacillin susceptibility testing and the detection of the surface protein A directly from swabs facilitates a specific antimicrobial therapy and prompt infection control. The expression of the 42 kDa protein A is encoded by the gene spa and is produced by more than 90% of the *S.aureus* strains [27-29]. Nevertheless not all *S. aureus* strains could be detected with the antibody directed against protein A. Using fibrinogen as binding partner to detect the clumping factor increased the sensitivity of the assay from 91.7 to 95.5%. Therefore, the

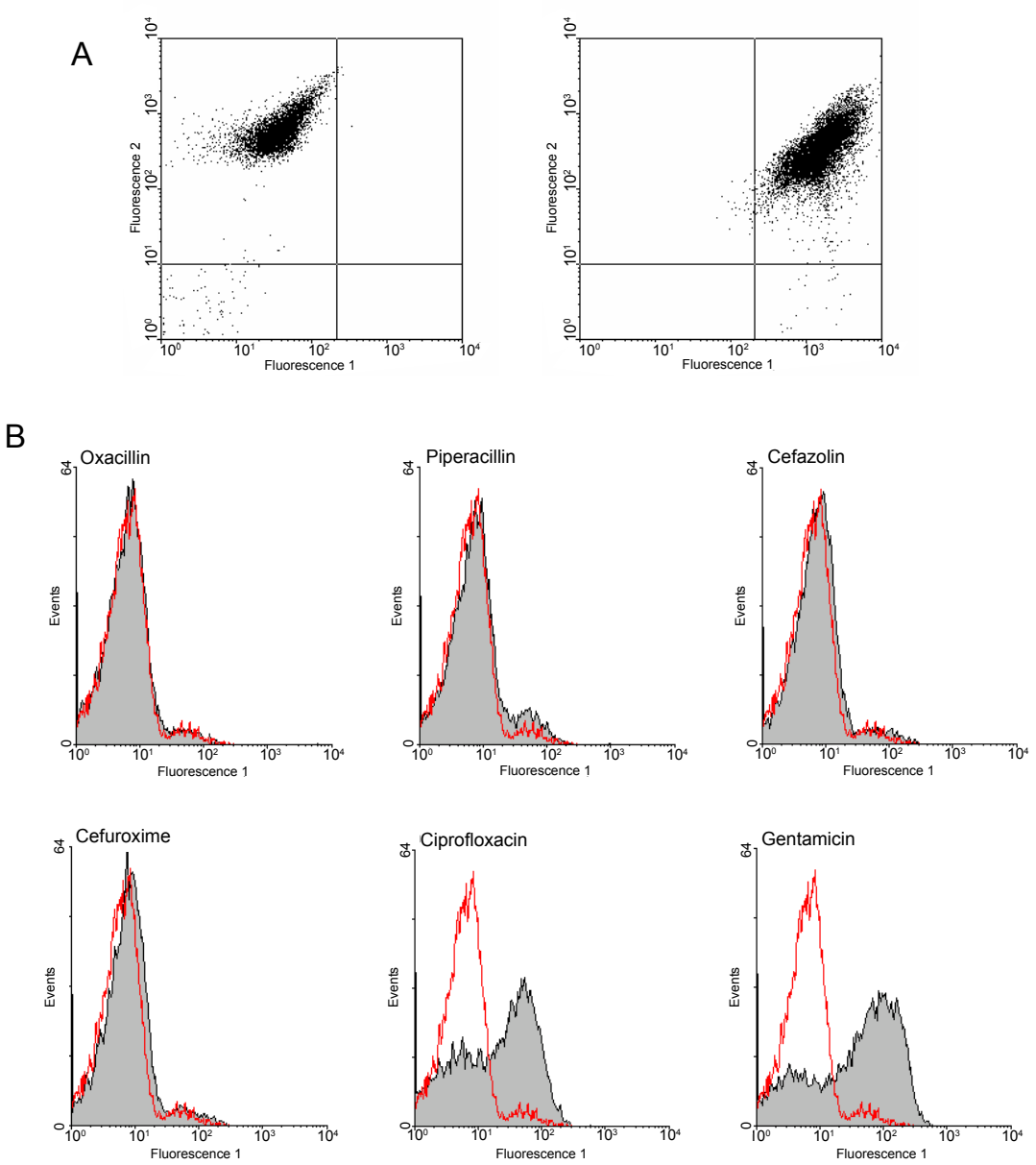



**Figure 6: Gram-determination und flow cytometric susceptibility testing from positive blood cultures.** Gram determination (A) with SYTO® 9 and Hexidium iodide of *S. hominis* 1, uptake of Hexidium iodide led to red fluorescence (left) and a determination as Gram-positive. *E. coli* showed green and red fluorescence due to the uptake of SYTO® 9 and Hexidium iodide (right) from blood culture and was determined as Gram-negative. Susceptibility testing (B) of *S. hominis* 1, red line: untreated control; filled grey histograms: samples with oxacillin, piperacillin/tazobactam, cefazolin, cefuroxime and gentamicin.

flow-cytometric MRSA-test provides results in 6 hours and permits an early specific therapy. Suller et al.[30] used flow cytometry to examine five MRSA strains and two MSSA strains, grown on solid media, with $DiBAC_4(3)$ but an identification was not done.

Using vancomycin as antibiotic, this assay may easily be transferable to the detection of vancomycin resistant MRSA and, where specific antibodies are available, even may be transferred to other bacterial species.

Basically flow cytometers were designed to analyse larger eukaryotic cells. The suitability of this technique for microbiological analyses depends on a good discrimination between background noise or debris and small bacteria cells as for example *Helicobacter spp.* Together with the rapidity of flow cytometry as discussed above, a further advantage is the universalism, because slight modifications of the assays lead to an extreme versatility. Flow cytometry has low costs, but although the cytometer can be automated due to many pipetting and washing steps it is personnel intensive compared to routine identification and susceptibility testing procedures. On the other hand there is rarely another method, which obtains various information about a large

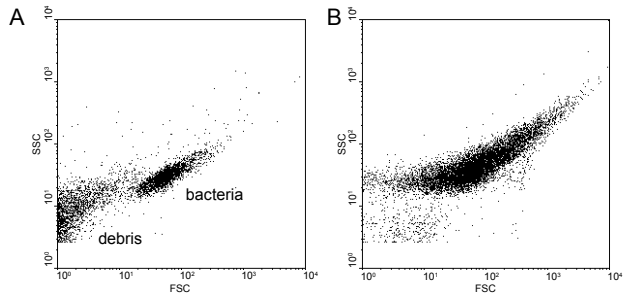



**Figure 7: Detection of bacteria in primary material by light scattering properties.** FSC-SSC Dot plot of a bacteria population of a throat swab after 3 (A) and 5 hours (B) incubation time in Schaedler broth. The bacteria could be well distinguished from debris or electronic noise.

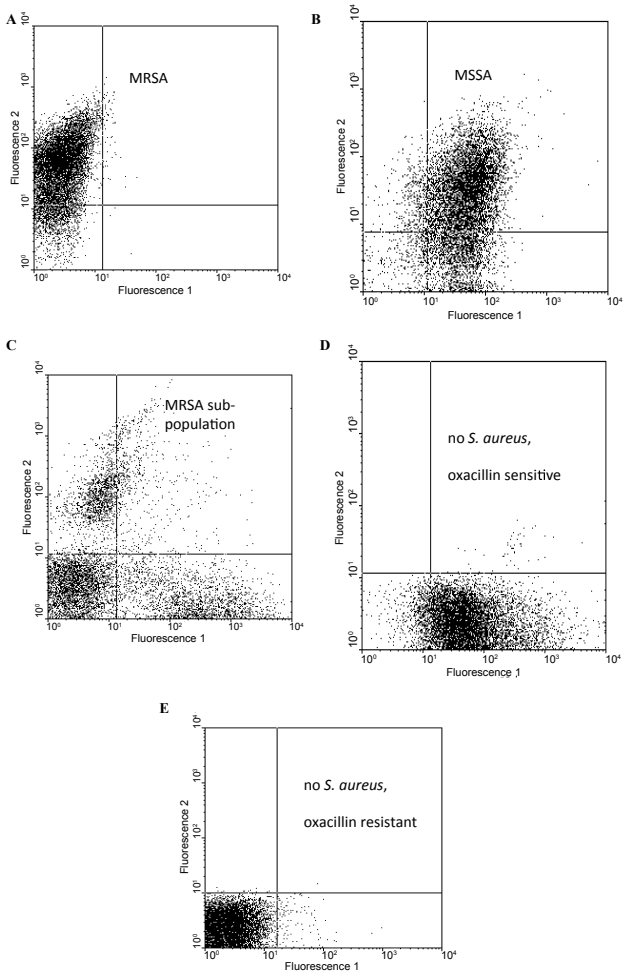



**Figure 8: Identification and susceptibility testing of *S. aureus*.** FL1-FL2-Dot Plots of bacteria cultivated from swabs. *S. aureus* was identified with a monoclonal anti protein A antibody and stained with $DiBAC_4(3)$ after incubation with oxacillin. Fluorescence 2 refers to the detection of protein A on the cell surface, increasing fluorescence 1 to the uptake of $DiBAC_4(3)$ due to oxacillin susceptibility.
A) MRSA population from a swab from throat.
B) MSSA from a swab of the nose.
C) Mixed culture with MRSA from nose.
D) Other species than *S. aureus* isolated from nose and oxacillin susceptible.
E) Other species than *S. aureus*, isolated from groin, oxacillin resistant.

number of bacteria with such a minimal effort. Therefore it is desirable that not only research and biotechnology, but also clinical microbiology laboratories employ this technique in a wider range.

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