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Original Article

Evaluation of real-time PCR and flow cytometry efficiency in rapid detection of carbapenemase-producing *Enterobacteriales*

İpek Koçer[1], Tekin Karsligil[2], Mustafa Sağlam[2], Uğur Arslanyürekli[3], İhsan Deveci[3], Emel Şahin[3]

[1] *Department of Medical Microbiology, Sanko University School of Medicine, Gaziantep, Turkey*
[2] *Department of Medical Microbiology, Gaziantep University School of Medicine, Gaziantep, Turkey*
[3] *Department of Medical Biology, Gaziantep University School of Medicine, Gaziantep, Turkey*

Abstract

Introduction: Infections due to carbapenem-resistant *Enterobacteriales*, which have increased worldwide in recent years, cause concern. This study aimed to rapidly detect carbapenemase gene region by using flow cytometry in *Enterobacteriales* isolates and to evaluate its efficiency and susceptibility by comparing it with polymerase chain reaction (PCR).

Methodology: In the study, 21 isolates obtained from the blood cultures of patients hospitalized in intensive care units and found to intermediate or resistant to at least one carbapenem in the automated system, and 14 isolates belonging to the carbapenem-susceptible *Enterobacteriales* family were included. Carbapenemase gene regions were investigated by PCR after their susceptibility was determined by disk diffusion method. Bacterial suspensions were treated with meropenem + specific carbapenemase inhibitors (EDTA or APBA) and Temocillin and stained with thiazole orange (TO) and propidium iodide (PI) to show dead/live cell differentiation. Dead/live cell percentages were calculated after reading on the flow cytometer device.

Results: In the ROC analysis of the flow cytometry method, the cut-off value, specificity, and susceptibility of PI staining rates for meropenem were found as 14.37%, 100%, and 65%, respectively. It was found that the flow cytometry method was well-compatible with PCR in the detection of the carbapenemase gene region.

Conclusions: Flow cytometry will continue to be a promising method for the detection of antimicrobial susceptibility and resistance due to its rapid analysis of many cells and its high compatibility with PCR results.

Key words: *Enterobacteriales*; carbapenemase; flow cytometry; meropenem.

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Introduction

Carbapenems are frequently used therapeutic options in the treatment of infections caused by strains belonging to multidrug-resistant *Enterobacteriales*. However, in recent years, carbapenemase-producing *Enterobacteriales* members, primarily *Klebsiella pneumoniae* and *Escherichia* coli, have been spreading in our country as well as in the rest of the world [1]. Carbapenemases, which are in the three molecular groups (A, B, and D) of the Ambler classification, can be detected by phenotypic methods by inhibiting them with various enzyme inhibitors [2]. The most common clinically encountered Class A carbapenemases are inhibited by aminophenyl boric acid (APBA), while the Class B carbapenemases with the strongest carbapenemase activity are inhibited by Ethylenediaminetetraacetic acid (EDTA) or dipicolinic acid (DPA) by various methods to demonstrate the presence of carbapenemases. The fact that class D beta-lactamases (OXA enzymes) have wide variants with similar amino acid sequences makes it difficult to find specific inhibitors [3].

High Temocillin resistance has been suggested as a good marker for the detection of the OXA enzyme group. It takes approximately 48-72 hours to suspect the presence of carbapenemase after the culture results and then to show the presence of carbapenemase by molecular methods. Today, flow cytometry is used as a rapid method alternative in studies on the detection of microorganisms in various body fluids, blood elements to be used in transfusion, and the effects of antimicrobial drugs [4,5]. The aim of this study is to evaluate the efficiency and susceptibility of the method created by flow cytometry to detect the presence of carbapenemases in a shorter time in species belonging to the *Enterobacteriales*, by comparing molecular methods.

Methodology

This study was carried out with the approval of Gaziantep University Clinical Research Ethics Committee (Decision no: 2019/348).

Bacterial isolates and antibiotic susceptibilities

The study comprised 35 *Enterobacteriales* isolates (14 carbapenem-susceptible, 21 intermediate, or resistant to at least one carbapenem group antibiotic) obtained from blood cultures collected between July 2019 and July 2020 from intensive care units at Gaziantep University Faculty of Medicine Hospital. Identification and susceptibility tests were performed in the first isolation of bacteria using conventional methods and an automated VITEK 2® (bioMerieux, France) system. The susceptibility of bacteria against carbapenem antibiotics was performed with the Kirby-Bauer disc diffusion method 10 µg meropenem (MEM) (Bioanalyse, Turkey), which is a phenotypic method. The results were interpreted as susceptible (S) and resistant (R) according to EUCAST criteria [6].

Investigation of Carbapenemases by PCR Method

The presence of carbapenemase was investigated by PCR method. KPC, NDM, IMP, VIM, OXA-48 gene regions positive isolates obtained from Dokuz Eylül University Medical Microbiology Department were used as positive control group. *E. coli* ATCC 25922 strain was used as a negative control. In the study, KPC, NDM, OXA-48, IMP, VIM gene presence was performed with MDR (KPC/OXA) Real-TM (SacaceBiotechnologie, Italy) and MBL (VIM, IMP, NDM) Real-TM PCR (SacaceBiotechnologie, Italy) kit by Rotor-Gene (QIAGEN, Germany) device.

Detection of carbapenemases by flow cytometry

Bacterial isolates stored at -20 °C were incubated at 37 °C for 18-24 hours after being brought to room temperature one day before the study and inoculated onto 5% sheep blood medium. Bacterial suspension was prepared at 0.5 McFarland density by taking a sterile loop from *E. coli* and *K. pneumoniae* colonies grown on 5% sheep blood agar. Each bacteria at 0.5 McFarland concentration was diluted 1:100 into 5 tubes containing Mueller-Hinton broth. 5×10^5 CFU/mL was obtained in each tube.

Then, 0.5 mL of bacterial suspension was taken and meropenem and carbapenemase inhibitors were added to 5 tubes at a ratio of 1:1 and incubated at 37 ºC for 1.5 hours. The first tube contained antibiotic-free bacterial suspension, the second tube meropenem + bacterial suspension, the third tube meropenem + EDTA + bacterial suspension, the fourth tube meropenem + APBA + bacterial suspension, and the fifth tube Temocillin + bacterial suspension. 500 µL was taken from each of the tubes containing the antibiotic bacterial suspensions removed from the incubator and transferred to the flow cytometry reading tubes. The BD® Cell viability kit (Becton Dickinson, USA) was used to demonstrate the dead/live cell differentiation of bacteria. Bacteria samples were first incubated with 5 µL of TO (thiazole orange) for 5 minutes, then with 5 µL of PI (propidium iodide) dye for 5 minutes at room temperature and in the dark. Stained cell suspensions were analyzed in a flow cytometry device.

Ten thousand cells were counted in each tube containing the sample, and the graphics of the samples were evaluated at the analysis stage. *E. coli* and *K. pneumoniae* were evaluated from common gates. Significant populations were gated on the dot blot plot in the SSC-H/FSC-H channel. Bacterial cells stained with TO in the SSC-H/FL1-H dot blot graph were plated and the percentages of live and dead cells were determined in a new dot blot graph created using the FL3-H (PI-A)/FL1-H (TO-A) channel in the selected cell group quadrant analysis was performed to determine. As a result of the analysis, the rate of staining with PI (%) of cells in which the parameters were double positive (TO + PI +), double negative (TO – PI -) or either positive and the other negative (TO + PI - / TO – PI +) were evaluated.

Statistical Analyses

ROC analysis was performed on the staining rates with PI (%) in bacterial suspensions. MedCalc (version 19.7) trial version was used for ROC analysis, and IBM SPSS Statistics 23 package program was used for other analyses.

Results

Polymerase Chain Reaction (PCR) results

The carbapenemase gene was found in 21 isolates by multiplex PCR method. Of the 21 isolates, 8 contained the OXA-48 gene region, 7 contained the KPC gene region, and 6 contained the NDM gene region. A total of 40 isolates were studied by flow cytometry, by taking the isolates whose gene regions were determined by PCR in the patient group, the positive control group (strains containing KPC, OXA-48, NDM, IMP, and VIM genes), and 14 carbapenem-susceptible isolates.

Figure 1. Dot blot graphic images of E. coli ATCC 25922 bacteria stained with TO and PI in flow cytometry without antibiotics.

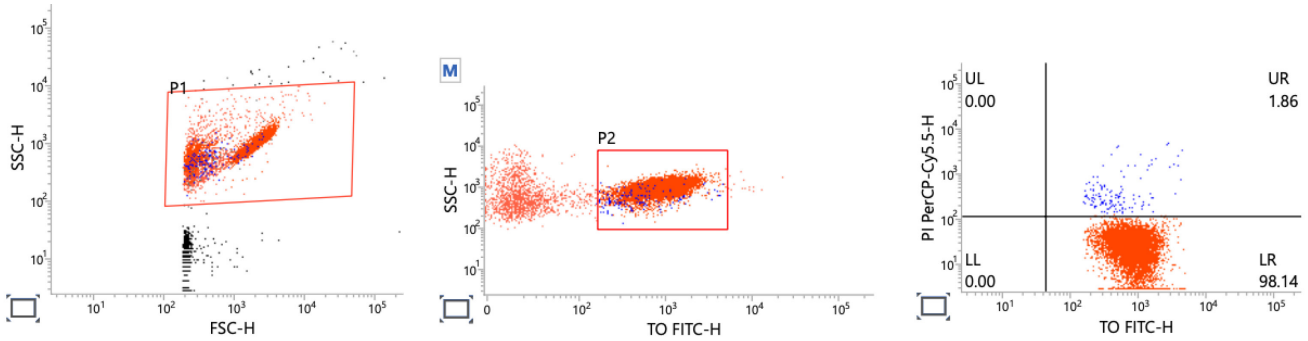


Figure 2. Dot blot graphic images of E. coli ATCC 25922 bacteria stained with TO and PI after treatment with meropenem antibiotic in flow cytometry.

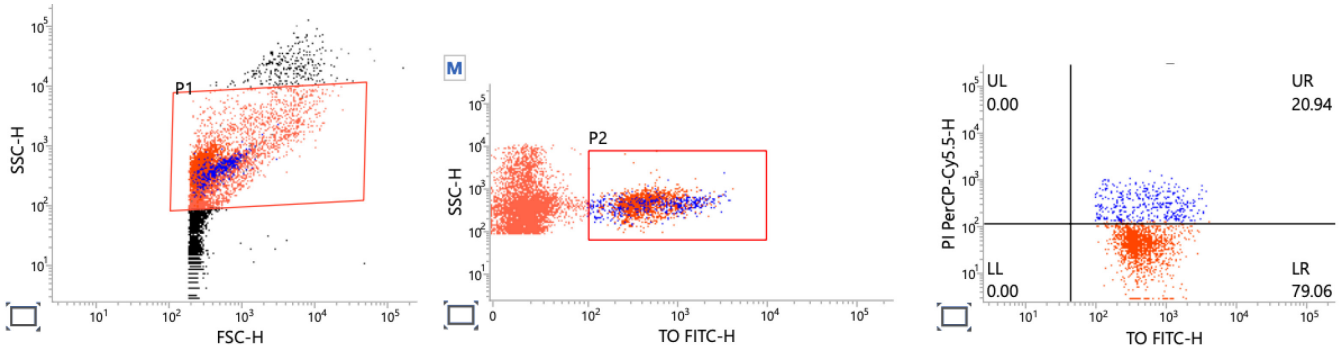


Figure 3. Dot blot graphic images of bacteria carrying the carbapenem-resistant KPC gene in flow cytometry, in a tube containing meropenem + bacteria suspension stained with TO and PI.

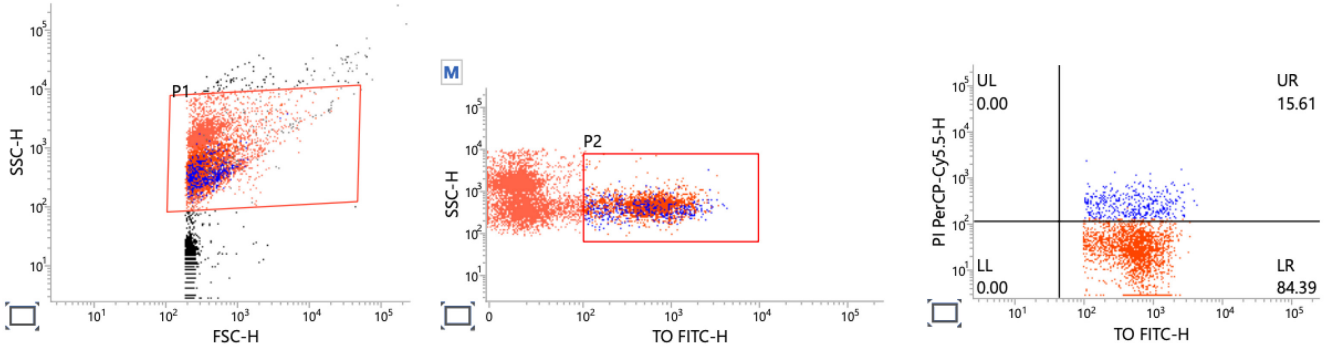
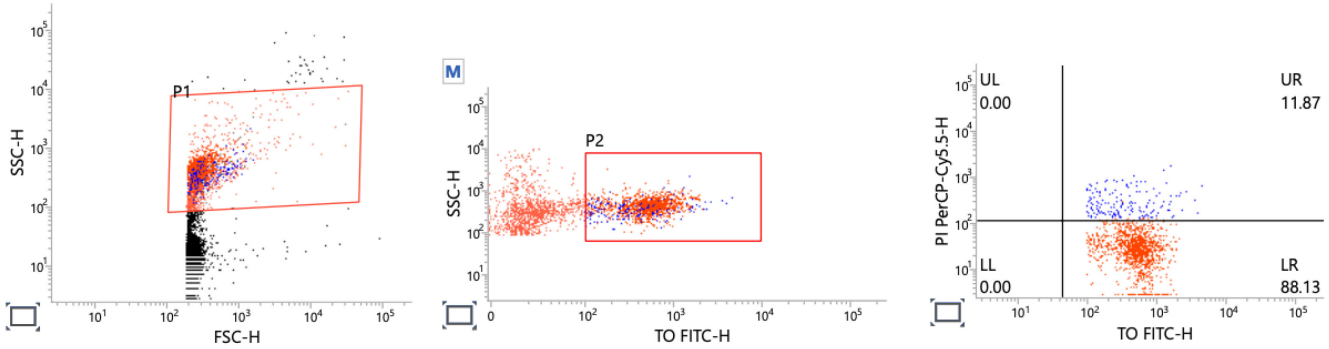


Figure 4. Dot blot graphic images of bacteria carrying the carbapenem-resistant KPC gene in flow cytometry, stained with TO and PI, in a tube containing meropenem + APBA + bacteria suspension.



A: Significant cells stained with fluorescent dyes are gated in the SSC-H (side scatter) /FSC-H (Forward scatter) plot. B: P2 gating was performed on the SSC-H/FL-1 (TO-FITC-H) dot blot in all cells stained with fluorescent dyes. C: TO-stained cells (red), PI-stained cells (blue) in the PI-H/TO FITC-H plot.

Flow Cytometry results

Carbapenem-susceptible *E. coli* ATCC 25922 strain and other carbapenem-susceptible strains with no gene region detected in the flow cytometry analysis showed a significant increase in the PI staining rate (%) of the bacterial suspension without antibiotics in the meropenem-containing tube ($p < 0.05$) (Figures 1 and 2). In all but one of the isolates containing the KPC gene and the positive control group carrying the KPC gene, in flow cytometry analysis, it was found that the staining rate (%) of the bacterial suspension without antibiotics showed a slight increase in the meropenem-containing tube, while it was significantly increased in the meropenem + ABPA-containing tube ($p = 0.038$) (Figures 3 and 4).

It was found that the positive control group carrying the NDM gene and the strains containing the NDM gene region showed a slight increase in the rate of staining (%) of the antibiotic-free bacterial suspension with PI in the tube containing meropenem, while it increased significantly in the tube containing meropenem + EDTA ($p = 0.002$) (Figures 5 and 6)

Positive control group carrying the OXA-48 gene and the strains containing the OXA-48 gene region showed an increase in the rate of staining (%) of the bacterial suspension without antibiotics with PI in the tube containing meropenem ($p < 0.001$), while the ratio of viable cells (TO + PI -) in the tube containing Temocillin (%), there was no statistically significant difference between the value of the bacterial suspension without antibiotics ($p = 0.062$). A statistically significant difference was found between meropenem + bacteria suspension and a bacterial suspension containing Temocillin alone in terms of PI staining rates (%) ($p = 0.026$). In the samples containing the other OXA-48 gene group, it was found that the rate of staining with PI (%) in meropenem + bacteria suspensions did not increase in tubes containing EDTA or APBA + bacteria suspensions. It was found that the rate of staining with PI (%) in the tubes containing meropenem in bacteria with other resistance genes (KPC and NDM) was higher than those in bacteria containing the OXA-48 gene region ($p < 0.05$). The viable cell (TO + PI -) ratio (%) in tubes containing Temocillin was found to be close to the value of bacterial suspension without antibiotics (Figures 7, 8, and 9).

Figure 5. Dot blot graphic images of bacteria carrying the carbapenem-resistant NDM gene in flow cytometry, in a tube containing meropenem + bacteria suspension stained with TO and PI.

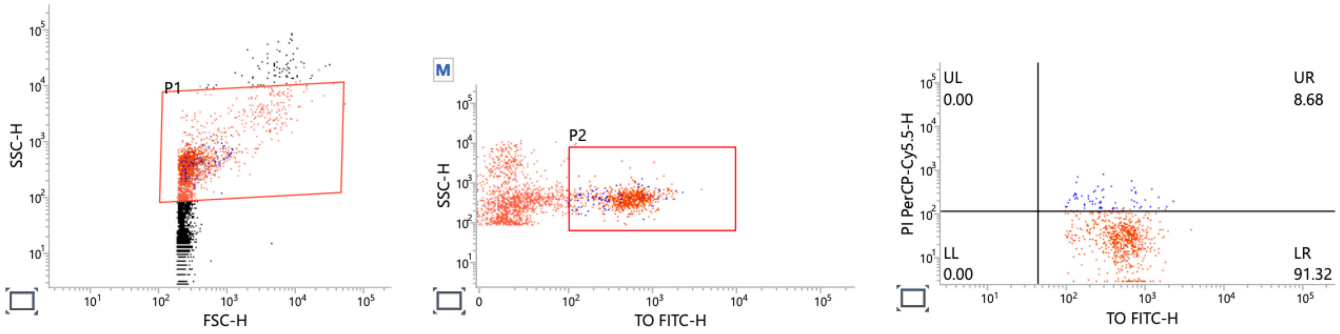
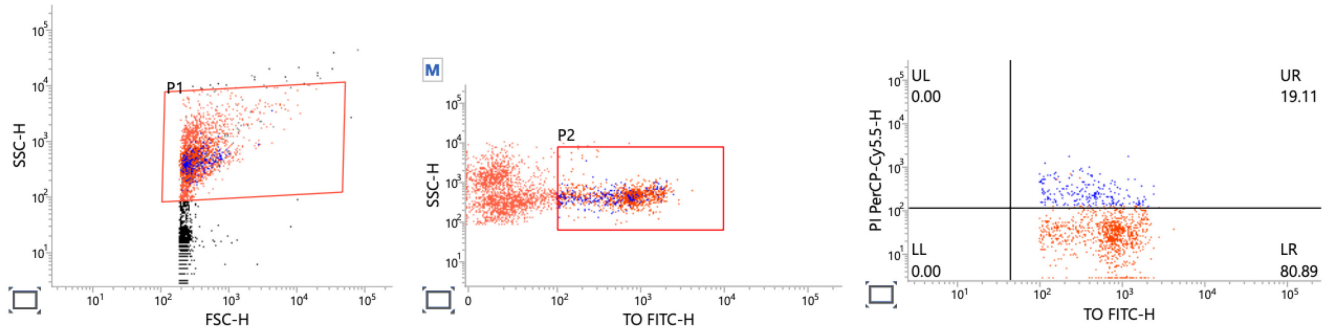


Figure 6. Dot blot graphic images of bacteria carrying the carbapenem-resistant NDM gene in flow cytometry, in a tube containing TO and PI stained meropenem + EDTA + bacteria suspension.



A: Significant cells stained with fluorescent dyes are gated in the SSC-H (side scatter) /FSC-H (Forward scatter) plot. B: P2 gating was performed on the SSC-H/FL-1 (TO-FITC-H) dot blot in all cells stained with fluorescent dyes. C: TO-stained cells (red), PI-stained cells (blue) in the PI-H/TO FITC-H plot.

In the positive control group carrying the VIM gene, it was found that the bacterial suspension without antibiotics showed a slight increase in the rate of staining with PI (%) in the tube containing meropenem, but it increased significantly in the tube containing meropenem + EDTA. In the positive control group carrying the IMP gene, it was found that the bacterial suspension without antibiotics showed a slight increase in the rate of staining with PI (%) in the tube containing meropenem, but there was no increase in the tube containing meropenem + EDTA.

The area under the curve (AUC) was as 0.766 (95% confidence interval [0.606-0.885%]) in the ROC analysis of all studied samples for meropenem alone and PI staining rates (%) in bacterial suspensions ($p < 0.001$). In determining the susceptibility of carbapenem antibiotics by flow cytometry, the rate of staining with PI for meropenem (%) cut-off value was found as 14.37% (differentiation between susceptible and resistant), specificity as 100% according to cut-off value, and susceptibility as 65% (Figure 10).

Figure 7. Dot blot graphic images of bacteria carrying the carbapenem-resistant OXA-48 gene in flow cytometry, in an antibiotic-free bacterial suspension tube stained with TO and PI.

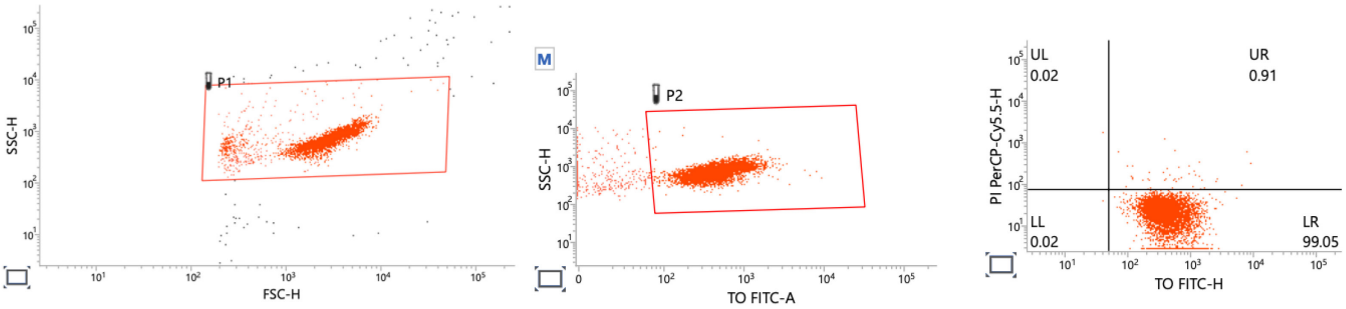


Figure 8. Dot blot graphic images of bacteria carrying the carbapenem-resistant OXA-48 gene in flow cytometry, in an antibiotic-free bacterial suspension tube stained with TO and PI.

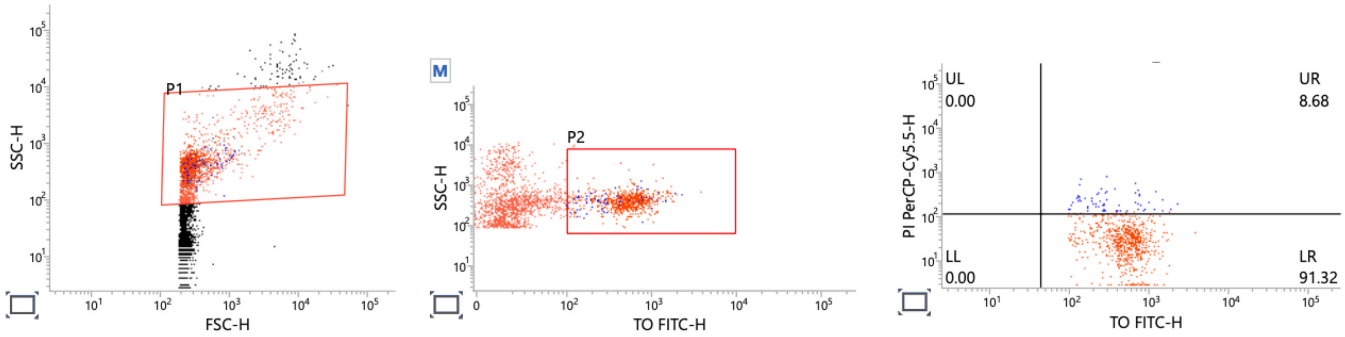
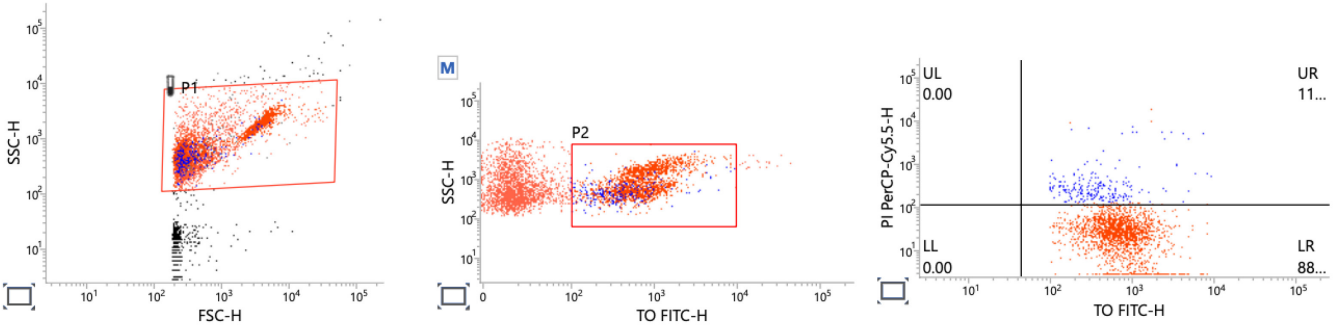


Figure 9. Dot blot graphic images of bacteria carrying the carbapenem-resistant OXA-48 gene in flow cytometry, in an antibiotic-free bacterial suspension tube stained with TO and PI.



A: Significant cells stained with fluorescent dyes are gated in the SSC-H (side scatter) /FSC-H (Forward scatter) plot. B: P2 gating was performed on the SSC-H/FL-1 (TO-FITC-H) dot blot in all cells stained with fluorescent dyes. C: TO-stained cells (red), PI-stained cells (blue) in the PI-H/TO FITC-H plot.

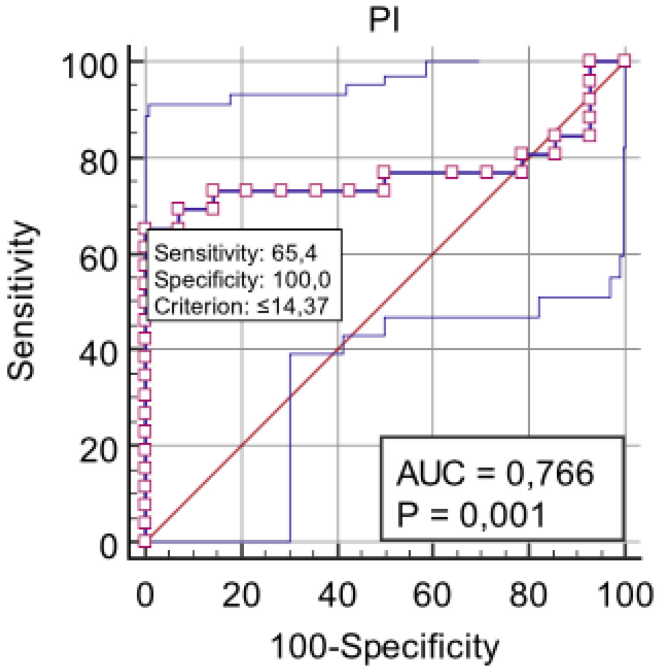
Discussion

The gradual increase in multi-drug resistance among *Enterobacteriales* has led to the use of carbapenem group antibiotics as a treatment option and the prevalence of resistance to this drug [7]. It has been shown that patients with carbapenem-resistant Gram-negative bacteremia are 3-4 times more mortal than the normal patient population without infection [8,9]. The most frequently isolated and endemic carbapenemase species in Turkey is OXA-48, followed by NDM type enzymes [10-12]. It was evaluated that the multiplex PCR method, which was used as a reference method in our study, was more disadvantageous in terms of time to obtain results compared to flow cytometry analysis. Although carbapenemase screening is not mandatory in antibiotic susceptibility tests according to EUCAST, it has been emphasized that it is important for infection control and public health (6). In their study comparing bacteremia, Tamma *et al.* found that carbapenemase-producing-CRE-related mortality was 32% and the non-carbapenemase-CRE mortality rate was 13% [13].

It is seen that the rational use of flow cytometry and optimization studies on bacteria have increased recently. It is thought that the ability to perform viability analyzes without cell culture will increase the accessibility of pathogenesis data in terms of bacteria and viruses [14]. In our study, a cell viability kit containing TO and PI was used to show bacteria's dead/live cell differentiation. Since the bacteria are much smaller in the optimization phase, it has been observed that better results are obtained when the reading speed is set to slower. When Kılıç *et al.* examined the susceptibility of meropenem in flow cytometry using gradient test and TO and PI dyes, they found that the results of flow cytometry were 99.4% compatible [15].

In our study, it was seen that the results of the detection of carbapenemase-producing *Enterobacteriales* were consistent when conventional methods were compared with flow cytometry. It was found that KPC and NDM enzyme-producing bacteria created synergy after treatment with a carbapenem and carbapenem + carbapenemase inhibitors, and the rate of staining with PI (%) increased more significantly than other enzyme-producing bacteria. Since there is no specific carbapenemase inhibitor in the detection of OXA-48 enzyme-producing bacteria, it was observed that they did not create synergy in these tubes and showed resistance to Temocillin. After treatment with carbapenem, bacteria producing OXA-48 enzyme showed much higher PI staining rates (%) than KPC and NDM enzyme producers compared to tubes without

Figure 10. ROC curve of PI staining rates (%) in tube containing meropenem + bacterial suspension.



antibiotics. The higher rates of staining with PI (%) in the tube containing the OXA-48 enzyme meropenem, and the lower MIC values of the strains containing OXA-48 compared to other carbapenemase producers suggested that they hydrolyze meropenem less. Antibiotic susceptibility tests for bacteria in flow cytometry are not standardized. Therefore, the resistance or susceptibility limit values have not yet been determined clearly. In the study, ROC analysis was performed by evaluating the rate of staining with PI (%) in the meropenem tube in order to evaluate the success of distinguishing carbapenem susceptibility and resistance of *Enterobacteriales* in flow cytometry. As a result of ROC analysis, the cut-off value (%) for PI staining determined for meropenem was determined as 14.37% (specificity 100% and susceptibility 65%). In *Enterobacteriales*, below this value was found to be carbapenem sensitive and above it as carbapenem-resistant. It is seen that the rate of staining with PI (%) of OXA-48 producers after treatment with meropenem is higher than the cut-off value. Kılıç *et al.* found the cut-off value to be 18.88% for detecting meropenem susceptibility in *K .pneumoniae* isolates [15]. As a result of the analysis by flow cytometry, studies with a much larger number of strains under the same optimal conditions are needed to decide on antibiotic susceptibility. Setting separate breakpoints for each carbapenemase group may increase the susceptibility of carbapenemase detection in terms of endemic regions.

In various flow cytometry studies, the effects of beta-lactam antibiotics, aminoglycosides, fluoroquinolones, and meropenem were examined at the morphological level, and flow cytometry analyzes were shown to be "well-matched" [16,17]. When Duyan *et al.* investigated the presence of beta-lactamase in 38 *Enterobacteriales* isolates by PCR and flow cytometry methods, they found that the consistency of both methods was 100% [18]. Silva *et al.* in their study, found that there was an "excellent" consistency when PCR and flow cytometry results were compared using antibiotics at various concentrations in the detection of carbapenem resistance in different enteric bacteria [19]. In our study, when PCR and flow cytometry results were compared in the detection of carbapenem resistance genes, it was found that flow cytometry provided an advantage in terms of time, its results were compatible with PCR, and its performance was good in detecting carbapenem susceptibility.

Conclusions

Flow cytometry provides an advantage over the molecular method as it can quantitatively show the amount of bacteria in the sample, distinguish between live/dead cells in the same study, and do not require culture for antibiotic susceptibility. Flow cytometry will continue to be a method open to development in the identification of microorganisms, antimicrobial susceptibility, and resistance in the field of clinical microbiology since it analyzes many versatile parameters at the same time at multiple cell levels and gives fast results, and its consistency with traditional methods is high.

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Corresponding author

Asst. Prof. İpek Koçer MD. Ph.D.
İncilipınar mah. Gazimuhtarpaşa bul. Sanko Üniversitesi Tıp Fakültesi No: 36
Şehitkamil / Gaziantep
Tel:+905326332033
Email: ipekkocer1@gmail.com

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