Research Article

BIOMEDICAL
Journal of Scientific & Technical Research

ISSN: 2574 -1241

DOI: 10.26717/BJSTR.2024.54.008565

Ultra-Rapid Flow Cytometry Assay for Methicillin Resistance Detection and Vancomycin MIC

R. Gomes[1], S. Cruz[1], I. Martins-Oliveira[1], A. Silva-Dias[1,2], Blanca Pérez-Viso[3], R. Cantón[3,4] and C. Pina-Vaz[1,2,5]*

[1]FASTinov S.A., Porto, Portugal

[2]CINTESIS-Center for health technology and services research, Faculty of medicine of the university of Porto, Portugal

[3]Servicio de Microbiología. Hospital Universitario Ramón y Cajal and Instituto Rámon y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain

[4]CIBER de Enfermedades Infecciosas (CIBERINFEC), Instituto de Salud Carlos III, 28029 Madrid, Spain

[5]Division of Microbiology, Department of Pathology, Faculty of Medicine, University of Porto, Porto, Portugal

***Corresponding author:** C Pina-Vaz, FASTinov SA, Porto, Portugal, CINTESIS-Center for health technology and services research, Faculty of medicine of the university of Porto, Portugal, Division of Microbiology, Department of Pathology, Faculty of Medicine, University of Porto, Porto, Portugal

ARTICLE INFO

Received: January 04, 2023

Published: May 02, 2024

Citation: R. Gomes, S. Cruz, I. Martins-Oliveira, A. Silva-Dias, Blanca Pérez-Viso, R. Cantón and C. Pina-Vaz. Ultra-Rapid Flow Cytometry Assay for Methicillin Resistance Detection and Vancomycin MIC. Biomed J Sci & Tech Res 54(3)-2024. BJSTR. MS.ID.008565.

ABSTRACT

MRSA are responsible for a high proportion of infections. Vancomycin resistance in *Staphylococcus aureus* is rare although, a decreased susceptibility has been described associated to worse outcome. An ultra-rapid antimicrobial susceptibility assay able to provide methicillin- resistance status and vancomycin MIC in *S. aureus* isolates in 2 h max, was evaluated. A two-sites susceptibility testing of cefoxitin and vancomycin was performed in 119 *S. aureus* isolates using reference methods and FASTinov® technology (Porto University spin-off, Portugal). Bacteria were incubated with a screening concentration of cefoxitin (4 mg/L) for the detection of methicillin- resistance and a serial concentration of vancomycin for MIC determination; a fluorescent probe was added to each drug and incubated for 1-hour. Flow cytometric analysis was performed and the susceptibility result was provided by a dedicated software. Sensitivity, specificity, essential agreement (EA) and bias were calculated comparing to the reference method using ISO guidelines. Sensitivity and specificity for detection of resistance to both drugs was 100%. Regarding vancomycin, the EA was 96.6% with +23.4% of bias. Reproducibility was 100% for both drugs. An ultra-rapid and accurate flow cytometry assay is described for the screening of methicillin-resistance and vancomycin MIC determination in *S. aureus* isolates with excellent correlation with standard reference methods.

Keywords: MRSA; Vancomycin MIC; Flow Cytometry Antimicrobial Susceptibility Assay

Abbreviations: MRSA: Methicillin-Resistance *Staphylococus aureus*; HA-MRSA: Hospital-Acquired Methicillin-Resistant *Staphylococcus aureus*; CA-MRSA: Community-Associated Methicillin-Resistant *Staphylococcus* aureus; MIC: Minimum Inhibitory Concentration; AST – Antimicrobial Susceptibility Test; BMD: Broth Microdilution; EUCAST: European Committee on Antimicrobial Susceptibility Testing; CLSI: Clinical & Laboratory Standards Institute; MBC: Minimal Bactericidal Concentration; BC: Blood Cultures; SSC: Side Scatter; FSC: Forward Scatter; EA: Essential Agreement; MSSA: Methicillin-Susceptible *Staphylococcus* aureus

Introduction

Staphylococcus aureus is one of the most common pathogens causing several infections. These infections are especially relevant particularly in case of methicillin-resistance, as they are resistant to all available beta-lactam drugs except ceftaroline and ceftobiprole. [1] Methicillin resistance *S. aureus* (MRSA) isolates are associated with longer hospitalizations, increased morbidity and mortality. Unlike traditional MRSA residing in hospitals (HA-MRSA), new clones have emerged in community settings (CA-MRSA) and infect people without predisposing risk factors. [2] Resistance to vancomycin, one of the most important anti-MRSA antibiotics is rare, although isolates with decreased susceptibility have been recovered in several geographic areas. In 1997, Hiramatsu et al. described the first documented case of infection caused by *S. aureus* with reduced susceptibility to vancomycin.[3] Reduction on its susceptibility was described as MIC creep and related with worse outcome. [4] High vancomycin MIC (>1.5 mg/L) was the only independent risk factor for development of complicated bacteriemia caused by methicillin-susceptible *S. aureus*. [5] However, Diaz et al., in a systematic review and meta-analysis, did not find evidence of MIC creep phenomenon but clinicians are now much more alert and often request for quantitative susceptibility result regarding vancomycin. [6] On the other hand, in conventional routine laboratory flow-chart, antimicrobial susceptibility testing (AST) results are reported after 16-24h from pure colonies using automated methods, disc diffusion or broth microdilution (BMD), but critical clinical situations demand faster answers. [7] Flow cytometry has been shown to be an excellent way to provide rapid AST results from colonies or even directly from positive blood cultures (BC) mainly as a qualitative assay but also allowing MIC determination. [8-10] In this study, we describe a rapid flow cytometry assay for the detection of methicillin resistance and vancomycin MIC determination in *S. aureus* from colonies providing results after maximum of 2 h since the initiation of the test.

Materials and Methods

Bacterial Isolates

A total of hundred and nineteen *S. aureus* were studied: 59 isolates belonging to the bacterial collection of the Microbiology Department of Porto School of Medicine (55 clinical isolates and 4 belonging to American Type Culture Collection-ATCC strains) isolated from different biological products (blood cultures, respiratory products and wounds) were tested in FASTinov, Porto, Portugal and 60 isolated obtained from patients with *S. aureus* bacteriemia were tested in the Microbiology department of Ramón y Cajal University Hospital in Madrid, Spain.

Reference Methods

Susceptibility of cefoxitin and vancomycin was performed from isolated colonies: disc diffusion method regarding cefoxitin and broth microdilution (BMD) regarding vancomycin MIC determination (range 0.125-64 mg/L). Results were interpreted according to both EUCAST and CLSI guidelines. [11-13] Moreover, minimal bactericidal concentration (MBC) for vancomycin were also determined for each isolate. Briefly, an aliquot of 10 µl from the wells with no visible growth in MIC panels were seeded on blood agar plates. The lower concentration with no bacterial colonies was considered the MBC.

Molecular Detection of *MecA* Gene

Both in Porto and Madrid isolates, molecular detection of *mecA* gene was carried out by polymerase chain reaction (PCR) using primers and conditions previously described.

Ultra-Rapid Antimicrobial Susceptibility (FASTinov® Panels)

Flow cytometry assay with cefoxitin was performed to infer methicillin-resistance and with vancomycin for MIC determination. Overnight cultures in blood agar plates (BioMériux, France) of *S. aureus* were used. A sub-culture in Brain-hearth broth (Sigma-Aldrich, US) was performed and incubated at 35±2°C with shake until turbidity (around 1.5h); a centrifugation step follows, and the suspension adjusted to 0.5 McFarland and diluted (1/2) in filtered Muller-Hinton broth cation adjusted (MH; Sigma-Aldrich, US). The FASTinov® AST panel, a conventional microplate with 12 wells including a screening concentration of cefoxitin (4 mg/L) for detection of methicillin-resistance and vancomycin at serial concentrations similar to a BMD panel, was inoculated adding 100 µl of the bacterial suspension to each well. A nucleic acid fluorescent probe, that only stain damaged cells, was added to each well. [8] As quality control, a negative well with non-treated bacterial cells and a positive control with citric acid, to assure fluorescent dyes performance, was used. After 1-hour incubation at 35±2°C with shaking and protected from light, the panel was analyzed using a flow cytometer, CytoFLEX (Beckman Coulter, US). Cell fluorescent intensity, number of event as well as morphological changes were recorded in a FSC (flow cytometer standard) file.

A specific software (BioFAST SW) analysed the FSC files and reported the MIC value as well as the clinical phenotypic category according to CLSI or EUCAST criteria. The number of events, the light scatter values and the fluorescence intensity of each well were incorporated on the algorithm to provide results based on cut-off values. Cut-off values for flow cytometry were previously calculated using ROC curves and introduced on the dedicated software [14].

Reproducibility

Ten *S. aureus* isolated were repeated 3 times in independent assays on the flow cytometry assay regarding both cefoxitin and vancomycin determinations and the results between repetitions were compared.

DOI: 10.26717/BJSTR.2024.54.008565

Data Analysis

Sensitivity and specificity for the detection of MRSA and vancomycin resistance was determined. Essential agreement (EA) and bias regarding MIC determination to vancomycin was calculated according ISO/DIS 20776-2:2021 for the cytometry assay.

Results

Classification of Isolates Regarding Susceptibility to Cefoxitin

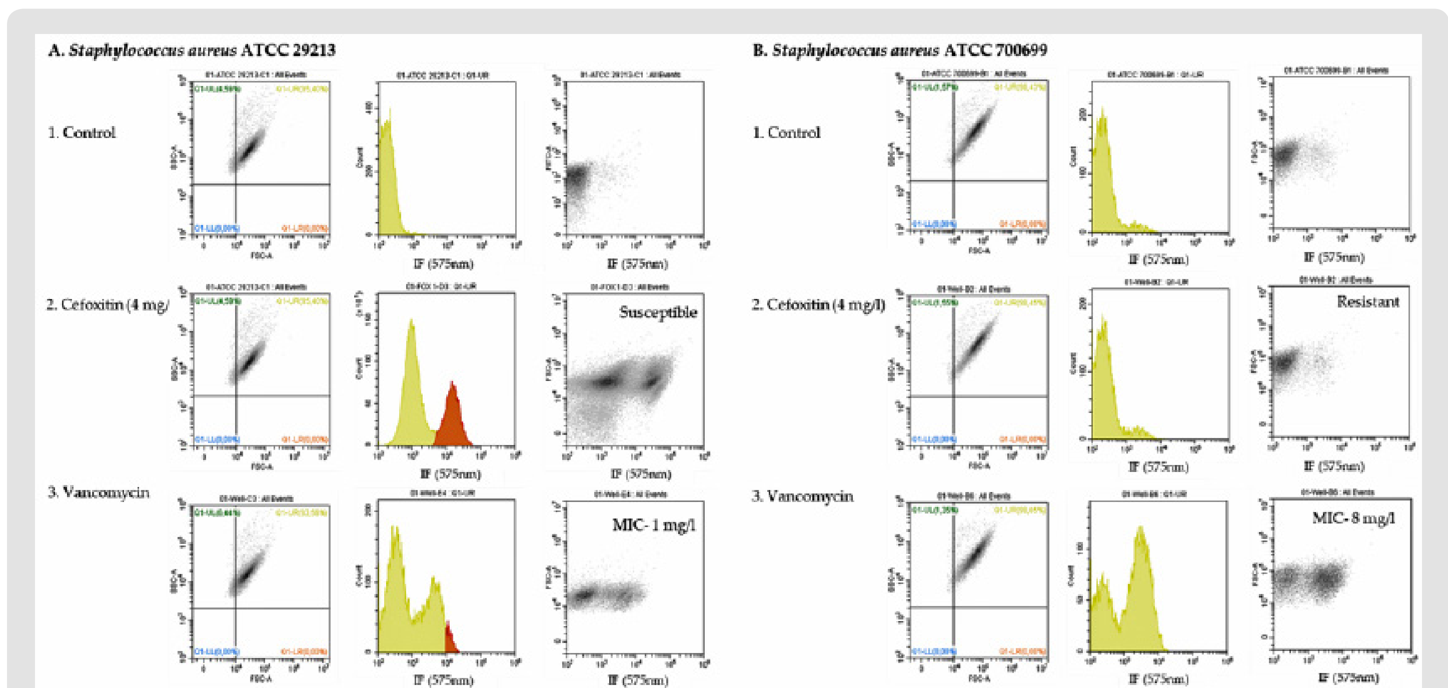
At Ramon y Cajal University Hospital in Madrid, Spain, molecular results for *mecA* gene detection were concordant with cefoxitin phenotypic ones in all the 60 studied isolates; 23 were classified as MRSA and 37 as MSSA (methicillin-susceptible S. aureus). Moreover, in Porto isolates (n=55), 33 were classified as MRSA and 20 as MSSA (Methicillin susceptible *S. aureus*), while the 2 remaining isolates were classified as methicillin-resistance by cefoxitin disc screen unlike the presence of *mecA* gene was not demonstrated.

Classification of Isolates Regarding Susceptibility to Vancomycin

The MICs varied between 0.25 and 8 mg/L, the upper range being obtained with the ATCC 700699 (Mu50) glycopepetide intermediate *S. aureus* (GISA) strain.

Flow Cytometric Results

Flow cytometer results are in Figure 1, that shows the result obtained with ATCC strain 29213 (MSSA; vancomycin MIC of 1 mg/L) and ATCC strain 700699 (MRSA; vancomycin MIC of 8 mg/l) incubated during 1-hour with cefoxitin at screening concentration and with different vancomycin concentrations. No changes on the size (SSC) or complexity (FSC) of the cells were evident after 1- hour incubation with both drugs, although a clear increase of the intensity of fluorescence (shift of the population to the right) could be observed on the susceptible strain. The sensitivity and specificity of the flow cytometry test for detecting MRSA was 100% comparing to phenotypic reference assay. Overall, the essential agreement (EA) of the flow cytometry test for determination of vancomycin MIC was 96.6%. Vancomycin MIC results of tested isolates determined by broth microdilution (BMD) and by flow cytometry assay are represented in Figure 2. The bias calculation is represented on Figure 2, representing how the flow cytometry results differ from the reference method and in which direction. The bias was +23.4% (Table 1) which is considered acceptable according the determined range for the bias calculation (-30% to +30%). [15] The minimum bactericidal concentrations (MBC) values for vancomycin were equal or one dilution above the MICs (only two strains had a difference of two dilutions above MIC value).



A. *S. aureus* ATCC 29213 susceptible to cefoxitin (MSSA) and with vancomycin MIC of 1mg/L and

B. *S. aureus* ATCC 700699 resistant to cefoxitin (MRSA) and with vancomycin MIC of 8mg/L.

1. Bacterial cells non-exposed to antibiotics (control);
2. Cells exposed to 4mg/L of cefoxitin (breakpoint concentration according EUCAST and CLSI);
3. Cells exposed to vancomycin MIC, the first concentration that shows an effect. SSC – side scatter (cell size), FSC – forward scatter (cell complexity); IF – intensity of fluorescence at log scale. A drift of the population to the right means an increase of the IF.

 DOI: 10.26717/BJSTR.2024.54.008565

Vancomycin MIC (mg/L) reference method

FASTinov MIC method	0,25	0,5	1	2	4	8
0,25	2	7	1			
0,5	3	42	19	3		
1		8	19	10		
2			2	2		
4						1
8						

Figure 2: Correlation between vancomycin MIC determination by FASTinov® technology and reference method in 119 *Staphylococcus aureus* isolates. MICs within essential agreement (within ±1 dilution of reference MIC) are highlighted in grey and MIC identical in both tests are within boxes. EUCAST and CLSI breakpoints are shown as lines: MIC ≤ 2mg/L means susceptible for both protocols, MIC > 2mg/L is resistant regarding EUCAST; according CLSI, MIC between 4-8mg/L are intermediate and MIC ≥ 16mg/L are considered resistant.

Table 1: MIC doubling dilution difference distribution to determine EA and bias. Cells highlighted in grey represented those that are in essential agreement.

≤-2	-1	0	+1	≥+2	EA
0	13/119	65/119	37/119	4/119	115/119
x	11%	54.6%	31.1%	3.3%	96.6%
11%		BIAS=+23.4%	34.4%		

Reproducibility

The reproducibility was 100% for both drugs.

Discussion

We described here a rapid flow cytometry assay for the detection of methicillin-resistance and vancomycin MIC values in *S. aureus* isolates. Detection of methicillin-resistance is crucial facing an infection due to *S. aureus*. It is of note that the time to perform flow cytometry assay is comparable to rapid molecular methods but, as it is a phenotypic assay, it could be more informative regarding patient treatment than molecular assay. Comparing with disc diffusion, flow cytometry assay is quite faster and gives the same phenotypic information. The main limitation of the described technology for the detection of methicillin resistance is the fact that it cannot be used directly on a polymicrobial sample such as a nose swab, respiratory secretions or a cutaneous wound. On that case, namely, to investigate for MRSA carries, a molecular assay is preferred whereas from colonies a phenotypic assay will be adequate if results can be offered in a short period of time. Molecular methods have been developed for *mecA* gene detection and more recently for mecC gene. Other unusual mec genes, such as mecB and mecD, and other homologues have been described also conferring methicillin- resistance. So, the absence of *mecA* or even mecC gene in a molecular assay, do not guarantee methicillin-susceptibility. [2] Two of the studied strains showed phenotypic result of resistance although negative for *mecA*. On phenotypic assays, cefoxitin has been proved to be the most sensitive drug to evaluate the susceptibility to methicillin in *S. aureus*. [16] Although the effectiveness of vancomycin in *S. aureus* infectious is supported by more than 5 decades of use, several challenges persist including the potential impact of the higher vancomycin MIC values and heteroresistance. Nevertheless, MIC values on *S. aureus* could be different according to the method used and heteroresistance is difficult to evaluate. [17] Due to mechanism of action of cefoxitin and vancomycin, cells must be at exponential growth phase, explaining the need for a broth incubation of 1.5-2 hours before incubation with the drugs.

Conclusion

In our study, flow cytometry has shown to be an excellent tool regarding a rapid evaluation of antimicrobial susceptibility from colonies providing not only the susceptibility phenotype to both drug but also vancomycin MIC values. Flow cytometry is a promising technology that could eventually change the microbiology diagnosis paradigm. An ultra-rapid phenotypical susceptibility assay could be performed directly from colonies but it also might be possible from positive blood cultures. [8,9,10,18] A fast and accurate susceptibility assay is here described for the phenotypic detection of methicillin-resistance and vancomycin MIC determination in *S. aureus* with excellent correlation with the conventional reference test saving almost one day.

Acknowledgment

This article was supported by National Funds through FCT - Fundação para a Ciência e a Tecnologia,I.P., within CINTESIS, R&D Unit (reference UIDB/4255/2020).

Conflicts of Interest

R-G, S-C, IM-O and AS-D are employees of FASTinov, S.A. CP-V is co-founders of FASTinov, S.A. Other authors do not declare conflict of interest.

References

1. Monaco M, Pimentel de Araujo F, Cruciani M (2017) Worldwide Epidemiology and Antibiotic Resistance of *Staphylococcus aureus*. Curr Top Microbiol Immunol 409: 21-56.
2. Lakhundi S, Zhang (2018) Methicillin-Resistant *Staphylococcus aureus*: Molecular characterization, Evolution, and Epidemiology. Clinical Microbiology Reviews 31(4): e00020-18.
3. KH, HH, TI (1997) Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. J Antimicrob Chemother 40: 135-136.
4. Yeh YC, Yeh KM, Lin TY (2012) Impact of Vancomycin MIC creep on patients with methicillin-resistant *Staphylococcus aureus* bacteriemia. Journal of Microbiology, Immunology and Infection 45(3): 214-220.
5. Aguado JM, San-Juan R, Lalueza A (2011) High Vancomycin MIC and Complicated methicillin-susceptible *Staphylococcus aureus* bacteriemia. Emerg Infect Dis 17(6): 1099-1102.
6. Diaz R, Afreixo V, Ramalheira E (2018) Evaluation of vancomycin MIC creep in methicillin- resistant *Staphylococcus aureus* infections – a systematic review and meta-analysis. Clin Microbiol Infect 24(2): 97-104.
7. Jonasson E, Matuschek E, Kahlmeter G (2020) The EUCAST rapid disc diffusion method for antimicrobial susceptibility testing directly from positive blood cultures bottles. J Antimicrob Chemother 75(4): 968-978.
8. Pina Vaz C, Cd OS, Silva Dias A (2017) Flow Cytomtry in Microbiology: The Reason and the Need. In: Single Ceçç Analysis. Singapore; Springer, pp. 155-169.
9. Van Belkum A, Burnham C AD, Rossen JWA (2020) Innovative and rapid antimicrobial susceptibility testing systems. Nature Reviews Microbiology 18: 299-311.
10. Fonseca e Silva D, Silva Dias A, Gomes R (2019) Evaluation of rapid colistin susceptibility directly from positive blood cultures using flow cytometry assay. International Journal of Antimicrobial Agents 54(6): 820-823.
11. Silva Dias A, Pérez Viso B, Martins Oliveira I (2021) Evaluation of FASTinov® ultra-rapid flow cytometry antimicrobial susceptibility testing directly from positive blood cultures. J Clin Microbiol 59(10): JCM0054421
12. (2020) European Committee on Antimicrobial Susceptibility Testing Clinical Breakpoints dosing of antibiotics Tenth Version.
13. (2018) National Committee for Clinical Laboratory Standards Methods for dilution Antimicrobial Susceptibility Test for Aerobically Grown Bacteria – EleventhEdition: Approved Standards M07.
14. (2018) Clinical and Laboratory Standards Institute Performance Standards for Antimicrobial Susceptibility Testing: Twenty-eight Informational Supplement M100.
15. International Organization for Standardization. ISO 20776-2:2007 Clinical Laboratory Testing and *In Vitro* Diagnostic Test System – Susceptibility Testing of Infectious Agents and Evaluation of Performance of Antimicrobial Susceptibility Test Devices – Part 2: Evaluation of Performance of Antimicrobial Susceptibility Test Devices.
16. Skov R, Larsen AR, Kearns A (2014) Phenotypic detection of mecC-MRSA: Cefoxitin is more reliable than Oxacillin. J Antimicrob Chemother 69(1): 133-135.
17. Catala C, Mir-Simon B, Feng X (2016) Online SERS Quantification of *Staphylococcus aureus* and the Application to Diagnosis in Human Fluids. Advance Materials Technologies 1(8): 600163.
18. Hoo ZH, Candlish J, Teare D (2017) What is an ROC curve? Emerg Med J 34: 357-359.

ISSN: 2574-1241

DOI: 10.26717/BJSTR.2024.54.008565

C Pina-Vaz. Biomed J Sci & Tech Res

This work is licensed under Creative *Commons* Attribution 4.0 License

Submission Link: https://biomedres.us/submit-manuscript.php

Assets of Publishing with us

- Global archiving of articles
- Immediate, unrestricted online access
- Rigorous Peer Review Process
- Authors Retain Copyrights
- Unique DOI for all articles

https://biomedres.us/