REVISTA UNINGÁ
ISSN 2318-0579

doi.org/10.46311/2318-0579.60.eUJ4398

Advantage of the use of oxyreductive dye and automated reading in the determination of pharmaceutical activity in bacteria of medical importance

Emprego de reveladores oxirredutores e de leitura automatizada na determinação da atividade de fármacos em bactérias de importância médica

Ana Beatriz Miranda, Luiz Henrique Reinert, Jean Eduardo Meneguello, Andressa Lorena Ieque, Vera Lucia Dias Siqueira, Regiane Bertin de Lima Scodro, Rosilene Fressatti Cardoso, Katiany Rizzieri Caleffi-Ferracioli*

ABSTRACT

Urinary Tract Infections (UTIs) are frequent bacterial infections in Brazil, with the *Escherichia coli* and *Staphylococcus aureus* bacilli being the most common. In the context of the pandemic caused by the SARS-CoV-2 virus, hospital infections have gained prominence, as they have been considered aggravating factors for the disease and have increased mortality rates. In this context, rapid and accurate diagnosis is essential in order to reduce costs, therapeutic errors, and development of resistant strains due to the inappropriate use of antimicrobials. The antibiotic sensitivity profile is determined by the Minimum Inhibitory Concentration (hereinafther, MIC), and the result is obtained from the observation of turbidity in the microplates with the naked eye. This technique has been improved with the use of redox indicators that can facilitate the interpretation of results through color changes. Thus, this study aimed to evaluate the performance of three dyes incorporated in the MIC for *E. coli* and *S. aureus* bacteria, in order to adopt them in routine laboratory work and to provide a better cost-benefit ratio as well as more appropriate reading. The visualization was based on the color change of the dyes and in the analysis of the absorbances emitted by a spectrophotometer, which were used in specific calculations for graph preparation. The results showed that it was possible to determine the MIC of the above-described bacteria in a minimum time of 40 minutes and to conclude that the indicators and automated reading facilitate the interpretation of bacterial growth.

Keywords: Automated reading. Dyes. *Escherichia coli*. MIC. *Staphylococcus aureus*.

RESUMO

As Infecções do Trato Urinário (ITU) são infecções bacterianas frequentes no Brasil, sendo os bacilos *Escherichia coli* e *Staphylococcus aureus* os mais incidentes. Diante do cenário pandêmico causado pelo vírus SARS-CoV-2, as infecções hospitalares ganharam destaque, já que foram consideradas agravantes da doença e aumentaram a mortalidade. Neste contexto, é indispensável o diagnóstico rápido e assertivo, a fim de se reduzirem custos, erros terapêuticos e desenvolvimento de cepas resistentes pelo uso inadequado de antimicrobianos. O perfil de sensibilidade a antibacterianos é determinado pela Concentração Inibitória Mínima (doravante, CIM), de modo que o resultado é obtido a partir da observação a "olho nu" de turvação das microplacas. Tal técnica tem sido aprimorada com o uso de reveladores oxirredutores que ajudam a facilitar a interpretação dos resultados por meio da alteração de cor. Desse modo, o presente estudo teve por objetivo avaliar o desempenho de três corantes incorporados na CIM para as bactérias *E. coli* e *S. aureus*, com o intuito de adotá-los na rotina laboratorial e proporcionar um melhor custo-benefício, bem como uma leitura mais apropriada. A visualização foi baseada na mudança de cor dos corantes e na análise das absorbâncias emitidas por espectrofotômetro, as quais foram utilizadas em cálculos específicos para elaboração de figuras. Os resultados mostraram que, no tempo mínimo de 40 minutos, foi possível determinar a CIM das bactérias acima descritas e a conclusão de que os reveladores e a leitura automatizada facilitam a interpretação do crescimento bacteriano.

Palavras-chave: CIM. *Escherichia coli*. Leitura automatizada. Reveladores. *Staphylococcus aureus*.

State University of Maringa – UEM, Maringa, PR, Brazil.
*krcferracioli2@uem.br

Received: August 08th, 2022.
Accepted: March 15th, 2023.
Published: June 12th, 2023.

INTRODUCTION

The World Health Organization (WHO) reports that about 1.4 million Healthcare-Associated Infections (HAIs) occur at any given moment around the world. These are opportunistic infections, as they can be caused by pathogens present in the patient's microbiota associated with immunosuppression, invasive hospital procedures, among others (Araújo, P. J. F. Freitas, Abreu, Freitas & Brandão, 2021). Urinary Tract Infections (UTIs) are among the most common hospital-acquired infections, with 90% of cases caused by gram-negative bacilli, with evidence for *Escherichia coli*, and about 6% by gram-positive cocci, particularly *Staphylococcus aureus* (Furlan et al., 2021). A study conducted in two New York hospitals with patients with COVID-19 revealed that although the rate of co-infection was relatively low (1.57%), the association of *S. aureus* with COVID-19 increased mortality from 54.8% to 66.7% within a period of 30 days (Cusumano et al., 2020).

Another important factor to consider regarding these pathogens is bacterial resistance, i.e., the emergence of new strains of *E. coli* and *S. aureus* resistant to available antimicrobial drugs on the market, as a result of their indiscriminate and inadequate use over the years, as well as the scarcity of new drugs. The scenario regarding bacterial resistance in Brazil has been alarming, as there has been a growing emergence of new multidrug-resistant bacterial strains in the hospital context (Menezes, Porto & Pimenta, 2013). In addition, hospital-acquired infections caused by multidrug-resistant strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA), are increasingly being documented worldwide (Feldhaus et al., 2016). Carbapenem-resistant Enterobacteriaceae (CRE), frequently identified in *E. coli*, are of great relevance, as this microorganism can cause severe infections, especially in the hospital environment (Gauthier et al., 2018).

These data indicate that an accurate diagnosis is indispensable for effective therapy, in order to reduce costs, therapeutic errors resulting in unfavorable disease outcomes, morbidity and mortality, and development of bacterial multidrug resistance (Santos, Porcy & Menezes, 2019). Therefore, the determination of the MIC, also known as the antibiogram test, is crucial for guiding antibacterial therapy. The evaluation of MIC, defined by the Clinical and Laboratory Standards Institute (CLSI) as the lowest concentration of the antimicrobial agent that inhibits the growth of the microorganism, can be obtained by the microdilution technique in broth with a naked-eye reading. This method involves the use of small volumes of broth in sterile microplates, in which the antimicrobials to be tested are diluted and placed in contact with the pathogen (Clinical & Laboratory Standards Institute, 2015).

Currently, research conducted at the Medical Bacteriology Laboratory at the State University of Maringa has shown that the use of revealing dyes can be useful, especially in determining the MIC result in bacteria whose bacterial growth provides a turbidity that is difficult to visualize with the naked eye in the culture medium. However, in certain situations, such as changes in the concentration of the revealing agent, reading time, and differences in color perception, a misinterpretation or uncertainty may occur. To confirm what is observed with the naked eye and to increase the degree of reliability of the results, it is possible to perform an automated reading through spectrophotometry, in which the device emits absorbance values that can be calculated and expressed in figures to accurately define the drug's MIC and the percentage of bacterial growth inhibition.

Thus, the present study arose from the difficulties observed in the clinical analysis laboratory, regarding the visualization and the analysis of the results of the MIC determination by the microdilution technique in broth in gram-negative and gram-positive bacteria. Given the importance of correctly determining the MIC for therapeutic efficacy, the present study aimed at evaluating the use of revealing dyes, as well as automated reading of the microdilution assay in broth, in order to facilitate quick and assertive visualization of the MIC.

MATERIALS AND METHODS

Selection and acquisition of dyes

Three redox indicators were selected, namely: triphenyl tetrazolium chloride (TTC), resazurin, and methylene blue. These indicators were chosen based on previous studies that have used them in broth microdilution assays for determining the MIC. These substances were acquired from the American company Sigma-Aldrich® and were already available in the Medical Bacteriology Laboratory at the State University of Maringa.

Studied bacteria

Escherichia coli (ATCC 25922) and *Staphylococcus aureus* (ATCC 29213) were used in this study as prototypes for the evaluation of gram-negative and gram-positive bacteria, respectively. The isolates were reactivated in tryptic soy broth (TSB - Difco Laboratories) for two to six hours at 35±2 °C, followed by plating on tryptic soy agar (TSA - Difco Laboratories) for up to 24 hours at 35±2 °C, to evaluate purity prior to the broth microdilution assay.

Modified broth microdilution assay

The drug ciprofloxacin was used as the standard drug of choice to evaluate the efficiency of the dyes. According to the CLSI, the MIC for *S. aureus* ATCC 25213 should be between: 0.12 - 0.5 µg/mL, and, for *E. coli* ATCC 25922, between 0.004-0.016 µg/mL. The MIC of this drug was determined by the microdilution method in broth, using Mueller Hinton Broth with cation adjustment (CAMHB, Difco Laboratories, Sparks, MD, USA),

according to the recommendation of the CLSI (2015). For adjustment, 200 µL of Ca^{2+} and 100 µL of Mg^{2+} were used. The isolates were first reactivated in TSB for six hours at 35 ºC. After that, they were plated on TSA for 24 hours at 35 ºC.

Then, 100 µL of the drug ciprofloxacin was serially diluted (twofold) in microplates containing MHB medium for each bacterium. Inoculums were prepared by comparison with the 0.5 McFarland scale and diluted to a final concentration of approximately 5.0 x 105 CFU/mL. After 18 hours of incubation, the lowest concentration of the drug without visible bacterial growth was considered as the MIC. Visualization was done before and after the use of the selected dyes. For each dye used as a revealer, the best conditions for visualization of the result were established based on different times (20, 40, 60 and 120 minutes). It is important to note that the experiments were carried out in duplicate to obtain consistent results with minimal variations.

Dye addition, reading and data tabulation

The assay was performed on 96-well microplates, in which the first two rows (A and B) were left without dye (visual reading - control). 20 µL of TTC reagent, prepared at a concentration of 0.4 g in 40 mL of distilled water, were added to each well in rows C and D. The resazurin was prepared at a concentration of 0.002 g in 10 mL, and the same 20 µL were added to wells in rows E and F. Finally, methylene blue was prepared at the same concentration as resazurin and 20 µL were added to each well in rows G and H, completing the microplate. The microplate model used is represented in Figure 1.

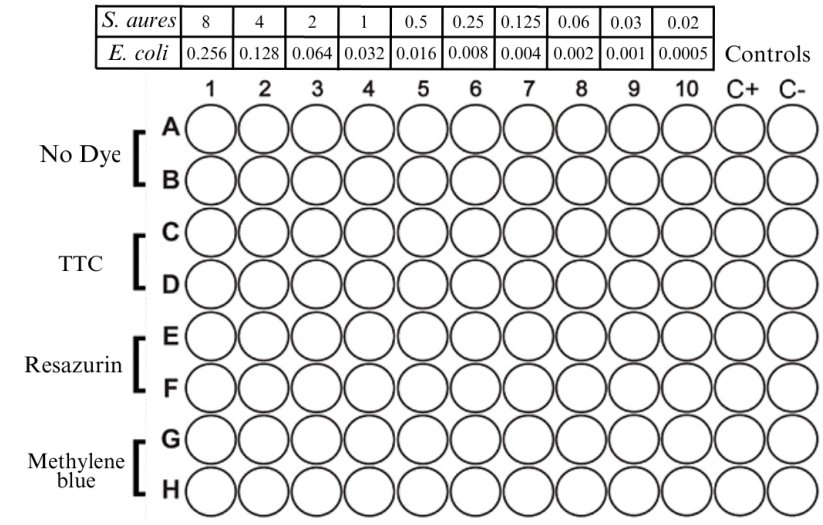
Photographs of the plates were taken at 20, 40, 60, and 120 minutes after the addition of the dyes for visual analysis, in addition to a reading on the spectrophotometer, which determined absorbance values based on specific wavelengths for each reagent. For TTC, the reading was performed at 480 nm, for resazurin at 570 and 600 nm, and for methylene blue at 600 nm. After that, the obtained data were transferred to an Excel® spreadsheet, in which figures were produced from specific calculations explained below.

Analysis and interpretation of results

Based on the results obtained, a spreadsheet was developed to standardize and facilitate the use of the methodology proposed in this study in the clinical analysis laboratory.

Figure 1

The model of the microplates used for the experiments on *S. aureus* and *E. coli*, highlighting the used colorimetric indicators and the different concentrations of ciprofloxacin employed.



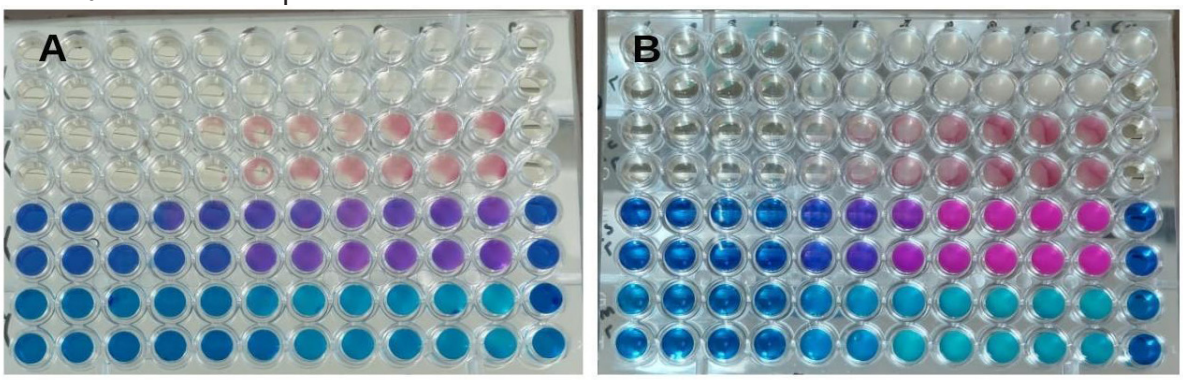
Source: The authors.

RESULTS AND DISCUSSION

Through the experiments conducted, it was possible to verify that the addition of colorimetric indicators allowed a better perception of color changes and the turbidity of the wells, and a more accurate determination of the MIC of ciprofloxacin for *S. aureus* and *E. coli*. For all the colorimetric indicators used, a minimum time of 40 minutes was sufficient to identify the MIC for both *S. aureus* and *E. coli*. Photographs of the microplates containing the two experiments were taken (Figure 2).

In both experiments, visual and colorimetric reactions characteristic of the presence of the indicators were observed due to their mechanisms of action (specified in Table 1 and illustrated in Figure 4). The MIC without indicator was determined according to the CLSI guidelines (2015), it was based on the turbidity of the corresponding well due to bacterial growth. The values obtained for ciprofloxacin, both for *E. coli* and *S. aureus*, without the use of a colorimetric indicator validated the methodology.

Figure 2
Photographs of the microplates containing the experiments on *S. aureus* (A) and *E. coli* (B) after 40 minutes of exposure to the colorimetric indicator.



Source: The authors.

Table 1
Oxidoreductase colorimetric indicators used related to their respective mechanisms and visual changes.

Compound	Mechanism and visual change
Tetrazolium Chloride (TTC)	It has a colorless oxidized form and is reduced to formazan (TPF) in the presence of dehydrogenases, turning red in color.
Resazurin	It has a purple color when in contact with microbial metabolism, it is reduced to resorufin, turning pink in color and presenting a violet hue at the transition of the well containing the MIC (Roca et al., 2019).
Methylene blue	It has a blue color and is reduced to "leuco-methylene," becoming colorless due to electrochemical reactions related to bacterial respiration (Duan, Fang & Wang, 2021).

Source: The authors.

After the visual interpretation, the two microplates containing the experiments with *S. aureus* and *E. coli* were sent to the spectrophotometer, which was configured with the defined wavelengths for each dye. In this way, the absorbance values of the wells were obtained through the Softmax® Pro 7 program. The automated reading was performed at the same time as the visual analysis, both established at 40 minutes after the end of the experiment. With 20 minutes of exposure to the dyes, the visual observation of the MIC could already be defined. However, the correspondence with the automated method was better established at the time of 40 minutes.

The absorbance values emitted by the spectrophotometer were transferred to a calculation chart adapted to the plate model, contained in a spreadsheet created in Excel®, according to the dye, the wavelength, and the well, represented respectively in Figures 5 and 6. With these data, it was possible to perform a quantitative analysis of the different dyes, determined by the percentage of bacterial inhibition of each well, through different formulas and tools within Excel®, automatically. The spreadsheet optimized the use of the proposed methodology, since, based on the averages of the positive or negative controls, corresponding figures were prepared, showing the relationship between the antimicrobial used and the percentage of bacterial inhibition as shown in Table 2 and 3.

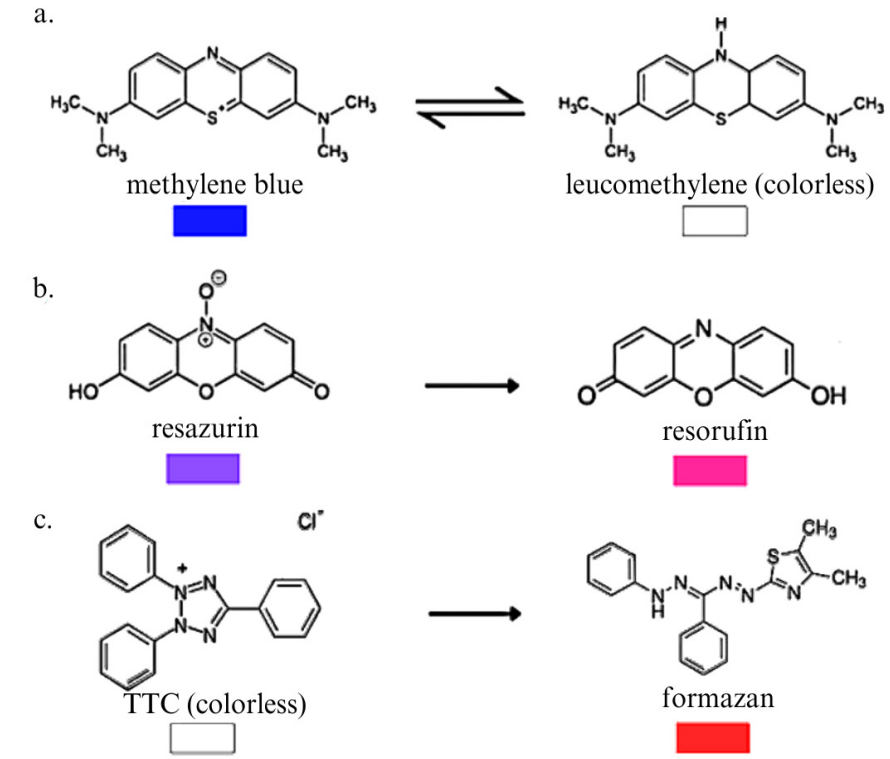
To obtain the bacterial inhibition percentages of both bacteria from wells without dye and with the TTC indicator, the formula below was used:

(1) % of Bacterial inhibition = 100 - (Duplicate Mean x 100)/ Mean of positive controls.

On the other hand, the calculation of bacterial inhibition percentage for wells containing the resazurin indicator was done using a different model, due to the use of two wavelengths in the experiments, as this compound changes in different colors depending on the bacterial growth rate. To calculate this, an adaptation of the cytotoxicity measurement formula presented in a protocol by the manufacturer of alamar Blue (the commercial name of resazurin), by Bio-Rad Laboratories, was performed. The percentage of bacterial growth inhibition with the resazurin dye is obtained from 100% minus the result of the formula below.

Figure 3

Redox reactions of the dyes used and their respective colorimetric reactions.



Source: Aiyker and Vijayakumar (2019), adapted by the authors.

Table 2

Table referring to the spectrophotometer reading after 40 minutes of the experiment with *Staphylococcus aureus*, containing the absorbance values for each well and the mean value of negative or positive controls for duplicates, following the 96-well microplate model.

Dye	Duplicate	1	2	3	4	5	6	7	8	9	10	C+	C-	Average*
No dye	A	0.003	0.002	0.002	0.000	0.234	0.442	0.476	0.347	0.473	0.458	0.461	0.000	0.461
	B	0.001	0.003	0.002	0.002	0.195	0.376	0.445	0.396	0.242	0.465	0.461	0.000	
TTC (480 nm)	C	0.011	0.003	0.002	0.003	0.336	1.129	0.449	1.029	1.138	1.163	0.982	0.043	0.982
	D	0.001	0.002	0.001	0.001	0.387	0.895	1.022	0.964	0.870	1.073	0.982	0.043	
Resazurin (570 nm)	E	1.619	1.645	1.780	1.798	2.309	2.325	2.538	2.273	2.317	2.542	2.481	1.752	2.481
	F	1.574	1.996	1.545	1.805	2.416	2.381	2.594	2.442	2.468	2.569	2.481	1.752	
Resazurin (600 nm)	G	2.176	2.209	2.361	2.378	1.207	1.005	0.939	0.653	0.788	0.871	1.127	2.340	1.127
	H	2.123	2.632	2.089	2.407	0.866	0.946	0.940	0.804	0.767	0.815	1.127	2.340	
MB (600 nm)	I	1.479	1.626	1.559	1.579	1.459	1.420	1.650	1.578	1.467	1.481	1.664	1.267	1.267**
	J	1.570	1.623	1.650	1.756	1.613	1.469	1.540	1.617	1.596	1.525	1.664	1.267	

Source: The authors.

Notes. MB - methylene blue, *Average of duplicates of positive controls (C+) or negative controls (C-). **Value of the mean referring to duplicate of negative controls, necessary only for calculations related to methylene blue dye.

Table 3

Table referring to spectrophotometer reading after 40 minutes of the *Escherichia coli* experiment, containing the absorbance values for each well and the mean value of negative or positive controls for duplicates, following the 96-well microplate model.

Dye	Duplicate	1	2	3	4	5	6	7	8	9	10	C+	C-	Average*
No dye	A	0.091	0.089	0.089	0.058	0.339	0.489	0.852	1.003	1.075	1.061	1.092	0.896	1.117
	B	0.093	0.091	0.092	0.114	0.439	0.557	0.868	0.943	0.951	0.986	1.141	0.082	
TTC (480 nm)	C	0.084	0.090	0.089	0.092	0.228	0.475	0.668	1.192	1.447	1.591	1.465	0.087	1.518
	D	0.082	0.089	0.094	0.090	0.247	0.482	0.763	1.094	1.304	1.403	1.571	0.087	
Resazurin (570 nm)	E	1.765	1.345	1.308	1.290	1.806	2.042	2.140	2.301	2.138	2.266	2.028	1.230	2.003
	F	1.197	1.219	1.251	1.368	1.606	1.973	2.369	2.338	2.252	2.238	1.978	1.215	
Resazurin (600 nm)	G	2.220	1.817	1.773	1.753	1.326	1.217	0.847	0.664	0.717	0.781	0.765	1.681	0.764
	H	1.619	1.650	1.695	1.858	1.251	1.179	0.789	0.768	0.866	0.896	0.763	1.663	
MB (600 nm)	I	2.047	2.344	2.359	2.362	1.984	1.586	1.419	1.323	1.294	1.232	1.022	2.027	2.279**
	J	2.412	2.641	2.648	2.554	1.867	1.829	1.306	1.251	1.427	1.264	1.389	2.531	

Source: The authors.

Notes. MB - methylene blue, *Average of duplicates of positive controls (C+) or negative controls (C-). **Value of the mean referring to duplicate of negative controls, necessary only for calculations related to methylene blue dye.

(2) % of Bacterial inhibition = 100 - [(117216 x Duplicate Mean at 570 nm) - (80586 x Duplicate Mean at 600 nm)]/ [(117216 x Mean of positive control at 570 nm) - Mean of positive control at 600 nm)] x 100.

Due to the fact that the methylene blue dye has a bleaching mechanism as bacterial growth occurs, the calculation was based on the mean of negative controls. Formula 3 represents the calculation performed.

(3) % of Bacterial inhibition = (Duplicate Mean x 100)/ Mean of negative controls.

The microdilution broth assay with visual reading and use of indicators (Figures 4 and 6) was based on the perceivable color change according to the respective dye used. On the other hand, the results obtained by automated reading were presented in the form of percentage of bacterial growth inhibition (Figures 5 and 7). Although MIC, defined by CLSI as the drug concentration in which ≥ 90% bacterial growth inhibition is observed, in the microdilution broth assay recommended by the reference, the value is determined based on visual reading.

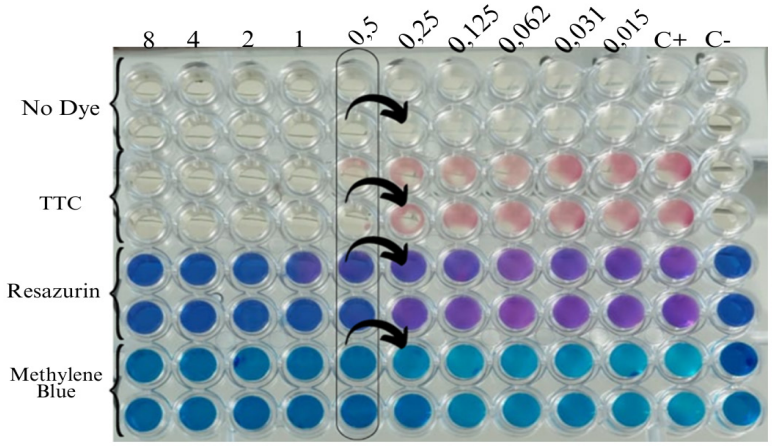
It is important to emphasize that, as a reliability criterion of the assays in the present study, the same plates were read by the spectrophotometer after visual reading and validation of the results obtained. Significant variations between the two reading methods are not expected. However, in laboratory practice, a 1-dilution variation in the MIC value is not considered significant and is attributed to an acceptable technical error in dilution (Brennan-Krohn, 2017).

The MIC for ATCC strains is typically stable and known within a range established by the CLSI (*S. aureus* ATCC 25213: 0.12-0.5 µg/mL and *E. coli* ATCC 25922: 0.004-0.016 µg/mL). Considering the higher sensitivity in detecting bacterial growth by the spectrophotometer to establish the standard inhibition ≥ 90%, a variation in MIC was observed comparing the two methodologies for the studied bacterial strains. We observed that for both strains the MIC on the graph was defined between ciprofloxacin concentrations of 0.5-1 µg/mL for *S. aureus* and 0.016-0.032 µg/mL for *E. coli*. According to Brennan-Krohn (2017), given an acceptable variation and that systematic errors could be minimized by using a series of finer dilutions, allowing greater precision in determining the MIC, our study shows that, although the use of indicators facilitates visualizing bacterial growth by traditional methodology and is an interesting gain for the laboratory, automated reading is more accurate, especially when combined with automated drug dilution.

With 40 minutes of exposure to the dyes, it was already possible to verify the color changes and to visually determine the MIC. Experimentally, the color change for *E. coli* was easier to visualize than for *S. aureus*. An exception was noticed for methylene blue for *S. aureus*, which, although with careful attention, a color change was noticed in the well referring to the MIC, but there was no correlation with the automated reading assay. Based on the ease of reading, the best dye we noted was TTC, followed by resazurin. If we consider the cost-benefit for laboratories that do not have a structure for automated MIC reading, the use of resazurin may be a good option for routine laboratory use, especially for bacterial isolates with difficult to growth.

Figure 4

Photograph of the microplate containing the *S. aureus* experiment at 40 minutes after the addition of the dyes.

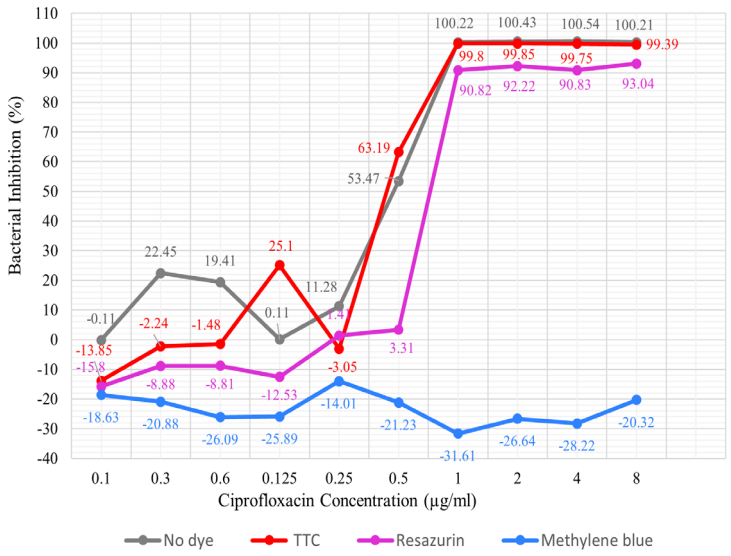


Source: The authors.

Note. The arrows indicate the color change of the tested dyes, indicating the minimum inhibitory concentration of the drug in the marked column.

Figure 5

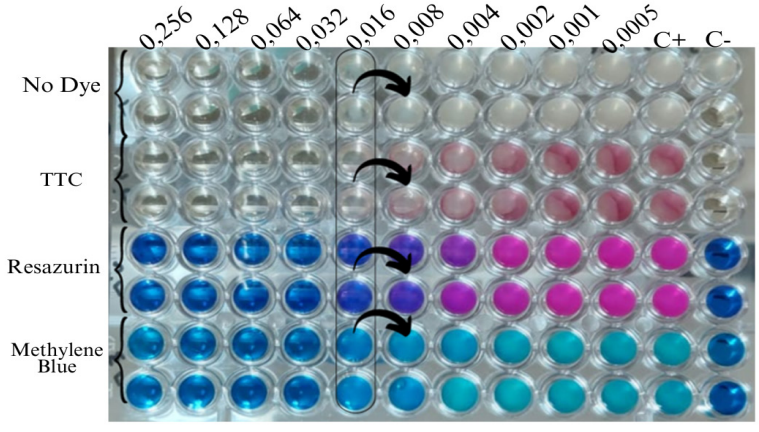
Figure generated from specific calculations for each dye at 40 minutes of incubation time for *S. aureus* bacteria.



Source: The authors.

Figure 6

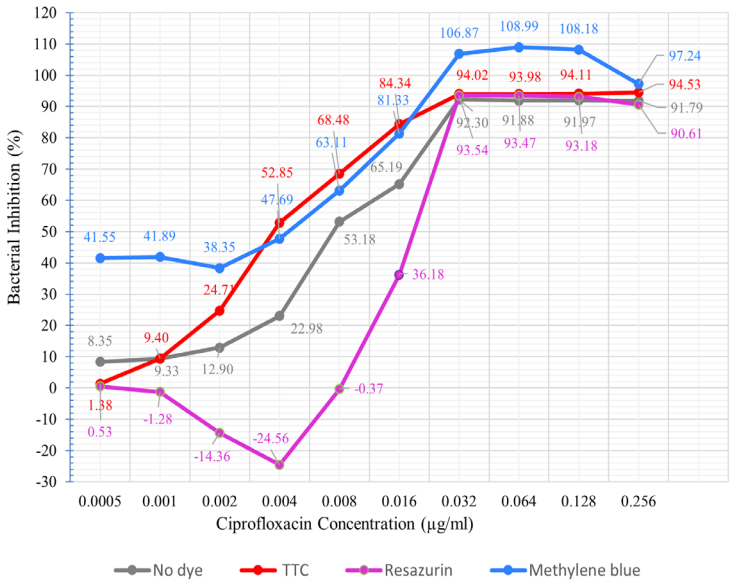
Photograph of the microplate containing the experiment on *E. coli* at 40 minutes after addition of the dyes.



Source: The authors.

Note. Arrows indicate the color change of the tested dyes indicating the minimum inhibitory concentration of the drug in the marked column.

Figure 7
Figure generated from specific calculations for each dye at 40 minutes for *E. coli* bacteria.



Source: The authors.

CONCLUSION

It can be concluded that the use of indicators assists in the interpretation of determining the MIC, especially when there is difficulty in visualizing bacterial growth. For *Escherichia coli* and *Staphylococcus aureus*, the shortest time for growth perception was 40 minutes for all dyes. Therefore, indicators allow for faster and more accurate results. Additionally, the analysis of microplates through spectrophotometry confirms the naked-eye reading and it is advantageous for accurately showing the minimum concentration of the drug that inhibits bacterial growth, providing even more precise and reliable results. The use of this methodology in laboratory routine will enable better exploration of the efficiency of antimicrobials in combating bacterial infections.

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