ORIGINAL ARTICLE

Optimization of Resazurin Microplate Assay (REMA) in Evaluating Anti-MRSA and Anti-MSSA Activities

Cayel Jurist C. Garong, Normela Patricia F. Burigsay, Renelyn S. Gapultos, John Lloyd B. Mandawe, Rae Martin V. Pedrosa and Geraldine B. Dayrit

Department of Medical Microbiology, College of Public Health, University of the Philippines Manila

ABSTRACT

Background and Objective. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the leading causes of hospital and community-acquired infections, showing antimicrobial resistance (AMR), which is an increasing public health concern. One of the commonly-used methods to evaluate resistance include the Kirby-Bauer disk diffusion method. However, this test is found to be time-consuming, lacking in terms of mechanization and automation, alongside its non-applicability to certain antibiotics such as vancomycin. Thus, the Clinical Laboratory Standards Institute (CLSI) recommends using the broth microdilution method in the evaluation of antibacterial activities against *S. aureus*. A rapid laboratory identification of MRSA is important in the treatment of patients. Therefore, this study aims to optimize and evaluate the effectiveness of a rapid microplate assay using resazurin dye as a colorimetric indicator in determining antibacterial activity against clinical isolates of MRSA and methicillin-susceptible *S. aureus* (MSSA).

Methods. Clinical isolates of MRSA and MSSA were obtained from the Philippine General Hospital (PGH) Microbiology Section, and American Type Culture Collection (ATCC) controls of both strains (ATCC 25923 and ATCC 43300) were acquired. These were then subjected to identification and confirmation procedures. A standardization of bacterial inoculum was performed by comparing its 24-hr growth in Mueller Hinton Broth to 0.5 McFarland Standard. The resazurin microplate assay (REMA) was set-up using two-fold serial dilution of control antibiotics such as oxacillin, vancomycin, and cefoxitin. Each plate was inoculated with standardized bacterial growth of controls and clinical isolates. To determine the time needed for the reduction of the resazurin dye, a qualitative assessment was conducted by comparing the reaction time between a 6.75 mg/mL dye with a 0.01 mg/mL dye. The plates were also subjected to different incubation times and dye concentrations, and the optical densities of the plates were compared using a microplate reader.

Results. Results showed that there were no significant differences between the optical densities of the wells of those incubated for 5 hours and for 24 hours ($p > 0.05$). Furthermore, there was a significant reduction in the reaction time of the dye (from 18 hours to 1 hour) when the dye concentration was reduced from 6.75 mg/mL to 0.01 mg/mL. The optimized REMA showed a significant difference between the minimum inhibitory concentrations (MICs) of the different antibiotics against the control and isolate strains of MRSA and MSSA, showing a W of -2.98 ($p < 0.05$) using the Wilcoxon Rank-Sum non-parametric test. Furthermore, the REMA has shown better illustration of anti-MRSA and anti-MSSA activities as compared to the Kirby Bauer disk diffusion method.

Conclusion. Based on the results presented, the researchers determined the optimal condition for the resazurin microtiter assay, which was 0.01 g/mL concentration of resazurin dye, at a 5-hour incubation period. This study has shown that an optimized REMA is an efficient and fast method to determine the antimicrobial activities of oxacillin, cefoxitin, and vancomycin against MRSA and MSSA.

Keywords: Resazurin Microplate Assay, Methicillin Resistant Staphylococcus aureus, Methicillin Susceptible Staphylococcus aureus, optical density, optimization

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Corresponding author: Cayel Jurist C. Garong
Department of Medical Microbiology
College of Public Health
University of the Philippines Manila
625 Pedro Gil Street, Ermita, Manila 1000, Philippines
Email: ccgarong@up.edu.ph

INTRODUCTION

Antimicrobial resistance (AMR) is an increasing public health concern, which corresponds to the evolution of microorganisms where they develop resistance to antimicrobial agents, making infections harder to treat and the spread of disease, severe illness, and fatality inevitable.[1]

Among the primary microorganisms that exhibit AMR are the common hospital-acquired bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), which has been found to be resistant to various antibiotics such as β-lactams, azithromycin, and cefoxitin. With this, Masim et al. assert that it is one of the leading causes of hospital-acquired and community-acquired infections.[2] More notably, the Philippines has maintained MRSA rates higher than 50% since 2010.[3] This creates a problem wherein its increasing rates and resistance entail worsening nosocomial infections in the country. Allegranzi et al. emphasized that the prevalence and density of nosocomial infections in developing countries, including the Philippines, were much higher than in the USA and Europe.[4]

MRSA is characterized by its high resistance to methicillin and other β-lactam antibiotics. This is caused by a mutation in the organism's gene, specifically the mecA gene, making it a major public health concern worldwide. The mecA gene codes for an enzyme called low-affinity penicillin-binding protein 2a (PBP2a), a transpeptidase that catalyzes cell-wall crosslinking.[5] The emergence of MRSA is mainly attributed to the frequent and irresponsible use of antibiotics in humans and animals as well. This can also be attributed to predisposing risk factors such as previous antibiotic therapy, previous hospital admission, usage of indwelling catheter.[6]

One of the treatments used for patients infected with MRSA is vancomycin, based on the National Antibiotic Guidelines of the Department of Health (DOH).[1] The mechanism of action of vancomycin is through inhibiting the polymerization of peptidoglycans in the bacteria's cell wall, which is similar to penicillin.[7] This can be used together with other antibiotics such as clindamycin, rifampin, gentamicin, or high-dose β-lactams to obtain their synergistic effect.

Evaluating the activities of antibacterials used against MRSA has been reported using various methods, some of which include agar screening method, e-test method, dilution methods (agar dilution or broth microdilution), and the Kirby-Bauer disk diffusion method. Hafez et al. contended that procedures like these require 24-48-hour incubation periods since they are culture-based.[8]

With the concern for antimicrobial resistance, the need to immediately look for appropriate antimicrobials against MRSA and MSSA is critical. Furthermore, Coban highlighted that the early identification of antibiotics against MRSA and MSSA is important in avoiding the excessive use of vancomycin and in the reduction of mortality, length of hospitalization, and costs associated with MRSA infection.[9] With this, a rapid laboratory identification of MRSA infection is a must in patient treatment. Molecular identification of mecA gene is one of the currently used methods, but is expensive and requires technical equipment.

Hence, a colorimetric method is suggested as a rapid and easy method, which still produces results concordant with molecular methods, also known as the Resazurin Microplate Assay (REMA). Coban pointed out that REMA has great potential for detecting MRSA early, especially when molecular techniques are unavailable.[9] Here, the duration needed to determine the Minimum Inhibitory Concentration (MIC), which is the compound's lowest concentration capable of suppressing the growth of the microorganism, was just six hours for a 5 μL of bacterial suspension in a turbidity of 0.5 McFarland standard.

This test uses resazurin as the colorimetric indicator. Sigma-Aldrich highlights resazurin as a blue, non-fluorescent dye that was originally used to detect bacterial and yeast contamination in milk and biological fluids. The dye's bioreduction decreases the quantity of the oxidized form (blue) while increasing the luminous intermediate (red).[10] Apart from its original use, it has been applied for the assessment of viability and bacterial contamination, as well as to test for antimicrobial activity.[9]

This preliminary study seeks to optimize and evaluate the effectiveness of the resazurin microplate assay in the rapid determination of antibacterial activity against MSSA and MRSA. Specifically, the study aimed to compare the effects of incubation time and dye concentration on the determination of the minimum inhibitory concentration (MIC) of the antibiotics against MRSA and MSSA.

MATERIALS AND METHODS

The MIC of the antibiotics against the MSSA and MRSA were determined in triplicate in 96-well microplates, using resazurin dye as the colorimetric indicator of cell viability. DMSO was used as a negative control, while cefoxitin, oxacillin, and vancomycin were used as drug controls.

Acquisition and Verification of Bacterial Isolates

Control isolates of MSSA 25923 and MRSA 43300 were obtained from CBCLINK Inc., while the clinical isolates of MRSA and MSSA were obtained from the Microbiology Section of the Department of Laboratories, Philippine General Hospital. The isolates were obtained without any patient information or any identifiers. They were already decoded prior to sample collection.

The clinical isolates and control strains of both MRSA and MSSA were subjected to various biochemical tests such as Gram stain, growth and fermentation in mannitol salt agar (MSA) and hemolytic pattern in blood agar plate (BAP), catalase and coagulase tests, and were evaluated with its antibacterial activity using the Kirby-Bauer disk diffusion method.

Preparation of Antibiotic Solutions

Using distilled water, cefoxitin and oxacillin were reconstituted to a 64 µg/mL working solution while vancomycin was reconstituted to a 128 µg/mL working solution.[11]

Preparation of Mueller-Hinton Broth

The Mueller-Hinton Broth (MHB) was prepared by dissolving 21 g of MHB powder in 1L of water used as a culture medium for MSSA. Another set of MHB was prepared and supplemented with 4% NaCl to be used as a culture medium for MRSA. This is the medium recommended by the Clinical and Laboratory Standards Institute (CLSI) for susceptibility testing of commonly isolated aerobic bacteria.[11] The supplemental NaCl in the medium prepared for MRSA increases the detection of oxacillin resistance in MRSA. The mixture is standardized to the turbidity similar to the 0.5-McFarland standard suspension, which was prepared by adding 0.005 L of 0.048 M BaCl2 to 0.0995 L of 0.18 M H_2SO_4 (1% [v/v]) with constant mixing.[11]

Preparation of Bacterial Suspension

Bacterial suspensions were prepared in the appropriate medium (MHB for MSSA; MHB with 4% NaCl for MRSA) and standardized to the turbidity similar to the 0.5 McFarland Standard prepared by adding 0.5 mL of 0.048 M BaCl2 to 99.5 mL of 0.18 M H_2SO_4 (1% [v/v]) with constant mixing, which was confirmed using UV-vis spectrophotometer, and had optical densities between 0.08 and 0.1 at 625 nm.[11]

Preparation of Dyes

Two concentrations of the resazurin dye were prepared: (1) 6.75 mg/mL solution recommended by the manufacturer of the dye and (2) 0.01 mg/mL solution following the study of Elshikh et al.[12] The dye was measured using an analytical balance to be able to precisely determine the measurements. These concentrations were compared to evaluate the reaction time of the bacteria and the dye.

Resazurin Microtiter Assay

Using 96-well plates, the resazurin microtiter assay method was performed following the study of Coban with modifications.[9]

Following our REMA plate map (Fig. 1), the upper border wells of the microplate were filled with sterile distilled water in order to account for evaporation as it undergoes incubation. Afterward, 50 µl of sterile MHB was transferred into each inner well of the microtiter plates from columns 1 to 12 of rows B to H for the MSSA isolates while MHB supplemented with 4% sodium chloride was used for the MRSA isolates.

Fifty microliters (50 µl) of control drugs were added to columns 1 and 2 — row B for oxacillin, while rows C and D for vancomycin, and rows E and F for cefoxitin. Meanwhile, for the negative control, 50 µl of DMSO was added to columns 1 and 2 of rows G and H. Serial two-fold dilutions of the drugs and negative controls were performed, starting from columns 2 to 11. Ten microliters (10 µL) of bacteria was added to columns 2 to 11, rows B to H. Columns 2 to 11 of rows G and H served as growth control for the bacterial strains, while column 12 contains MHB only. The plates were wrapped in an opaque paper and cling wrap to minimize light exposure.

After the two separate incubation times (5 hours and 24 hours), 5 µL of resazurin dye was added to columns 1 to 12 of rows B to G only of the microplates and was observed for color change. Row H contains both the DMSO and bacteria without the resazurin dye serving as a negative control as well. A change from blue to pink was indicative of bacterial growth due to the reduction of resazurin. The minimum inhibitory concentration (MIC), known as the lowest concentration of drug, was recorded as the last well that did not change from color blue to pink.

Different factors were evaluated to test the effects on the antibacterial activities of the MSSA and MRSA strains: incubation period and dye concentration. For the incubation period, the effect was evaluated by comparing the optical densities of the plate setup incubated at 5 hours and 24 hours. These were performed using the microplate reader Thermo Fisher Scientific Multiskan Go with its wavelength set at 625 nm. Meanwhile, for the qualitative evaluation of the effects of resazurin dye concentration on their corresponding reaction time with the bacteria, two different concentrations of dye, 6.75 mg/mL and 0.1 mg/mL, were added to the wells of separate plates after the incubation period.

Statistical Analysis

Statistical analysis was performed using Jamovi software and the following statistical treatments were utilized: Kruskal-Wallis non-parametric test and Dwass-Steel-Critchlow-Fligner pairwise comparison tests (for the incubation time), and Wilcoxon Rank-Sum non-parametric test (for the minimum inhibitory concentrations).

Ethical Considerations

An ethical clearance was secured from the UP Manila Research Ethics Board (UPMREB) prior to the conduct of the study. Another clearance from the Institutional Biosafety and Biosecurity Committee (IBBC) of UP Manila was obtained as the study involves resistant organisms such as MRSA, and must be performed in a Biosafety Level 2 laboratory.

RESULTS

The clinical isolates and control strains of MSSA and MRSA were subjected to various biochemical tests and the Kirby-Bauer disk diffusion method (Table 1).

The incubation time of the microplates subjected to REMA was compared based on the standard method

(24-hour incubation) and based on the 5-hour incubation period set by the protocol of Coban.[9] (Table 2).

The microplates were subjected to two different concentrations of dye: 6.75 mg/mL and 0.01 mg/mL. 6.75 mg/mL of resazurin dye, following the protocol of Coban, was qualitatively compared with 0.01 mg/mL of resazurin dye based on the reaction period that will happen between the bacteria and the dye.[9]

After evaluating the effects of the factors related to REMA, an optimized REMA was conducted to determine the antibacterial activity of the antibiotics oxacillin, vancomycin, and cefoxitin against the control and clinical strains of MRSA and MSSA. Table 3 illustrates the observed MIC of the antibiotics against the aforementioned strains.

With these results, a Wilcoxon Rank-Sum non-parametric test was performed to determine if there is a significant difference between the MICs of these antibiotics against the two strains. Results showed that there is a significant difference between the minimum inhibitory concentrations (MICs) of the different antibiotics (oxacillin, vancomycin, cefoxitin) against the control and isolate strains of MRSA and MSSA, depicting a W of -2.98 ($p < 0.05$).

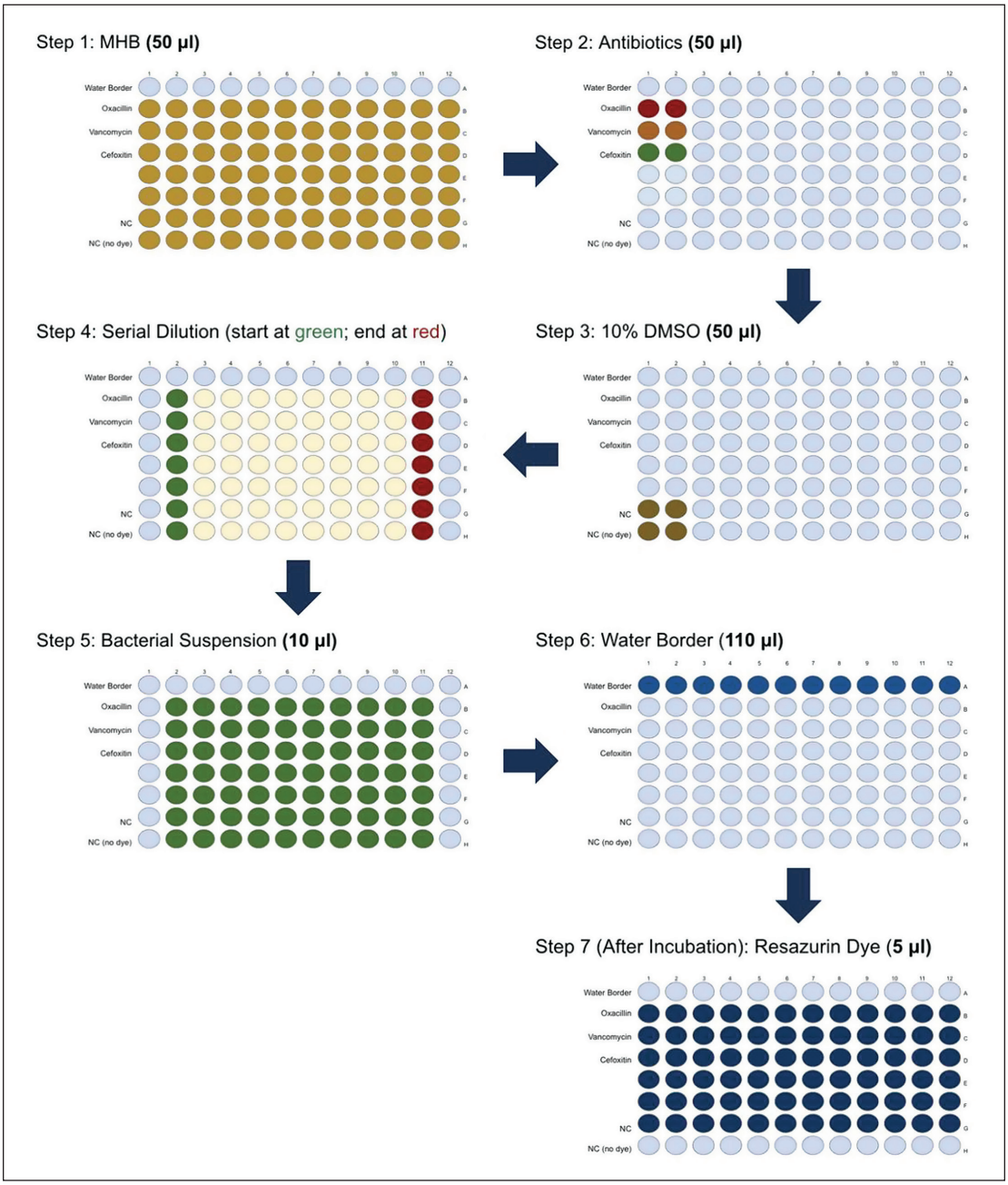

Figure 1. Resazurin microplate assay plate map.

Table 1. Biochemical and Antimicrobial Susceptibility Test Results for MSSA and MRSA

Bacterial Isolate	Gram Stain	Catalase	Coagulase	MSA	BAP	Kirby-Bauer (ZOI in mm)		
						CEF	OXA	VAN
MSSA-ATCC	Gram +	+	+	+	+	30	22	17
MRSA-ATCC	Gram +	+	+	+	+	15	no zone	18
MSSA-CLIN	Gram +	+	+	+	+	32	25	19
MRSA-CLIN	Gram +	+	+	+	+	15	no zone	19

CEF = cefoxitin; OXA = oxacilin; VAN = vancomycin

Table 2. Results of Dwass-Steel-Critchlow-Fligner pairwise comparison test among values of 24 HR and 5 HR incubation time

Bacterial Isolate	*p*-value	Interpretation
MRSA-ATCC	0.958	Not significant
MRSA-CLIN	0.791	Not significant
MSSA-ATCC	0.996	Not significant
MSSA-CLIN	0.774	Not significant

Table 3. Results of MIC Values for the Optimized Resazurin Microtiter Assay

Bacterial Isolate	Minimum Inhibitory Concentration (in µg/mL)		
	Oxacillin	Vancomycin	Cefoxitin
MRSA-ATCC	16	2	16
MSSA-ATCC	0.0625	0.5	0.5
MRSA-CLIN	8	1	32
MSSA-CLIN	2	2	4

DISCUSSION

Biochemical Tests

Results show that the bacteria strains exhibited the following biochemical test results for *Staphylococcus aureus*: Gram-positive, catalase-positive, coagulase-positive, hemolytic on blood agar plate, and yellow-forming colonies on mannitol salt agar. These results are at par with the study of Karmakar et al., wherein they were able to phenotypically identify *S. aureus* isolates in a hospital, which were Gram-positive, catalase-positive, coagulase-positive, and blood agar plate hemolytic.[13] They also highlighted that these tests, along with thermonuclease tests, are important phenotypic identifying markers for the said organism. Meanwhile, results of the MSA test were also similar to the findings of the study of Sharp and Searcy, where they elucidated that the colonies of *S. aureus* appear yellow (from red) on MSA plates.[14] MSA serves as a selective media for *S. aureus*, and is at par with the coagulase test; coagulase-negative *Staphylococci* will remain color red.

Meanwhile, for the Kirby-Bauer disk diffusion method, results show that for the clinical isolate of MSSA, the zone of inhibition against cefoxitin is at 32 mm, and 25 mm for oxacillin. This is at par with the results of the ATCC control strain, which is at 30 mm for cefoxitin and 22 mm for oxacillin. CLSI standards state that for both oxacillin and cefoxitin, isolates are considered susceptible if the zone of inhibition is at ≥22 mm, while at the zones of inhibition of ≤21 mm, CLSI classifies the isolate as resistant.[15] Given these results, it is noted that the control and clinical isolates of MSSA are susceptible to cefoxitin and oxacillin. Moreover, with the same classification, both the control and clinical isolates of MRSA were found to be resistant to the antibiotics.

For vancomycin, it was noted that resistant strains are regarded when the zone of inhibition is at ≤17 mm.[16] With this contention, it may be noted that all of the control and clinical isolates except for the control strain of MSSA is regarded as sensitive. However, current CLSI guidelines do not recommend the use of the Kirby-Bauer disk diffusion method in determining antimicrobial resistance of *S. aureus* against vancomycin and recommends the use of broth microdilution methods in the determination of antimicrobial resistance.[17] With this, given that vancomycin serves as a drug of choice for MRSA infections here in the Philippines, this highlights the importance to further assess the resistance with the minimum inhibitory concentration (MIC) as a measure.

Effect of the Incubation Time

The optical densities of MSSA-CLIN, MSSA-ATCC, MRSA-CLIN, MRSA-ATCC treated with oxacillin, vancomycin, and cefoxitin were subjected to Kruskal-Wallis non-parametric test to determine if there is a statistically significant difference between the means of the optical densities of the plates subjected to 24-hour and 5-hour incubation times. Table 2 reveals that there is no significant difference between the optical densities of the wells incubated for 24 hours and 5 hours ($p > 0.05$). This might be attributed to the antibacterial effects of the treatment and the time it takes for the microorganisms to resume growth.[18] With the results presented below, it must be noted that since there is no significant difference between the optical densities of the two incubation times, a 5-hour incubation period should be utilized instead of the 24-hour incubation period as it is efficient in terms of the time needed.

Effect of the Dye Concentration

Results showed that between the two different concentrations, 0.01 mg/mL concentration of resazurin dye required the least amount of time for incubation which deviates against the limitations of REMA such as exposure to light for an extended amount of time.[19]

Minimum Inhibitory Concentration of the Bacterial Isolates

Results show that for oxacillin, the strains had a recorded MIC of 16 μg/mL for MRSA ATCC, 8 μg/mL for the MRSA clinical isolate, 0.0625 μg/mL for the MSSA ATCC, and 2 μg/mL for the MSSA clinical isolate. With the results, it is noted that the MRSA isolates are classified as resistant to oxacillin, based on the latest CLSI guidelines which declared the MIC breakpoint for oxacillin resistance at ≥4 μg/mL. Furthermore, considering the current CLSI guidelines, the MSSA isolates are classified as susceptible to oxacillin, having a MIC breakpoint of ≤2 μg/mL.[17] These results were at par with the classifications based on the previously conducted Kirby-Bauer disk diffusion method (Table 1).

As for cefoxitin, results show that the clinical and control isolates of MRSA are classified as resistant to cefoxitin, with the CLSI dictating cefoxitin resistance with the MIC breakpoint of ≥8 μg/mL. In contrast, the MSSA isolates were classified as susceptible to cefoxitin as they meet the MIC breakpoint of ≤4 μg/mL.[17] These results were also at par with the classifications based on the previously conducted Kirby-Bauer disk diffusion method (Table 1).

Finally, for vancomycin, the optimized REMA presented the following MICs for the strains: 2 μg/mL for MRSA ATCC, 1 μg/mL for the MRSA clinical isolate, 0.5 μg/mL for the MSSA ATCC, and 2 μg/mL for the MSSA clinical isolate. Based on the CLSI guidelines, all of the strains are considered susceptible to vancomycin, with MIC breakpoint of ≤2 μg/mL.[17]

CONCLUSION

This preliminary report aimed to evaluate and optimize the conditions used in the resazurin microtiter assay to determine the anti-MRSA and anti-MSSA activities using different factors: incubation time and resazurin dye concentration. Based on the results presented, the researchers determined the optimal condition for the resazurin microtiter assay which was 0.01 g/mL concentration of Resazurin dye, at a 5-hour incubation period.

This preliminary study also highlighted the importance of REMA as a rapid and cost-effective assay that will be used in the immediate detection of multidrug-resistant *Staphylococcus aureus*, which will in turn provide an avenue to screen the important treatment protocol against MRSA infection, and assist in the discovery of alternative sources of treatment such as in the natural products of plants and marine alga.

The researchers recommend providing a regression analysis on the optical densities of bacteria over time to determine the rate of growth in 5 hours. Furthermore, future studies may also investigate the accuracy of the resazurin microtiter assay in determining methicillin resistance by comparing it with molecular tools detecting the mecA gene.

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Statement of Authorship

All authors certified fulfillment of ICMJE authorship criteria.

Author Disclosure

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