ORIGINAL ARTICLE

# *In-Vitro* Determination of Minimum Inhibitory Concentration (MIC) and Contact Time of Povidone-Iodine against *Staphylococcus aureus* and *Klebsiella aerogenes* Using Micro Suspension Test, Colorimetric Resazurin Microplate Assay, and Dey Engley Neutralizer Assay

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## ABSTRACT

**Background and Objective.** The human nasal passages host major human pathogens. Recent research suggests that the microbial communities inhabiting the epithelial surfaces of the nasal passages play a key factor in maintaining a healthy microenvironment by affecting both resistance to pathogens and immunological responses. Colonization of the nasal cavity by different pathogens such as *Staphylococcus aureus* and *Klebsiella aerogenes*, is associated with a higher postoperative infection morbidity. Povidone-iodine (PVP-I) as an antiseptic has been proven to display high antibacterial, antiviral, and antifungal properties even at low concentrations, and was shown to be effective in the control of infections to limit their impact and spread. It can be used as a topical antiseptic for skin decontamination and wound management, as a nasal spray, or as a gargle. There are different methods in testing the efficacy of potential antimicrobial suspensions. This study aimed to determine the concentration of PVP-I that is most effective in nasal decolonization using microsuspension test and colorimetric minimum inhibitory concentration (MIC) determination assays, resazurin microtiter assay (REMA), and Dey-Engley (D/E) neutralizer assay. The findings of this study will contribute to knowledge regarding the intended use of PVP-I in microbial control, particularly in bacterial infections.

**Methods.** Several dilutions (2.0%, 1.0%, 0.5%, 0.25%, 0.1% and 0.09%) of commercially bought 10% (10 mg per 100 ml) povidone-iodine were prepared and tested against a standardized inoculum ($1x10^5$) of *Staphylococcus aureus and Klebsiella aerogenes* at different contact times (5 seconds, 10 seconds, 30 seconds, 1 minute, and 5 minutes). Microdilution suspension test was performed to determine the log reduction per variable, while REMA and D/E neutralizer assay were used to determine the MIC. A value of greater than or equal to 5 log reduction was considered effective for microdilution suspension test. Estimates of agreement statistics were used to interpret the results of the assay in which the overall percent agreement (OPA), positive percent agreement (PPA), negative percent agreement (NPA), and Cohen's kappa statistics were calculated.

**Results.** Povidone-iodine concentration of 0.25% exhibited ≥5 log reduction against *K. aerogenes* at the minimum contact time of 5 seconds. On the other hand,



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a slightly higher PVP-I concentration was required to achieve ≥5 log reduction for *S. aureus* at 0.5% concentration and a minimum contact time of 1 minute. There was an observed concordance of the results of REMA and D/E neutralizer as MIC colorimetric indicators, which yielded an overall test percent agreement of 90.30% (95% CI: 84.73–94.36), and a strong level of agreement (K = 0.8, $p$<0.0001). A lower overall percent agreement for both REMA and D/E neutralizer versus the microsuspension test was observed at 79.17% (K = 0.57, $p$<0.0001) and 78.18% (K = 0.55, $p$<0.0001), respectively.

**Conclusion.** Low povidone-iodine concentrations (i.e., 0.5% against *S. aureus* and 0.25% against *K. aerogenes*) were observed to have bactericidal activity of at least 5 log reduction as rapid as the minimum contact time of 5 seconds. Furthermore, D/E and REMA, as colorimetric indicators, had comparable performance (OPA = 90.30%; K = 0.8, $p$<0.0001) suggesting that both REMA and D/E neutralizer assay may detect the same range of minimum inhibitory concentration for the organisms and disinfectant tested in this study.

*Keywords: Povidone-Iodine, microbial sensitivity tests, local anti-infective agents, Enterobacter aerogenes, Staphylococcus aureus*

## INTRODUCTION

The human body is composed of a diverse microbiota which is important in maintaining the host's overall health. These microorganisms, including bacteria, compete in order to survive. Commensal bacteria inhibit the colonization of invasive and potentially pathogenic bacteria, referred to as opportunistic bacteria, through complex processes. The disruption of microbiota, especially of beneficial commensals, may favor the proliferation of opportunistic bacteria and eventually cause disease.[1] These diseases include pneumonia, meningitis, systemic infections, and hospital-acquired infections; which makes opportunistic pathogens a major cause of morbidity and mortality.[2]

One of the opportunistic pathogens harbored in the nasal cavities is *Staphylococcus aureus,* a Gram-positive bacterium which frequently colonizes the nasal cavity. It has been estimated that around 30% of the human population is colonized asymptomatically by *S. aureus*, and the carriage of the organism increases the risk of infection.[3] *S. aureus* can cause infections ranging from milder skin and soft tissue infections to serious invasive infections, that includes osteomyelitis and septic arthritis, pneumonia, endocarditis, septicemia, and necrotizing fasciitis.[4,5] Infections may be opportunistic as community-acquired or hospital-acquired. The emergence of drug-resistant strains, including the Methicillin-Resistant *S. aureus* (MRSA), continues to make *S. aureus* a public health threat. The World Health Organization included *S. aureus* as a high priority bacteria in the Priority Pathogen List for Research and Development of New Antibiotics due to its resistance to various antimicrobials at a global level.[6]

Another common opportunistic pathogen causing respiratory infections is *Klebsiella aerogenes,* a Gram-negative bacterium that belongs to the family Enterobacteriaceae. It has been reclassified from the genus *Enterobacter* to the genus *Klebsiella* due to its genotypic characteristics.[7] *K. aerogenes* is an opportunistic pathogen, often acquired via nosocomial infections, that causes soft tissue infections, osteomyelitis, endocarditis, severe urinary tract infections, septicemia, and pneumonia.[8,9] *K. aerogenes* is intrinsically resistant to ampicillin and exhibits extended-spectrum beta-lactamases (ESBLs) resistance.[8] It is also included as a critical priority bacteria, under the family *Enterobacteriaceae,* in the Priority Pathogen List for Research and Development of New Antibiotics, and is part of ESKAPE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter spp.*), which are significant causes of nosocomial infection.[6,10]

With the emergence of multidrug resistant bacterial strains, especially in hospital settings, nosocomial infections have been getting harder to treat and control. Efforts towards the intervention for these infections have proved to become a burden in the healthcare system. These infections are usually associated with invasive procedures, which most commonly include surgery, central line-associated infections, in-dwelling catheterization, and ventilator-associated pneumonia. Prevention of hospital-acquired infections calls for prompt detection and isolation, proper hygiene, application of aseptic techniques when performing procedures, and environmental disinfection.[11]

In terms of skin and mucosal antiseptic, povidone-iodine (PVP-I), as an antiseptic, has been proven to have high antibacterial, antiviral, and antifungal properties even at low concentrations, and were shown to be effective in the control of infections to limit their impact and spread. It can be used as a topical antiseptic for skin decontamination and wound management, as a nasal spray, or gargle.[12] While resistance against decolonization agents such as mupirocin, alcohol-based sanitizers, and other antiseptics have been found in some bacteria, there has been no observed significant resistance for iodine.[12,13] Moreover, PVP-I is a broad spectrum antiseptic in which the free iodine is released directly into the bacterial cell membrane, resulting in cell death. Studies have demonstrated the powerful bactericidal activity of PVP-I through the increased release of free iodine at lower concentrations of around 0.08% to 0.1% than at a higher concentration (i.e., 10%).[14] Long term utilization of lower concentrations of PVP-I have also been found to exhibit lesser hormonal effects in patients.

Bactericidal activity of disinfectants and antiseptics are measured by standard suspension tests such as the EN13727:2012+A2:2015. These tests are utilized to

determine efficacy of various concentrations of disinfectants against microorganisms at incremental time periods by determining the minimum inhibitory concentration (MIC). A sterile neutralizer is added to inactivate the disinfectant, and the time increments are referred to as the contact time. Interfering substances are added to simulate contaminants non-sterile conditions.[15] For most bacterial disinfectant challenge assays, bacterial reduction of at least 5 log is considered effective.[16] However, conventional suspensions tests particularly macrodilution broth assays are more tedious methods of testing antiseptic efficacy requiring more resources such as media, reagents, and time to obtain accurate results. The microbroth dilution assay utilized in this study, follows the macrodilution protocol and dilutions, but utilized a smaller volume of reagents and the samples. Furthermore, MIC detection for dark-colored solutions such as povidone-iodine or plant extracts often present problems in terms of visual or spectrophotometric detection, wherein the color of the solution interferes with absorbance readings. The presence of bacterial growth is determined instead through spread plate technique.

To circumvent the problems of MIC detection with colored solutions such as povidone-iodine, colorimetric indicators such as resazurin and Dey/Engley (D/E) neutralizer were used in detecting bacterial growth. Colorimetric assays such as Resazurin Microplate Assay (REMA) can also be utilized as an indicator of bacterial growth. REMA has been used to determine MIC by the ability of bacteria to reduce resazurin to resorufin, indicated by a color change from blue to pink.[17] Optimization and standardization of the protocol on REMA is needed to address gaps in the differences in dye concentrations, solvents used, and incubation times.[18–20]

The D/E neutralizer solution is formulated as a neutralizing solution to most disinfectants. Tween 80 and lecithin are the components that carry out the neutralizing function. Commercial D/E neutralizers also have dextrose and other compounds that can support bacterial growth, as well as bromcresol purple added as an indicator dye. Positive bacterial growth from dextrose fermentation will produce acid byproducts that will then facilitate a color change of the indicator from purple to yellow.[21] Given these properties of resazurin and D/E neutralizer as a possible indicator for bacterial growth, the researchers explored the possibility of using them as an MIC indicator in contrast with the results of a conventional MIC with spread plate method.

This study aimed to determine the minimum bacterial concentration of povidone-iodine at different concentrations and contact times using Microsuspension Assay, and MIC using the colorimetric assays, REMA, and D/E Assay. The findings of this study will help to facilitate efficient, fast, and cost-effective antimicrobial testing of potential antiseptics and disinfectants to combat emerging and re-emerging infectious diseases and antimicrobial resistance.

## MATERIALS AND METHODS

### Preparation of Materials

Test organisms included Methicillin-susceptible *S. aureus* ATCC 25923 and *K. aerogenes* obtained from the bacterial repository of the Department of Medical Microbiology, College of Public Health, University of the Philippines Manila. Organisms were subcultured in a general growth medium such as Brain Heart Infusion Agar (Biomark, India). The interfering substance for the suspension test was prepared by mixing 3 g of Bovine Serum Albumin powder and 3 mL of washed red blood cells in 1 L distilled water.

For REMA, 100 mg of resazurin was mixed with 50 mL distilled water. Other culture media and reagents such as Mueller Hinton Broth (Biomark, India), Mueller Hinton Agar (Biomark, India), and Dey-Engley (D/E) Neutralizer Broth (BD Difco, USA) were prepared according to manufacturer's instructions.

### MIC Determination using Microsuspension Test, REMA, and D/E Assay

An overnight culture of the bacteria was prepared in Brain Heart Infusion Broth (BHIB) (Biomark, India). The 24-hour culture of the organism was standardized into an organism load of 0.5 McFarland approximately containing 1.5 x $10^8$ CFU/mL. Ten-fold serial dilutions were then made from $10^7$, $10^6$, and $10^5$ bacterial suspensions. Twenty microliters of the $10^5$ bacterial suspension were plated out to serve as the reference for the baseline count. The same prepared dilutions were used for the test.

PVP-I test concentrations (2.0%, 1.0%, 0.5%, 0.25% 0.1% and, 0.09%) were prepared from a commercially available 10% w/v (10 mg in 100 ml) PVP-I solution (10% Betadine solution, Lot# 23BWS-27, Interphil Laboratories, Inc.). Contact times tested were 5 seconds, 10 seconds, 30 seconds, 1 minute, and 5 minutes. Three tests were employed in the study namely: Microdilution Suspension Test, REMA, and D/E Neutralizer Assays as alternative MIC indicators.

For the quantitative microdilution suspension test, the study followed the EN 13727:2012+A2:2015 protocol of the bactericidal quantitative suspension test in a lower dilution scale.[15] Briefly, 200 uL of desired PVP-I concentration and interfering substance solution were dispensed in a microtiter well reacted with 100 uL of the organism adjusted to a bacterial density of approximately 1x$10^5$ CFU/mL. After the prescribed contact time, 50 uL of the bacteria-PVP-I mixture was reacted with 150 uL of D/E neutralizer. After 5 minutes incubation time, 20 uL of the neutralizer-bacteria-PVP-I mixture was plated onto Mueller Hinton Agar (Biomark). The test was done in triplicates. MHA plates and the microtiter plate containing neutralizer-bacteria-PVP-I mixture were then incubated at 37°C for 18 to 24 hours. After the incubation period, growth of colony forming units (CFU) per MHA plate were counted and encoded. MHA plates with no colony growth after 24 hours were

further incubated. The colony count after a total of 48 hours incubation was taken as the final reading and used in data encoding.

For the REMA and D/E Assay, colorimetric change was observed after the 24-hour incubation period. Color change of the D/E neutralizer from light blue to yellow green was noted, indicative of growth of the organisms. On a replicate well of the neutralizer and test solution, 10 uL of resazurin was added and incubated for 15 to 30 minutes until color change was visible to the naked eye. Wells exhibiting color change from blue to pink or colorless was noted. For both REMA and D/E Assay, the lowest concentration at which no color change occurred was taken as the MIC value.

### Log Reduction

For the Microsuspension Test, average CFU/ml for each contact time and PVP-I concentration was calculated and log reductions were computed based on the average of the growth of the triplicate colonies in 48 hours versus the baseline organism count based on the following formula[22]:

$$\text{Log Reduction} = \log_{10}\left(\frac{A}{B}\right)$$

Where:
A = number of viable microorganisms before treatment
B = number of viable microorganisms after treatment

**Figure 1.** Formula of log reduction.

A cutoff point of greater than or equal to 5 log reduction was considered effective in this study in accordance with the EN 1040:2005.[16]

Microsoft Excel for Windows, version 2403 (Microsoft Corp., Redmond, W.A., USA) and EpiTools Epidemiological Calculators, available at https://epitools.ausvet.com.au/ (AusVet Animal Health Services, Fremantle, WA Australia), were used in the encoding and analysis of the data. Average data from each parameter are tabulated and presented in graphs.

### Determination of Agreement between Microsuspension Test, D/E Assay and REMA

To determine the agreement of MIC detection by REMA and D/E Assay, a 2x2 contingency table was constructed wherein the diagnostic outcomes of the two tests were compared against the minimum bactericidal concentration (MBC) results from the Microsuspension Test. Estimates of agreement parameters such as overall percentage agreement (OPA), positive percentage agreement (PPA), and negative percentage agreement (NPA) were computed, and the Cohen's kappa statistic was used to determine the strength of agreement (Table 1).[23–25]

Lastly, the McNemar's Chi-square values were used to determine the significance between the frequency of disagreement between the two test results. Data were treated as pairs, with the null hypothesis being the absence of significant difference between the results of both tests. Hence, a p-value of less than 0.05 indicates a statistically significant disagreement between the two test results.[26,27]

**Table 1.** Interpretation of Cohen's Kappa (κ). Adapted from McHugh[24]

| Kappa value | Level of Agreement | % Reliability of Data |
|---|---|---|
| ***0 – .20*** | None | 0–4% |
| ***.21 – .39*** | Minimal | 4–15% |
| ***.40 – .59*** | Weak | 15–35% |
| ***.60 – .79*** | Moderate | 35–63% |
| ***.80 – .90*** | Strong | 64–81% |
| ***Above .90*** | Almost Perfect | 82–100% |

### Ethical Considerations

The research ethics approval was obtained from the University of the Philippines Manila Research Ethics Board (Code: UPMREB 2024-0268-EX). A permit to handle biohazardous organisms was obtained from the Institutional Biosafety and Biosecurity Committee of UP Manila (Approval Number: 2024-016).

## RESULTS

This study evaluated the MIC of a commercially available povidone-iodine solution diluted to lower concentrations against *S. aureus* and *K. aerogenes*. Resazurin and D/E neutralizer were also evaluated as alternative MIC indicators, and the results for each assay were compared against the results of a Microsuspension Test.

One of the variables measured in the study was the log reduction of the tested organisms against lower PVP-I concentrations from 2% to 0.09% and contact time from 5 seconds to 5 minutes. Aside from log reduction, inter-assay agreement statistics were determined to measure the concordance of results of REMA and D/E Assay against the standard Suspension Assay. The inter-assay agreement statistics measured by the 2x2 contingency table, Cohen's kappa coefficient, and McNemar's Chi-square test were derived from the statistics used for inter-assay variability.

### Log Reduction and Contact Time of Povidone-Iodine

Colony forming units recovered from each contact time and PVP-I concentration were computed for log reduction against baseline counts (Appendix A), which were visualized into a graph (Figures 2 and 3).

For *S. aureus* ATCC 25923, the MIC that consistently produced ≥5 log reduction at any contact time started at 1.0% povidone-iodine concentration. A lower concentration of 0.5% was effective in significantly reducing *S. aureus* colonies after a 1-minute contact time (Figure 2). For *K.*

*aerogenes,* PVP-I concentration as low as 0.25% was found to have satisfactory inhibitory effects across all the contact times tested (Figure 3). Overall, a contact time of at least 30 seconds of the effective concentration of PVP-I provided ≥5 log reduction against the test organisms.

## Determination of Diagnostic Agreement of D/E Assay and REMA as MIC Indicators

The potential of D/E neutralizer broth versus resazurin in detecting the MIC of PVP-I was also evaluated in the study (Figure 4). The colorimetric change of the indicators was noted visually, and the results were tabulated against the result of the Microsuspension Test. Categorical grading of "positive" for color change, and "negative" for the absence of color change were assigned for the colorimetric indicators; whereas for the Microsuspension Test, bacterial colony isolates recovery in the plates were considered positive, while the absence of bacterial growth was considered as negative.

Based on the tabulated results of categorical "positive" or "negative" results, the contingency tables for each pair of tests are constructed and the overall percent agreement, positive percent agreement, negative percent agreement, kappa values, and McNemar's Chi-square between the tests were also reported per organism (Tables 2 to 8).

The inter-assay agreement between REMA and D/E Assay was rated as high with an overall percent agreement of

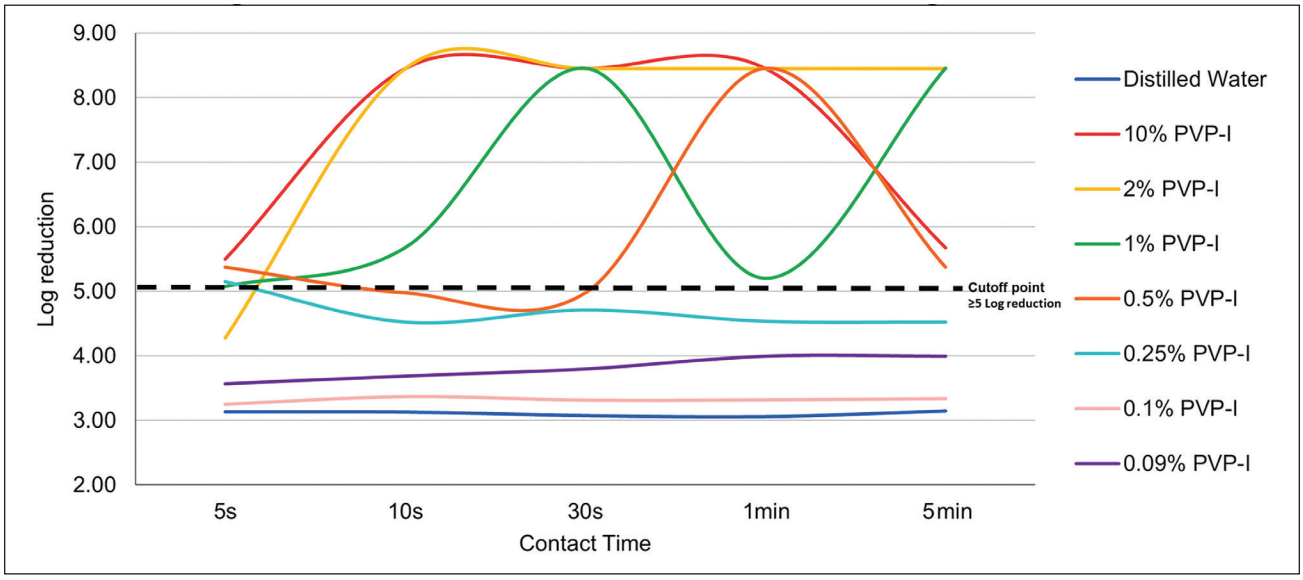


**Figure 2.** Log reduction versus contact time of PVP-Iodine concentrations against *Staphylococcus aureus*.

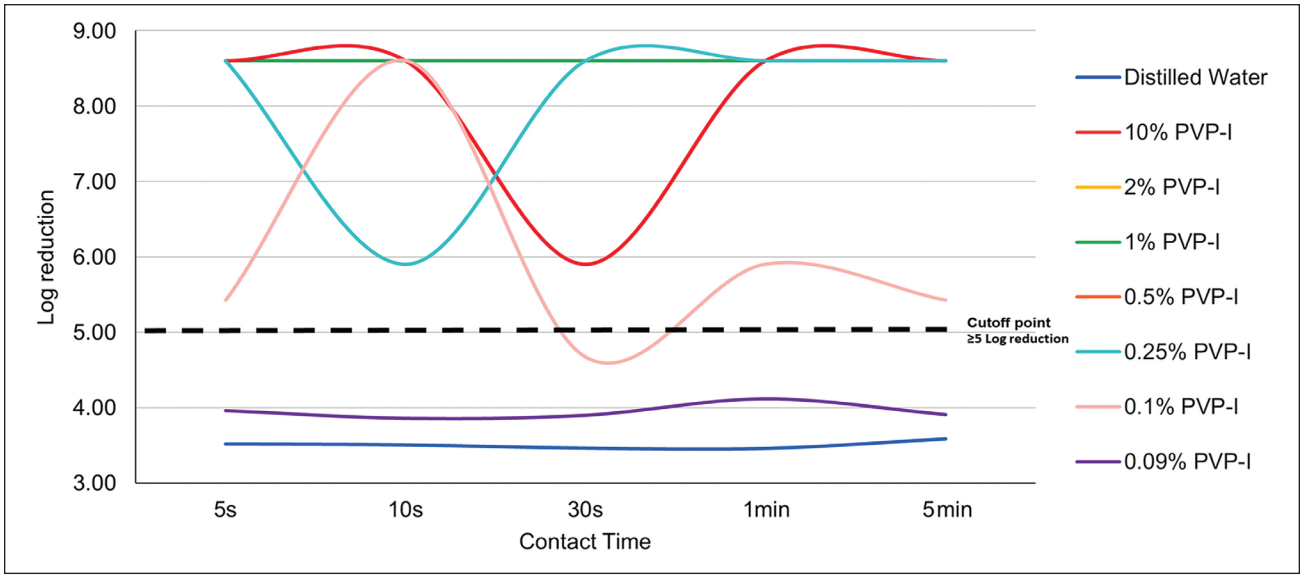


**Figure 3.** Log reduction versus contact time of PVP-iodine concentrations against *Klebsiella aerogenes*.

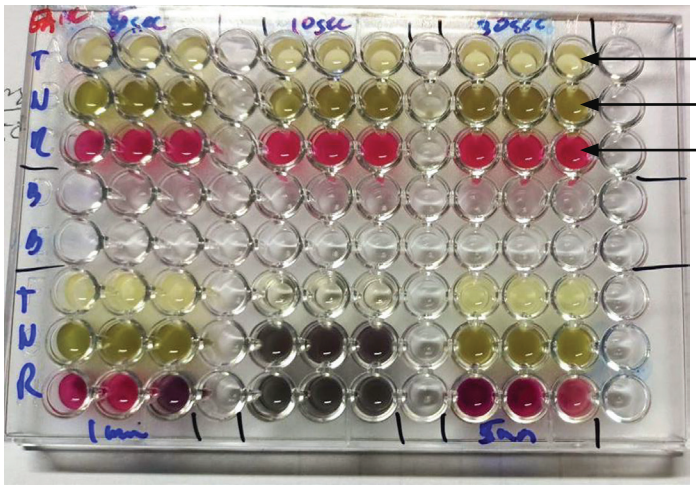


**Figure 4.** Representative 96-well plate of REMA and D/E Assay.

**Table 2.** 2x2 Contingency Table of Assay Agreement against *Klebsiella aerogenes*

| Test Conducted: Microsuspension | Neutralizer | | | REMA | | |
|---|---|---|---|---|---|---|
| | Positive | Negative | Total | Positive | Negative | Total |
| ***Positive*** | 29 | 1 | 30 | 28 | 11 | 39 |
| ***Negative*** | 24 | 66 | 90 | 14 | 67 | 81 |
| ***Total*** | 53 | 67 | 120 | 42 | 78 | 120 |

*Note: Microsuspension Test positive and negative samples were classified based on the presence and absence of bacterial CFU, respectively. REMA and D/E Assay positive and negative samples were classified based on the presence or absence of color change, respectively.*

**Table 3.** 2x2 Contingency Table of Assay Agreement against *Staphylococcus aureus* ATCC 25923

| Test Conducted: Microsuspension | Neutralizer | | | REMA | | |
|---|---|---|---|---|---|---|
| | Positive | Negative | Total | Positive | Negative | Total |
| ***Positive*** | 20 | 10 | 30 | 23 | 7 | 30 |
| ***Negative*** | 1 | 14 | 15 | 2 | 13 | 15 |
| ***Total*** | 21 | 24 | 45 | 25 | 20 | 45 |

*Note: Microsuspension Test positive and negative samples were classified based on the presence and absence of bacterial CFU, respectively. REMA and D/E Assay positive and negative samples were classified based on the presence or absence of color change, respectively.*

**Table 4.** Overall 2x2 Contingency Table of Assay Agreement against both *K. aerogenes* and *S. aureus* ATCC 25923

| Test Conducted: Microsuspension | Neutralizer | | | REMA | | |
|---|---|---|---|---|---|---|
| | Positive | Negative | Total | Positive | Negative | Total |
| ***Positive*** | 49 | 11 | 60 | 51 | 18 | 69 |
| ***Negative*** | 25 | 80 | 105 | 16 | 80 | 96 |
| ***Total*** | 74 | 91 | 165 | 67 | 98 | 165 |

*Note: Microsuspension Test positive and negative samples were classified based on the presence and absence of bacterial CFU, respectively. REMA and D/E Assay positive and negative samples were classified based on the presence or absence of color change, respectively.*

**Table 5.** 2x2 Contingency Table of Agreement between REMA and D/E Assay against *K. aerogenes*

| Test Conducted: REMA | Neutralizer | | |
|---|---|---|---|
| | Positive | Negative | Total |
| ***Positive*** | 48 | 3 | 51 |
| ***Negative*** | 5 | 64 | 69 |
| ***Total*** | 53 | 67 | 120 |

*Note: REMA and D/E Assay positive and negative samples were classified based on the presence or absence of color change, respectively.*

**Table 6.** 2x2 Contingency Table of Agreement between REMA and D/E Assay against *S. aureus*

| Test Conducted: REMA | Neutralizer | | |
|---|---|---|---|
| | Positive | Negative | Total |
| ***Positive*** | 16 | 4 | 20 |
| ***Negative*** | 4 | 21 | 25 |
| ***Total*** | 20 | 25 | 45 |

*Note: REMA and D/E Assay positive and negative samples were classified based on the presence or absence of color change, respectively.*

**Table 7.** 2x2 Contingency Table of Agreement between REMA and D/E Assay against both *K. aerogenes* and *S. aureus* ATCC 25923

| Test Conducted: REMA | Neutralizer | | |
|---|---|---|---|
| | Positive | Negative | Total |
| ***Positive*** | 64 | 7 | 71 |
| ***Negative*** | 9 | 85 | 94 |
| ***Total*** | 73 | 92 | 165 |

*Note: REMA and D/E Assay positive and negative samples were classified based on the presence or absence of color change, respectively.*

**Table 8.** Computed Percentage Agreement and Kappa Values for each Antimicrobial Assay

| Test Compared | Test Organism | Overall Percentage Agreement (95% CI) | Positive Percentage Agreement (95% CI) | Negative Percentage Agreement (95% CI) | Kappa (κ) Value (*p*-value) | McNemar's $\chi^2$ (*p*-value)* |
|---|---|---|---|---|---|---|
| ***Microsuspension and D/E Assay*** | *K. aerogenes* | 79.17 (70.80–86.04) | 54.72 (40.45–68.44) | 98.51 (91.96–99.96) | 0.5575 (<0.0001) | 19.3600 (<0.0001) |
| | *S. aureus* | 75.56 (60.46–87.12) | 95.24 (76.18–99.88) | 58.33 (36.64–77.89) | 0.5289 (0.0001) | 5.8182 (0.0159) |
| | *K. aerogenes* and *S. aureus* | 78.18 (71.10–84.23) | 66.22 (54.28–76.81) | 87.91 (78.32–93.07) | 0.5510 (<0.0001) | 4.6944 (0.0303) |
| ***Microsuspension and REMA*** | *K. aerogenes* | 79.17 (70.80–86.04) | 66.67 (50.45–80.43) | 85.90 (76.17–92.74) | 0.5345 (<0.0001) | 0.1600 (0.6892) |
| | *S. aureus* | 80.00 (65.40–90.42) | 92.00 (73.97–99.02) | 65.00 (40.78–84.61) | 0.5846 (<0.0001) | 1.7778 (0.1824) |
| | *K. aerogenes* and *S. aureus* | 79.17 (72.41–85.29) | 66.67 (64.14–85.69) | 85.90 (72.53–88.74) | 0.5748 (<0.0001) | 0.0294 (0.8638) |
| ***REMA and D/E Assay*** | *K. aerogenes* | 93.33 (87.29–97.08) | 94.12 (83.76–98.77) | 92.75 (83.89–97.61) | 0.8643 (<0.0001) | 0.1250 (0.7237) |
| | *S. aureus* | 82.22 (67.95–92.00) | 80.00 (56.34–94.27) | 84.00 (63.92–95.46) | 0.6400 (<0.0001) | 0.8222 (0.1250) |
| | *K. aerogenes* and *S. aureus* | 90.30 (84.73–94.36) | 90.14 (80.74–95.94) | 90.43 (82.60–95.53) | 0.8029 (<0.0001) | 0.0625 (0.8026) |

**Note: McNemar's Chi-square for paired data was applied to determine the significance of the disagreements between the results of the two assays. p<0.05 indicates a statistically significant disagreement between two test results.*

90.30% (95% CI: 84.73–94.36) and exhibited a strong level of agreement based on the kappa values (K= 0.8, p<0.0001). Additionally, the McNemar Chi-square test (0.0625, p=0.80) confirmed that the disagreements among the results were not significant. When compared against the bacterial recovery from Microsuspension Test, both REMA and D/E Assay exhibited a lower overall agreement of 79.17% (95% CI: 72.41–85.29), K= 0.57, and 78.18% (95% CI: 71.10–84.23), K= 0.55, respectively. The disagreement between the Microsuspension Test and REMA were not significant (0.0294, p=0.86). However, the disagreement between the Microsuspension Test and D/E Assay were found to be significant (4.6944, p=0.03). For both assays, detection of negative results had a higher agreement with the results of the Microsuspension Test.

## DISCUSSION

Iodine solutions or tinctures have long been established as antiseptics, with various preparations as topical ointment, ophthalmic solution, nasal spray, and gargle. Among other disinfectant compounds, povidone-iodine was also found to have low cytotoxicity, broad spectrum activity against organisms and at the moment, bacteria have not developed mechanisms of resistance against it, making it a suitable compound in combating bacterial infections.[13,28-30] In this study, the MIC of a commercially-prepared Povidone-Iodine was determined against a representative Gram-positive *(S. aureus)* and Gram-negative (*K.aerogenes)* which are organisms commonly harbored in the nasal cavity. Furthermore, alternative assays using resazurin and D/E Neutralizer as indicators for MIC were evaluated in this study. MIC determination for dark colored solutions such as povidone-iodine and crude plant extracts present a problem on the accurate visual detection of MIC and would often have to rely on spread plate methods to evaluate the growth or inhibition of organisms. This study explored the capacity of resazurin and D/E neutralizer in correctly indicating bacterial growth without the need for spread plate method by comparing the results against a standard microsuspension spread plate method.

### Povidone-Iodine as an Antiseptic

Due to the instability of free iodine, common iodine solutions or iodophors marketed as antiseptic often have carriers or solubilizing agents. The best-known and most widely used iodophor is povidone-iodine, a compound of polyvinylpyrrolidone with iodine (PVP-I). PVP-I formulation retains the germicidal activity of iodine but is non-staining and relatively free of toxicity and irritancy.[31] The mechanism of action of PVP-I relies on the release of free iodine in aqueous solution from the PVP-I complex that serves as an iodine reservoir. The free iodine molecules penetrate bacteria and oxidize vital bacterial structures, including amino and fatty acids, nucleic acids, and membrane components, eventually causing cell death.[32] Subsequently, this process entails the consumption of free iodine and causes the release of more free iodine from the PVP-I complex until equilibrium is achieved.[33] The sustained release of free iodine from the complex results to a prolonged antimicrobial effect, while providing better patient tolerability.[12]

## MIC and Contact Time of PVP-I

In this study, a commercial 10% Povidone-iodine (10mg iodine in 100 mL) was diluted at different concentrations to determine the concentration that is low enough to be safely administered to the sites of high viral shedding in the mouth and nose, and as a potential use as nasal and oral antiseptic. The researchers found that for *K. aerogenes,* a concentration of 0.25% PVP-I (0.25mg iodine per 100mL) was effective even as fast as 5 seconds contact time. On the other hand, a higher concentration of 0.5% PVP-I at 1 minute contact time was effective for *S. aureus*. Alternatively, 1% PVP-1 can be used as fast as 5 seconds based on the results of the study. These findings correspond to MIC ranges reported by other studies[34–36] with PVP-I concentration as low as 0.05% exhibiting activity as demonstrated by Grzybowski et al.[14], 0.25% povidone-iodine is also well within the concentration range that is non-toxic[35,36]. Low concentration PVP-I with more frequent administration is also more advisable than high concentration PVP-I especially for use in sensitive organs such as the eyes and nasal cavity.[14,35]

With regard to the observed higher concentration required for *S. aureus* as bactericidal, it has also been postulated that free iodine penetrates porins that are present in plasma membranes of Gram-positive bacteria, whereas in Gram-negative bacteria, iodine penetrates porins present in both outer membrane and plasma membrane[28], thus requiring lower concentration and an observed lower contact time needed.

## Stability of Low Concentration PVP-I

The low effective concentration of PVP-I needed to disinfect organisms bodes well in terms of lower potential as irritant and cytotoxic effects especially in sensitive organs such as eyes or nasal passages. However, the stability of low concentration PVP-I must also be noted. According to a study by Moniruzzaman et al.[37], as low as 0.5% and 0.6% PVP-I formulation is stable for a period of one year and sufficiently homogeneous at 25˚C and 60% relative humidity storage conditions. PVP-I lower concentrations have the tendency to degrade rapidly, and its concentration cannot be effectively maintained during storage, hence, the possibility of loss of activity over time at concentrations below 0.5%. The minimum shelf life before reduction or loss of activity may be explored in future studies. Interestingly, some studies suggest that lower PVP-I concentrations are more effective and act faster as bactericidal and virucidal than 10% PVP-I solutions.[38,39] This can be attributed to more free iodine readily generated from its molecular complex in diluted PVP-I solutions compared to concentrated solutions.

## D/E Neutralizer and Resazurin as MIC Indicators

An additional component of this study is exploring the potential of the D/E neutralizer as an MIC indicator in comparison with resazurin. The D/E neutralizing broth neutralizes a broad spectrum of antiseptics and disinfectants including iodine and has wide application in bactericidal and disinfectant challenge assays.[40,41] It also supports the growth of organisms characterized by turbidity and a color change from an opalescent purple solution to yellow or yellow green through the fermentation of dextrose. The color change is facilitated by the bromcresol purple indicator. Growth of non-dextrose fermenting bacteria such as *P. aeruginosa* can be detected by a pellicle formation in the broth.[21] These properties of the neutralizer solution make it a promising candidate as an alternative MIC indicator for MIC studies, thus results of the D/E Assay as MIC indicator is compared against REMA which is another colorimetric assay.

Resazurin or Alamar Blue is considered as a low cytotoxic dye and can be reduced by viable cells to resorufin. The direct correlation between the resazurin reduction and the viable cell quantity was commonly used to assess the active growth of bacteria and cells.[42] This is characterized by the reduction of the blue, non-fluorescent, and non-toxic resazurin, to pink, fluorescent resorufin. REMA allows the detection of microbial growth in extremely small volumes of solution in microtiter plates with or without the use of a spectrophotometer or fluorometer. In some cases such as high bacterial load, the solution might turn colorless if resazurin is completely reduced. In this study, 100 mg of resazurin was diluted with 50 mL of distilled water. Bacterial recovery after contact time with the PVP-I was incubated for 24 hours prior to addition of 10 uL resazurin. The change in color was observed 15 to 30 minutes after the addition of resazurin. One aspect noted by the researchers in the usage of resazurin is the lack of standardized or recommended concentration for use. Literature also suggests a wide variety of concentration, volume to be added as well as recommended incubation time.[43-45] The researchers found out through these experiments that prolonged incubation would result in high cell concentration and may lead to rapid depletion of the resazurin pool and loss of the accurate correlation between the resazurin reduction and the viable cell number. Thus, optimization of REMA in terms of resazurin concentration and incubation time is recommended before being used as a test system for MIC. Furthermore, in this study the color change was noted only visually by the naked eye for a maximum of 30 minutes after addition of the resazurin. The researchers also tried to measure the absorbance at a recommended dual wavelength of 570 nm and 600 nm to detect both peak absorbance for oxidized resazurin (blue color) and reduced resorufin (pink color) as used in literature.[46,47] However, no cutoff point to say positive or negative reading can be established due to the very close absorbance values observed. Picture of a trial assay and the spectrophotometry values are shown in Appendix B.

## Inter-assay Agreement

The comparability of REMA and D/E Assay as MIC indicators were assessed against the presence or absence of CFU in the MHA plates, specifically the MBC, after the

microsuspension test. Corresponding categorical counts were tabulated in a contingency table and the inter-assay agreement was computed along with the Cohen's kappa (K) statistic which measures degree of agreement between the assays. The McNemar Chi-square test was used to determine the significance of the disagreements between the test result of the assays. The findings of the study show that REMA and D/E Assay have comparable performance as MIC indicators (OPA=90.30%) and a kappa score of 0.80 ($p<0.0001$) that indicates a strong agreement between the two tests. This suggests that use of D/E neutralizer may be used as a substitute indicator in conducting MIC tests especially for disinfectant challenges, wherein after the neutralization step, the color change of the neutralizer broth can already directly act as indicator for MIC.

However, when compared with the microsuspension tests which assess bacterial recovery, the percent agreement and kappa statistics of both indicator tests were found to be low. This might be explained by the fact that the amount of bacteria needed to facilitate color change in the colorimetric indicators is high, while suspension tests allow for the detection of bacteria at even lower load.[48,49] This results in a false negative reading, i.e., negative color change but has low bacterial CFU allowing for the recovery colonies in suspension test. In fact, in a study by Sandberg et.al.[18], they found that a minimum bacterial load of $5x10^7$ CFU/mL of planktonic and biofilm *S. aureus* is needed for a significant fluorescence detection of resorufin from resazurin. Another study[48] constructed calibration curves of bacterial densities of *E. coli* and *E. faecalis* using REMA and found that the limit of quantification in terms of fluorescence is around $10^3$ bacterial density. Unfortunately, there were no studies found regarding the limit of quantification or the minimum bacterial load needed for the neutralizer broth to facilitate color change at a set number of hours of incubation. A kinetic assay on the bacterial load and incubation time might be explored in future studies to fully assess D/E neutralizer broth as an alternative MIC indicator.

## CONCLUSION

The determination of an effective concentration and contact time through the use of an antimicrobial assay is crucial in the assessment of the effectiveness of an antiseptic solution, such as PVP-I, to combat common respiratory pathogens causing morbidity and mortality among affected individuals. Through this study, bactericidal activity of PVP-I concentrations at 0.5% against *S. aureus,* and 0.25% against *K. aerogenes* were observed with log reduction values greater than 5. Furthermore, resazurin and D/E neutralizer as colorimetric indicators had comparable performance in terms of identifying the minimum inhibitory concentration tested against the results of the microsuspension test based on the computed inter-assay agreement and K statistic. However, detection of positive results in the microsuspension test (at least 1 CFU growth noted in the plates) is quite low between the MIC colorimetric tests with only 66.22% and 66.67% positive percentage agreement for D/E Assay and REMA, respectively. Thus, the limit of quantification or limit of detection tests must be established in future studies for the colorimetric determination of MIC using these indicators.

Further tests must be performed with the use of other disease-causing microorganisms and antimicrobial resistant strains to assess the efficacy of PVP-I as antiseptic and disinfectant. Moreover, further optimization of the antimicrobial assays used in this study should also be explored such as the use of spectrophotometric measurements for a quantitative assay for resazurin and D/E neutralizer as MIC indicators in the determination of antimicrobial efficacy.

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### Statement of Authorship

All authors certified fulfillment of ICMJE authorship criteria.

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# APPENDICES

**Appendix A.** Tables of Log Reduction Values of PVP-I against *S. aureus* and *E. aerogenes*

**Table A1.** Log Reduction of various PVP-I Concentrations against *Staphylococcus aureus* ATCC 25923

| PVP-I Dilution | Log Reduction of *S. aureus* per Contact Time | | | | |
|---|---|---|---|---|---|
| | 5 seconds | 10 seconds | 30 seconds | 1 minute | 5 minutes |
| ***10%*** | 5.50 | 8.45 | 8.45 | 8.45 | 5.67 |
| ***2%*** | 4.28 | 8.45 | 8.45 | 8.45 | 8.45 |
| ***1%*** | 5.07 | 5.67 | 8.45 | 5.20 | 8.45 |
| ***0.5%*** | 5.37 | 4.97 | 4.97 | 8.45 | 5.37 |
| ***0.25%*** | 5.15 | 4.52 | 4.71 | 4.54 | 4.52 |
| ***0.1%*** | 3.25 | 3.37 | 3.31 | 3.32 | 3.34 |
| ***0.09%*** | 3.57 | 3.68 | 3.79 | 3.99 | 3.99 |
| ***Distilled Water*** | 3.13 | 3.13 | 3.07 | 3.06 | 3.14 |

*Note: Distilled water and 10% PVP-I served as the negative and the positive controls, respectively.*

**Table A2.** Log Reduction of various PVP-I Concentrations against *Klebsiella aerogenes*

| PVP-I Dilution | Log Reduction of *K. aerogenes* per Contact Time | | | | |
|---|---|---|---|---|---|
| | 5 seconds | 10 seconds | 30 seconds | 1 minute | 5 minutes |
| ***10%*** | 8.60 | 8.60 | 8.60 | 8.60 | 8.60 |
| ***2%*** | 8.60 | 8.60 | 8.60 | 8.60 | 8.60 |
| ***1%*** | 8.60 | 8.60 | 8.60 | 8.60 | 8.60 |
| ***0.5%*** | 8.60 | 8.60 | 5.90 | 8.60 | 8.60 |
| ***0.25%*** | 8.60 | 5.90 | 8.60 | 8.60 | 8.60 |
| ***0.1%*** | 5.42 | 8.60 | 4.67 | 5.90 | 5.42 |
| ***0.09%*** | 8.60 | 8.60 | 4.90 | 4.99 | 5.42 |
| ***Distilled Water*** | 3.52 | 3.51 | 3.46 | 3.46 | 3.59 |

*Note: Distilled water and 10% PVP-I served as the negative and the positive controls, respectively.*

**Appendix B.** REMA Standard Curve Determination

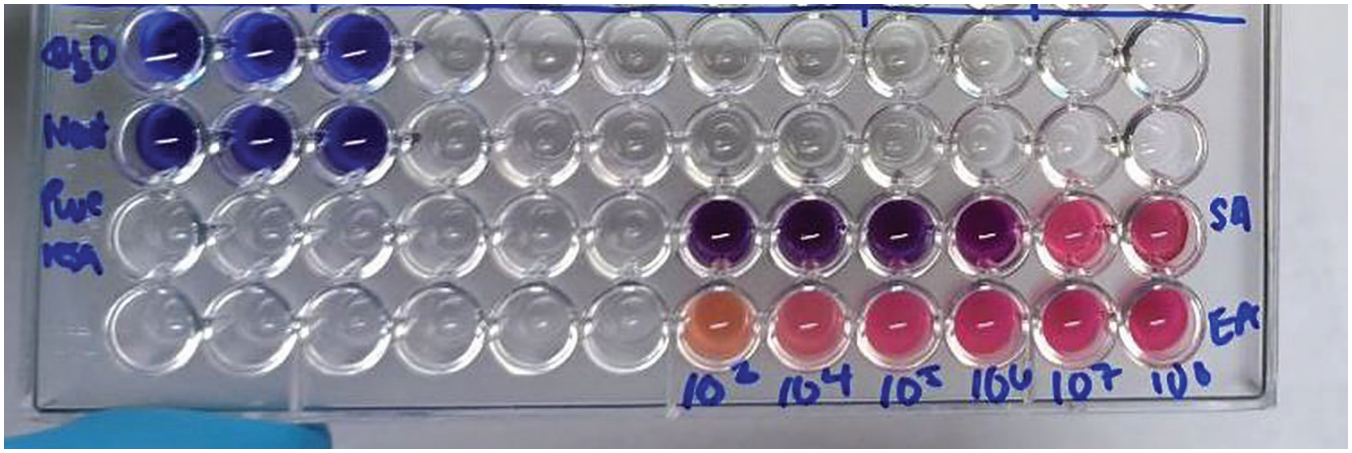


**Figure B1.** Plate layout and results of different bacterial density concentrations against REMA.

**Table B2.** Absorbance Values by Bacterial Density of *S. aureus* and *K. aerogenes*

| Plate layout for *S. aureus* | Bacterial load | *S. aureus* | | Plate layout for *K. aerogenes* | Bacterial load | *K. aerogenes* | |
|---|---|---|---|---|---|---|---|
| | | REMA Absorbance at 570 nm | REMA Absorbance at 600 nm | | | REMA Absorbance at 570 nm | REMA Absorbance at 600 nm |
| ***G7*** | $10^3$ | 0.056 | 0.055 | ***H7*** | $10^3$ | 0.063 | 0.063 |
| ***G8*** | $10^4$ | 0.064 | 0.063 | ***H8*** | $10^4$ | 0.060 | 0.059 |
| ***G9*** | $10^5$ | 1.312 | 1.279 | ***H9*** | $10^5$ | 1.529 | 1.011 |
| ***G10*** | $10^6$ | 1.221 | 1.186 | ***H10*** | $10^6$ | 1.201 | 0.978 |
| ***G11*** | $10^7$ | 1.478 | 1.445 | ***H11*** | $10^7$ | 1.194 | 1.059 |
| ***G12*** | $10^8$ | 0.093 | 0.091 | ***H12*** | $10^8$ | 0.068 | 0.067 |

*As observed from the data above, for S. aureus, at least $10^5$ bacterial density is needed to see a notable color change. Absorbance values for both 570 nm and 600 nm does not correspond to the intensity of color change as well. For K. aerogenes, although color development is quite noticeable even at a lower bacterial density of $10^3$ the same problem with S. aureus is noted in which the absorbance readings do not correspond to the intensity of color or bacterial load. Thus, the researchers deemed that further optimization is necessary to be able to utilize absorbance readings for Resazurin Microplate Assay.*