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Laboratory Certification
AIHA LAP #183867
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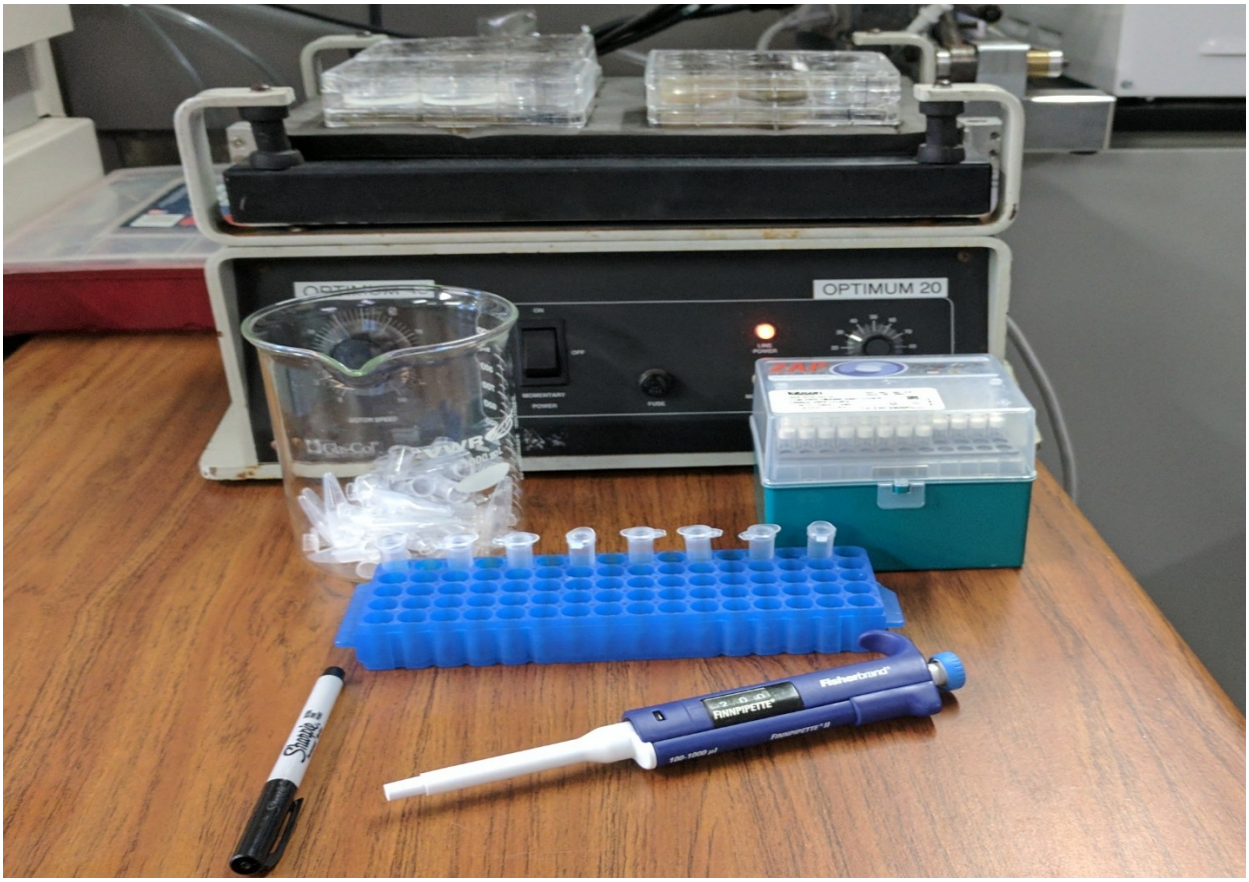
NY State Legionella Certified #12050
State of TN #03147

CAZ Solution Degradation of Methicillin-resistant *Staphylococcus aureus* (MRSA).



Product Testing Lab
May 29, 2017

Overview

- Assured Bio Labs, LLC was contracted by Clean Air Zone, Inc. (CAZ) to conduct time series analysis to determine the capacity of CAZ Solution to degrade viable Methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria.

- A stock solution of MRSA (strain: ATCC 700699) was prepared at a 7 Log concentration. The mixture was inoculated at a 1:100 dilution into five different solutions.

    - 80% CAZ Solution
    - 80% CAZ Solution Autoclaved (121°C @ 19psi for 30 minutes) to kill all microbes present in the CAZ solution.
    - Tap Water
    - 20% BioAiRx
    - 20% CAZ Solution Autoclaved (121°C @ 19psi for 30 minutes) to kill all microbes present in the CAZ solution.

- The inoculated CAZ Solution and water solutions were aerated during incubation by gently mixing on a Glass Col. multiwell plate mixer at a 65 rpm.

- Samples were collected for DNA analysis immediately following inoculation (T_0) and every 12 hours thereafter ($T_1 \ldots T_{10}$).

Key Findings

- DNA analysis allowed for the detection and quantification of the specific target (MRSA) when added to the complex microbial CAZ Solution mixture. DNA analysis uses high fidelity quantitative polymerase chain reaction (qPCR) technology that has superior detection range compared to standard microbial culture analysis.

- MRSA DNA was significantly reduced within 36 hours in 80% CAZ solution (see Figures 2 and 3). MRSA DNA stayed at its lowest observed concentration for the rest of the study.

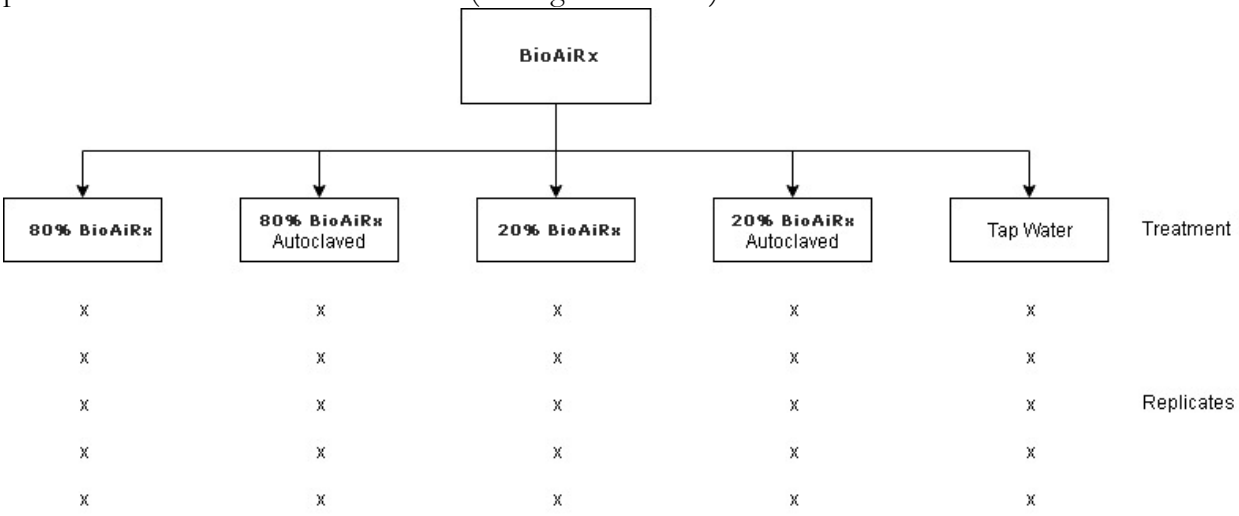
- Residual activity persisted in autoclaved solutions. The findings indicate that even when the living fraction of the CAZ solution is killed, the enzymatic fraction continues to function and is capable of temporary neutralizing the bacteria.

- The cell concentration growth was found in all but 80% CAZ solution within 72 hours and 96 hours for 20% CAZ Solution following inoculation. The observed behavior is either due to slight MRSA growth or random cell clumping during sampling.

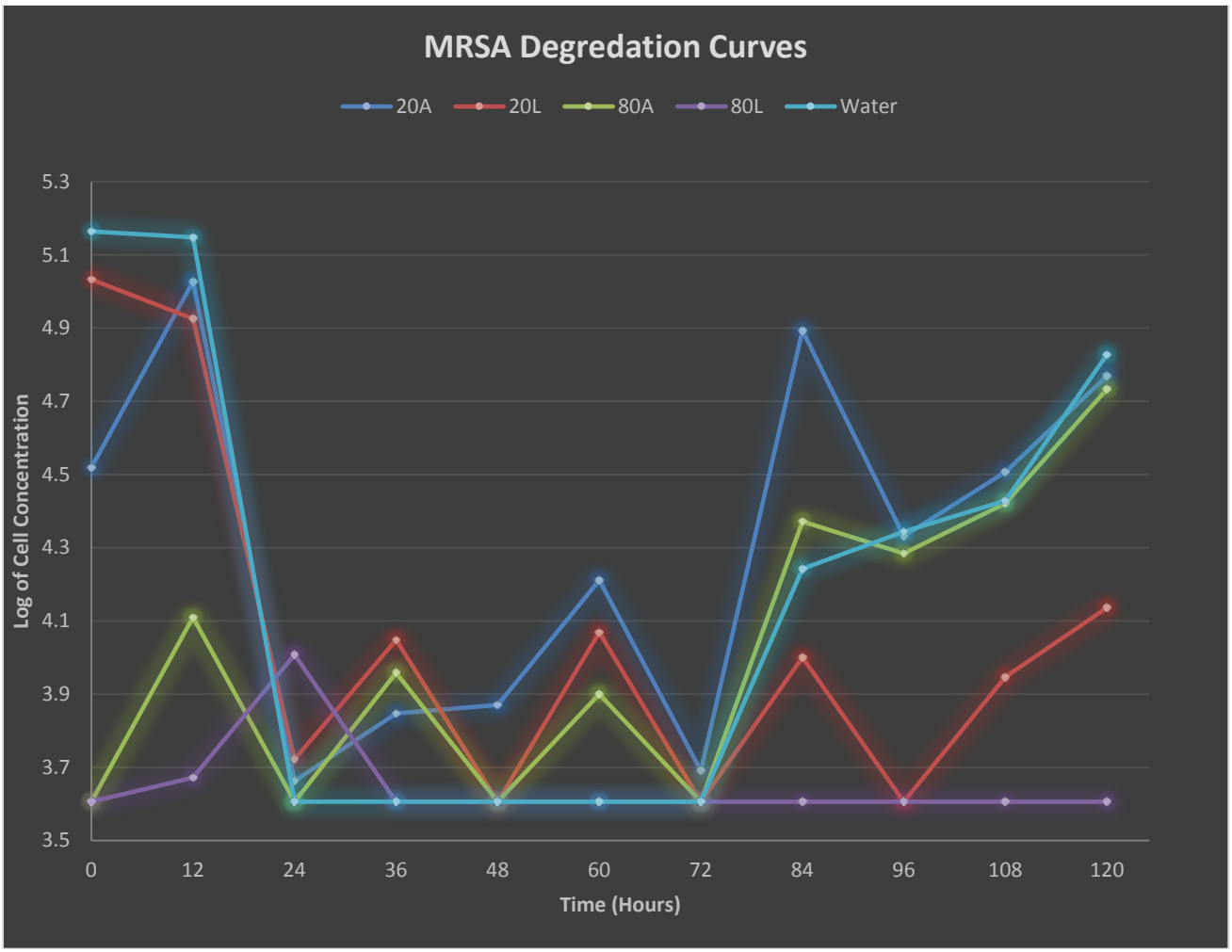
Experiment Methods

Stock Solution. A culture of Methicillin-resistant Staphylococcus aureus (MRSA) was obtained from the American Type Culture Collection (ATCC). The ATCC identifier is # 700699. The freeze dried culture was rehydrated and cultured onto Tryptic Soy Agar with 5% Sheep Blood, and incubated for 48 hours at 37°C. The culture was subsequently subcultured onto multiple plates of TSA with 5% Sheep Blood and incubated. A sterile cell scraper was used to remove the cells from the subcultured plates after 48 hours and to prepare a 10^7 Log concentration of cells suspended in sterile saline. That stock solution of MRSA was quantified using a hemocytometer.

Inoculations. Five solutions were prepared. Four solutions included 45 ml of 80% and 20% CAZ Solution dispensed into 4 separated sterile 100 ml screw-top bottles. Two of the bottles, one 80% CAZ Solution and the other 20% CAZ Solution were autoclaved for 30 minutes at 121°C and 19 psi to kill all viable CAZ Solution microbes. Two bottles contained either 45 ml of 80% viable CAZ Solution or 20% viable CAZ Solution. The fifth bottle contained 45 ml of tap water. The bottles were placed in a Bio-level 2 safety cabinet for inoculations. Exactly 0.5 ml of the 10^7 Log concentration of MRSA cells was dispensed into each of the five bottles, and the bottles were shaken for 30 seconds to evenly disperse the cells. Afterwards, 25, 1.5 ml snap top tubes were filled with 120 µl from the bottles. Five tubes per bottle. The tubes were immediately frozen and stored at -81°C. Then each bottle was dispensed in 6 ml aliquots into 5 of the 6 wells in a 6-well multiwell plate (Griener). The plates were placed on a mixing device at 65 rpm at room temperature and ambient light. The multiwell plates remained on the mixing device for 120 h. Every 12 hours, 25, 1.5 ml tubes were collected and frozen for the duration of the experiment. Hence, there were five replicates for each 12 hour time period for each of the 5 solutions (see Figure 1 below).

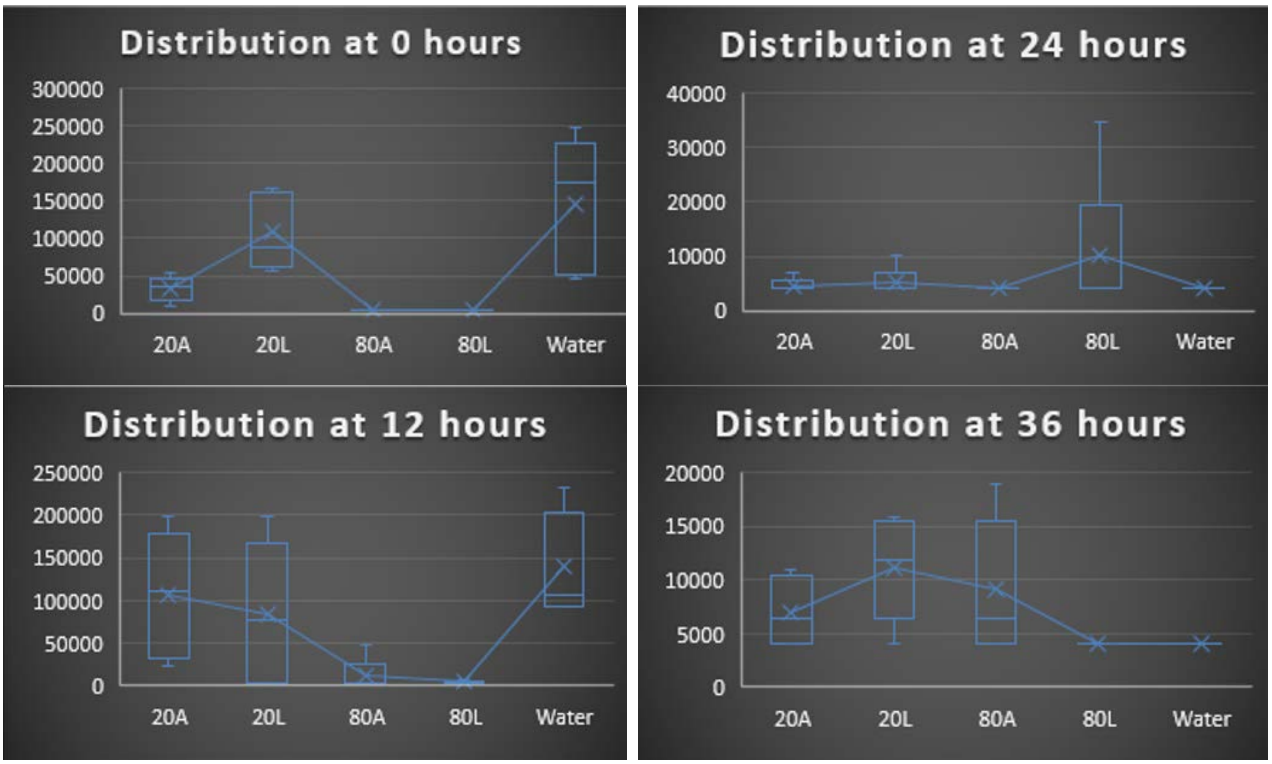


DNA analysis: Two hundred and fifty samples (n=250) were analyzed using qPCR specifically for MRSA. The qPCR assay provided both detection and quantification of MRSA cell units. Twenty-five samples were analyzed for each time sampling period from T0-T120 with a frequency of 12 hours between collections.



NOTE: Logarithm of cell concentration values (ranged from 4,037 to 145,956) was taken for better visual representation.

Figure 2. Rapid degradation of MRSA is observed in 80% CAZ Solution (purple line) within 36 hours following inoculation, some bacteria persisted up to 24 hours. The MRSA concentration fluctuated in all other solutions. Sadden continuous increase in cell concentration in 20% CAZ Solution Autoclave (dark blue line), 80% CAZ Solution Autoclave (green line), and Tap Water (light blue line) occurred after 72 hours since inoculation. The cell concentration increase is also observed in 20% CAZ Solution after 96 hours following inoculation. The observed behavior is either due to MRSA growth or cell clumping during sampling.



NOTE: Four time periods are displayed for demonstration purposes.

Figure 3. Distribution of cell concentrations in different solutions within 0, 12, 24, and 36 hours following inoculation. The 80% CAZ Solution outperformed all other solutions while 20% CAZ Solution Autoclave displayed poorest performance over the span of the study.

Repeated Measures Analysis of Variance

Overview

When several measurements are taken on the same experimental unit the measurements tend to be correlated with each other. When the measurements can be thought of as responses to levels of an experimental factor of interest, such as time, treatment, the correlation can be taken into account by performing a repeated measures analysis of variance.

The Effects of interest

- between-subject effects (GROUP)
- within-subject effects (TIME)
- interactions between the two types of effects (GROUP*TIME)

Data Description

Variable	Description
Group	Five levels of the Group are: 20A, 20L, 80A, 80L, and Water.
Time0* Time1-Time10**	*The immediate samples drawn at Time0. ** The samples drawn at Time1 through Time10. There are eleven levels of Time.
LogTime0-LogTime10	Logarithms are applied to time measurements to minimize correlation between the mean and the variance of the data.

Results

MANOVA Test Criteria and Exact F Statistics for the Hypothesis of no Time Effect H = Type III SSCP Matrix for Time E = Error SSCP Matrix S=1 M=4 N=4.5					
Statistics	**Value**	**F Value**	**Num DF**	**Den DF**	**Pr>F**
Wilks' Lambda	0.01302	83.37	10	11	<.0001
Pillai's Trace	0.98698	83.37	10	11	<.0001
Hotelling-Lawley Trace	75.7935	83.37	10	11	<.0001
Roy's Greatest Root	75.7935	83.37	10	11	<.0001

NOTE: MANOVA stands for Multivariate analysis of variance.

p-values <.0001 consistent across four statistical methods indicate that we can reject Null Hypothesis of No Time effect. We have strong evidence to suggest that Time has significant effect on observed cell concentration measurements.

MANOVA Test Criteria and F Approximations for the Hypothesis of no Time*Group Effect H = Type III SSCP Matrix for Time*Group E = Error SSCP Matrix S=4 M=2.5 N=4.5					
Statistics	**Value**	**F Value**	**Num DF**	**Den DF**	**Pr>F**
Wilks' Lambda	0.00042826	7.33	40	43.566	<.0001
Pillai's Trace	3.12263337	4.98	40	56	<.0001
Hotelling-Lawley Trace	40.56604699	10.11	40	20.61	<.0001
Roy's Greatest Root	27.74951114	38.85	10	14	<.0001
NOTE: F Statistic for Roy's Greatest Root is an upper bound.					

p-values <.0001 consistent across four statistical methods indicate that we can reject Null Hypothesis of No Time-Group interaction effect. We have strong evidence to suggest that the Time-Group interaction has significant effect on observed cell concentration measurements.

Tests of Hypotheses for Between Subjects Effects					
Statistics	**DF**	**Type III SS**	**Mean Square**	**F Value**	**Pr>F**
Group	4	57.53235791	14.38308948	82.67	<.0001
Error	20	3.47976667	0.17398833		

p-value <.0001 indicates that we can reject Null Hypothesis of No Between subject effect (effect of different solutions). We have strong evidence to suggest that the solution type has significant effect on observed cell concentration measurements.

Univariate Tests of Hypotheses for Within Subject Effects							
Statistics	DF	Type III SS	Mean Square	F Value	Pr>F	Adj Pr>F	
						G - G	H-F-L
Time	10	120.5513058	12.0551306	26.25	<.0001	<.0001	<.0001
Time*Group	40	121.6212850	3.0405321	6.62	<.0001	<.0001	<.0001
Error(Time)	200	91.8384544	0.4591923				

Greenhouse-Geisser Epsilon (G-G)	0.4824
Huynh-Feldt-Lecoutre Epsilon (H-F-L)	0.6544

p-value <.0001 consistent with MANOVA results indicate that we have strong evidence to suggest that both Time and Time-Group interaction has significant effect on observed cell concentration measurements.

The Greenhouse-Geisser and Huynh-Feldt epsilon adjustments alter the p level by the extent to which the assumptions of the repeated measures analysis of variance are violated. The results indicate that the assumption of sphericity (the condition where the variances of the differences between all combinations of related groups (levels) are equal) was met.