KILL-TIME PROTOCOL
Antimicrobial Activity of Five Silver-based Solutions
Using Methicillin-resistant *Staphylococcus aureus* (MRSA)
Test solutions: A, B, C, D, E

Submitted May 16, 2014

May 23, 2014

BY:

Richard A. Robison, Ph.D.
Professor and Director
Biosafety Level-3 laboratory
Department of Microbiology and Molecular Biology
Brigham Young University
Provo, UT 84602

I. PURPOSE.

The purpose of this study was to determine the relative antimicrobial activity of five silver-based formulations on methicillin-resistant *Staphylococcus aureus* (MRSA). This will be accomplished by performing standard kill-time suspension tests, using a 2 minute contact time.

II. MATERIALS AND METHODS.

A. Test organism.

The test suspension was prepared by growing a 5 ml culture of methicillin-resistant *Staphylococcus aureus* (MRSA), ATCC 43300, in Nutrient Broth at 37 °C for 24 hr. The 5 ml culture was pelleted by centrifugation, washed with five ml sterile 18 MΩ purified water, centrifuged again, and re-suspended in a final volume of one ml sterile purified water.

B. Neutralizers.

The neutralizer solution consisted of 9-ml tubes of the following: 12.7% Tween 80, 6.0% Tamol SN, 1.7% lecithin, 1% Peptone, 1.0% Cysteine, and 500 mM Tris (pH 7.0).

C. Kill-Time Test Procedure.

1. 9.9 ml of each solution was added to separate 50 ml polypropylene sterile centrifuge tubes.
2. Tubes of solution were equilibrated in a 20 °C water bath. Then, 0.1 ml of the MRSA test suspension was added to each at time zero.
3. After the specified contact time (2 min), 1 ml of this solution/organism mixture was added to 9 ml of neutralizer solution. The tube was mixed thoroughly.
4. After two min, the neutralized suspension was serially diluted in 9 ml blanks of physiological saline solution (PSS).
5. The number of viable organisms in selected dilution tubes was assayed by membrane filtration. One ml aliquots were plated in triplicate. The membranes were washed with about 100 ml of sterile PSS and removed to Columbia Agar plates. The plates were incubated at 37 °C for 24 and 48 hours.
6. The number of colonies on each filter was counted, and log reduction and percent kill values were computed.

D. Controls.

1. A titer of the MRSA test suspension was computed by performing membrane filtration assays on selected 1:10 dilutions in PSS of the test suspension. The test suspension titer was 3.53×10^9 CFU/ml.
2. A neutralizer control for each solution was performed by inoculating a mixture of 9.0 ml of neutralizer and 1 ml of solution with 0.1 ml of the $1{:}1 \times 10^5$ dilution of the titer. This produced about 350 CFU / ml in the tube, which was allowed to stand for 20 minutes prior to dilution and assay by membrane filtration using triplicate 1 ml samples. These results were compared to the expected counts to insure neutralizer efficacy.
3. Sterility controls were performed on all solutions and media used in this assay.

III. RESULTS.

***S. aureus* (MRSA) suspension:**

Titer.

Dilution:	$1:1x10^7$	$1:1x10^8$
Number of colonies:	242	29
	257	34
	225	43

Solution A:

(Received 05/16/14)

Exposure	Dilution of spore/Solution suspension:			
Time	$1:1x10^1$	$1:1x10^2$	$1:1x10^3$	$1:1x10^4$
2 min	TNC	~520	63	6
	TNC	~420	50	6
	TNC	~520	49	3

Neutralization Control		**Expected Counts**:		**Percent of Expected:**
Undiluted	1:10	Undiluted	1:10	78.2
207	25	350	35	
215	27			
217	22			

Solution B:

(Received 05/16/14)

Exposure	Dilution of spore/Solution suspension:			
Time	$1:1x10^1$	$1:1x10^2$	$1:1x10^3$	$1:1x10^4$
2 min	Lawn	Lawn	TNC	~1240
	Lawn	Lawn	TNC	~1720
	Lawn	Lawn	TNC	~1740

Neutralization Control		**Expected Counts**:		**Percent of Expected:**
Undiluted	1:10	Undiluted	1:10	85.4
239	28	350	35	
246	22			
238	28			

Solution C:
(Received 05/16/14)

Exposure Time	Dilution of spore/Solution suspension: 1:1x10^1	1:1x10^2	1:1x10^3	1:1x10^4
2 min	2	0	0	0
	0	0	0	0
	2	1	0	0

Neutralization Control		**Expected Counts**:		**Percent of Expected:**
Undiluted	1:10	Undiluted	1:10	84.4
227	22	350	35	
260	26			
249	27			

Solution D:
(Received 05/16/14)

Exposure Time	Dilution of spore/Solution suspension: 1:1x10^1	1:1x10^2	1:1x10^3	1:1x10^4
2 min	Lawn	Lawn	TNC	~1180
	Lawn	Lawn	TNC	~1260
	Lawn	Lawn	TNC	~1280

Neutralization Control		**Expected Counts**:		**Percent of Expected:**
Undiluted	1:10	Undiluted	1:10	92.2
225	30	350	35	
221	34			
238	30			

Solution E:
(Received 05/16/14)

Exposure Time	Dilution of spore/Solution suspension: 1:1x10^1	1:1x10^2	1:1x10^3	1:1x10^4
2 min	Lawn	Lawn	TNC	~1880
	Lawn	Lawn	TNC	~1680
	Lawn	Lawn	TNC	~1820

Neutralization Control		**Expected Counts**:		**Percent of Expected:**
Undiluted	1:10	Undiluted	1:10	91.5
266	26	350	35	
235	35			
223	28			

Sterility Controls:

Material	Counts
PSS-1	0, 0, 0
PSS-2	0, 0, 0
PSS-3	0, 0, 0
PSS-4	0, 0, 0
Neutralizer	0, 0, 0
Solution A	0, 0, 0
Solution B	0, 0, 0
Solution C	0, 0, 0
Solution D	0, 0, 0
Solution E	0, 0, 0
Media	0, 0, 0

IV. DISCUSSION.

Results of the titer showed a viable *S. aureus* (MRSA) concentration of 2.97×10^9 organisms per ml in the original suspension. Inoculation of 9.9 ml of a solution with 0.1 ml of this suspension produced an initial concentration of 2.97×10^7 CFU per ml in the assay tube.

Results from these procedures allowed log reduction (LR) and percent kill (PK) values to be calculated using the formulas: 1) $LR = -Log(S/S_0)$; where S = concentration of viable organisms after the specified contact time; and S_0 = the initial concentration of viable organisms at time zero. 2) $PK = (1 - (S/S_0)) \times 100$. These values are shown below.

Test Solution	Contact Time	Log Reduction (LR)	Percent Kill (PK)
Solution A	2 min	2.74	99.82%
Solution B	2 min	~0.28	~47.1%
Solution C	2 min	6.35	99.999955%
Solution D	2 min	~0.38	~58.3%
Solution E	2 min	~0.22	~39.7%

Neutralization control data revealed that the neutralizer was able to adequately neutralize the test solutions. Observed counts were 78.2-92.2% of those expected.

A wide disparity in antimicrobial activity between the five test solutions was observed. Solution C had the highest antimicrobial activity, producing a 6.35 log reduction of MRSA in 2 minutes. Solution A had the next highest activity, producing a 2.74 log reduction in 2 minutes. It should be kept in mind that these values are log reductions, and thus, the antimicrobial activity of Solution C was roughly 4,000 times greater than that of Solution A. Solutions B, D, and E all displayed little or no antimicrobial activity against MRSA in 2 minutes. Counts were so high, that the number of CFU had to be estimated on the 1:10,000 dilution of the reaction mixture. Thus, the log reduction and percent kill values are also estimates. That said, all log reduction estimates for Solutions B, D, and E were less than 0.4, indicating relatively low antimicrobial activity against MRSA in 2 minutes.

Test Dates: May 16 – 22, 2014

Performed By:

Woan Lowe
Research Associate

Supervised by:

Richard A. Robison, Ph.D.
Professor
851 WIDB
Brigham Young University
Provo, Utah 84602