Test Protocol for Verifying Antimicrobial Effectiveness on Printed Surfaces

Results from Execution of Protocol P-2014-002-ACE

10/22/2014 to 10/29/2014

Protocol: A detailed protocol is provided in Appendix A.

Results: A picture of the experimental setup is shown in Figure 1. Prior to starting the experiments, all plates were checked for proper current. Of the plates tested, 24 resulted in a current of 80µA without the addition of liquid to the surface. These plates were not used for the study. Only plates that resulted in a current of 0µA without liquid applied to the surface were used for the study. The bacterial counts resulting from the experiment are listed in the tables below. The first four tables contain the results from four independent runs with *Escherichia coli* ATCC 8739 and the last four tables contain the results from four independent runs with methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC BAA-44.

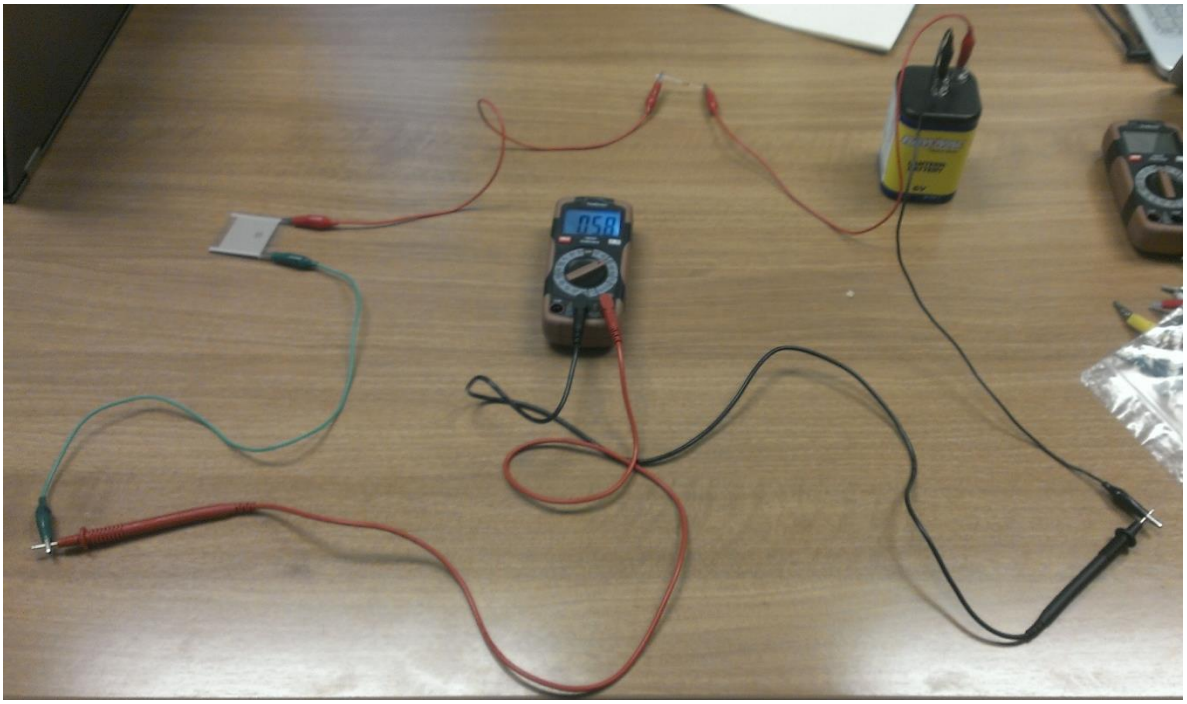

Figure 1. Experimental set-up for protocol P-2014-002-ACE.

Plate geometry 1 Ink PE826 against *E. coli* ATCC 8739: Run #1

Test sample	Time (min)	*E. coli* ATCC 8739 (CFU/mL)	% Bacterial reduction	Charge (µA)
Positive control growth (no system setup)	5	1.0×10^6	N/A	N/A
System 1	5	*0	100	60
System 2	5	*0	100	60
System 3	5	*0	100	58
System 4	5	*0	100	62
Positive control growth (no system setup)	10	1.0×10^6	N/A	N/A
System 1	10	*0	100	60
System 2	10	*0	100	60
System 3	10	*0	100	58
System 4	10	*0	100	62
Positive control growth (no system setup)	20	1.0×10^6	N/A	N/A
System 1	20	*0	100	60
System 2	20	*0	100	60
System 3	20	*0	100	58
System 4	20	*0	100	62

*limit of detection is less than ten cells in the initial 50µL sample
"positive growth control (no system setup)" is collected from a bacterial sample that is untreated (spotted on a sterile petri dish); % bacterial reduction is calculated as the % in bacterial reduction compared to the positive growth control

Plate geometry 1 Ink PE826 against *E. coli* ATCC 8739: Run #2

Test sample	Time (min)	*E. coli* ATCC 8739 (CFU/mL)	% Bacterial reduction	Charge (µA)
Positive control growth (no system setup)	5	6.0×10^5	N/A	N/A
System 1	5	*0	100	60
System 2	5	*0	100	61
System 3	5	*0	100	56
System 4	5	*0	100	61
Positive control growth (no system setup)	10	2.4×10^4	N/A	N/A
System 1	10	*0	100	59
System 2	10	*0	100	67
System 3	10	*0	100	57
System 4	10	*0	100	61
Positive control growth (no system setup)	20	8.0×10^5	N/A	N/A
System 1	20	*0	100	61
System 2	20	*0	100	68
System 3	20	*0	100	56
System 4	20	*0	100	61

*limit of detection is less than ten cells in the initial 50µL sample
"positive growth control (no system setup)" is collected from a bacterial sample that is untreated (spotted on a sterile petri dish); % bacterial reduction is calculated as the % in bacterial reduction compared to the positive growth control

Plate geometry 1 Ink PE826 against *E. coli* ATCC 8739: Run #3

Test sample	Time (min)	*E. coli* ATCC 8739 (CFU/mL)	% Bacterial reduction	Charge (µA)
Positive control growth (no system setup)	5	6.0×10^6	N/A	N/A
System 1	5	*0	100	60
System 2	5	*0	100	70
System 3	5	*0	100	57
System 4	5	*0	100	60
Positive control growth (no system setup)	10	1.6×10^6	N/A	N/A
System 1	10	*0	100	60
System 2	10	*0	100	68
System 3	10	*0	100	58
System 4	10	*0	100	60
Positive control growth (no system setup)	20	1.6×10^6	N/A	N/A
System 1	20	*0	100	59
System 2	20	*0	100	69
System 3	20	*0	100	57
System 4	20	*0	100	60

*limit of detection is less than ten cells in the initial 50µL sample
"positive growth control (no system setup)" is collected from a bacterial sample that is untreated (spotted on a sterile petri dish); % bacterial reduction is calculated as the % in bacterial reduction compared to the positive growth control

Plate geometry 1 Ink PE826 against *E. coli* ATCC 8739: Run #4

Test sample	Time (min)	*E. coli* ATCC 8739 (CFU/mL)	% Bacterial reduction	Charge (µA)
Positive control growth (no system setup)	5	6.0×10^5	N/A	N/A
System 1	5	*0	100	60
System 2	5	*0	100	71
System 3	5	*0	100	57
System 4	5	*0	100	61
Positive control growth (no system setup)	10	1.6×10^6	N/A	N/A
System 1	10	*0	100	60
System 2	10	*0	100	71
System 3	10	*0	100	57
System 4	10	*0	100	60
Positive control growth (no system setup)	20	2.0×10^6	N/A	N/A
System 1	20	*0	100	60
System 2	20	*0	100	71
System 3	20	*0	100	57
System 4	20	*0	100	60

*limit of detection is less than ten cells in the initial 50µL sample
"positive growth control (no system setup)" is collected from a bacterial sample that is untreated (spotted on a sterile petri dish); % bacterial reduction is calculated as the % in bacterial reduction compared to the positive growth control

Plate geometry 1 Ink PE826 against MRSA ATCC BAA-44: Run #1

Test sample	Time (min)	MRSA ATCC BAA-44 (CFU/mL)	% Bacterial reduction	Charge (µA)
Positive control growth (no system setup)	10	2.0×10^4	N/A	N/A
System 1	10	4.0×10^3	80	59
System 2	10	8.0×10^3	60	66
System 3	10	6.0×10^2	97	56
System 4	10	2.0×10^2	99	61
Positive control growth (no system setup)	20	1.2×10^5	N/A	N/A
System 1	20	2.2×10^4	82	60
System 2	20	6.0×10^4	50	67
System 3	20	4.0×10^4	67	57
System 4	20	*0	100	61
Positive control growth (no system setup)	60	1.6×10^5	N/A	N/A
System 1	60	*0	100	60
System 2	60	*0	100	67
System 3	60	*0	100	58
System 4	60	*0	100	61

*limit of detection is less than ten cells in the initial 50µL sample
"positive growth control (no system setup)" is collected from a bacterial sample that is untreated (spotted on a sterile petri dish); % bacterial reduction is calculated as the % in bacterial reduction compared to the positive growth control

Plate geometry 1 Ink PE826 against MRSA ATCC BAA-44: Run #2

Test sample	Time (min)	MRSA ATCC BAA-44 (CFU/mL)	% Bacterial reduction	Charge (µA)
Positive control growth (no system setup)	10	4.0×10^5	N/A	N/A
System 1	10	*0	100	62
System 2	10	*0	100	69
System 3	10	*0	100	58
System 4	10	*0	100	61
Positive control growth (no system setup)	20	2.6×10^5	N/A	N/A
System 1	20	*0	100	60
System 2	20	*0	100	69
System 3	20	*0	100	56
System 4	20	*0	100	60
Positive control growth (no system setup)	60	4.8×10^5	N/A	N/A
System 1	60	*0	100	60
System 2	60	*0	100	69
System 3	60	*0	100	57
System 4	60	*0	100	60

*limit of detection is less than ten cells in the initial 50µL sample
"positive growth control (no system setup)" is collected from a bacterial sample that is untreated (spotted on a sterile petri dish); % bacterial reduction is calculated as the % in bacterial reduction compared to the positive growth control

Plate geometry 1 Ink PE826 against MRSA ATCC BAA-44: Run #3

Test sample	Time (min)	MRSA ATCC BAA-44 (CFU/mL)	% Bacterial reduction	Charge (µA)
Positive control growth (no system setup)	10	2.0×10^5	N/A	N/A
System 1	10	*0	100	61
System 2	10	*0	100	72
System 3	10	*0	100	58
System 4	10	2.0×10^2	99.9	61
Positive control growth (no system setup)	20	6.0×10^5	N/A	N/A
System 1	20	*0	100	62
System 2	20	*0	100	71
System 3	20	2.0×10^2	99.97	58
System 4	20	*0	100	63
Positive control growth (no system setup)	60	4.0×10^4	N/A	N/A
System 1	60	*0	100	60
System 2	60	*0	100	70
System 3	60	*0	100	58
System 4	60	*0	100	61

*limit of detection is less than ten cells in the initial 50µL sample
"positive growth control (no system setup)" is collected from a bacterial sample that is untreated (spotted on a sterile petri dish); % bacterial reduction is calculated as the % in bacterial reduction compared to the positive growth control

Plate geometry 1 Ink PE826 against MRSA ATCC BAA-44: Run #4

Test sample	Time (min)	MRSA ATCC BAA-44 (CFU/mL)	% Bacterial reduction	Charge (µA)
Positive control growth (no system setup)	10	1.4×10^5	N/A	N/A
System 1	10	*0	100	60
System 2	10	*0	100	74
System 3	10	*0	100	58
System 4	10	*0	100	61
Positive control growth (no system setup)	20	4.0×10^5	N/A	N/A
System 1	20	2.8×10^3	99.3	60
System 2	20	1.4×10^4	96.5	74
System 3	20	1.4×10^4	96.5	57
System 4	20	1.0×10^4	97.5	61
Positive control growth (no system setup)	60	2.4×10^5	N/A	N/A
System 1	60	*0	100	60
System 2	60	*0	100	74
System 3	60	*0	100	57
System 4	60	2.0×10^2	99.95	60

*limit of detection is less than ten cells in the initial 50µL sample
"T_0" is the initial zero time point collected from the bacterial sample at the very start of each test run; "positive growth control (no system setup)" is collected from a bacterial sample that is untreated (spotted on a sterile petri dish); % bacterial reduction is calculated as the % in bacterial reduction compared to the positive growth control

Objective and Scope of study

The purpose of this study is to evaluate the antimicrobial properties of surfaces by measuring bacterial reduction of *Escherichia coli* ATCC 8739 and methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC BAA-44 over the course of three time points. The amount of viable bacteria present in the samples will be quantified using standard plating methods. Antimicrobial activity will be determined by comparing the number of viable bacteria (colony forming units, CFUs) in the experimental samples to the untreated controls at each time point. This protocol will be used to perform two experiments as outlined in **Table 1**. Experiments A and B will be performed a total of four times with one geometry/surface and one amperage. Within a single experiment, each time point will be assessed with four separate surfaces/test set-ups.

Experiment	Screen printed surface	Amperage	Bacteria	Time Points (minutes)
A	geometry #1 ink PE826	60µA	*E. coli*	5, 10, 20
B			MRSA	10 ,20, 60

Table 1. Bacteria and time points to be evaluated in Experiments A and B.

Description of testing method

Supplies:

1) Bacteria:

The following species of bacteria will be used for the tests: a) MRSA ATCC BAA-44 and b) *Escherichia coli* ATCC 8739.

2) Growth medium:

S. aureus and *E. coli* will be grown in tryptic soy broth (TSB; Becton Dickinson; 211825). TSB agar will be made with the addition of agar to 15g/L. Media will be made according to manufacturer's protocols.

3) Experimental surfaces:

Screen printed surfaces will be provided by the Sponsor. Additionally, power supplies, connectors, and meters needed to create and monitor the current for each surface will be provided by the Sponsor.

Procedure:

1) Pre-culture of bacteria:

Using a sterile inoculating loop, 5mL of broth will be inoculated with a colony of bacteria grown overnight on an agar plate. The inoculated broth will be placed at 37°C with shaking (180 rpm) for 16 hours.

2) Preparation of test inoculum:

The overnight culture will be diluted 1:100 in 10mL of growth medium and placed in a 37°C shaker for 2-4 hours for the bacteria to reach the exponential phase of growth. The actively growing culture will then be diluted to $1x10^6$ CFU/mL in 1X PBS.

3) Inoculation of screen printed surfaces:

A 50µL aliquot of the bacterial suspension will be pipetted onto the designated surface within the active area. A new printed surface (with no previous bacterial suspensions) will be used for each test. Surfaces will be attached to the proper power source prior to inoculation. Once inoculated, the printed surfaces will remain at room temperature on a bench top until completion of the study. Amperage of the printed surface will be noted at 90 seconds after placement of the 50µL aliquot of the bacterial suspension. A 50µL aliquot of bacterial suspension pipetted on a sterile, plastic surface will be used as the positive growth control.

4) Collection and plating of viable bacteria:

At the specified time points (see **Table 1**), the 50µL aliquots of the bacterial suspension will be collected using a pipette. The suspension will be diluted in 1X PBS and undiluted and diluted samples will be plated on TSB plates. Plates will be incubated at 37°C for approximately 16 hours. Colonies will be counted to determine CFU/mL of each sample.

Reporting of Results

Results will be reported as the percent of bacterial reduction at each time point compared to the control samples. These results will be supplied in a final report that will also describe any modifications of this protocol.

Approvals

Angie Pollard – Agile Sciences, Inc.	Study Director	Date
Sponsor representative	Sponsor	Date

Protocol number: P-2014-0002-ACE
10/08/2014