Final Study Report

Study Title

The Antimicrobial Activity of Stalosan-F in Moist and Wet Conditions Using *S.aureus*.

Data Requirements

Research and Development

Author

Jennifer Ortiz, B.S.

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Performing Laboratory

MicroChem Laboratory, Inc.
1107-C S. Airport Circle
Euless, TX 76040

Laboratory Project ID Numbers

040114-1, 040114-2, 040120-2

Records

The original records of this report are recorded in Lab notebook NMC-131 pages 99-125. This notebook, protocol, and final study report are stored in the archives of MicroChem Laboratory, Inc.

Sponsor

Archangel L.L.C.
636 Hampshire St.
Quincy, IL 62301

Summary

Study Title

The Antimicrobial Activity of Stalosan-F in Moist and Wet Conditions Using *S.aureus*.

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In test 1, *S.aureus* was labeled onto 2x2 cm paper squares to simulate damp straw that might be found in animal quarters, and three paper squares apiece were placed in a sterile plastic petri plates. One set of *S. aureus* labeled paper squares in petri plates was immediately sprinkled with 0.365g per petri plate of Stalosan-F per label directions using a saltshaker and another set was left untreated as a control. After exposure times of 0.0 minutes, 20.0 minutes, 4.0, 8.0 24.0, and 48.0 hours at ambient temperature, the three paper squares from each petri plate were removed and placed into 10.0 ml neutralizing medium and agitated on a vortex mixer for 60 seconds to release surviving microbes. Serial ten-fold dilutions were made as 1.0 ml into 9.0 ml neutralizing medium, and 0.5 ml portions of the dilutions were placed onto nutrient agar in petri plates to measure the number of surviving CFU of *S. aureus* at each exposure time. Stalosan-F was found to kill 100% of *S.aureus* within 8.0 hours at ambient temperature.

In test 2 and 3, *S.aureus* was labeled as 1.0 ml in a sterile plastic petri plate to simulate pooled water or urine. Three petri plates were immediately sprinkled with 0.365g per petri plate per the label directions using a saltshaker and three other petri plates were left untreated as a control. For test 2, after being mixed and held for an exposure time of 20.0 minutes, the petri plates were tilted on their side, 0.8-0.3 ml was removed and placed into a 9.0 ml tube of neutralizing medium. For test 3, after being mixed and held for an exposure time of 20.0 minutes, 4.0 ml of neutralizing medium was placed into the petri plate and mixed for 30 seconds. The petri plates were tilted on their side and 1.0 ml was removed and placed into a 5.0 ml tube of neutralizing medium. The tubes were agitated on a vortex mixer for 30 seconds and serial ten-fold dilutions were made as 1.0 ml into 9.0 ml neutralizing medium. One-half (0.5) ml portions of the dilutions were placed onto nutrient agar in petri plates to measure the number of surviving CFU of *S.aureus* at an exposure time of 20.0 minutes. Stalosan-F was found to kill 76%-99.8% of *S.aureus* within 20.0 minutes at ambient temperature.

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Objective

The purpose of test 1 was to measure the antimicrobial activity of Stalosan-F in moist conditions. To simulate moist straw, 2x2 cm paper squares were cut, labeled with *S.aureus* and immediately sprinkled with Stalosan-F in order to test Stalosan-F according to its probable environmental use. The purpose of test 2 and 3 was to measure the antimicrobial activity of Stalosan-F in wet conditions.

Materials

Stalosan-F
Midstates Stalosan, Inc.
Quincy, IL

Microbes
Staphylococcus aureus ATCC# 6538
American Type Culture Collection
Manassas, VA 20110

Nutrient Broth (NB)
Lot No. 2196531, Exp. 01/07
Difco Laboratories
Detroit, MI

Nutrient Agar (NA)
Lot No. 3183317, Exp. 05/08
Difco Laboratories

Dey Engley Broth (DE)
Lot No. 3141414, Exp. 04/06
Difco Laboratories

Glycine
Lot No. 02613CB, Exp.06/06
Aldrich Chemical Company
Milwaukee, WI

Sterilized Deionized Water (SDIW)
MicroChem Laboratory, Inc.
Euless, TX

Petri Plates, Plastic, Disposable 15x100mm
Fox Scientific
Alvarado, TX

Incubators capable of $35 \pm 2^{o}C$

Steam Sterilizer, Model STM-E
Market Forge

Glassware:
Test tubes, closures, and support racks
Beakers and flasks a variety of sizes
French square bottles (FSQB)

Pipettes 1.0 ml. 10.00 ml, 50.0 ml, Glass
VWR Scientific
Sugarland, TX

Nichrome Wire Loops
Baxter Diagnostic, Inc.
McGraw Park, IL

Black felt tip marking pen

Scissors

Vortex Mixer
Fisher Scientific

Construction paper

Forceps

Procedures

1. **Grow the Bacterial Cultures:**

The bacterial stock was obtained fresh from the American Type Culture Collection within six months from use in this test. *S.aureus* stocks were maintained on nutrient agar slants at 3±2ºC after monthly transfers to fresh nutrient agar slants grown for 48±8 hours at 35±2ºC. From the bacterial stock, one loopful of bacteria was added to 10.0 ml of nutrient broth and incubated at 35±2ºC for 24±4 hours.

For tests 1-3, *S.aureus* was diluted 100 fold as 1.0 ml into 99.0 ml of NB.

2. **Label the 2x2 cm Paper Square Carriers and Petri Plates**

In test 1, sterile 2x2 cm paper square carriers were soaked in the diluted culture of *S.aureus* for 60 seconds. The 2x2 cm paper square carriers were removed with flamed forceps and were placed in a sterile plastic 15x100 mm petri plate. Three 2x2 cm paper square carriers were placed into one petri plate per exposure time. Each plate was immediately sprinkled with Stalosan-F except for the controls.

In test 2 and 3, each sterile petri plate was labeled with 1.0 ml of the culture by using a sterile 1.0 ml glass pipette.

3. **Application of Stalosan-F**

For tests 1-3, 0.365g per petri plate was applied to the test petri plates. The Stalosan-F label directs to use 1.6oz/10.5ft^2. Then covert it to 1 ft^2: 1.6oz = 45.36g/10.5ft^2

$$\frac{45.36\ g}{10.5\ ft^2} = \frac{1\ ft^2}{0.0929\ m^2} = 46.5\ g/m^2$$

Next, to find the area of the petri plate. $A= (\pi)(r^2)$

$$A= 3.14(50)^2 = 7850\ mm^2$$

To find how much Stalosan-F is needed per petri plate.

$$\frac{46.5g}{1\ m^2} = \frac{1m^2}{10^6\ mm^2} = \frac{7850\ mm^2}{1} = 0.365g/\text{petri plate}$$

4. **Measure the Number of Surviving CFU of *S.aureus* After Exposure to Stalosan-F**

For test 1, after exposure times of 0.0 minutes, 20.0 minutes, 4.0, 8.0, 24.0, and 48.0 hours for the test, a 2x2 cm paper square carrier was removed from the petri plate using flamed forceps and placed into a 10.0 ml tube of DE + 1% Glycine. The tube was agitated for 60 seconds on a vortex mixer. Serial ten-fold dilutions were made as 1.0 ml into 9.0 ml DE. One-half (0.5) ml samples of dilutions were placed onto nutrient agar (NA) in petri plates. The test tubes and petri plates were incubated for 48±8 hours at 35±2ºC. Colonies were counted and multiplied by dilution factors to determine the number of surviving CFU for each 2x2 cm paper square carrier. Three squares were removed and assayed per exposure time and averaged for the test.

In test 2, after an exposure time of 20.0 minutes for the test, the petri plate was slightly tilted on its side. With a 1.0 ml sterile glass pipette, 0.8-0.3 ml was removed from the petri plate and placed into a 9.0 ml tube of DE. In test 3, after an exposure time of 20.0 minutes for the test, 4.0 ml of neutralizing recovery medium was placed into the petri plate and mixed for 30 seconds. The petri plate was slightly tilted on its side, 1.0 ml was removed by using a sterile 1.0 ml glass pipette and was placed into a 5.0 ml tube DE. The tube was agitated for 30 seconds on a vortex mixer. Serial ten-fold dilutions were made as 1.0 ml into 9.0 ml DE. One-half (0.5) ml samples of dilutions were placed onto nutrient agar (NA) in petri plates. The test tubes and petri plates were incubated for 48±8 hours at 35±2ºC. Colonies were counted and multiplied by dilution factors to determine the number of surviving CFU for each petri plate. The three petri plates were averaged for the test.

As a control, the above procedures were repeated for each test to determine the number of surviving CFU of bacteria at each exposure time without exposure to Stalosan-F.

5. **Calculation of Percent Kill by Stalosan-F**

C= Control Colony Forming Units T= Test Colony Forming Units

For test 1: Total % Kill = $\frac{C_0 - T_{48}}{C_0} \times 100$

For test 2 and 3: Total % Kill = $\frac{C_0 - T_{20}}{C_0} \times 100$

6. **Validation of Neutralization and Viability**

To validate the neutralization of Stalosan-F, two 2x2 cm paper squares were soaked in SDIW for 60 seconds. The 2x2 cm paper squares were removed with flamed forceps, placed in a sterile plastic petri plate and immediately sprinkled with 0.365g of Stalosan-F. With flamed forceps, each square was removed and placed into a 10.0 ml neutralizing recovery medium. A ten-fold dilution was made as 1.0 ml into 9.0 ml of neutralizing recovery medium. Each dilution tube was spiked with about 1000 CFU in 1.0 ml and 0.5 ml of each tube was plated onto nutrient agar (NA) in petri plates.

In test 2, to validate the neutralization of Stalosan-F, two sterile plastic petri plates were labeled with 1.0 ml of SDIW and 0.365g of Stalosan-F was immediately sprinkled in the petri plate. With a sterile 1.0 ml glass pipette, 0.45 ml was removed from the petri plate and placed into 9.0 ml of neutralizing recovery medium. In test 3, two sterile plastic petri plates were labeled with 1.0 ml of SDIW and 0.365g of Stalosan-F was immediately sprinkled in the petri plate. After an exposure time of 20.0 minutes, 4.0 ml of neutralizing medium was placed into the petri plate and mixed for 30 seconds. The petri plate was tilted on its side and 1.0 ml was removed and placed into 5.0 ml of DE. A ten-fold dilution was made as 1.0 ml into 9.0 ml of neutralizing recovery medium. Each dilution tube was spiked with about 1000 CFU in 1.0 ml and 0.5 ml of each tube was plated onto nutrient agar (NA) in petri plates.

For a comparative number in tests 1-3, a ten-fold dilution was made as 1.0 ml into 9.0 ml tube of neutralizing recovery medium. Each dilution tube was spiked with about 1000 CFU in 1.0 ml and 0.5 ml of each dilution tube was plated onto nutrient agar (NA) in petri plates.

7. **Test Dates**

Test 1:	January 14, 2004
Test 2:	January 14, 2004
Test 3:	January 20, 2004

Results

There were three tests with Stalosan-F versus *S.aureus*. The results of the number of surviving CFU of *S.aureus* in moist conditions after exposure to Stalosan-F on 2x2 cm paper square carriers placed in a sterile petri plate as a function of time is recorded in **Table 1**. The results of the number of surviving CFU of *S.aureus* in wet conditions after exposure to Stalosan-F in a sterile petri plate as a function of time is recorded in **Table 2** and **Table 3**.

The percent kill of *S. aureus* in moist conditions was 100% in the presence of Stalosan-F within 8.0 hours. The percent kill of *S.aureus* in wet conditions was 76%-99.8% in the presence of Stalosan-F within 20.0 minutes.

Conclusions

Stalosan-F killed 100% of *S.aureus* in moist conditions after an exposure time of 8.0 hours. In wet conditions, Stalosan-F killed 76%-99.8% of *S. aureus* after an exposure time of 20.0 minutes.

Table 1. Results of the Number of Surviving CFU of *S.aureus* After Exposure to Stalosan-F on 2x2 cm Paper Square Carriers As a Function of Time.

***S. aureus* After Exposure to Stalosan-F**

Exposure Time	Surviving CFU/Carrier	Avg. Surviving CFU/Carrier
20.0 min.	3.08×10^4	6.1×10^5
20.0 min.	7.8×10^4	
20.0 min.	5.06×10^4	
4.0 hrs.	1.22×10^5	1.6×10^5
4.0 hrs.	9.0×10^3	
4.0 hrs.	3.38×10^4	
8.0 hrs.	0	0
8.0 hrs.	0	
8.0 hrs.	0	
24.0 hrs.	0	0
24.0 hrs.	0	
24.0 hrs.	0	
48.0 hrs.	0	0
48.0 hrs.	0	
48.0 hrs.	0	

Controls for *S. aureus*

Exposure Time	Surviving CFU/Carrier	Avg. Surviving CFU/Carrier
0.0 min.	1.02×10^5	1.88×10^5
0.0 min.	3.10×10^5	
0.0 min.	1.52×10^5	
20.0 min.	4.96×10^4	5.03×10^4
20.0 min.	3.54×10^4	
20.0 min.	6.6×10^4	
4.0 hrs.	2.48×10^6	1.53×10^6
4.0 hrs.	1.18×10^6	
4.0 hrs.	9.4×10^5	
8.0 hrs.	3.64×10^5	3.68×10^5
8.0 hrs.	2.40×10^5	
8.0 hrs.	5.00×10^5	
24.0 hrs.	1.04×10^7	2.18×10^7
24.0 hrs.	1.92×10^7	
24.0 hrs.	3.58×10^7	
48.0 hrs.	8.84×10^7	1.11×10^8
48.0 hrs.	1.11×10^8	
48.0 hrs.	1.35×10^8	

Table 1 Notes:

1. The percent kill of *S. aureus* was 100% in the presence of Stalosan-F within 8.0 hours.

Table 2. Results of the Number of Surviving CFU of *S.aureus* After Exposure to Stalosan-F in Plastic Sterile Petri Plates As a Function of Time.

***S. aureus* After Exposure to Stalosan-F**

Exposure Time	Surviving CFU/Plate	Avg. Surviving CFU/Plate
20.0 min.	6.0×10^2	5.4×10^3
20.0 min.	6.2×10^3	
20.0 min.	9.4×10^3	

Controls for *S. aureus*

Exposure Time	Surviving CFU/Plate	Avg. Surviving CFU/Plate
20.0 min.	2.92×10^6	2.38×10^6
20.0 min.	2.12×10^6	
20.0 min.	2.10×10^6	

Table 2 Notes:

1. The percent kill of *S. aureus* was 99.8% in the presence of Stalosan-F within 20.0 minutes.

Table 3. Results of the Number of Surviving CFU of *S.aureus* After Exposure to Stalosan-F in Plastic Sterile Petri Plates As a Function of Time.

***S. aureus* After Exposure to Stalosan-F**

Exposure Time	Surviving CFU/Plate	Avg. Surviving CFU/Plate
20.0 min.	2.85×10^5	7.02×10^5
20.0 min.	5.61×10^5	
20.0 min.	1.26×10^6	

Controls for *S. aureus*

Exposure Time	Surviving CFU/Plate	Avg. Surviving CFU/Plate
20.0 min.	2.25×10^6	2.94×10^6
20.0 min.	3.36×10^6	
20.0 min.	3.21×10^6	

Table 3 Notes:

1. The percent kill of *S. aureus* was 76% in the presence of Stalosan-F within 20.0 minutes.

End of Final Study Report

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This study was begun on January 14, 2004, the date the Study Director signed the Study Protocol, and completed on January 22, 2004, the signature date of the Study Director on this Final Study Report.

_____	_____
Lead Scientist	Date of Signature
_____	_____
Assisting Scientist	Date of Signature
_____	_____
Norman Miner, Ph. D. Study Director MicroChem Laboratory, Inc. 1107-C S. Airport Circle Euless, TX 76040	Date of Signature