



Final Study Report

Study Title

The Antimicrobial Activity of Stalosan F and Various Competitive Products in Moist Conditions Using *S.aureus* Test 2.

Data Requirements

Research and Development

Author

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Performing Laboratory

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Laboratory Project ID Numbers

050405-1, 050412-2

Records

The original records of this report are recorded in Lab notebook NMC-146 pages 1-12 & 52-66. This notebook, protocol, and final study report are stored in the archives of MicroChem Laboratory, Inc.

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Summary

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The Antimicrobial Activity of Stalosan F and Various Competitive Products in Moist Conditions Using *S.aureus* Test 2.

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Stalosan F and various competitive products are used to sanitize and deodorize animal quarters such as barns or stalls where an animal might inhabit. At different times, these animal quarters can contain moist straw or hay due to urine or other environmental effects that could cause unsanitary conditions for the animals. In this study, the antimicrobial activity of Stalosan F and various competitive products were measured as found in moist conditions.

To simulate moist straw, 2x2 cm paper squares were cut, sterilized, labeled with *S. aureus*, and three paper square carriers were placed in sterile petri plates. One set of squares were immediately sprinkled with each of the products per the label directions and one set of squares were left untreated as a control. After various exposure times at ambient temperature, three paper squares from each petri plate were assayed to measure the surviving number of colony-forming units (CFU) of *S. aureus*. The percent reduction and the percent inhibition were measured. The moisture of this test method is similar to the products probable environmental use. Stalosan F (On Guard) and one new competitive product and four old competitive products were tested.

The competitive products killed 0% and inhibited 0% - 95.73% of *S. aureus* within 24.0 hours at ambient temperature.

Stalosan F was found to kill 92.25%-99.45% and inhibit 99.97%-99.9996% of *S. aureus* within 24.0 hours at ambient temperature. Stalosan F killed at least 80% and inhibited at least 4% more than the competitive products. Stalosan F has more antimicrobial activity than Staldren, Barn Fresh, Mistral, Stable Boy, and Sweet PDZ.

Study Title

The Antimicrobial Activity of Stalosan F and Various Competitive Products in Moist Conditions Using *S.aureus* Test 2.

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Objective

The purpose of this test was to measure the antimicrobial activity of Stalosan F and various competitive products in moist conditions by simulating moist straw found in barns/animal stalls using *S.aureus*.

Materials

Stalosan F

Directions for use: 1 lb/100ft²
Conc. Tested: 0.383g/petri plate
Midstates Stalosan, Inc.
Quincy, IL

Competitive Products:

Sweet PDZ

Directions for use: 5-6 cups/144ft²
Conc. Tested: 0.798g/petri plate
RockPointe Corporate Center
Spokane, Washington

Poultry Litter Treatment (PLT)

Directions for use: 75lbs/1000²
Jones-Hamilton Company
Walbridge, OH

EnviroShield

Directions for use: 50g/m²
R&D Distribution
Marshalltown, IA

Staldren

Directions for use: 50g/m²
Conc. Tested: 0.393g/petri plate
Trading Company LDL
Spinnerstraat 36
7461 TT Rijssen
Holland

Stable Boy

Directions for use: 2kg/9m²
Conc. Tested: 1.74g/ petri plate
Westhawk Traders Ltd.
Vancouver, B.C.

Barn Fresh

Directions for use: 18kg/45m²
Conc. Tested: 3.14g/petri plate
Western Industrial Clay Products LTD.
Kamloops, B.C.

Mistral

Directions for use: 100g/m²
Conc. Tested: 0.785g/petri plate
Mistral Olmix
LeLintan 56580 Brehan
France

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

Nutrient Broth (NB)

Lot No. 4223326, Exp. 7/09
Difco Laboratories
Detroit, MI

Nutrient Agar (NA)

Lot No. 4237960, Exp. 1/09
Difco Laboratories

Dey Engley Broth (DE)

Lot No. 4362309, Exp. 9/07
Difco Laboratories

Glycine

Lot No. 02613CB, Ass. Exp. 6/06
Aldrich Chemical Company
Milwaukee, WI

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

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

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

Steam Sterilizer, Model STM-E
Market Forge

Test tube racks, stainless steel closures, and test tubes 18x 150 mm

Pipettes 1.0 ml, 10.00 ml, Glass, Sterile
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

Metal Forceps

Saltshakers

Procedures

1. Grow the Bacterial Culture

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 $3\pm 2^{\circ}\text{C}$ after monthly transfers to fresh nutrient agar slants grown for $48\pm 8^{\circ}\text{C}$ hours at $35\pm 2^{\circ}\text{C}$. From the bacterial stock, one loopful of bacteria was added to a 10.0 ml tube of nutrient broth and incubated at $35\pm 2^{\circ}\text{C}$ for 24 ± 4 hours.

A 100-fold dilution of *S.aureus* was made as 1.0 ml of culture into 99.0 ml of nutrient broth and this dilution was used in the test.

2. Prepare and Label the 2x2 cm Paper Square Carriers and Place in Petri Plates

Paper squares were cut 2x2 cm and steam sterilized in a covered beaker for approximately 20.0 minutes at 121°C . The sterile 2x2 cm paper square carriers were soaked in the diluted culture of *S.aureus* for about 60 seconds. Using flamed forceps, the paper square carriers were removed, shaken to remove any excess and were placed in a sterile plastic petri plate. Three squares were placed in each petri plate.

3. Application of Stalosan F and the Competitive Products

Stalosan F and the competitive products were immediately sprinkled from a saltshaker onto the bacteria-labeled paper squares in the petri plates. The appropriate amount of each test product added to a petri plate is determined as follows:

Find the area of the petri plate. $A = (\pi)(r^2)$

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

On Guard (New Stalosan F Product)

Directions for use: 1 lb. per 100 sq. ft.

First convert lbs. to g: $\frac{1\text{lb.}}{1} \times \frac{453.6\text{g}}{1\text{lb.}} = 453.6\text{g}$

Next convert ft^2 to 1 m^2 : $\frac{100\text{ ft}^2}{1} \times \frac{0.0929\text{ m}^2}{1\text{ ft}^2} = 9.29\text{m}^2$

Convert 9.29 m² to 1 m²: $\frac{453.6\text{g}}{9.29\text{m}^2} = 48.83\text{g/m}^2$

Find how much is needed per petri plate.

$$\frac{48.83\text{g}}{1\text{ m}^2} \times \frac{1\text{m}^2}{10^6\text{ mm}^2} \times \frac{7850\text{ mm}^2}{1} = 0.383\text{g/petri plate}$$

Staldren

Directions for use: 50g/m².

Find how much is needed per petri plate.

$$\frac{50\text{g}}{1\text{ m}^2} \times \frac{1\text{m}^2}{10^6\text{ mm}^2} \times \frac{7850\text{ mm}^2}{1} = 0.393\text{g/petri plate}$$

Stable Boy

Directions for use: 2kg/9m².

First convert kg to g: $\frac{2\text{kg}}{1} \times \frac{1000\text{g}}{1\text{kg}} = 2000\text{g}$

Next convert 9 m² to 1 m²: $\frac{2000\text{g}}{9\text{m}^2} = 222\text{g/m}^2$

Find how much is needed per petri plate.

$$\frac{222\text{g}}{1\text{ m}^2} \times \frac{1\text{m}^2}{10^6\text{ mm}^2} \times \frac{7850\text{ mm}^2}{1} = 1.74\text{g/petri plate}$$

Barn Fresh

Directions for use: 18kg/45m².

First convert kg to g: $\frac{18\text{kg}}{1} \times \frac{1000\text{g}}{1\text{kg}} = 18,000\text{g}$

Next convert 9 m² to 1 m²: $\frac{18,000\text{g}}{45\text{m}^2} = 400\text{g/m}^2$

Find how much is needed per petri plate.

$$\frac{400\text{g}}{1\text{ m}^2} \times \frac{1\text{m}^2}{10^6\text{ mm}^2} \times \frac{7850\text{ mm}^2}{1} = 3.14\text{g/petri plate}$$

Mistral

Directions for use: 100g/m² per calf pen.

Find how much is needed per petri plate.

$$\frac{100\text{g}}{1\text{ m}^2} \times \frac{1\text{m}^2}{10^6\text{ mm}^2} \times \frac{7850\text{ mm}^2}{1} = 0.785\text{g/petri plate}$$

Sweet PDZ

Directions for use: 5-6 cups/144ft², 6 cups = 48 oz

Convert oz to g:

$$\frac{48 \times 28.35\text{g}}{1\text{oz}} = 1360.8\text{g}$$

Convert 144ft² to m²:

$$\frac{144\text{ft}^2 \times 0.0929\text{m}^2}{1\text{ ft}^2} = 13.38\text{m}^2$$

Convert 13.38m² to 1 m²:

$$\frac{1360.8\text{g}}{13.38\text{m}^2} = 101.7\text{g/m}^2$$

Find how much is needed per petri plate.

$$\frac{101.7\text{g}}{1\text{ m}^2} \times \frac{1\text{m}^2}{10^6\text{ mm}^2} \times \frac{7850\text{ mm}^2}{1} = 0.785\text{g/petri plate}$$

4. **Measure the Number of Surviving CFU of *S.aureus* After Exposure to Stalosan F and Various Competitive Products**

For test 1, after exposure times of 30.0 minutes, 8.0 hours, and 24.0 hours each 2x2 cm paper square carrier was removed from the petri plate using flamed forceps and placed into a 10.0 ml tube of DE + 0.5% glycine. The tube was agitated on a vortex mixer for 60 seconds to remove any surviving colonies of *S. aureus*. Serial ten-fold dilutions were made as 1.0 ml to 9.0 ml DE. One-half (0.5) ml samples from various dilution tubes were transferred onto the surface of nutrient agar (NA) in petri plates. The plates were incubated for 48±8 hours at 35±2°C. The colonies were counted and multiplied by the appropriate dilution factor to determine the number of surviving CFU for each 2x2 cm paper square

carrier. Three squares were removed and assayed per exposure time for each product.

For test 2, this procedure was repeated in the same manner as test 1 except had an exposure time of only 24.0 hours.

As a control, the above procedures were repeated, including a zero time point, using untreated (no exposure to Stalosan F or competitor products) bacteria-labeled paper squares to determine the number of surviving CFU of bacteria at each exposure time. To prevent death of the *S.aureus* due to drying out, about 0.1 ml of sterile nutrient broth was added to the squares if they appeared to be dry.

5. **Calculation of Percent Kill and Inhibition by Stalosan F and the Competitive Products**

The percent kill of *S.aureus* by the various test products was calculated with the following formula:

$$\% \text{ Kill} = 1 - \frac{S_0 - S}{S_0} \times 100$$

S = The number of surviving CFU after exposure to test chemistry.

S₀ = The original number of CFU labeled onto a paper square (control) at time zero.

Whereas the percent inhibition of *S.aureus* by the various test products was calculated according to the following formula:

$$\% \text{ Inhibition} = 1 - \frac{S_T - S}{S_T} \times 100$$

S = The number of surviving CFU after each exposure time to test chemistry.

S_T = The number of CFU labeled onto a paper square (control) at each exposure time.

6. **Validation of Neutralization and Viability**

To validate the neutralization of Stalosan F and the competitive products, two 2x2 cm paper squares were soaked in sterile deionized water about 60 seconds. Using flamed forceps, the squares were placed to a sterile plastic petri plate and quickly sprinkle with a test product. Each square was transferred to a 10.0 ml tube containing neutralizing recovery medium at the concentration as used in the test. One ten-fold dilution was made as 1.0 ml into 9.0 ml of DE and each tube was spiked with approximately 1000 CFU in 1.0 ml of broth. One-half (0.5) ml of each dilution tube was transferred onto the surface of nutrient agar in petri plates, incubated with the test, and the colonies of *S. aureus* were counted. This was done twice for each product tested.

For a comparative number, two tubes of 10 ml neutralizing recovery medium were spiked with about 1000 CFU in 1.0 ml of broth. One-half (0.5) ml of each tube was transferred onto nutrient agar in petri plates, incubated with the test, and the colonies were counted. Similar numbers of colonies on all plates validated that neutralization occurred. Neutralization of each test product must occur for a valid test.

Results

Test 1

In test 1, one new competitive product was tested, Sweet PDZ, alongside Stalosan F (On Guard) with exposure times of 30.0 minutes, 8.0 and 24.0 hours. Stalosan F killed 21.83% 89.01%, and 92.25% of *S. aureus* within 30.0 minutes, 8.0 hours, and 24.0 hours, respectively. Sweet PDZ killed 3.52% of *S. aureus* within 30.0 minutes and 0% of *S. aureus* within 8.0 and 24.0 hours.

Stalosan F inhibited 3.50%, 97.91% and 99.97% of *S. aureus* within 30.0 minutes, 8.0 hours, and 24.0 hours, respectively. Sweet PDZ inhibited 0% of *S. aureus* within 30.0 minutes, 8.0 hours, and 24.0 hours.

Test 2

In test 2, four competitive products were tested again alongside Stalosan F (On Guard) with an exposure time of 24.0 hours. Stalosan F killed 99.45% of *S. aureus* and all four competitive products killed 0% of *S. aureus* within 24.0 hours.

Stalosan F inhibited 99.9996% of *S. aureus* within 24.0 hours. Within 24.0 hours, 88.42%, 95.73%, 91.52%, and 56.90% of *S. aureus* CFU were inhibited by Staldren, Barn Fresh, Mistral, and Stable Boy, respectively.

Discussion

The percent kill and inhibition were found for each product per test because we observed that with longer exposure times the control would increase in the number of surviving colony-forming units (CFU), meaning that the *S. aureus* on the control grew. To calculate the percent kill, the treated squares (sprinkled with a test product) are compared to the untreated squares (control) at the zero time whereas the percent inhibition compares the treated squares to the untreated squares at the same various exposure times.

For test 1, one (Sweet PDZ) of the three new competitive products (that have not previously been tested) was tested alongside Stalosan F for various exposure times. Stalosan F killed 92.25% and inhibited 99.97% of *S. aureus* within 24.0 hours. Sweet PDZ killed 0% and inhibited 0% of *S. aureus* within 24.0 hours.

EnviroShield and Poultry Litter Treatment (PLT) were the other two new competitive products. EnviroShield was highly contaminated in a preliminary neutralization test and we could not get adequate numbers to determine if neutralization occurred. Therefore, we consulted the ingredients in EnviroShield and found that *Saccharomyces cerevisiae* (yeast) was an ingredient. An R&D study was done to measure the amount of *S. cerevisiae* found in 0.5g of EnviroShield and to determine if we could do sufficient testing with the product. The results indicated that there were an average of 1.03×10^5 CFU of *S. cerevisiae* in 0.5g sample of EnviroShield. Due to the high concentration of *S. cerevisiae*, we determined that we would not have a viable test and the product was not tested.

The PLT competitive product was not tested because we were unable to neutralize it. The pH of PLT was observed to be below or right at 1. This competitive product is very acidic and may not be safe for the animals due to its toxicity.

For test 2, four of the competitive products that were previously tested were re-tested with a longer exposure time of 24.0 hours. Within the 24.0 hour exposure time, the four competitive products killed 0% of *S. aureus* and inhibited 88.42% (Staldren), 95.73% (Barn Fresh), 91.52% (Mistral), and 56.90% (Stable Boy) of *S. aureus*. The difference in the percent inhibition by the competitive products may be due to the various concentrations tested per product, meaning that the varying amounts of each product could contribute to the *S. aureus* drying out and dying.

Conclusion

Stalosan F was found to kill 92.25%-99.45% and inhibit 99.97%-99.9996% of *S. aureus* within 24.0 hours at ambient temperature. Stalosan F killed at least 80% and inhibited at least 4% more than the competitive products. Stalosan F has more antimicrobial activity than Staldren, Barn Fresh, Mistral, Stable Boy, and Sweet PDZ.

Table 1. (Test 1) The Average Percent (%) Kill and Inhibition of *S. aureus* After Exposure to Stalosan F and One Competitive Product on 2x2cm Paper Square Carriers As a Function of Time.

Product Name	Exposure Time	Avg. Percent (%) Kill	Avg. Percent (%) Inhibition
Stalosan F	30.0 min.	21.83%	3.50%
	8.0 hrs.	89.01%	97.91%
	24.0 hrs.	92.25%	99.97%
Sweet PDZ	30.0 min.	3.52%	0%
	8.0 hrs.	0%	0%
	24.0 hrs.	0%	0%

Table 2. (Test 2) The Average Percent (%) Kill and Inhibition of *S. aureus* After Exposure to Stalosan F and Various Competitive Products on 2x2cm Paper Square Carriers As a Function of Time.

Exposure Time	Product Name	Avg. Percent (%) Kill	Avg. Percent (%) Inhibition
24.0 hrs.	Stalosan F	99.45%	99.9996%
	Staldren	0%	88.42%
	Barn Fresh	0%	95.73%
	Mistral	0%	91.52%
	Stable Boy	0%	56.90%

End of Final Study Report

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050405-1, 050412-2

This study was begun on April 5, 2005 and completed on April 21, 2005, the signature date of the Study Director on this Final Study Report.

Lead Scientist – Jennifer Dunham

Date of Signature

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