Test Report

Stormøllen A/S

EVALUATION OF ANTIMICROBIAL ACTIVITY against Campylobacter jejuni

STALOSAN F

September 2007

Client: **Stormøllen A/S**
Ringsbjergvej 16
4682 Tureby

Date: 1 October 2007

Testing Laboratory: Eurofins Environment A/S
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SUMMARY

An assay was conducted on test substance STALOSAN F in order to determine its antimicrobial effectiveness against *Campylobacter jejuni*, according to the protocol provided by the Sponsor.

The following microbial strain was used for the test:
Campylobacter jejuni ATCC 49349

After sterilization, 2x2 cm paper squares were contaminated with an inoculum of *Campylobacter jejuni* and were then placed in sterile petri plates (three carriers for each plate). One set of squares were treated with the test substance and one set were left untreated as a control.
At various exposure times (30 minutes, 8 hours, 24 hours), three paper squares exposed to the test substance were removed from each petri plate and assayed for the microbial viable count.
The same procedure was performed for the untreated control.
The test was performed in moist conditions to simulate the probable environmental use conditions.

After the incubation time, the results are expressed as *Percent Kill (% Kill)* and *Percent Inhibition (%Inhibition)*; they are listed in the following table.

Exposure time	Average % Kill	Average % Inhibition
30 minutes	88,21	71,55
8 hours	99,99	99,93
24 hours	100,00	99,98

INTRODUCTION

A study was conducted on behalf of Stormøllen A/S in order to evaluate the antimicrobial effectiveness, in compliance with the protocol provided by the Sponsor.

The study was conducted in Eurofins Biolab S.p.A. Test Facility located in Vimodrone (MI), via Bruno Buozzi, 2.

The experimentation started on September 21st, 2007 and was completed on September 24th, 2007.

REFERENCES

1. Jennifer Dunham, B.S. Microchem Laboratory, Inc. – *The antimicrobial Activity of Stalosan-F and Various Competitive Products in Moist Conditions Using S. aureus Test 2* (Project ID Numbers 050405-1, 050412-2) – April 21, 2005.

2. EN 13697 August 2001 – Chemical disinfectants and antiseptics – Quantitative test for not porous surfaces for the evaluation of bactericidal and/or fungicidal activity of chemical disinfectants used in food, industrial, domestic and institutional areas - Test method and requirements without mechanical action (phase 2/step2).

FILING

The study program, all raw data, the final report with some possible reviews, are kept in the archives of Biolab S.p.A. for 10 years from the conclusion of the study.
The control sample of the test substance will not be kept.
The Sponsor, upon drafting a suitable contract, may request an extension of the conservation of all or part of the substances for a further period or their restitution.

PROCEDURES

The procedures used in the study are documented in the Procedure Handbook of Biolab S.p.A.

TEST SUBSTANCE

The test substance consisted of a product used to sanitize and deodorize the animal quarters.

Name:	STALOSAN F
Stability:	not provided
Composition provided by the Sponsor:	not provided

TESTED SAMPLE

The analysed sample, representative of the test substance, consisted of a brown powder contained in a plastic bag.

Batch nr.:	not provided
Date of preparation:	not provided
Expiry date:	not provided
Analytical certificate:	not provided
Id Nr:	07.20531 / 760700
Receiving nr.:	R04527.07
Receiving date:	17/09/2007

The characterisation of the test substance is responsibility of the Customer.

EVALUATION OF ANTIMICROBIAL ACTIVITY against Campylobacter jejuni

PRIMARY RESEARCHER: Dr. L. Brambilla

EXPERIMENTAL PROCEDURE

1 ASSAY SYSTEM

1.1 Microorganisms

Identification
The following test strain was used:
Campylobacter jejuni ATCC 49349

Centre of origin
The strain was acquired from the Institute Pasteur, Paris.

Conservation
The bacterial strain was kept frozen; before use, it was transplanted on TSA with sheep blood and kept in a refrigerator at 4°C ±2°C.

Preparation of the bacterial suspension
The bacterial strain was transplanted on TSA with sheep blood twice consecutively and incubated at 37°C ±1°C for 48 hours in microaerophilic conditions.
Within two hours from the beginning of the test, the final culture was suspended in the diluent using glass beads, and the suspension was diluted to a concentration of $1.5x10_8$-$5.0x10_8$ cfu/ml.
The colony number was determined performing the counting.

2 CULTURE MEDIA AND REAGENTS

2.1 Tryptone Soya Agar (TSA) with defibrinated sheep blood

Tryptone Soya Agar	30 g	MERCK
Defibrinated sheep blood	50 ml	OXOID
Distilled water q.s. to	1000 ml	

2.2 Diluent

Tryptone, pancreatic digestion of casein	1.0 g	MERCK
NaCl	8.5 g	MERCK
Distilled water q.s. to	1000 ml	

2.3 Neutraliser

Lecithin	3 g	MERCK
Polysorbate 80	30 g	MERCK
Sodium Thiosulfate	5 g	MERCK

L-histidine	1 g	MERCK
Saponin	30 g	SIGMA
Triptone-treated water q.s. to	1000 ml	

2.4 Steril water

3 EQUIPMENT

Standard microbiology laboratory equipment:

- Dry sterilization oven	MEMMERT
- Steam autoclave	FEDEGARI
- Incubator	MEMMERT
- pHmeter	BECKMAN
- Vortex stirrer	VELP
- Chronometer	ARBORE
- Micropipettes	GILSON
- CampyGen	OXOID
- Petri plates 90 mm	TARGET
- Paper squares (2x2 cm)	GHIARONI

4 EXPERIMENTAL DESIGN

4.1 Test temperature
The test was performed at 20°C ±1°C.

4.2 Concentration
The test substance was tested at the following concentration:
As such

4.3 Contact times
The following contact times were used:
30 minutes – 8 hours – 24 hours

4.4 Carriers
Paper squares (2x2 cm) had been used to the performing the assay.

5 EXECUTION OF THE ASSAY

5.1 Assay
After sterilization, 2x2 cm paper squares were contaminated with 0.05 ml of the suspension 1.5 x10^8-5.0x10^8 cfu/ml and were then placed in sterile petri plates (three carriers for each plate).
One set of squares (3 plates, 9 carriers) were treated with the test substance that was sprinkled uniformly on the surface of the plate with a final dosage of 0.310 g/petri plate.
One set (4 plates, 12 carriers) were left untreated as a control.

At various exposure times (30 minutes, 8 hours, 24 hours), three paper squares exposed to the test substance were removed from each petri plate and tranferred to a test tube containing 10 ml of neutraliser and 5 g of glass beads. The test tubes had been strongly stirred on a vortex mixer in order to take off the bacteria from the carrier.
After a neutralization time of 5 minutes, the mixture had been diluted with serial ten-fold dilutions and 0.5 ml from each dilution had been transferred onto the surface of Tryptone Soya Agar (TSA) with defibrinated sheep blood plate.
The plates were incubated at 37±1°C for 48 hours in microaerophilic atmosphere.
The same procedure was performed for the untreated control, including a zero time point, to evaluate the number of surviving cfu at different exposure times.
At the end of the incubation time, the number of cfu/plate was determined and multiplied by the appropriate dilution factor to determine the number of surviving cfu for each carrier.
All the test was performed in moist conditions to simulate the probable environmental use conditions.

5.2 Validation of neutralization and viability

Two 2x2 cm sterile paper squares were soaked in sterile deionized water for about 60 seconds. The carriers were then placed in a sterile plate and quickly sprinkled with the test substance.
Each square was transferred to a test tube containing 10 ml of neutralizer and 5 g of glass beads. The test tubes had been strongly stirred on a vortex mixer in order to take off the bacteria from the carrier.
After a neutralization time of 5 minutes, the mixture had been diluted with serial ten-fold dilutions and each tube had been inoculated with approximatly 1000 cfu/ml of the test strain; 0.5 ml from each dilution had been transferred onto the surface of Tryptone Soya Agar (TSA) with defibrinated sheep blood plate.
The plates were incubated at 37±1°C for 48 hours in microaerophilic atmosphere.
At the same time two tubes of 10 ml of neutralizer were inoculated with approximatly 1000 cfu/ml of the test strain; 0.5 ml of this suspension had been transferred onto the surface of Tryptone Soya Agar (TSA) with defibrinated sheep blood plate.
The plates were incubated at 37±1°C for 48 hours in microaerophilic atmosphere.

6 CALCULATION AND EXPRESSION OF THE RESULTS

After the incubation time, the results are expressed as *Percent Kill (% Kill)* and *Percent Inhibition (%Inhibition)*:

$$\%\ Kill = \left| \frac{S_0 - S}{S_0} \right| \times 100$$

S = number of surviving cfu after exposure to the test substance
S_0 = original number of cfu at the time zero (control)

$$\%\ Inhibition = \left| \frac{S_T - S}{S_T} \right| \times 100$$

S = number of surviving cfu after exposure to the test substance

S_T = number of cfu at each exposure time (control)

ASSAY VALIDITY CRITERIA

In the validation of neutralization and viability test, similar numbers of colonies on all plates must occur for a valid assay.

RESULTS

1. Validation of neutralization and viability (Table N. 1)
The validation test complies with the assay validity criteria.

2. Assay (Table N. 2)
The average Percent Kill (% Kill) and average Percent Inhibition (%Inhibition) at the different contact times are listed in the following table.

Exposure time	Average % Kill	Average % Inhibition
30 minutes	83,21	71,55
9 hours	99,99	99,93
24 hours	100,00	99,98

DEVIATIONS

No deviations occured during the study.

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TABLES

TABLE N.1: Validation test
Average count for the validation test (cfu/plate)

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Average count for the validation test (cfu/plate)

Neutralization	Viability
80.7	97.0

TABLE N. 2: Assay
Average count for the assay (cfu/carrier)

Exposure time	Control	Test
Time zero	4.67×10^5	//
30 minutes	1.93×10^5	5.50×10^3
8 hours	6.33×10^3	4.13×10^2
24 hours	5.73×10^3	1.33×10^2