Final Report N. 2010/882 SAMi

SUSPENSION BACTERICIDAL EFFECTIVENESS FOR VETERINARY FIELD ON SAGEWASH SANITIZER 250ppm

Study Program No: 2010/882 SAM

Contract No: PPR12010029702

Sponsor: EUROFINS LABORATORIES LTD
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Test substance: SAGEWASH SANITIZER 250ppm

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SUMMARY

A series of assays were conducted on the test substance SAGEWASH SANITIZER 250ppm in order to determine its bactericidal effectiveness for the uses for which the product is specifically intended. The test substance, consists of white powder compressed in a cylindrical form that was put in a spray gun before the use. According to the Sponsor instructions, this device was connected to the tap water and the test solution was obtained after 10 minutes of supply.

The bactericidal effectiveness has been verified with the following experimentation:
- phase 2/step 1 bactericidal activity in suspension for veterinary field – Method of dilution-neutralization in which 4 different bacterial strains, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 15442, *Proteus vulgaris* ATCC 13315, *Enterococcus hirae* ATCC 10541, have been exposed to the following conditions test:
- final test concentrations: as such (80% maximum concentration testable) – 50% – 25%
- contact time: 5 minutes
- test temperature: 30°C ± 1°C
A solution of skim milk has been used as interfering substance with final concentration of 1%.

On the basis of obtained results, in compliance with the assay validity criteria, the test substance SAGEWASH SANITIZER 250ppm results **BACTERICIDAL** with the concentration of 80% after 5 minutes of contact, using a 1% final concentration of skim milk, in compliance with the provisions of EN1656:2000.

See Experimental Report 2010/882 for more details.

INTRODUCTION

A study has been conducted on behalf of EUROFINS LABORATORIES Ltd in order to prove the bactericidal and effectiveness in compliance with European regulations.
The study was performed at the Test Facility Biolab S.p.A. of Vimodrone (MI) – via B. Buozzi n. 2 (Italy).

In this report:
- Doses are expressed as grams of the test substance for 100 milliliters of water (%)
- the number of microorganisms, counted in colony-forming units per milliliter of test solution, are expressed as colony-forming units per milliliter (cfu/ml).

EXPERIMENTATION	*START*	*END*	*RESEARCHER*
Bactericidal activity in suspension for veterinary field – Method of dilution-neutralization	Sept 17th 2010	Sept 20th 2010	C. Meroni

TERMS AND DEFINITIONS

Bactericidal: a chemical agent or formulation, which kills vegetative bacterial forms under certain conditions.

Bactericidal activity: capability of a product to produce a reduction in the number of bacteria under certain conditions.

BIBLIOGRAPHY

1. EN 1656 January 2000 - Chemical disinfectants and antiseptics -Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in veterinary field. Test methods and requirements (Phase 2/Step1).

FILING

The study program, all raw data are filed in the archives of Eurofins Biolab S.r.L for ten years after the issuing of the final report.
No retained sample will be kept.
At the end of the conservation period, the Sponsor may request an extension of the conservation of all or part of the products for a further period, or their restitution. A suitable agreement shall be drafted in this case.

PROCEDURES

All procedures used during this study are recorded in the Eurofins Biolab S.r,L Procedures Manual.

TEST SUBSTANCE

The test substance consists of a disinfectant product in veterinary field.

Product	SAGEWASH SANITIZER 250ppm
Stability	Not provided
Composition	See Attachment #1

ANALYSED SAMPLE

The analysed sample, representative of the test substance, consists of white powder compressed in a cylindrical form, protected by transparent colourless plastic covering. Before the start of the experimentation, the tablet was put in a spray gun that was also provided by the Sponsor. This device was connected to the tap water and according to the Sponsor instructions, the test solution was obtained after 10 minutes of supply.

Batch	Not provided
Manufacturing date	Not provided
Expiry date	Not provided
CoA	Not provided
Receiving	EUITVI-11232
Date	Sept 13th 2010
#ID	10.1286-S

The characterization of the test substance is under Sponsor responsibility.

Experimentation Report 2010/882 - EVALUATION OF BACTERICIDAL ACTIVITY IN SUSPENSION FOR VETERINARY FIELD – Method of dilution-neutralization (EN 1656:2000)

EXPERIMENTAL PROCEDURE

1. ASSAY SYSTEM

Microorganisms
The following test strains have been used:

Pseudomonas aeruginosa	ATCC 15442
Staphylococcus aureus	ATCC 6538
Enterococcus hirae	ATCC 10541
Proteus vulgaris	ATCC 13315

Maintenance
Bacterial strains have been kept frozen; before their use they have been transplanted on TSA slants and kept in a refrigerator at 4°C ± 2°C.

Preparation of the bacterial suspensions
Bacterial strains have been transplanted on TSA slants two times in a row and incubated at 37°C ±1°C for 18 hours.
In two hours from the beginning of the test the final culture has been suspended in diluent using glass beads and the suspension has been diluted in order to reach a count between 1.5 x10^8 and 5x10^8 cfu/ml.
The number of colonies has been determined doing the counting.

2. CULTURAL MEDIA AND REAGENTS

Tryptone Soya Agar (TSA) MERCK

Diluent

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

Sterile water for injection (WFI) EUROSPITAL

Interfering substances
The interfering substance used was prepared a concentration 10 times higher than the final concentration.

Skim milk 10% solution

Skim milk (free of antibiotics and additives)	10 g
Distilled water q.s. to	100 ml

Sterilization at 105°C for 30 minutes

The final concentration test of skim milk solution was 1%.

3. EQUIPMENT

Dry sterilization oven	MEMMERT
Steam autoclave	FEDEGARI
Thermostat	MEMMERT
pHmeter	BECKMAN
Vortex stirrer	VELP
Chronometer	ARBORE
Micropipettes	GILSON

4. EXPERIMENTAL DESIGN

Test temperature
The test was conducted at 30°C ± 1°C.

Concentrations and contact time
The test substance has been tested at the following conditions:
- final test concentrations: 80% (maximum concentration testable) – 50% – 25%
- contact time: 30 minutes

Interfering substances
A skim milk solution having a final concentration of 1% has been used as interfering substance.

Neutralizer
The following neutralizer was selected:

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	

5. ASSAY EXECUTION

5.1 Preliminary assay

A preliminary assay was conducted prior to the execution of the assay.
The test sample, the bacterial suspension and the interfering substances have been previously stabilized at the test temperature, while the neutraliser and the water have been previously stabilized at the temperature of 20°C ±1°C.

Count of bacterial suspensions
Bacterial suspensions have been diluted to a concentration between $6x10^2$ and $3x10^3$ cfu/ml.
This suspension has been additionally diluted with a decimal dilution and then the number of colonies, by inclusion in Agar, after an incubation period of 48 hours at 37°C ±1°C has been determined; then the $\mathbf{N_v}$ value has been calculated.

Preparation of the test substance
The test substance has been diluted to the maximum concentration tested in the assay.

Validation of experimental conditions
1 ml of interfering substances and 1 ml of bacterial suspension containing between $6x10^2$ and $3.0x10^3$ cfu/ml, have been put in a test tube.
The components have been left in contact for 2 minutes and after 8 ml of hard water have been added, left in contact at the temperature planned by the test for the longest contact time among the ones to be tested. At the end of the contact time the mixture has been vortex-stirred and a count in double by inclusion in Agar has been made.
After an incubation period of 48 hours at 37°C ±1°C the number of colony-forming units/ml of the mixture has been determined and the **A** value has been calculated.

Validation of neutralizer toxicity
For each test strain in a test tube have been mixed 8 ml of neutralizer, 1 ml of distilled water and 1 ml of bacterial suspension containing between $6x10^2$ and $3x10^3$ cfu/ml, left in contact, at the temperature of 20°C ±1°C, for 5 minutes. At the end of the contact time the mixture has been vortex-stirred and a count in double by inclusion in Agar has been made.
After an incubation period of 48 hours at 37°C ±1°C the number of colony-forming units/ml of the mixture has been determined and the **B** value has been calculated.

Validation of the dilution-neutralisation
For each test strain in a test tube have been mixed 1 ml of interfering substances, 1 ml of diluent and 8 ml of the test substance at the highest concentration used in the test, left in contact, at the temperature planned by the assay for the longest contact period. . At the end of the contact time, 1 ml of the mixture has been transferred into a test tube containing 8 ml of neutralizer; left in contact for 5 minutes. Afterwards 1 ml of bacterial suspension containing between $6x10^2$ and $3x10^3$ cfu/ml, has been added, left in contact, at the temperature of 20°C ± 1°C, for 30 minutes.
At the end of the contact time, the mixture has been vortex-stirred and a count in double by inclusion in Agar has been made.
After an incubation period of 48 hours at 37°C ±1°C the number of colony-forming units/ml has been determined and the **C** value has been calculated.

5.2 Assay

Count of the bacterial suspensions
Dilutions up to 10^{-6}, 10^{-7} of bacterial suspensions with a concentration between 1.5×10^8 and 5×10^8 cfu/ml have been prepared. A count in double by inclusion in Agar has been made. The number of colony-forming units per ml of the suspension after an incubation period of 48 hours at 37°C ±1°C has been determined; then the **N** value has been calculated.

Assay execution
The assay sample, the bacterial suspensions and the interfering substance have been previously stabilized at the test temperature, while the neutraliser and the water have been stabilized at the temperature of 20°C ±1°C.
The test substance has been prepared with a concentration 1.25 times the requested test concentrations.
For each bacterial strain and each concentration of the test substance a test tube has been prepared containing 1 ml of interfering substance and 1 ml of bacterial suspension with a concentration between $1.5 x10^8$ and $5.0x10^8$ cfu/ml, at the temperature planned by the test. After a contact period of 2 minutes 8 ml of test substance have been added and left in contact for the planned periods and at the test execution temperature.
After the contact time, 1 ml of the mixture has been put in a test tube containing 8 ml of neutralizer and 1 ml of distilled water. After 5 minutes ±10 sec. of neutralization the mixture has been stirred and a count in double by inclusion in Agar has been made.
After an incubation period of 48 hours at 37°C ±1°C the number of cfu/dish has been determined and the **Na** value has been calculated.

6. CALCULATION AND EXPRESSION OF THE RESULTS

The count is made using the number of colonies counted on both dishes.
Only dishes containing between 15 and 300 colonies have been used to calculate the results.
In the assay, where the number of cfu on all counted dishes was <15, the number of cfu/ml has been registered as $<1.5 \times 10^2$. Where the number of cfu on all counted dishes was >300 the number of cfu/ml has been registered as $>3.0 \times 10^3$.

Calculation of live units (cfu/ml)

Test bacterial suspension

For the test bacterial suspension the calculation of the bacterial count is made in the following way:

$$N(ufc/ml) = \frac{c}{(n_1 + 0.1n_2)d}$$

c	=	sum of colonies counted on both dishes
n_1	=	number of counted dishes of the first dilution
n_2	=	number of counted dishes of the second dilution
d	=	dilution factor correspondent to the first dilution

Assay and preliminary assay

For the assay (Na) and for the preliminary assay (A,B,C e N_v) the calculation of the bacterial count is done in the following way:

$$ufc/ml = \frac{c}{n \times V \times d}$$

c	=	sum of colonies counted on both dishes
n	=	number of counted dishes
V	=	volume used
d	=	dilution factor correspondent to the performed dilution

Calculation of vitality reduction

Vitality reduction was calculated for each organism and test concentration using the following formula:

$$R = \frac{N \times 10^{-1}}{Na}$$

ASSAY VALIDITY CRITERIA

N : must be included between 1.5×10^8 and 5×10^8 cfu/ml
N_V : must be included between 6×10^2 and 3×10^3 cfu/ml
B : is equal or higher than 0.05 times **Nv**
C : is equal or higher than 0.5 times **B**
A : is equal or higher than 0.05 times **Nv**

where:
N = count in cfu/ml of the bacterial suspension in the preliminary assay
A = count in cfu/ml of the solution to verify the experimental conditions
B = count in cfu/ml for the control of neutralizer toxicity
C = count in cfu/ml of neutralizer effectiveness.

The test substance is considered bactericidal when for each bacterial strain 10°C (30°C in the case of disinfectants for breasts) after 5 minutes of contact, it causes a vitality reduction of at least 10^5.

RESULTS

Preliminary assay
The N, A, B and C values for each bacterial strain are included in the validity criteria. The specific values are shown in Attachment #2.

Assay
The vitality reduction values at the different concentrations are shown below and in Attachment #2:

TEST MICROORGANISMS	CONTACT TIME AND TESTED CONCENTRATIONS		
	5 minutes		
	80%	50%	25%
***Staphylococcus aureus* ATCC 6538**	$>2.5\times10^5$	$<1.2\times10^4$	$<1.2\times10^4$
***Pseudomonas aeruginosa* ATCC 15442**	$>3.3\times10^5$	$<1.7\times10^4$	$<1.7\times10^4$
***Proteus vulgaris* ATCC 13315**	$>2.6\times10^5$	$<1.3\times10^4$	$<1.3\times10^4$
***Enterococcus hirae* ATCC 10541**	1.7×10^5	$<1.5\times10^4$	$<1.5\times10^4$

DEVIATIONS

No deviations occurred during the study.

CONCLUSIONS

On the basis of obtained results, in compliance with the assay validity criteria, the test substance SAGEWASH SANITIZER 250ppm results **BACTERICIDAL** with the concentration of 80% after 5 minutes of contact, using a 1% final concentration of skim milk, in compliance with the provisions of EN1656:2000.

ATTACHMENTS

ATTACHMENT	TITLE	NUMBER OF PAGES
N.1	COMPOSITION	1
N.2	RAW DATA EXPERIMENTATION 2010/882	2

Eurofins Biolab S.r.l.
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SAGEWASH MASTER PARTS LIST

SWS-01	Sagewash Sanitizer
SWS-02	Masterpack 6 x SWS-01
SWST-01	HTH Calcium Hypochlorite Tablets – One Six Pack
SWST-02	HTH Calcium Hypochlorite Tablets – Six Six Packs
SWST-COMBO	SWS-01 + SWST-01
SWST-COMBO-M	Masterpack – six x COMBO
SWS-104	Female Quick Connector
SWS-105	Male Quick Connector
SWS-106	Flow Control Handle
SWS-107	Pivot Connector
SWS-108	**Hydro Body**
SWS-109	**Hydro Venturi Plate**
SWS-110	**Hydro Venturi Tube**
SWS-111	**Tablet Container**
SWS-112	**Adjustable Nozzle**
SWS-113	Sweep Nozzle
SWS-114	Stainless Steel (SS) Fasteners – Set of Five
SWS-115	Hydro Seal

bold items not available separately

eurofins | biolab	**Prova quantitativa in sospensione per la valutazione dell'attività battericida dei disinfettanti chimici e antisettici utilizzati nel campo veterinario**
	(Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in veterinary areas)
Mod. PS/MIC/011.E	**Norma *(Standard)*** : UNI EN 1656:2000 - phase2/step1
Rev.3	Pagina 1 di 2 *(Page 1 of 2)*

Data inizio *(Started on)*: 17/09/10

ID. studio *(ID. Study)*: 2010/882 SAM

ID. campione *(ID. sample)*: 10.1286-S

Test Microrganisms		N		Nv		A		B		C	
	Dil.	*cfu/plate*	*cfu/plate*	*cfu/plate*	*cfu/plate*	*cfu/plate*	*cfu/plate*	*cfu/plate*	*cfu/plate*	*cfu/plate*	*cfu/plate*
Staphylococcus aureus ATCC6538	-6	>300	>300	78	68	82	67	54	58	63	64
	-7	31	43								
		3.7E+08		7.3E+02		7.5E+01		5.6E+01		6.4E+01	
Pseudomonas aeruginosa ATCC15442	-6	>300	>300	96	102	86	108	92	94	97	86
	-7	51	48								
		5.0E+08		9.9E+02		9.7E+01		9.3E+01		9.2E+01	
Proteus vulgaris ATCC13315	-6	>300	>300	86	92	80	76	74	72	68	78
	-7	36	43								
		4.0E+08		8.9E+02		7.8E+01		7.3E+01		7.3E+01	
Enterococcus hirae ATCC10541	-6	>300	>300	106	102	96	102	104	108	98	95
	-7	46	43								
		4.5E+08		1.0E+03		9.9E+01		1.1E+02		9.7E+01	

N: conteggio sospensione batterica ufc/ml *(N: count of the bacterial suspension cfu/ml)*
Nv: conteggio sospensione batterica per il saggio preliminare ufc/ml *(Nv: count of the bacterial suspension in the preliminary assay cfu/ml)*
A: conteggio nella convalida delle condizioni sperimentali ufc/ml *(A: count in the experimental conditions verification solution cfu/ml)*
B: conteggio nel controllo di tossicità del neutralizzante ufc/ml *(B: count in the neutraliser toxicity control cfu/ml)*
C: conteggio nel controllo dell'efficacia del neutralizzante ufc/ml *(C: count in the neutraliser effectiveness control cfu/ml)*

eurofins | biolab	**Prova quantitativa in sospensione per la valutazione dell'attività battericida dei disinfettanti chimici e antisettici utilizzati nel campo veterinario**
	(Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in veterinary areas)
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Rev.3	Pagina 2 di 2 *(Page 2 of 2)*

Data inizio *(Started on)*: 17/09/10

ID. studio *(ID. Study)* : 2010/882 SAM

ID. campione *(ID. sample)* : 10.1286-S

Test Microrganisms	***CONCENTRATIONS AND CONTACT TIMES (cfu/plate)***					
	80%	**5 MIN**	**50%**	**5 MIN**	**25%**	**5 MIN**
Staphylococcus aureus ATCC6538	0	0	>300	>300	>300	>300
	Na=	<1,5E+02	**Na=**	>3,0E+03	**Na=**	>3,0E+03
	R=	> 2.5E+05	**R=**	< 1.2E+04	**R=**	< 1.2E+04
Pseudomonas aeruginosa ATCC15442	0	0	>300	>300	>300	>300
	Na=	<1,5E+02	**Na=**	>3,0E+03	**Na=**	>3,0E+03
	R=	> 3.3E+05	**R=**	< 1.7E+04	**R=**	< 1.7E+04
Proteus vulgaris ATCC13315	0	0	>300	>300	>300	>300
	Na=	<1,5E+02	**Na=**	>3,0E+03	**Na=**	>3,0E+03
	R=	> 2.6E+05	**R=**	< 1.3E+04	**R=**	< 1.3E+04
Enterococcus hirae ATCC10541	19	34	>300	>300	>300	>300
	Na=	2.7E+02	**Na=**	>3,0E+03	**Na=**	>3,0E+03
	R=	1.7E+05	**R=**	< 1.5E+04	**R=**	< 1.5E+04

Na = count of the test mixture (cfu/ml)
R = vitality reduction

Sigla tecnico *(Technician signature)*:

Data fine *(Finished on)*: 20/09/10

Sigla Approvazione *(Approval signature)*:

Data *(Date)* : 20/09/10