Final report 2011/2134 AMi

SUSPENSION BASIC BACTERICIDAL EFFECTIVENESS AGAINST Salmonella enterica AND Salmonella typhimurium ON VIRES 5

Study Program No: 2011/2134 AM

Contract No: PARA2011040201

Sponsor: VIRES5 BVBA
BREDABAAN 926
2990 WUUSTWEZEL (BELGIUM)

Study monitor: BSL BIOSERVICE SCIENTIFIC LABORATORIES GmbH
BEHRINGSTRASEE 6/8
82152 PLANEGG

Test substance: VIRES 5

Director of the Study: .....
(Laura Brambilla)

Released on: Jan 09th 2012

Eurofins Biolab S.r.l.
Società con Socio unico sottoposta
a direzione e coordinamento della società
Eurofins Scientific Italia S.r.l.
parte di Eurofins Scientific Group
http://pharma.eurofins.com/

Via Bruno Buozzi, 2
20090 Vimodrone (MI) - Italia
Tel. + 39-022507151
Fax + 39-0225071599
biolab@eurofins.com
www.eurofins.it www.biolab.it

C.SOC. € 100.000 i.v.
P. IVA 00762140960
C.F. 03765750157
REA MI 966696
D-U-N-S 429117112
CIT005 4-385

INDEX

COMPLIANCE WITH GOOD LABORATORY PRACTICE

I the undersigned declare that the studies described in this report have been conducted under my supervision and in compliance with the following standards of Good Laboratory Practice:

- OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring - OECD principles of Good Laboratory Practice (as revised in 1997) – Environment Directorate – Organisation for Economic Co- Operation and Development , Paris 1998.

- Legislative decree n. 50 of March the 2nd, 2007. Enforcement of Community Directives 2004/9/CE e 2004/10/CE, concerning the inspection and verification of Good Laboratory Practice and the drawing of the legislative, regulatory and administrative dispositions relative to the application of Good Laboratory Practice rules, to the control of their application on the assays performed on the chemical substances (GU n.86 of April the 13th, 2007).

- Decree of the Italian Ministry of Health October the 12th 2010, certification N. 121/2010 authorizing Eurofins Biolab S.r.l. to perform analyses in compliance with the principles of good laboratory practices (http://www.biolab.it).

There were no circumstances that may affected the quality or integrity of the study

Laura Brambilla

Study Director
(Laura Brambilla)

Jan 09th 2011
DATE

QUALITY ASSURANCE STATEMENT

The study was assessed for compliance with the approved study program and the Standard Operating Procedures of Eurofins Biolab Srl.
The study and/or the test facility were periodically inspected by the Quality Assurance unit according to the corresponding SOPs. These inspections and audit were carried out by the Quality Assurance unit, personnel independent of staff involved in the study.

The undersigned hereby certifies the dates on which the inspections have been carried out and reported to the Director of the Study and to Eurofins Biolab's S.r.l. Management:

PHASE OF STUDY	DATE OF INSPECTION / REPORTING
Pre-experimental period	//
Experimental period	//
Post-experimental period	//
Documentation:	
- Study program	December, 20th 2011
- Amendment #1 to the Study program	December, 22nd 2011
- Raw data	January, 9th 2012
- Final report	January, 9th 2012

QA MANAGER
(Patrizia Custode)

Jan. 09th, 2012
DATE

Eurofins Biolab S.r.l.
Società con Socio unico sottoposta
a direzione e coordinamento della società
Eurofins Scientific Italia S.r.l.
parte di Eurofins Scientific Group
http://pharma.eurofins.com/

Via Bruno Buozzi, 2
20090 Vimodrone (MI) - Italia
Tel. + 39-022507151
Fax + 39-0225071599
biolab@eurofins.com
www.eurofins.it www.biolab.it

C.SOC. € 100.000 i.v.
P. IVA 00762140960
C.F. 03765750157
REA MI 966696
D-U-N-S 429117112
CIT005 4-385

SUMMARY

An assay was conducted on test substance VIRES 5 in order to determine its basic bactericidal effectiveness against *Salmonella enterica subsp. enterica abony* and *Salmonella typhimurium* for the uses for which the product is specifically intended.

The bactericidal effectiveness has been evaluated with the following experimentation:
- **phase 1, basic bactericidal activity suspension test for chemical disinfectants and antiseptics** in which two additional bacterial strains, *Salmonella enterica subsp. enterica abony* NCTC 6017 and *Salmonella typhimurium* ATCC 13311, have been exposed to the test substance in the following conditions:
- final concentrations: 80% (maximum concentration testable) – 50% – 25%.
- contact times: 5 - 15 minutes
- test temperature: 20°C±1°C

On the basis of the obtained results, in compliance with the **assay validity criteria, the test substance VIRES 5 causes a reduction >5Log** against all the test strains with the concentration of 50% after 5 minutes of contact and with the concentration of 25% after 15 minutes of contact, in compliance with the provisions of EN 1040:2005.

See Experimental Report 2011/2134 for more details.

INTRODUCTION

A study was conducted on behalf of VIRES5 BVBA in order to demonstrate the basic bactericidal effectiveness against *Salmonella enterica subsp. enterica abony* and *Salmonella typhimurium*, in accordance with European regulations and Sponsor requirements.
The study was performed at the Test Facility Eurofins Biolab S.r.l. of Vimodrone (MI) – via B. Buozzi n. 2 (Italy).

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

EXPERIMENTATION	*START*	*END*	*RESEARCHER*
Basic bactericidal activity suspension test for chemical disinfectants and antiseptics	Dec 28th 2011	Dec 30th 2011	C. Meroni

On December 22nd, 2011 an amendment to the Study Program 2011/2134 AMi was issued in order to add some information about the analyzed sample provided by the Sponsor and to correct the foreseen study ending date for a clerical error.

TERMS AND DEFINITIONS

Bactericidal: a chemical agent or formulation capable of killing vegetative bacteria under given conditions.

Bactericidal activity: the ability of a product of reducing the number of bacteria under given conditions.

REFERENCES

EN 1040, December 2005 - Chemical disinfectants and antispetics – Quantitative suspension test for the evaluation of basic bactericidal activity of chemical disinfectants and antiseptics - Test method and requirements (phase 1).

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.
The retained sample will be not kept because consists in an aliquot of the test substance of the study 2011/2133 AMi.
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 to improve water quality in veterinary field.

Name	VIRES 5
Product	Purified water with increased OrpV value
Stability	3 years
Composition	Hypochlorous acid (CAS-No: 7790-92-3) <1% Water (CAS-No: 7732-18-5) 50-100% Other additives <10%

ANALYSED SAMPLE

The analysed sample, representative of the test substance, consists in an aliquot of the test substance of the study 2011/2133 AM that consist in a transparent colourless liquid contained into a plastic transparent container.

Batch	23107
Code	05231
Manufacture date	July 2011
Expiry date	July 2014
CoA	Not provided
Receiving n.	EUITVI-21918
Receiving date	Dec 14th 2011
Id number	11.3173-S

The characterisation of the test product is under Sponsor's responsibility

Experimental Report 2011/2134 – EVALUATION OF BASIC BACTERICIDAL ACTIVITY IN SUSPENSION- DILUTION – NEUTRALIZATION METHOD (EN1040:2005)

EXPERIMENTAL PROCEDURE

1. ASSAY SYSTEM

Microorganisms
The following test strains were used:

Salmonella enterica subsp. enterica abony	NCTC 6017
Salmonella typhimurium	ATCC 13311

Conservation
The bacterial strains were kept frozen; before they were used, they were transplanted on TSA slants and kept in a refrigerator at 5°C ±3°C.

Preparation of the bacterial suspensions
The bacterial strains were transplanted on TSA slants twice consecutively and incubated at 37°C ±1°C for 18 hours.
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 about 1.5×10^8-5×10^8 cfu/ml.
The colony number was determined performing the counting.

2. COLTURE MEDIA AND REAGENTS

Tryptone Soya Agar (TSA)		MERCK
Diluent		
Tryptone, pancreatic digestion of casein	1.0 g	MERCK
NaCl	8.5 g	MERCK
Distilled water q.s. to	1000 ml	
Water for injections (WFI)		EUROSPITAL

3. EQUIPMENT

Dry sterilization oven	MEMMERT
Steam autoclave	FEDEGARI
Incubator	BINDER
pHmeter	BECKMAN
Vortex stirrer	VELP
Chronometer	GHIARONI
Micropipettes	GILSON
Spectrophotmeter	SHIMADZU

4. EXPERIMENTAL DESIGN

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

Experimental conditions
The test was performed at the following conditions:
- final concentrations: 80% (maximum concentration testable) – 50% – 25%
- contact times: 5 - 15 minutes

The test substance was prepared with a concentration 1.25 times higher than the concentration required to perform the test.

Neutralizer
The following neutraliser 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. EXECUTION OF THE ASSAY

5.1 *Preliminary assay*

The bacterial suspensions had previously been stabilized at the test temperature while the neutralizer and the water had been stabilized at 20°C ± 1°C .

Count of the validation bacterial suspensions
The bacterial suspensions were diluted to a concentration of about $3.0x10^2$ to $1.6x10^3$ cfu/ml.
This suspension was further diluted using a decimal dilution and the number of colonies was then determined through inclusion in agar after 48 hours' incubation period at 37°C ± 1°C. **Nv** value was then calculated.

Preparation of test substance
The test substance was diluted at the highest concentration tested during the assay.

Validation of the experimental conditions
1 ml of sterile water and 1 ml of bacterial validation suspension containing $3.0x10^2$ to $1.6x10^3$ cfu/ml were placed in a test tube.
The components were left in contact for 2 minutes; then 8 ml of water were added and left in contact at the temperature adopted during the assay for the longest period to be tested.
At the end of the contact time, the mixture was vortex-stirred and a double count was performed by inclusion in agar.
The number of colony-forming units per ml of the mixture was determined following incubation for 48 hours at 37°C ± 1°C and **A** was calculated.

Validation of the neutralizer non-toxicity
For each test strain, 8 ml of neutraliser, 1 ml of distilled water and 1 ml of bacterial validation suspension ($3.0x10^2$ to $1.6x10^3$ cfu/ml) were mixed in a test tube and left in contact for 5 minutes at 20°C ± 1°C temperature. At the end of the contact time, the mixture was vortex-stirred and a double count was performed by inclusion in agar.
The number of colony-forming units per ml mixture was determined following incubation for 48 hours at 37°C ±1°C and **B** was calculated.

Validation of the dilution-neutralization test

For each test strain, 1 ml of sterile water, 1 ml of the diluent and 8 ml of the test substance at the highest tested concentration were mixed in a test tube and left in contact at the temperature adopted during the assay for the longest period chosen. At the end of the contact time, 1 ml of the mixture was transferred into a test tube containing 8 ml of neutraliser and left in contact for 5 minutes. Then 1 ml of bacterial validation suspension $3.0x10^2$ to $1.6x10^3$ cfu/ml were added and left in contact for 30 minutes at 20°C ±1°C. At the end of the contact time, the mixture was vortex-stirred and a double count was performed by inclusion in agar.

The number of colony-forming units per ml mixture was determined following incubation for 48 hours at 37°C ±1°C and **C** value was calculated.

5.2 *Assay*

Counting of bacterial suspension

The bacterial suspension showing concentrations in a $1.5x10^8$ to $5x10^8$ cfu/ml range were diluted up to 10^{-6} and 10^{-7}.

A double counting through inclusion in agar was performed. The number of colony-forming units per ml of the suspension was determined following incubation for 48 hours at 37°C ± 1°C and **N** value was calculated.

Assay performing

The assay sample, the bacterial suspensions, the neutraliser agent and the water had previously been stabilised at the test temperature of 20°C ±1°C.

For each bacterial strain and for each concentration of the test substance, one test tube containing 1 ml of sterile water and 1 ml of bacterial test suspension showing concentrations in a $1.5x10^8$ to $5.0x10^8$ cfu/ml range, was prepared at the temperature adopted during the assay.

After 2 minutes of contact, 8 ml of test substance were added and left in contact again for the selected times at the test temperature.

At the end of the contact time (5 minutes), 1 ml of mixture was transferred into a test tube containing 8 ml of neutraliser and 1 ml of distilled water.

After 5 minutes ±10 sec. of neutralization procedure, the mixture was vortex-stirred and a double count was performed by inclusion in agar.

The number of cfu per plate was determined following incubation for 48 hours at 37°C ± 1°C, and **Na** value was then calculated.

6. CALCULATION AND EXPRESSION OF THE RESULTS

Calculation of the viable count (cfu/ml)

The count was performed using the number of colonies counted on both plates.

Only the plates showing a number of colonies included in a 15-300 range were used to perform the result calculation. A deviation of 10% is accepted, so the limits are 14 and 330.

In the assay, where the number of cfu on every plate counted is <14, the number of cfu/ml should be recorded as $<1.4 \times 10^2$.

Where the number of cfu on every plate counted is >330, the number of cfu/ml should be recorded as $>3.3 \times 10^3$.

Test suspension

The calculation of the bacterial count for the suspension test (**N**) is performed applying the following formula:

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

where:

c	=	sum of colonies counted on both plates
n_1	=	number of counted plates in the lower dilution
n_2	=	number of counted plates in the highter dilution
d	=	dilution factor corresponding to the lower dilution

Assay and preliminary assay

For the calculation of the bacterial count for the assay (**Na**) and for the preliminary assay (**A**, **B**, **C** and **N_V**) is performed applying the following formula:

$$cfu/ml = \frac{C}{n \times V \times d}$$

where:

C	=	total of colonies counted on both plates
n	=	number of counted plates
V	=	volume used
d	=	dilution factor corresponding to the relevant dilution

Calculation of vitality reduction

Vitality reduction is expressed in logarithm and was calculated for each organism and test concentration using the following formula:

$$\lg R = \lg N_0 - \lg Na$$

where:

R	=	Reduction of vitality
N_0	=	N/10
Na	=	bacterial counting for the test mixture at the end of the contact time

ASSAY VALIDITY CRITERIA

Verify the following:

N: must be included between $1.5x10^8$ and $5.0x10^8$ cfu/ml

N_V: must be included between $3.0x10^2$ and $1.6x10^3$ cfu/ml

A, B, C: must be equal to, or higher than 0.05 times **Nv**

Control Of Weighted Mean Counts: quotient is not lower than 5 and not higher than 15

where:

N: count of cfu/ml in the bacterial test suspension

N_V: count of cfu/ml in the bacterial validation suspension in the preliminary assay

A: count of cfu/ml in the experimental conditions validation

B: count of cfu/ml in the neutraliser toxicity control

C: count of cfu/ml of the neutraliser effectiveness

Weighted Mean Counts: weighted mean of two subsequent dilutions (e.g. "*N*")

The test substance is considered bactericidal when the bacterial count for each bacterial strain is reduced by at least 5 Log following 5 minutes' contact at 20°C.

The test substance is considered effective against the test microorganisms when the bacterial count for each bacterial strain is reduced by at least 5 Log following the chosen contact time at 20°C.

RESULTS

Preliminary assay
The N, N_v, A, B, C and the control of weighted mean counts of each bacterial strain comply with the validity criteria. The specific values are shown in Attachment #1.

Assay
The vitality reduction values at the different concentrations tested are shown below and in the Attachment #1:

TEST MICROORGANISMS	CONTACT TIME AND TESTED CONCENTRATIONS		
	80%	**50%**	**25%**
	5 minutes		
***Salmonella enterica subsp. enterica abony* NCTC 6017**	>5.55	>5.55	4.85
***Salmonella typhimurium* ATCC 13311**	>5.51	>5.51	4.98
	15 minutes		
***Salmonella enterica subsp. enterica abony* NCTC 6017**	>5.55	>5.55	>5.55
***Salmonella typhimurium* ATCC 13311**	>5.51	>5.51	>5.51

DEVIATIONS

The study did not undergo deviations compared to the study program.

CONCLUSIONS

On the basis of the obtained results, in compliance with the **assay validity criteria, the test substance VIRES 5 causes a reduction >5Log** against all the test strains with the concentration of 50% after 5 minutes of contact and with the concentration of 25% after 15 minutes of contact, in compliance with the provisions of EN 1040:2005.

ATTACHMENTS

ATTACHMENT	TITLE	NUMBER OF PAGES
N.1	EXCEL ELABORATION OF EXPERIMENTATION 2011/2134	3

eurofins | biolab	**Prova quantitativa in sospensione per la valutazione dell'attività battericida di base dei disinfettanti chimici e antisettici** *(Quantitative suspension test for the evaluation of basic bactericidal activity of chemical disinfectants and antiseptics)*
Mod. PS/MIC/001.E	**Norma *(Standard)*** : EN 1040:2005- phase1/step1
Rev.3	Pagina 1 di 3 *(page 1 of 3)*

ID. studio *(ID. Study)* : 2011/2134 AM

ID. campione *(ID. sample)* : 11.3173-S

Data inizio *(Started on)*: 28/12/11

Microrganismi test *(Test Microrganisms)*		**N**		**Nv**		**A**		**B**		**C**	
	Dil	ufc/piastra *(cfu/plate)*	ufc/piastra *(cfu/plate)*	ufc/piastra *(cfu/plate)*	ufc/piastra *(cfu/plate)*	ufc/piastra *(cfu/plate)*	ufc/piastra *(cfu/plate)*	ufc/piastra *(cfu/plate)*	ufc/piastra *(cfu/plate)*	ufc/piastra *(cfu/plate)*	ufc/piastra *(cfu/plate)*
Salmonella ent subps enterica abony NCTC 6017	-6	>330	>330	80	100	90	87	76	95	94	90
	-7	54	46								
		8.70	VALIDO (VALID)	9.0E+02		8.9E+01		8.6E+01		9.2E+01	
Salmonella typhimurium ACC 13311	-6	>330	>330	84	126	65	110	92	80	72	84
	-7	39	52								
		8.66	VALIDO (VALID)	1.1E+03		8.8E+01		8.6E+01		7.8E+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)*

ID. studio *(ID. Study)* : 2011/2134 AM

ID. campione *(ID. sample)* : 11.3173-S

Data inizio *(Started on)*: 28/12/11

Microrganismi test *(Test Microrganisms)*		**CONCENTRAZIONI E TEMPI DI CONTATTO ufc/piastra *(CONCENTRATIONS AND CONTACT TIMES cfu/plate)***					
		80.0%	**5 min**	**50.0%**	**5 min**	**25.0%**	**5 min**
Salmonella ent subps enterica abony NCTC 6017		0	0	0	0	76	64
		Na= < 2.15		**Na=** < 2.15		**Na=**	2.85
		R= > 5.55		**R=** > 5.55		**R=**	4.85
Salmonella typhimurium ACC 13311		0	0	0	0	50	46
		Na= < 2.15		**Na=** < 2.15		**Na=**	2.68
		R= > 5.51		**R=** > 5.51		**R=**	4.98

Na = conteggio della miscela test ufc/ml *(Na = count of the test mixture cfu/ml)*
R = riduzione della vitalità *(R = vitality reduction)*

Sigla tecnico *(Technician signature)*:

Data fine *(Finished on)*: 30/12/11

Sigla Approvazione *(Approval signature)*:

Data *(Date)* : 30/12/11

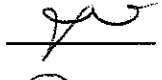
ID. studio *(ID. Study)* : 2011/2134 AM

ID. campione *(ID. sample)* : 11.3173-S

Data inizio *(Started on)*: 28/12/11

Microrganismi test *(Test Microrganisms)*		**CONCENTRAZIONI E TEMPI DI CONTATTO ufc/piastra *(CONCENTRATIONS AND CONTACT TIMES cfu/plate)***					
		80.0%	**15 min**	**50.0%**	**15 min**	**25.0%**	**15 min**
Salmonella ent subps enterica abony NCTC 6017		0	0	0	0	3	5
		Na= < 2.15		**Na=** < 2.15		**Na=** < 2.15	
		R= > 5.55		**R=** > 5.55		**R=** > 5.55	
Salmonella typhimurium ACC 13311		0	0	0	0	2	0
		Na= < 2.15		**Na=** < 2.15		**Na=** < 2.15	
		R= > 5.51		**R=** > 5.51		**R=** > 5.51	

Na = conteggio della miscela test ufc/ml *(Na = count of the test mixture cfu/ml)*
R = riduzione della vitalità *(R = vitality reduction)*

Sigla tecnico *(Technician signature)*: 

Data fine *(Finished on)*: 30/12/11

Sigla Approvazione *(Approval signature)*: 

Data *(Date)* : 30/12/11