

Final report 2009/690 AMi

VIRUCIDAL EFFECTIVENESS ON IGEN HYGIENIC HANDRUB

Study Program No: 2009/690 AM

Contract No: PPR12009019202

Sponsor: EUROSPITAL SPA
VIA FLAVIA, 122
34147 TRIESTE

Test substance: IGEN



Authorized accurate copy

Number

001

Director of the Study: *Laura Brambilla*
(Dr. L. Brambilla)

Date of issue: *27th Oct 2009*

This report cannot be partially reproduced without written approval of the Test Facility.

INDEX

INDEX	2
COMPLIANCE WITH GOOD LABORATORY PRACTICE.....	3
QUALITY ASSURANCE STATEMENT	4
SUMMARY	5
INTRODUCTION	6
TERMS AND DEFINITIONS.....	6
REFERENCES	6
FILING	6
PROCEDURES.....	6
TEST SUBSTANCE.....	7
ANALYZED SAMPLE	7
Experimental Report 2009/690.A1 – QUANTITATIVE SUSPENSION TEST FOT THE EVALUATION OF VIRUCIDAL ACTIVITY AGAINST Influenza A virus (H1N1), (EN14476, April 2005+A1, October 2006).....	8
EXPERIMENTAL PROCEDURE.....	8
ASSAY VALIDITY CRITERIA	11
RESULTS	11
DEVIATIONS	12
CONCLUSIONS.....	12
ATTACHMENTS.....	12

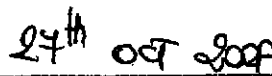
COMPLIANCE WITH GOOD LABORATORY PRACTICE

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:

- Organisation for Economic Co-operation and Development, ISBN 92-64-12367-9, Paris 1982.
- Italian Ministerial Decree of June 26th, 1986 "Application of Good Laboratory Practice on chemical substances and criteria for issuing the authorizations foreseen by Presidential decree n. 927/81 art. 6".
- Legislative decree of January 27th, 1992, n. 120. Enforcement of Community Directives n. 88/320/CEE, n. 90/18/CEE and following decree of 5 August 1999 Enforcement of Community Directives n. 1999/11/CE and 1999/12/CE concerning inspection and verification of Good Laboratory Practice.
- Directive 2004/10/CE issued by European Parliament and Council dated February 11th concerning 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.
- Decree of the Italian Ministry of Health March 10th, 2005, Certification 158/245/2005 authorizing Biolab S.p.A. to perform analyses in compliance with the principles of good laboratory practices.
- 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).



STUDY DIRECTOR
Dr. L. Brambilla



DATE

QUALITY ASSURANCE STATEMENT

I the undersigned below certify the dates on which the inspections on this study have been done and the relevant observations have been reported to the Director of the Study and to the assay centre Management:

STUDY PHASE	CHECK DATE / NOTIFICATION
Pre-experimental	/
Experimental	/
Post- experimental	/
Documentation: Study program Raw data Final report	Oct 15 th 2009 Oct 27 th 2009 Oct 27 th 2009

Carolina Ronaghi
 QA BPL
 Dr. Carolina Ronaghi

Oct 27th 2009
 DATE

	Pharma Services	Test Facility Certification M.S. 85/2009	Report No.:	2009/690 AMI
			Version:	English
			Page:	5 of 12
			Print date:	Oct 27 th , 2009

SUMMARY

A series of assays were performed on the test substance IGEN to determine the virucidal effectiveness for the specific uses provided for the product (hygienic handwash and handrub).

For this purpose the following test was performed:

- **virucidal activity in suspension. Filtration method** in which one viral suspension of *Human Influenza virus H1N1*, was incubated with the test substance in the following conditions test:

- final concentrations: 80% (maximum concentration testable) – 50% - 25%
- contact times: 1 minute
- temperature test: 20°C ±1°C.

This test was conducted using as interfering substance a Phosphate Buffer Solution (PBS).

After inactivation of disinfectant activity for each contact time, the virus suspension was inoculated on MDCK monolayer cells (Madin Darby canine kidney cells), epithelial cells from kidney dog.

Cell cultures were held on 37°C ± 1°C and CO₂ 5% for 6 day and the virus positive cells culture were identify by HA-testing (Haemoagglutinine).

Before checking virucidal activity, a test to validate the method based on 5 steps was performed, in order to verify the compliance to the EN 14476 requirements:

1. CHECK OF CYTOTOXICITY OF THE TEST SUBSTANCE
2. ASSAY OF VIRAL ACTIVITY (virus titration)
3. CHECK OF CELLULAR SENSITIVITY TO VIRUS
4. CHECK OF SUPPRESSION OF DISINFECTANT ACTIVITY
5. CHECK OF VIRUS CONTROL VITALITY (Virus control)

Due to the level of cytotoxicity observed at test concentrations and the virus titration, the test substance has been subjected to filtration S400 HR Columns MicroSpin™ before contact with the cells in order to reduce the cytotoxicity level and permit to evaluate a logarithmic reduction ≥ 4 Log according to the requirements of acceptability criteria of the test.

The entire test was carried out by means of filtration, with positive results.

The requirements of validity assay criteria were satisfied.

On the basis of obtained results, in the experimental adopted conditions, the test substance IGEN **causes a Log reduction ≥ 4 Log** against *Influenza A virus* (subtype H1N1) at the test concentrations of 80% (maximum concentration testable) and 50%, after 1 minute of contact time, in compliance with EN 14476: April 2005+A1:2006.

See *Experimental Report 2009/690.A1* for more details.

 eurofins	Pharma Services	Test Facility Certification M.S. 85/2009	Report No.:	2009/690 AMI
			Version:	English
			Page:	6 of 12
			Print date:	Oct 27 th , 2009

INTRODUCTION

A study was conducted on behalf of EUROSPIRAL SPA to demonstrate the disinfectant effectiveness, in accordance with European regulations.

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

EXPERIMENTATION	START	END	RESEARCHER
Virucidal activity in suspension. Filtration method (2009/690.A1)	October 09 th 2009	October 15 th 2009	F. Faccioli

In this report:

- doses are expressed as grams of the test substance per 100 millilitres of water (%)
- the virus titres are expressed as TCID₅₀: 50% infecting dose of a virus suspension or that dilution of the virus suspension that induce a CPE in 50% of cell culture units.

TERMS AND DEFINITIONS

Virucidal: a chemical agent or a formulation that inactivates viruses under certain conditions.

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

REFERENCES

1. EN 14476 – Chemical disinfectants and antiseptics – Quantitative test in virucidal suspension for chemical disinfectants and antiseptics used in human medicine – Test method and requirements (phase2, step1) April 2005.
2. Addendum A1 – October 2006

FILING

The study program, with possible modifications, raw data, a copy of final report and its possible revisions will be stored in the archives of Biolab S.p.A. for 10 years from the issuing of the final report.

The control sample of the test substance will be kept until June 2012 according to the expiry date provided by the Sponsor.

The Sponsor upon drafting a suitable contract, may request an extension of the period of conservation of the substances (or of parts of them), or their restitution.

PROCEDURES

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

 eurofins	Pharma Services	Test Facility Certification M.S. 85/2009	Report No.:	2009/690 AMI
			Version:	English
			Page:	7 of 12
			Print date:	Oct 27 th , 2009

TEST SUBSTANCE

The test substance is an hygienic handrub disinfectant.

Name: IGEN

Stability: 3 anni

Composition declared on the label:

COMPOSANTS	AMOUNT (g)
Triclosan	0.05
Ethyl alcohol	70
Emollients, water and excipients	29.95

ANALYZED SAMPLE

The analysed sample, representative of the test substance, consists of transparent fluid gel contained into transparent plastic container.

Batch	0907370
Manufacturing date	June 2009
Expiry date	June 2012
Receiving	EUITVI-4337
Date	September 14 th 2009
#ID	09-1060-S

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

**Experimental Report 2009/690.A1 – QUANTITATIVE SUSPENSION TEST FOT
THE EVALUATION OF VIRUCIDAL ACTIVITY AGAINST Influenza A virus (H1N1),
(EN14476, April 2005+A1, October 2006)**

EXPERIMENTAL PROCEDURE

1. ASSAY SYSTEM

Virus

Influenza A virus (H1N1) ATCC VR-1469

Classification: Orthomyxoviridae, Influenzavirus A.

Original Source: clinical specimen – human.

Depositors: ATCC & M. Coleman.

Cellular culture

MDCK (NBL-2) ATCC-CCL-34

Original Source: MDCK cell line was derived from a kidney of an apparently normal adult female dog.

Depositors: Madin, NB Darby.

2. MEDIA AND REAGENTS

Culture Medium

EMEM: Eagle's minimal essential medium

FBS Fetal Bovine Serum

Erythrocytes (Type 0)

BIOWHITTAKER - LONZA

BIOWHITTAKER - LONZA

Lampire Biological Laboratories

Culture Medium

PBS Phosphate Buffer Saline

WFI Water for injection

BIOWHITTAKER - LONZA

EUROSPITAL

3. EQUIPMENT AND MATERIALS

- Incubator
- CO₂ incubator
- Vortex
- Chronometer
- Micropipettes
- Microdishes 96 wells
- Inverted microscope
- Water bath
- Centrifuge MicroSpin
- Columns MicroSpin™ S400 HR

ARBORE
PBI
VELP
ARBORE
GILSON
CAMBREX
LEITZ
ARBORE
EPPENDORF
GE HEALTHCARE

4. EXPERIMENTAL DESIGN

Test temperature

The test was conducted at 20°C ±1°C.

Concentration

The test substance was used not diluted (100%) and diluted into sterile water at the following concentrations:

final test concentrations of 80% (maximum concentration testable, 50% and 25%.

The test substance was prepared at concentrations 1.25 times higher than the concentrations requested by the test.

Contact times

The following contact time was used: 1 minute.

Interfering substance

PBS (Phosphate Buffer Saline).

5. ASSAY EXECUTION**Check of cytotoxicity of the test substance (Preliminary assay)**

Assuming a likely high level of cytotoxicity of the test substance, two cytotoxicity tests were conducted: one for the test substance to the concentration of the test itself, and another on the test substance at the test concentration after filtration with S400 HR columns MicroSpin™.

A 8 ml of the test sample were mixed with 2 ml of sterile water two times. One test mixture was treated with ultrafiltration technique, the other not, then of each one, serial dilutions were performed and 0.1 ml were transferred, sixfold in 96 wells microplate containing the cellular confluent monolayer (>90%) and incubated at 37°C ±1°C for 1 hour, then 0.1 ml of culture Medium were added. Immediately after the addition of the test substance and daily for 2 days, the cellular culture was observed with inverted microscope to detect any cytopathic effect (CPE) due to test substance.

Assay of viral activity (virus titration)

Two virus titration were performed, one for the virus suspension as such and one upon the virus suspension after filtration with S400 HR columns MicroSpin™. Of each one, serial dilutions were prepared, as per the following scheme: 0.5 ml of viral suspension was added to 4.5 ml ice cold culture Medium. 0.1 ml were transferred sixfold in microplate with 96 wells containing the cellular confluent monolayer (>90%) without culture Medium. The 12 wells of the upper part and the 12 wells of the lower part of the dish did not receive the viral inoculum and were used as control of cellular line.

After 1 hour of incubation at 37°C ±1°C 0.1ml of culture Medium were added to viral inoculum.

The microplates were incubate for 6 days and virus positive cell cultures were identified by HA-testing (Haemagglutinine) using erythrocytes as substrate. The recorded results were used for calculate the infecting activity (TCID₅₀ evaluation) by means of Spaerman – Karber method.

Check of cellular sensitivity to virus

To verify if the test substance modifies the cellular sensitivity to viral infection, 0.1 ml of the assay sample, at each test concentration after filtration with S400 HR columns MicroSpin™, were put on microplate in parallel with 0.1 ml of PBS. After 1 hour of incubation at 37°C ±1°C, the test substance and PBS were removed and 0.1ml of the dilutions of virus suspension were plated, after 1 hour of incubation at 37°C ±1°C 0.1 ml of culture Medium were added to the viral inoculum.

The microplates were incubate for 6 days and virus positive cell cultures were identified by HA-testing (Haemagglutinine) using erythrocytes as substrate. The recorded results were used for calculate the infecting activity (TCID₅₀ evaluation) by means of Spaerman – Karber method.

Check of suppression of disinfectant activity

1 ml of interfering substance was added to 1 ml of viral suspension and to 8 ml of the assay sample at the test concentrations. At time zero, 0.5 ml of the afore described solution was diluted into 4.5 ml of culture Medium + 2% FBS in iced bath and filtrated with S400 HR columns MicroSpin™.

Then the solution was left in iced bath for 30 minutes ±10 seconds, serial dilutions in culture Medium were performed and 0.1 ml of each dilutions were plated sixfold and incubated at 37°C ±1°C for 1 hour, then 0.1 ml of culture Medium were added to the viral inoculum.

The microplates were incubate for 6 days and virus positive cell cultures were identified by HA-testing (Haemagglutinine) using erythrocytes as substrate. The recorded results were used for calculate the infecting activity (TCID₅₀ evaluation) by means of Spaerman – Karber method.

Check of viral inactivation (Test)

1 ml of interfering substances was mixed with 1 ml of viral suspension, then vortexed.

To this solution 8 ml of the assay sample were added and left for the test contact time, then 0.5 ml of the solution was mixed with 4.5 ml of culture Medium in iced bath, filtrated with S400 HR columns MicroSpin™, then serial dilutions with ice cold Medium were performed and 0.1 ml were transferred sixfold in microplate 96 wells containing the cellular confluent monolayer (>90%) and incubated at 37°C ±1°C for 1 hour, then 0.1 ml of culture Medium were added to the viral inoculum.

The same test described above, was performed with water instead of the test substance, (virus control). The microplates were incubate for 6 days and virus positive cell cultures were identified by HA-testing (Haemagglutinine) using erythrocytes as substrate. The recorded results were used for calculate the infecting activity (TCID₅₀ evaluation) by means of Spaerman – Karber method.

HA-test

The hemagglutination assay (HA-assay) is widely used for titration of virus and takes advantage of the fact that many viruses contain proteins that can bind to red blood cells (RBCs). Among the viruses that share this ability we can find the influenza virus, which binds to RBCs' surface thanks to its hemagglutinin protein. In the HA-assay, RBCs were added to the serial dilution of virus suspension in a U-bottomed microtiter plate. Normally, RBCs sediment to the bottom of each well. If viruses are present, the erythrocytes bind to the virus particles forming a network and preventing the formation of the precipitate red button at the bottom of each well.

In order to perform a HA-assay, a RBC a 0.5% suspension was used. After the 6 incubation days 50 µl of the suspension in each plated wells were transferred into a microplate 96-well with U-bottomed, then 100 µl of RBCs (0.5% suspension) are added to each well. The plates were incubated for 2 hours at 5 ± 3°C, then the results were evaluated for the absence/presence of virus (-/+).

6. CALCULATIONS AND EXPRESSION OF THE RESULTS**Determination of TCID₅₀.**

The infecting activity was determined by means of Spaerman – Karber method that uses the following formula to calculate the value of TCID₅₀:

$$-\text{Log}_{10} \text{TCID}_{50} = -(-x_0) - \{[R/100] - 0.5\} \times \log_{10} \text{dilution factor}$$

where:

x_0 = \log_{10} of the lowest dilution with 100% of positive reaction

R = sum (%) of positive cultures

Log TCID₅₀ values are rounded to two significant ciphers as shown in Annex D table D.1 of EN 14476. Rounding is automatically performed by excel datasheet.

Evaluation of the virucidal activity

The virucidal activity of the product test solution was performed for each exposure time.

The log-reduction was performed by subtracting the logarithmic titre TCID₅₀ at any contact time from the logarithmic titre TCID₅₀ of the virus control.

	Pharma Services	Test Facility Certification M.S. 85/2009	Report No.:	2009/690 AMI
			Version:	English
			Page:	11 of 12
			Print date:	Oct 27 th , 2009

ASSAY VALIDITY CRITERIA

The test of virucidal activity is valid when in the preliminary tests the following results are obtained:

Check of cytotoxicity of the test substance

The test substance shall not be cytotoxic at the concentrations tested, in any case, the cytotoxicity shall be low enough to at least enable a titre reduction of 4 Log to verify the method.

Assay of viral activity (virus titration)

The minimum titre of the virus suspensions is at least 10^6 TCID₅₀/ml; in any case, it shall be sufficiently high to at least enable a titre reduction of 4 Log to verify the method.

Check of cellular sensitivity to virus

The difference of the value of TCID₅₀ among the cellular cultures treated with the test substance and the ones not treated with the test substance (i.e. PBS only) must be $< 1 \log_{10}$.

Check of suppression of disinfectant activity (ice inactivation)

The difference of the value of TCID₅₀ among the cellular cultures treated with the inactivated test substance and the ones treated only with the viral inoculum must be ≤ 0.5 Log.

RESULTS

The validity criteria were satisfied.

1. VALIDATION

Check of cytotoxicity of the test substance

The test substance is cytotoxic at the concentrations tested so the entire test was carried out by means of filtration, so that the cytotoxicity is enough low to at least enable a titre reduction of 4 Log to verify the method.

Assay of viral activity (virus titration)

The virus titre was sufficiently high to at least enable a titre reduction of 4 Log to verify the method.

Check of cellular sensitivity to virus

The test substance had not influenced the MDCK cells sensitivity to *Influenza A virus* (subtype H1N1).

Check of suppression of disinfectant activity (ice inactivation)

The suppression of disinfectant activity was validated, the difference of the value of TCID₅₀ among the cellular cultures treated with the inactivated test substance and the ones treated only with the viral inoculum satisfy the validity criteria.

 eurofins Pharma Services	Test Facility Certification M.S. 85/2009	Report No.:	2009/690 AMI
		Version:	English
		Page:	12 of 12
		Print date:	Oct 27 th , 2009

2 VIRUCIDAL ACTIVITY

The results obtained in the adopted experimental conditions are reported at the Attachment N. 1. The results of Log reduction at the different contact times are reported following and at the Attachment N.1.

Influenza A virus (subtype H1N1)

Product	Experimental Condition		Contact time (minutes)
	Concentrations (%)	Interfering substance	Log Reduction
IGEN	80%	PBS	4.50
	50%	PBS	4.17
	25%	PBS	2.67

DEVIATIONS

No deviations to the study program occurred.

CONCLUSIONS

On the basis of obtained results, in the experimental adopted conditions, the test substance IGEN causes a Log reduction ≥ 4 Log against *Influenza A virus (subtype H1N1)* at the test concentrations of 80% (maximum concentration testable) and 50%, after 1 minute of contact time, in compliance with EN 14476: April 2005+A1:2006.

ATTACHMENTS

ADDENDUM	TITLE	NUMBER OF PAGES
N.1	Raw Data: Experimentation 2009/690.A1	8