Toulouse, April 9th 2021

STUDY 20-2778/2
REPORT 21-1668

DETERMINATION OF VIRICUDAL ACTIVITY OF CHEMICAL ANTISEPTICS AND DISINFECTANTS USED IN HUMAN MEDECINE STANDARD NF EN 14476+A2 (JULY 2019) DISINFECTION SURFACE CLEAN OBLIGATORY CONDITIONS

Promotor of Study

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I - IDENTIFICATION OF TESTING LABORATORY

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II - SAMPLE IDENTIFICATION

Product name	CIEL VERT 300B
Batch number	20201010
Active substance	Benzalkonium
Expiry date	December/2021
Receipt date	Oct/10/2020
Internal code	20-2778/2-1
Supplier	CIEL VERT
Analysis period	February - March 2020

Storage conditions during period of testing: Ambient temperature

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The COFRAC accreditation only attests the capability of the laboratory for assays covered by the accreditation. COFRAC is a signatory of the multilateral recognition agreements of EA[1] (European co-operation for Accreditation) and/or of ILAC[2] (International Laboratory Accreditation Cooperation) and/or of IAF[3] (International Accreditation Forum) for testing activities.

[1] European cooperation for Accreditation
[2] International Laboratory Accreditation Cooperation
[3] International Accreditation Forum

III - TEST METHODE

III-1 Virus test

Name:	ADENOVIRUS TYPE 5
Origin:	ATCC
Reference:	VR-5
Supplier Batch number:	58486654
Internal Batch number :	SS-1-181212 (Passage N°1) and SS-3-290518 (Passage N°3)

Name:	MURINE NOROVIRUS
Origin:	Freidrich-Loeffler-Institut
Reference:	FLI-RVB-651
Batch number	4/200409/220409
Internal batch number:	SS-5-110119 (Passage N°5) and SS-4-271118 (Passage N°4)

Name:	Poliovirus type 1
Origin:	CNR des Entérovirus - Hospices civiles de Lyon
Reference supplier:	NIBSC 01/528
Autorization number ANSM:	ADE-097692018-7
Internal batches number:	SS-7-110978 (Passage N°7)

III-2 Recipient cells

Name:	HELA Cells (Adenovirus)
Origin:	ATCC
Reference ATCC:	CCL-2
Batch number ATCC:	4440136
Internal batch number:	WCB-140613 (Passages N°20 and 36)

Name:	RAW 264-7 (Murine Norovirus)
Origin:	ATCC
Reference ATCC:	TIB-71
Batch number ATCC:	5822175
Internal batch number:	WCB-210912 (Passages N°25 and N°55)

Name:	VERO cells (Poliovirus type 1)
Origin:	ATCC
Reference ATCC:	Cellules VERO - CCL-81
Batch number ATCC:	3372621
Internal batch number :	WCB-141215 (Passages N°34 and 45)

IV -EXPERIMENTAL CONDITIONS

IV-1 Analysis conditions

- **Test concentrations:** **30% (V/V), 10% (V/V), and 3% (V/V).**
- **Contact times:** 60 minutes ± 10
- **Test temperature:** 30°C ± 1°C
- **Incubation temperature:** 36°C ± 1°C
- **Interfering substance:** BSA 0.3g/L (Batches N°337, N° 338, N°361, N°364 and N°367)
- **Molecular sieve filter:** Sephadex LH20 (Batches N° 9803, N°10106 and N°10177)
- **Reference substance:** Formaldehyde (Batch N°9672)
- **Product diluent:** Hard water (Batches N°780, N°781, N°769, N°776, N°782 and N°788)
- **Culture medium:** MEM (Batches N°2655, N°2763, N°2767, N°2768, N°2781 and N°2782) HELA cells and VERO cells
  DMEM (Batches N° 2700 and N°2752) RAW 264-7 cells
- **Saline Phosphate Buffer (PBS):** Batches N° MS00J1, N°MS00PN and N°MS00I0

- **Aspect dilution product:** Homogeneous preparation

- **Stability and appearance of the test mixture during the test**: Preparation homogeneous during the test

IV-2 Methods

IV-2-1 Virus suspension title

The assay was performed by the microplate method of suspension cells. The cytopathic effect was determined at least 4 days of culture.
The number of infectious units is estimated by the SPEARMAN-KÄRBER method by calculating the negative logarithm of the 50% limit point ($lgDICT_{50}$) using the following formula:

$lgDICT_{50}$ = Negative logarithm of the highest concentration of virus used - [(Sum of % assigned to each dilution/100 - 0.5) X (lg of dilution)]

IV-2-2 Cytotoxicity of product solutions

IV-2-2-1 Direct cytotoxicity

This test determines the concentration of disinfectant that does not induce signs of cytotoxicity to the cell line for the demonstration of the effect of virus.

IV-2-2-2 Indirect Cytotoxicity: molecular sievfilter

The activity of the product is stopped by the molecular sieving method using a sieve filter, i.e. Sephadex LH 20.

The neutralization of the product was validated by passing it through Sephadex at a dilution 1/20.

The neutralization of formaldehyde was validated by passing it through Sephadex at a dilution 1/120 (HELA cells) and 1/30 (RAW 264-7 cells and VERO cells).

IV-2-3 Sensitivity of cells to viruses

Comparative assays were performed on cells treated or not with the disinfectant to ensure that the disinfectant subcytotoxic concentration does not change the behavior of the virus to receive cell.

IV-2-4 Verification of effectiveness of stopping the disinfectant activity at T0

Immediately after preparation of the test mixture at time 0, the reaction was stopped in ice for 30 min ± 10 s and then infectious virus titre was determined.

IV-2-5 Reference virus inactivation

Virucidal effect of formaldehyde is tested to control the uniformity of the behavior of virus to chemical agents over time (30 min and 60 min).

V- RESULTS

ADENOVIRUS TYPE 5

V-1 Validations

V-1-1 Cytotoxicity of product solutions

V-1-1-1 Direct cytotoxicity

No-cytotoxic concentration from 3.0 10^{-3}% (V/V)

V-1-1-2 Indirect cytotoxicity

No-cytotoxic concentration from 1.5 10^{-1}% (V/V)

V-1-2 Sensitivity of cell to virus

S1 (in presence of product): lg $DICT_{50}$ = 7.38
S2 (in absence of product): lg $DICT_{50}$ = 7.13
S2 - S1 = 0.25
Sensitivity valid (limit S2 - S1 < 1 lg)

V-2 Assay

V-2-1 Validations

V-2-1-1 Verification of control virus suspension assay in presence or absence of Sephadex

- Before Sephadex
lg $DICT_{50}$ = 6.50
- After Sephadex
lg $DICT_{50}$ = 6.38
Difference ≤ 0.5 lg (verification valid)

V-2-1-2 Verification of effectiveness of stopping the disinfectant activity at T0

I1 (In presence of product): lg $DICT_{50}$ = 7.25
I2 (In absence of product): lg $DICT_{50}$ = 7.63
Difference ≤ 0.5 lg (verification valid)

V-2-1-3 Assay of inactivation of virus reference

V-2-1-3-1 Direct cytotoxicity

No-cytotoxic concentration from 7 10^{-7}% (V/V)

V-2-1-3-2 Indirect cytotoxicity

No-cytotoxic concentration from 3.5 10^{-4}% (V/V)

V-2-1-3-3 Title of the PBS viral control

Before Sephadex
lg $DICT_{50}$ PBS = 6.88
After Sephadex
lg $DICT_{50}$ PBS = 6.50
Difference ≤ 0.5 lg (verification valid)

V-2-1-3-4 Title result of formaldehyde

Contact time	30 min	60 min
lg $DICT_{50}$	2.75	2.38
Logarithmic reduction	3.75	4.12

V-2-2 Results of assay product:

Adenovirus type 60 min ± 10S - 30°C ± 1°C								
	In presence of dilutions of test product						Control	
-log Dilution	Degree of cytopathogenic effect*			% positive wells			Degree of cytopathoge effect*	% positiv wells
	30 %(V/V)	10%(V/V)	3%(P/V)	30%(V/V)	10 %(V/V)	3 %(V/V)		
1	30000000	43221021	44444444	12.5%	84.5%	100%	44444444	100%
2	00000000	00200010	44444444	0%	25%	100%	44444444	100%
3	00000000	00000000	44444444	0%	0%	100%	44444444	100%
4	00000000	00000000	44444444	0%	0%	100%	44444444	100%
5	00000000	00000000	44444444	0%	0%	100%	44444444	100%
6	00000000	00000000	02230000	0%	0%	37.5%	10002132	62.5%
7	00000000	00000000	00000000	0%	0%	0%	00000003	12.5%
8	00000000	00000000	00000000	0%	0%	0%	00002000	12.5%
9	00000000	00000000	00000000	0%	0%	0%	00000000	0%
10	00000000	00000000	00000000	0%	0%	0%	00000000	0%
lg $DICT_{50}$				**0.63**	**1.63**	**5.88**		**6.38**

* 1 to 4: Presence of virus, with cytopathogenic effect observed in cell wells
0: Absence of virus, with absence of cytopathogenic effect

V-2-3 Logarithmic reduction

Contact time	60 minutes ± 10 S		
Concentrations	30% (V/V)	10% (V/V)	3% (V/V)
Lg reduction	**5.75**	**4.75**	0.5

MURINE NOROVIRUS

V-1 Validations

V-1-1 Cytotoxicity of product solutions

V-1-1-1 Direct cytotoxicity

No-cytotoxic concentration from 3.0 10^{-3}% (V/V)

V-1-1-2 Indirect cytotoxicity

No-cytotoxic concentration from 0.01% (V/V)

V-1-2 Sensitivity of cell to virus

S1 (in presence of product): lg $DICT_{50}$ = 7.88
S2 (in absence of product): lg $DICT_{50}$ = 8.0
S2 - S1 = 0.12
Sensitivity valid (limit S2 - S1 < 1 lg)

V-2 Assay

V-2-1 Validations

V-2-1-1 Verification of control virus suspension assay in presence or absence of Sephadex

- Before Sephadex
lg $DICT_{50}$ = 5.13
- After Sephadex
lg $DICT_{50}$ = 5.25
Difference ≤ 0.5 lg (verification valid)

V-2-1-2 Verification of effectiveness of stopping the disinfectant activity at T0

I1 (In presence of product): lg $DICT_{50}$ = 6.88
I2 (In absence of product): lg $DICT_{50}$ = 7.0

Difference ≤ 0.5 lg (verification valid)

V-2-1-3 Assay of inactivation of virus reference

V-2-1-3-1 Direct cytotoxicity
No-cytotoxic concentration from 7 10^{-7}% (V/V)

V-2-1-3-2 Indirect cytotoxicity
No-cytotoxic concentration from 3.5 10^{-4}% (V/V)

V-2-1-3-3 Title of the PBS viral control
Before Sephadex
lg $DICT_{50}$ PBS = 5.63
After Sephadex
lg $DICT_{50}$ PBS = 5.50
Difference ≤ 0.5 lg (verification valid)

V-2-1-3-4 Title result of formaldehyde

Contact time	30 min	60 min
lg $DICT_{50}$	3.0	1.88
Logarithmic reduction	2.50	3.62

V-2-2 Results of assay product:

Murine Norovirus 60 min ± 10S - 30°C ± 1°C								
	In presence of dilutions of test product						Control	
-log Dilution	Degree of cytopathogenic effect*			% positive wells			Degree of cytopathoge effect*	% positiv wells
	30 %(V/V)	10%(V/V)	3%(P/V)	30%(V/V)	10 %(V/V)	3 %(V/V)		
1	00000000	03210310	44444444	0%	62.5%	100%	44444444	100%
2	00000000	00000000	44444444	0%	0%	100%	44444444	100%
3	00000000	00000000	430440132	0%	0%	75%	44444444	100%
4	00000000	00000000	03030200	0%	0%	37.5%	44444444	100%
5	00000000	00000000	00000000	0%	0%	0%	32021033	75%
6	00000000	00000000	00000000	0%	0%	0%	00000000	0%
7	00000000	00000000	00000000	0%	0%	0%	00000000	0%
8	00000000	00000000	00000000	0%	0%	0%	00000000	0%
9	00000000	00000000	00000000	0%	0%	0%	00000000	0%
10	00000000	00000000	00000000	0%	0%	0%	00000000	0%
lg $DICT_{50}$				**≤0.5**	**1.13**	**3.63**		**5.25**

* 1 to 4: Presence of virus, with cytopathogenic effect observed in cell wells
0: Absence of virus, with absence of cytopathogenic effect

V-2-3 Logarithmic reduction

Contact time	60 minutes ± 10 S		
Concentrations	30% (V/V)	10% (V/V)	3% (V/V)
Lg reduction	**≥ 4.75**	**4.12**	1.63

POLIOVIRUS TYPE

V-1 Validations

V-1-1 Cytotoxicity of product solutions

V-1-1-1 Direct cytotoxicity

No-cytotoxic concentration from 3.0 10^{-5}% (V/V)

V-1-1-2 Indirect cytotoxicity

No-cytotoxic concentration from 0.1% (V/V)

V-1-2 Sensitivity of cell to virus

S1 (in presence of product): lg $DICT_{50}$ = 7.88
S2 (in absence of product): lg $DICT_{50}$ = 7.38
S2 - S1 = 0.50
Sensitivity valid (limit S2 - S1 < 1 lg)

V-2 Assay

V-2-1 Validations

V-2-1-1 Verification of control virus suspension assay in presence or absence of Sephadex

- Before Sephadex
lg $DICT_{50}$ = 5.13
- After Sephadex
lg $DICT_{50}$ = 4.88
Difference ≤ 0.5 lg (verification valid)

V-2-1-2 Verification of effectiveness of stopping the disinfectant activity at T0

I1 (In presence of product): lg $DICT_{50}$ = 6.63
I2 (In absence of product): lg $DICT_{50}$ = 7.13
Difference ≤ 0.5 lg (verification valid)

V-2-1-3 Assay of inactivation of virus reference

V-2-1-3-1 Direct cytotoxicity

No-cytotoxic concentration from 7 10^{-6}% (V/V)

V-2-1-3-2 Indirect cytotoxicity

No-cytotoxic concentration from 3.5 10^{-3}% (V/V)

V-2-1-3-3 Title of the PBS viral control

Before Sephadex
lg $DICT_{50}$ PBS = 5.88
After Sephadex
lg $DICT_{50}$ PBS = 5.88
Difference ≤ 0.5 lg (verification valid)

V-2-1-3-4 Title result of formaldehyde

Contact time	30 min	60 min
lg $DICT_{50}$	4.13	4.38
Logarithmic reduction	1.75	2.50

V-2-2 Results of assay product:

Poliovirus type 1 60 min ± 10S - 30°C ± 1°C								
	In presence of dilutions of test product						Control	
-log Dilution	Degree of cytopathogenic effect*			% positive wells			Degree of cytopathoge effect*	% positiv wells
	30 %(V/V)	10%(V/V)	3%(V/V)	30%(V/V)	10 %(V/V)	3 %(V/V)		
1	00000000	00000000	44444444	0%	0%	100%	44444444	100%
2	00000000	00000000	44444444	0%	0%	100%	44444444	100%
3	00000000	00000000	04234430	0%	0%	75%	44444444	100%
4	00000000	00000000	00000000	0%	0%	0%	44444444	100%
5	00000000	00000000	00000000	0%	0%	0%	30000340	37.5%
6	00000000	00000000	00000000	0%	0%	0%	00000000	0%
7	00000000	00000000	00000000	0%	0%	0%	00000000	0%
8	00000000	00000000	00000000	0%	0%	0%	00000000	0%
9	00000000	00000000	00000000	0%	0%	0%	00000000	0%
10	00000000	00000000	00000000	0%	0%	0%	00000000	0%
lg $DICT_{50}$				≤0.5	≤0.5	3.75		4.88

* 1 to 4: Presence of virus, with cytopathogenic effect observed in cell wells
0: Absence of virus, with absence of cytopathogenic effect

V-2-3 Logarithmic reduction

Contact time	60 minutes ± 10 S		
Concentrations	30% (V/V)	10% (V/V)	3% (V/V)
Lg reduction	≥ 4.38	≥ 4.38	1.13

VI- CONCLUSION

According to standard NF EN 14476 +A2 (July 2019), the product CIEL VERT 300B (batch N° 20201010) for disinfection surface - in clean condition - has a virucidal activity at the concentrations 30% (VV) and 10% (V/V) at the contact time 60 minutes ± 10 S at320°C ± 1°C against Adenovirus type 5, Murine Norovirus and Poliovirus type 1

These results hold only to the product under assay and apply to the sample as received.