



Toulouse, April 13th 2015

STUDY 15-1780

**Determination of bactericidal, fungicidal, sporicidal, mycobactericidal and virucidal activity for aerial surface disinfection processes
According to the method described
in the standard NF T 72-281 (November 2014)**

Medical area

Promotor

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1. Test Laboratory

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2. Identification of the aerial disinfection system

Appartus : NOCOSPRAY
Serial number : 37S347

Disinfectant : ONE SHOT
Batches : 231214N+ (peremption 12/2016)
160315N+F (peremption 03/2017)

Concentration du produit dans la pièce : 3 ml/m³
One treatment or two treatments with one or two hours of wait (carriers recovery after waiting)
Amount of disinfectant diffusion \approx 155 mL/treatment of 3mL/m³.

Promotor : OXY'PHARM

Storage conditions: Ambient temperature
Period of testing: January-April 2015
Actives Substances: Hydrogen peroxide

3. Experimental Conditions

a. Tests micro-organisms

- Bactericidal activity :
 - o *Pseudomonas aeruginosa* CIP 103467
 - o *Staphylococcus aureus* CIP 4.83
 - o *Enterococcus hirae* CIP 58.55
 - o *Escherichia coli* CIP 54.127

- Fungicidal, yeastidal activity :
 - o *Candida albicans* DSM 1386
 - o *Aspergillus brasiliensis* CBS 733.88

- Sporicidal activity :
 - o *Bacillus subtilis* (spores) CIP 52.62

- Mycobactericidal activity :
 - o *Mycobacterium terrae* ATCC 15755

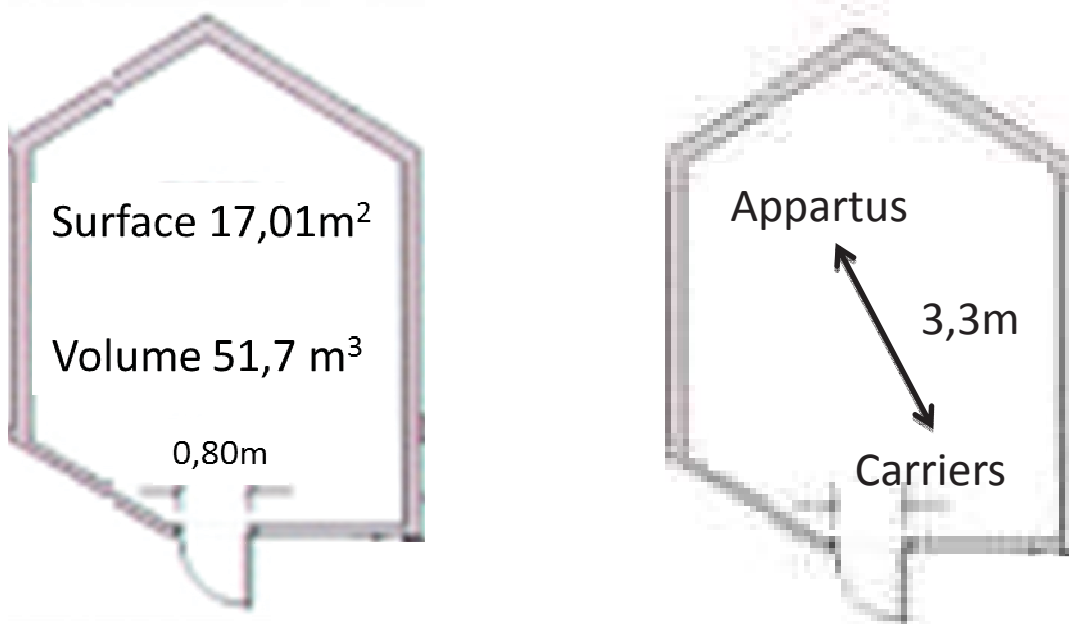
- Virucidal activity :
 - o *Adenovirus type 5/HELA* :
 - Origin : ATCC
 - Supplier reference: VR-5
 - Batch number supplier : 58486654
 - Internal batch number : SS-1-240112 (passage N°1)
 - o Cells
 - Origin : ATCC
 - Name : HELA
 - ATCC reference: CCL-2
 - Batch number ATCC: 4440136
 - Internal batch number : WCB-080713 (passage N°52)
 - o *Murine Norovirus S99/RAW264.7*:
 - Origin : Friedrich Loeffler Institut Berlin
 - Supplier reference: RVB-651
 - Batch number supplier: 4/200409/220409
 - Internal batch number: SS-1-240512 (passage N°1)
 - o Cells
 - Origin : ATCC
 - Name : Raw-264.7
 - ATCC reference: CCL-2
 - Batch number ATCC: 5822175
 - Internal number Batch: WCB-031012 (passage N°14)

The selected tests surfaces are stainless steel discs, flats, corresponding to the requirements of paragraph 5.2.3.1 of the standard. The supplier is CONTIGIANI (Toulouse).

Les supports sélectionnés sont des disques en acier inoxydable, plats correspondant aux exigences du paragraphe 5.2.3.1 de la norme. Le fournisseur est l'entreprise CONTIGIANI.

b. Conditions of aerial disinfection system use

- Room :



Relative humidity ranging from 40% to 50%.

Initial temperatures ranging from 19°C to 22°C.

Test room volume : 51,7m³.

Distance between the appartus and the carriers : 3,3m (tableau B.1).

Diluants and culture media

Interfering substances

1/20 reconstituted milk (batch 5763, exp. 23/03/15; batch 5811, exp. 17/04/2015)

BSA fraction V at 0,3g/l (Batch N°126)

Diluants

Suspension preparation : EPPI (Cooper, batch 19GG17GC, exp. 06/2016)

Diluant for *A. brasiliensis* (batch 30, exp. 05/04/15)

Recovery solution (batches 5760, 5761, 5765, 5783, 5789, 5804)

Filtration membranes

Nitrocellulos membranes 0,45µm (Millipore, batch F4NA10079, exp. 10/2016; batch F4NA18553, exp. 03/04/2015)

Culture media

Malt extract agar (batch 5730, exp. 04/03/15; batch 5773, exp. 24/03/2015; batch 5782, exp. 02/04/15; batch 5787, exp. 03/04/15)

Trypcase soy agar (Biomérieux, batch 1003531150, exp. 01/06/2016 ; batch 1003701520, exp. 20/07/2016)

Milieu GGL (batch 5856, exp. 09/05/2015)

Middlebrook Agar + OAPC (batch 5797, exp. 10/04/2015)

EMEM 2% SVF batch N°1304 (Adenovirus) - DMEM 2% SVF batch N°1314 (Murine Norovirus)

4. Assays

a. Bactericidal activity

- Treatment 3 mL / m³ - waiting 1H

Tests microorganisms	N Test suspension (CFU/mL)	Preliminary tests			T Control (CFU/spot - 50µL)	n'1 + n'2 UFC/ spot 50µL (dilution/filtration - disc in agar)	Log reduction - Mean
		n1/N1	n2/N2	n3/N1			
	5.10 ⁷ - 2.10 ⁹	n1 > 0.5 N1	n2 > 0.5 N2	n3 > 0.5 N1	≈ 10 ⁶		
<i>P. aeruginosa</i> Date 25/02/15 B: 19,9°C/RH 41% E: 20,5°C/RH 46%	0,97.10 ⁹	d1 : 75/97 d2 : 81/97	d1 : 75/98 d2 : 79/98	d1 : 74/97 d2 : 79/97	d1 : 0,51.10 ⁶ d2 : 0,68.10 ⁶ T = 0,60.10 ⁶	d1 : 6 + 0 d2 : 12 + 0 d3 : 3 + 0	R1 : 5,00 R2 : 4,70 R3 : 5,30 R = 5,00
<i>E. hirae</i> Date 26/02/15 B: 20,3°C / RH 43% E: 20,3°C / RH 49%	1,7.10 ⁹	d1 : 170/170 d2 : 145/170	d1 : 123/150 d2 : 133/150	d1 : 187/170 d2 : 149/170	d1 : 0,98.10 ⁸ d2 : 0,97.10 ⁸ T = 0,97.10 ⁸	d1 : 0 + 0 d2 : 0 + 0 d3 : 0 + 0	R1 : 7,99 R2 : 7,99 R3 : 7,99 R = 7,99
<i>E. coli</i> Date 19/03/15 B: 19,6°C / RH 45% E: 19,8°C / RH 53%	2.34.10 ⁸	d1 : 25/23 d2 : 22/23	d1 : 21/21 d2 : 32/21	d1 : 27/23 d2 : 26/23	d1 : 0 ;76.10 ⁶ d2 : 0,86.10 ⁶ T = 0,81.10 ⁶	d1 : 0 + 0 d2 : 0 + 0 d3 : 0 + 0	R1 : 5,91 R2 : 5,91 R3 : 5,91 R = 5,91

• Treatment 3 mL / m³ - waiting 2H - Treatment 3 mL / m³ - waiting 2H

Tests microorganisms	N Test suspension (CFU/mL)	Preliminary tests			T Control (CFU/spot - 50µL)	n'1 + n'2 UFC/ spot 50µL (dilution/filtration - disc in agar)	Log reduction - Mean
		n1/N1	n2/N2	n3/N1			
<i>S. aureus</i>	5.10 ⁷ - 2.10 ⁹	n1 > 0.5 N1	n2 > 0.5 N2	n3 > 0.5 N1	≈ 10 ⁶		
Essai du 18/03/15 D: 19,0°C/RH 44% F: 19,6°C/RH 53%	3,2.10 ⁸	d1 : 40/32 d2 : 33/32	d1 : 42/44 d2 : 46/44	d1 : 41/32 d2 : 31/32	d1 : 1,63.10 ⁷ d2 : 1,47.10 ⁷ T = 1,55.10 ⁷	d1 : 0 + 1 d2 : 30 + 0 d3 : 300 + 1	R1 : 7,19 R2 : 5,71 d3 : 4,71 R = 5,87

T: counting of micro-organisms on the discs.

N₁: counting of test suspension by pour plate technique - N₂: counting of test suspension by filtration method

n₁: counting to search inhibitor effect in agar medium - n₂: counting to search inhibitor effect on membrane filtration - n₃: counting to search inhibitor effect after inclusion of disc in agar medium

n₁: number of survival micro-organisms in 100mL of tryptone-salt - n'₂: number of micro-organisms after inclusion of the disc in agar medium.

n₁ + n₂: total number of survival micro-organisms on the carrier surface.

d1 : disc N°1 / d2 : disc N°2 / d3 : disc N°3

b. Fungicidal activity

- Treatment 3 mL / m³ - waiting 1H

Test microorganisms	N Test suspension (CFU/mL)	Preliminary assay			T Control (CFU/spot - 50µL)	n ¹ + n ² UFC/ spot 50µL (dilution/filtration - disc in agar)	Log reduction - Mean
		n ¹ /N ¹	n ² /N ²	n ³ /N ¹			
	2.10 ⁷ - 1.10 ⁸	n ¹ > 0.5 N ¹	n ² > 0.5 N ²	n ³ > 0.5 N ¹	≈ 10 ⁵		
<i>C. albicans</i>							
Date 25/02/15	0,98.10 ⁸	d1 : 94/98	d1 : 86/97	d1 : 113/98	d1 : 1,13.10 ⁶	d1 : 0 + 0	R1 : 6,03
B: 19,9 °C/RH 41%		d2 : 95/98	d2 : 102/97	d2 : 87/98	d2 : 1,00.10 ⁶	d2 : 0 + 0	R2 : 6,03
E: 20,5 °C/RH 46%					T = 1,06.10 ⁶	d3 : 0 + 0	R3 : 6,03
	5.10 ⁶ - 1.10 ⁷	n ¹ > 0.5 N ¹	n ² > 0.5 N ²	n ³ > 0.5 N ¹	≈ 10 ⁵		R = 6,03
<i>A. brasiliensis</i>							
Date 03/03/15	0,93.10 ⁷	d1 : 245/93	d1 : 240/80	d1 : 200/93	d1 : 4,2.10 ⁵	d1 : 1 + 0	R1 : 5,64
B: 19,1 °C/RH 47%		d2 : 200/93	d2 : 330/80	d2 : 260/93	d2 : 4,6.10 ⁵	d2 : 2 + 0	R2 : 5,34
E: 19,7 °C/RH 53%					T = 4,38.10 ⁵	d3 : 0 + 0	R3 : 5,64
							R = 5,54

T: counting of micro-organisms on the discs.

N₁: counting of test suspension by pour plate technique - N₂: counting of test suspension by filtration method

n₁: counting to search inhibitor effect in agar medium - n₂: counting to search inhibitor effect on membrane filtration - n₃: counting to search inhibitor effect after inclusion of disc in agar medium

n'₁: number of survival micro-organisms in 100mL of tryptone-salt - n'₂: number of micro-organisms after inclusion of the disc in agar medium.

n₁ + n'₂: total number of survival micro-organisms on the carrier surface.

d1 : disc N°1 / d2 : disc N°2 / d3 : disc N°3

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c. Sporicidal activity

- Treatment 3 mL / m³ - waiting 1H - Treatment 3 mL / m³ - waiting 1H

Tests microorganisms	N Test suspension (CFU/mL)	Preliminary assay			T Control (CFU/spot - 50µL)	n ¹ + n ² UFC/ spot 50µL (dilution/filtration - disc in agar)	Log reduction - Mean
		n1/N1	n2/N2	n3/N1			
	2.10 ⁵ - 5.10 ⁵	n1 > 0.5 N1	n2 > 0.5 N2	n3 > 0.5 N1	≈ 10 ⁴		
B. subtilis		d1 : 27/20 d2 : 27/20	d1 : 17/22 d2 : 23/22	d1 : 16/20 d2 : 26/20	d1 : 0,90.10 ⁴ d2 : 1,06.10 ⁴ T = 0,98.10 ⁴	d1 : 0 + 0 d2 : 0 + 0 d3 : 0 + 0	R1 : 3,99 R2 : 3,99 R3 : 3,99 R = 3,99
Date 12/03/15	2,24.10 ⁵						
B: 19,6 °C/RH 47%							
E: 20,4 °C/RH 57%							

T: counting of micro-organisms on the discs.

N₁: counting of test suspension by pour plate technique - N₂: counting of test suspension by filtration method

n₁: counting to search inhibitor effect in agar medium - n₂: counting to search inhibitor effect on membrane filtration - n₃: counting to search inhibitor effect after inclusion of disc in agar medium

n₁: number of survival micro-organisms in 100mL of tryptone-salt - n₂: number of micro-organisms after inclusion of the disc in agar medium.

n₁ + n₂: total number of survival micro-organisms on the carrier surface.

d1 : disc N°1 / d2 : disc N°2 / d3 : disc N°3

d. Mycobactericidal activity

- Treatment 5 mL / m³ - waiting 2H

Souche	N Titre suspension initiale (UFC/mL)	Essai préliminaire			T Témoins (UFC/dépôt 50µL)	n'1 + n'2 UFC/ dépôt 50µL (dilution/filtration - inclusion)	Réduction log - Moyenne
		n1/N1	n2/N2	n3/N1			
	1.10 ⁷ - 1.10 ⁸	n1 > 0.5 N1	n2 > 0.5 N2	n3 > 0.5 N1	≈ 10 ⁵		
<i>M. terrae</i> Essai du 11/03/15 D: 19,5°C / 44 RH F: 20,1°C / 51 RH	7,64.10 ⁸	d1 : 58/91 d2 : 56/91	d1 : 41/65 d2 : 43/65	d1 : 58/91 d2 : 53/91	d1 : 3,22.10 ⁷ d2 : 2,78.10 ⁷ T = 3,15.10 ⁷	d1 : 2 + 0 d2 : 0 + 0 d3 : 0 + 0	R1 : 7,20 R2 : 7,50 R3 : 7,50 R = 7,40

T: counting of micro-organisms on the discs.

N₁: counting of test suspension by pour plate technique - N₂: counting of test suspension by filtration method

n₁: counting to search inhibitor effect in agar medium - n₂: counting to search inhibitor effect on membrane filtration - n₃: counting to search inhibitor effect after inclusion of disc in agar medium

n'₁: number of survival micro-organisms in 100mL of tryptone-salt - n'₂: number of micro-organisms after inclusion of the disc in agar medium.

n'₁ + n'₂: total number of survival micro-organisms on the carrier surface.

d1 : disc N°1 / d2 : disc N°2 / d3 : disc N°3

e. Virucidal activity

○ **Validation Protocols**

Control of sensitivity of cells to virus

- Add one volume of solution S or PBS + one volume of cellular suspension at 2.10^5 cellules/ml for one hour in water bath at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$
- The cells are centrifuged and resuspended in culture media
- The virus is diluted from 1/4 to 1/4 on a 96-well microplate (15 dilutions)
- Add 100 μl of cell suspension treated (Solution S) or not treated (PBS control) to each well of the microplate
- Incubate for 48 hours at 72 hours

The difference of titre reduction between cells treated by the solution S and cells treated by PBS shall be $< 1 \text{ lg}$.

Control of efficiency for suppression of disinfectant activity

- Add 1 volume of BSA + 1 volume of virus suspension + 1 volume of solution S or distilled water
- Leave the mixture in the ice bath for 30 min at room temperature

Titration method

- Titrate the virus (method titration on cell in suspension) by following steps :
- Serial dilutions (1/4) are realized with culture medium in the glass tube
- Transfer 0,1 ml of each dilution into eight wells of a microplate plaque
- The last row of eight wells will receive 0,1 ml of culture medium (control untreated cells)
- Add 0,1 ml of cell suspension cells at 2.10^5 cell/ml.
- Incubate for 48 or 72 hours at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$ under 5% $\text{CO}_2 \pm 2\%$.
- The viral cytopathic effect is read by using an inverted microscope

The estimated of infectious unite is determined by method KARBBER-SPAERMAN calculating the negative logarithm of 50% endpoint (lgDIC50) by the following formula :

$\text{lgDICT50} = \text{negative logarithm of the highest concentration of virus} - [(\text{Sum of \% affected to each dilution}/100 - 0.5) \times (\text{lg dilution})]$

- **Results**

Adenovirus type 5

Virus suspension titre: IgDICT50 = 8,7

No cytotoxicity was observed on the carrier without treatment which has been pretreated with the aerial disinfection system according to treatment 1 and 2.

Treatment 3ml/m³ - waiting 1H

	Degree of cytopathogenic effect (log)	Logarithmic reduction
Sensitivity of cells to virus		
- With treatment (S1)		
Carrier1	8,1	
Carrier 2	8,0	
Average	8,1	
- Without traitement (S2)		
Carrier 1	8,0	
		Différence <1 lg.
Efficiency for suppression of disinfectant activity		
- With treatment (D1)		
Carrier1	7,7	
Carrier 2	7,1	
Average	7,4	
- Without traitement (D2)		
Carrier 1	7,1	
		Différence <0,5 lg.
Test control		
Carrier1	4,4	
Carrier 2	4,3	
Average	4,4	
Assay		
Support 1	0,3	
Support 2	0,3	4,1
Support 3	0,3	
Average	0,3	

Murine Norovirus

Virus suspension titre: IgDICT50 = 8,5

No cytotoxicity was observed on the carrier without treatment which has been pretreated with the aerial disinfection system according to treatment 1 and 2.

Treatment 3ml/m³ - waiting 1H - 3ml/m³ - waiting 1H

	Degree of cytopathogenic effect (log)	Logarithmic reduction
Sensitivity of cells to virus		
- - With treatment (S1)		
Carrier1	7,0	
Carrier 2	7,1	
Average	7,1	
- Without traitement (S2)		
Carrier 1	6,9	
		Différence <1 lg.
Efficiency for suppression of disinfectant activity		
- With treatment (D1)		
Carrier1	7	
Carrier 2	7,1	
Average	7,1	
- Without traitement (D2)		
Carrier 1	7,3	
		Différence <0,5 lg.
Test control		
Carrier1	5,0	
Carrier 2	5,1	
Average	5,1	
Assay		
Support 1	0,5	
Support 2	0,5	
Support 3	0,5	
Average	0,5	
		4,6

5. Conclusion

According to the conditions of test, the couple apparatus/product led to:

- **A bactericidal activity (log reduction ≥ 5)**
 - o **After a 3 mL/m³ treatment and 1 hour of wait on the following strains :**
 - *P. aeruginosa* CIP 103467
 - *E. coli* CIP 54.127
 - *E. hirae* CIP 58.55
 - o **After a 3 mL/m³ treatment - 2 hours of wait and a second 3 mL/m³ treatment - 2 hours of wait on the following strain :**
 - *S. aureus* CIP 4.83
- **A fungicidal activity (log reduction ≥ 4)**
 - o **After a 3 mL/m³ treatment and 1 hour of wait on the following strains :**
 - *C. albicans* DSM 1386
 - *A. brasiliensis* CBS 733.88
- **A sporicidal activity (log reduction ≥ 3)**
 - o **After 3 mL/m³ treatment - 1 hour of wait and a second 3 mL/m³ treatment - 1 hour of wait on the following strain :**
 - *B. subtilis* spores CIP 52.62
- **A mycobactericidal activity (log reduction ≥ 4)**
 - o **After a 5 mL/m³ treatment and 2 hours of wait on the following strain :**
 - *M. terrae* ATCC 15755
- **A virucidal activity (log reduction ≥ 4)**
 - o **After 3 mL/m³ treatment - 1 hour of wait on the following strain :**
 - *Adenovirus type 5*
 - o **After 3 mL/m³ treatment - 1 hour of wait a second 3 mL/m³ treatment - 1 hour of wait on the following strain :**
 - *Murine Norovirus*