



Toulouse, June 4th 2015

STUDY 15-1806

Determination of bactericidal, fungicidal, yeasticidal, 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

Apparatus : NOCOSPRAY
Serial number: 37S347

Disinfectant : NOCOLYSE®
Batch: 070415N (Expiry date - 04/2017)

Concentration of product in the room: 5 mL/m³ or 7 mL/m³
One treatment or two treatments with two hours of wait (carriers recovery after 2H)
Amount of disinfectant diffusion \approx 260 mL/treatment of 5 mL/m³ or 360 mL/treatment of 7 mL/m³.

Promotor : OXY'PHARM

Storage conditions: Ambient temperature
Period of testing: April - May 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, yeasticidal 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 :

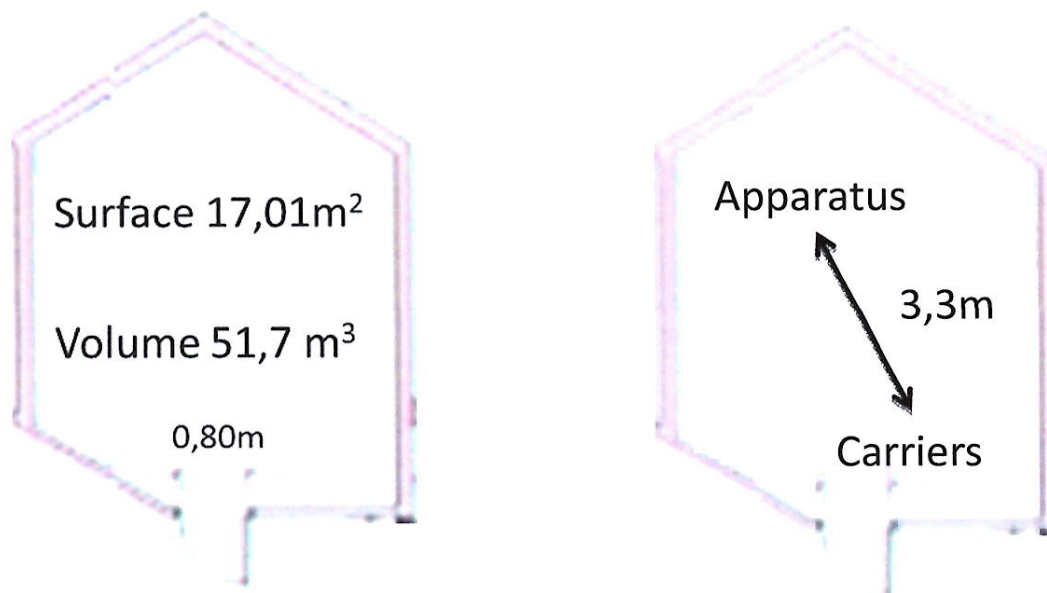
- *Adénovirus type 5/HELA* :
 - Origin : ATCC
 - Supplier reference: VR-5
 - Batch number supplier : 58486654
 - Internal batch number : SS-1-171012 (passage N°1)
- Cells
 - Origin : ATCC
 - Name : HELA
 - ATCC reference: CCL-2
 - Batch number ATCC: 4440136
 - Internal batch number : WCB-1704415 (passage N°30)
- *Murine Norovirus S99/RAW264.7*:
 - Origin : Friedrich Loeffler Institut Berlin
 - Supplier reference: RVB-651
 - Batch number supplier: 4/200409/220409
 - Internal batch number: SS-1-220811 (passage N°1)
- Cells
 - Origin : ATCC
 - Name : RAW-264.7
 - ATCC reference: TIB-71
 - Batch number ATCC: 5822175
 - Internal number Batch: WCB-170415 (passage N°16)

b. Carriers

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).

c. Conditions of aerial disinfection system use

- Room :



Relative humidity ranging from 42% to 70%.

Initial temperatures ranging from 19,4°C to 20,9°C.

Test room volume : 51,7m³.

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

Diluants and culture media

Interfering substances

1/20 reconstituted milk (Internal preparation - Batch 5866 Exp. May/13/2015)

BSA fraction V at 0,3g/l (Internal preparation - Batch N°129)

Diluants

Suspension preparation: EPPI (Cooper - Batch 19HD03GA Exp. March/2017)

Diluant for *A. brasiliensis* (Internal preparation - Batch 31 Exp. May/13/2015)

Recovery solution (Internal preparation - Batches 5881, 5889, 5898, 5905 and 5907)

Filtration membranes

Nitrocellulose membranes 0,45µm (Millipore - Batch F4SA32924 Exp. December/2016)

Culture media

Malt extract agar (Internal preparation - Batch 5904 Exp. May/24/2015)

Trypcase soy agar (Biomérieux - Batch 1003773960 Exp. August/14/2015)

Milieu GGL (Internal preparation - Batch 5902 Exp. May/23/2015)

Middlebrook Agar + OAPC (Internal preparation - Batch 5894 Exp. May/21/2015)

EMEM 2% SVF Batch N°1354 (Adenovirus) - DMEM 2% SVF Batch N°1341 (Murine Norovirus)

4. Assays

a. Bactericidal activity

- Treatment 5 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>E. hirae</i> Date April/28/15 B: 19,9°C / RH 53% E: 20,1°C / RH 62%	5.10 ⁷ – 2.10 ⁹ 2,25.10 ⁸	n1 > 0.5 N1 d1 : 21/22 d2 : 21/22	n2 > 0.5 N2 d1 : 19/24 d2 : 15/24	n3 > 0.5 N1 d1 : 18/22 d2 : 28/22	≈ 10 ⁶ d1 : 1,17.10 ⁷ d2 : 1,19.10 ⁷	d1 : 0 + 0 d2 : 0 + 0 d3 : 0 + 0	R1 : 7,07 R2 : 7,07 R3 : 7,07 R = 7,07
<i>E. coli</i> Date April/28/15 B: 19,9°C / RH 53% E: 20,1°C / RH 62%	2,64.10 ⁸	d1 : 27/27 d2 : 24/27	d1 : 28/25 d2 : 34/25	d1 : 23/27 d2 : 28/27	d1 : 1,69.10 ⁶ d2 : 1,24.10 ⁶ T = 1,46.10 ⁶	d1 : 0 + 0 d2 : 0 + 0 d3 : 0 + 0	R1 : 6,16 R2 : 6,16 R3 : 6,16 R = 6,16

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

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- **Treatment 5 mL / m³ - waiting 2H and Treatment 5 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>							
Date April/23/15							
B: 20,7°C/RH 52%							
E: 20°C/RH 70%							
	5.10 ⁷ - 2.10 ⁹	n1 > 0.5 N1	n2 > 0.5 N2	n3 > 0.5 N1	≈ 10 ⁶		
	2,81.10 ⁸	d1 : 135/143 d2 : 139/143	d1 : 141/127 d2 : 137/127	d1 : 131/143 d2 : 135/143	d1 : 1,30.10 ⁷ d2 : 1,15.10 ⁷	d1 : 0 + 0 d2 : 0 + 0 d3 : 0 + 0	R1 : 7,09 R2 : 7,09 d3 : 7,09 R = 7,09

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

- Treatment 7 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>P. aeruginosa</i> Date April/24/15 B: 20,6°C/RH 47% E: 20,6°C/RH 61%	5.10 ⁷ - 2.10 ⁹ 8,75.10 ⁸	n1 > 0.5 N1 d1 : 78/88 d2 : 77/88	n2 > 0.5 N2 d1 : 86/77 d2 : 97/77	n3 > 0.5 N1 d1 : 70/88 d2 : 76/88	≈ 10 ⁶ d1 : 0,76.10 ⁶ d2 : 0,74.10 ⁶ T = 0,75.10 ⁶	d1 : 0 + 0 d2 : 0 + 0 d3 : 0 + 0	R1 : 5,88 R2 : 5,88 R3 : 5,88 R = 5,88

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

b. Fungicidal activity

- Treatment 5 mL / m³ - waiting 2H

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
		n1/N1	n2/N2	n3/N1		
<i>C. albicans</i> Date Apr/30/15 B: 19,8 °C/RH 42% E: 19,9 °C/RH 52%	2.10 ⁷ - 1.10 ⁸ 0,89.10 ⁸	n1 > 0.5 N1 d1 : 93/87 d2 : 94/87	n2 > 0.5 N2 d1 : 97/90 d2 : 92/90	n3 > 0.5 N1 d1 : 89/87 d2 : 82/87	Control d1 : 6,35.10 ⁵ d2 : 5,90.10 ⁵ T = 6,13.10 ⁵	d1 : 8 + 0 d2 : 111 + 0 d3 : 1 + 0 R1 : 4,88 R2 : 3,74 R3 : 5,79 R = 4,81

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

- Treatment 5 mL / m³ – waiting 2H and Treatment 5 mL / m³ – waiting 2H

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
		n1/N1	n2/N2	n3/N1			
<i>A. brasiliensis</i> Date Apr/27/15 B: 20,9°C/RH 52% E: 19,9°C/RH 70%	5.10 ⁶ – 1.10 ⁷ 1,32.10 ⁷	n1 > 0.5 N1 d1 : 54/26 d2 : 47/26	n2 > 0.5 N2 d1 : 38/29 d2 : 36/29	n3 > 0.5 N1 d1 : 78/26 d2 : 89/26	≈ 10 ⁵ d1 : 1,99.10 ⁶ d2 : 1,84.10 ⁶ T = 1,91.10 ⁶	d1 : 510 + 0 d2 : 1750 + 0 d3 : 1 + 0	R1 : 3,58 R2 : 3,04 R3 : 6,28 R = 4,3

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

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

c. Sporidical activity

- Treatment 7 mL / m³ - waiting 2H

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			
<i>B. subtilis</i>							
Date Apr/24/15							
B: 20,6°C/RH 47%							
E: 20,6°C/RH 61%							
	2.10 ⁵ - 5.10 ⁵	n1 > 0.5 N1	n2 > 0.5 N2	n3 > 0.5 N1	≈ 10 ⁴		
	2.10.10 ⁵	d1 : 32/28 d2 : 28/28	d1 : 28/20 d2 : 24/20	d1 : 31/28 d2 : 16/28	d1 : 1,11.10 ⁴ d2 : 1,00.10 ⁴ T = 1,06.10 ⁴	d1 : 0 + 0 d2 : 0 + 0 d3 : 0 + 0	R1 : 4,03 R2 : 4,03 R3 : 4,03 R = 4,03

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

d. Mycobactericidal activity

- Treatment 5 mL / m³ - waiting 2H

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 > 0.5 N2	n3 > 0.5 N1			
<i>M. terrae</i>							
Date Apr/22/15							
B: 19,4°C/RH 55%	3,09.10 ⁷	d1 : 24/45 d2 : 40/45	d1 : 57/54 d2 : 40/54	d1 : 25/45 d2 : 38/45	d1 : 1,04.10 ⁵ d2 : 0,86.10 ⁵	d1 : 0 + 0 d2 : 0 + 0 d3 : 0 + 0	R1 : 4,98 R2 : 4,98 R3 : 4,98
E: 19,4°C/RH 68%					T = 0,95.10 ⁵		R = 4,98

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

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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 titer reduction between cells treated by the solution S and cells treated by PBS shall be $< 1 \lg$.

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 ($\lg \text{DIC}_{50}$) by the following formula:

$\lg \text{DICT}_{50}$ = negative logarithm of the highest concentration of virus - $[(\text{Sum of \% affected to each dilution}/100 - 0.5) \times (\lg \text{ dilution})]$

○ **Results**

Adenovirus type 5

Virus suspension titer: lgDICT50 = 8,6

No cytotoxicity was observed on the carrier without treatment which has been pretreated with the aerial disinfection system.

Treatment 5ml/m³ – waiting 2H

Date May/11/2015 B : 19,4°C / 48% RH E : 19,8°C / 59% RH	Degree of cytopathogenic effect (log)	Logarithmic reduction
Sensitivity of cells to virus - With treatment (S1) Carrier1 Carrier 2 Average - Without traitement (S2) Carrier 1	8,0 7,9 8,0 8,0	Difference < 1 lg
Efficiency for suppression of disinfectant activity - With treatment (D1) Carrier1 Carrier 2 Average - Without traitement (D2) Carrier 1	7,8 8,1 8,0 8,0	Difference < 0,5 lg
Test control Carrier1 Carrier 2 Average	5,9 5,8 5,9	
Assay Support 1 Support 2 Support 3 Average	0,8 0,9 0,9 0,9	5,0

Murine Norovirus

Virus suspension titer: lgDICT50 = 8,5

No cytotoxicity was observed on the carrier without treatment which has been pretreated with the aerial disinfection system.

Treatment 5ml/m³ – waiting 2H

Date Apr/30/2015 B : 19,8°C / 42% RH E : 19,9°C / 52% RH	Degree of cytopathogenic effect (log)	Logarithmic reduction
Sensitivity of cells to virus - - With treatment (S1) Carrier1 Carrier 2 Average - Without traitement (S2) Carrier 1	7,5 7,4 7,5 7,4	Difference < 1 lg
Efficiency for suppression of disinfectant activity - With treatment (D1) Carrier1 Carrier 2 Average - Without traitement (D2) Carrier 1	7,2 7,1 7,2 7,4	Difference < 0,5 lg
Test control Carrier1 Carrier 2 Average	5,9 5,8 5,9	
Assay Support 1 Support 2 Support 3 Average	0,8 0,8 0,6 0,7	5,2

5. Conclusion

According to the conditions of test, the couple apparatus/product for a use in medical area led to:

- A bactericidal activity (log reduction ≥ 5)
 - After a 5 mL/m³ treatment and 2 hours of wait on the following strains :
 - *E. coli* CIP 54.127
 - *E. hirae* CIP 58.55
 - After a 5 mL/m³ treatment - 2 hours of wait and a second 5 mL/m³ treatment - 2 hours of wait on the following strain :
 - *S. aureus* CIP 4.83
 - After a 7 mL/m³ treatment - 2 hours of wait on the following strain :
 - *P. aeruginosa* CIP 103467
- A yeasticidal activity (log reduction ≥ 4)
 - After a 5 mL/m³ treatment and 2 hours of wait on the following strains :
 - *C. albicans* DSM 1386
- A fungicidal activity (log reduction ≥ 4)
 - After a 5 mL/m³ treatment and 2 hours of wait on the following strains :
 - *C. albicans* DSM 1386
 - After a 5 mL/m³ treatment and 2 hours of wait and a second 5 mL/m³ treatment - 2 hours of wait on the following strains :
 - *A. brasiliensis* CBS 733.88
- A sporicidal activity (log reduction ≥ 3)
 - After 7 mL/m³ treatment - 2 hours of wait on the following strain :
 - *B. subtilis* spores CIP 52.62
- A mycobactericidal activity (log reduction ≥ 4)
 - 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)
 - After 5 mL/m³ treatment - 2 hours of wait on the following strain :
 - *Adenovirus type 5*
 - *Murine Norovirus*