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Efficacy of the Nocolyse/Nocospray System against the spores of *Clostridium sporogenes ATCC 3584* and *Bacillus subtilis ATCC 19659* according to the ASTM E 2197:2002

Study done by :

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1. Summary of the test

Tested product: Nocolyse/Nocospray System
Manufacturer: Airel
Method used: Derivative of ASTM E 2197-02
Carriers: Polystyrene petri dishes
Testing temperature: Room temperature 22 ± 2°C
Dilution: None required
Four rooms (2 rooms per batch) were studied with 5 positions per room

Results :

Table I: Test results of the Nocolyse sporicide activity against *B. subtilis* ATCC 19659 and *C.sporogenes* ATCC 3584 using the ASTM 2197:2002 test method (vapour phase)

Strains	**Positions**	**Rooms**			
		310	**311**	**304**	**305**
B. subtilis ATCC 19659	1	7,2	7,7	6,9	8,0
	2	6,9	6,1	6,9	6,9
	3	8,0	6,9	6,9	8,0
	4	6,9	7,2	8,0	6,9
	5	6,9	6,9	6,9	6,9
C.sporogenes ATCC 3584	1	6,6	7,5	7,5	7,5
	2	6,6	7,5	7,5	6,0
	3	6,6	7,5	7,5	7,5
	4	6,6	7,5	7,5	7,2
	5	6,6	7,2	6,1	6,0

As the testing was not done in the laboratory, closable petri dishes were chosen in order to facilitate the transportation of the inoculum to and from the hospital test site.

2. Introduction

The objective of the study is to determine the efficacy, in a hospital milieu, of the Nocolyse/Nocospray Sytem against *B. subtilis* ATCC 19659 and *C.sporogenes* ATCC 3584.

The Nocolyse/ Nocospray System consists of a portable plug-in machine (Nocospray) and a liquid hard surface disinfectant (Nocolyse).The disinfecting liquid Nocolyse is nebulized at 37ºC to form a dry vapour which is then propelled by the Nocospray turbine at 80m/sec permitting a rapid distribution throughout the target space.

Test Method

The testing of the vapour was done in four standard hospital rooms with petri dishes of each strain located in five different positions. The population of each strain was counted on the carriers and compared to the control carriers not exposed to the Nocolyse/Nocospray System. The test was done using a derivative of the quantitative carrier test method, ASTM E 2197 (2002).

3. Test Product

Name: NOCOLYSE

Manufacturer: Airel, Champigny-Sur-Marne, France

Active Material: 6 % solution of hydrogen peroxide

Principal: Liquid Nocolyse is vaporized at 37ºC into a dry vapour within the space to be disinfected.

4. Tested microorganisms

The microbial strains used for the testing are illustrated in Table II.

Table II: Microbial strains utilized

Microorganisms	**Strains**	**Provider**	**Lot Numbers**
Clostridium sporogenes	ATCC 3584	Microbiologics Kwikstik c (Saint Cloud – USA)	67630
Bacillus subtilis	ATCC 19659	Microbiologics Kwikstik c (Saint Cloud – USA)	54002

The recovery medium used for each strain is stated below :
Columbia Blood Agar for *Clostridium sporogenes* and Trypticase Soy Agar for *Bacillus subtilis.*

5. Study Facility

The hospital test site was at the Iris Sud Hospital, Bracops in Anderlecht (Bruxelles). Four standard hospital rooms were used for the test (refer to Annexe 1). The carriers were prepared and analyzed at the Laboratory of ‘Ecologie microbienne et d'Epuration des Eaux de la Faculté Universitaire des Sciences agronomiques de Gembloux’ at a Biosafety Level 2 (P2) lab by a qualified staff.

6. Study Period

The test was done from the 7th of August to the 18th of September 2009.

7. Experimental Procedure

The testing done was based on a derivative of the ASTM E2197:2002 standard.

Carriers :

The testing was done on 35mm diameter sterile petri dishes.

Culture Media and solutions :

Columbia Broth (Reference 7127A)
Double Concentration : 70 g/L
1/10 : 3.5 g/L
Columbia Agar with 5 % Sheep Blood (AES - R& : AEB 520680) ready to use petri dishes
Trypticase Soy Agar (AES – Réf : AEB 152852) prepared according to manufacturer's recommendations

Saline Solution: 8.5 g/L NaCl solution p.a. (Merck)

Sterile normal saline-T: Saline Solution + 1 mL/L Tween 80 (BDH Prolabo)

The above culture Medias and solutions were sterilized at 121°C for 15 minutes.

0.1M Phosphate Buffer: 13.8 g/L $NaH_2PO_4.H_2O$ (Fluka) solution, 7.2 pH (NaOH 1M)

Mucin 0.4%: 4 mg/L mucin bovine solution (Sigma) in 0.1M phosphate buffer pH 7.2. Sterilization by membrane filtration at 0.2 µm (Sartorius-Minisart)

Bovine serum albumine 4% (m/v): bovine serum albumine solution at 40 mg/ml in 0.1M phosphate buffer. Sterilization by membrane filtration at 0.2 µm (Sartorius-Minisart)

Tryptone 5 %(m/v): 50 mg/mL tryptone solution (Oxoid) in 0.1M phosphate buffer, 7.2 pH. Sterilization by membrane filtration at 0.2 µm (Sartorius-Minisart)

Preparation of Inocula :

The drying of the test organisms is illustrated in Table V.

Table V: The Cultivation of the Various Test Organisms to be Used in the Carrier Test

Microorganisms	**Culture Media**	**Temperature**	**Time**
Clostridium sporogenes	Columbia Broth double concentration	30°C	5 days
Bacillus subtilis	Columbia Broth 1/10 + $MnSO_4$	35°C	72 h

After incubation, the cells were washed (3 centrifugation cycles at 4°C and resuspended in sterile distilled water). The concentration was verified by a Bürker Counting Chamber.

The suspension was heated at 80°C for 10 minutes to inactivate vegetative cells. This should yield 10^9 CFU/mL.

340 µL of the microbial suspension was mixed with
100 µL of mucin 0.4% in phosphate buffer
35 µL of tryptone stock 5% in phosphate buffer
32 µL of BSA 4%[1] in phosphate buffer

Preparation of the Test Carriers :
Each 23 mm disk was inoculated with 20 µL of test organism. Allow the inoculum to become visibly dry (2 h in a desiccator under vacuum at room temperature of 25°C).

The concentration was twice as much the one in the standard in order to ensure obtaining a big enough population. The quantity of solution was changed to take into account the lowest concentration of test organism.

A 5% solution could not be prepared due to the limited solubility of the Bovine Serum Albumin.

Disinfection Testing

Disinfection testing took place at the Anderlecht Bracops Hospital under the supervision of the doctor J.M. Hubrechts, hygienist doctor of the institution.

5 dishes of each strain were located in various positions in each of the 4 rooms used for the testing. The rooms were standard hospital rooms with a $50m^3$ volume (refer to Annexe 2).

Even though a neutralization process was not necessary due to the rapid degradation of hydrogen peroxide vapour particles at a temperature of 37°C, a neutralization was done in order to follow lab procedures. A neutralization was done right after exposure of the carriers to the Nocolyse/Nocospray System with 10mL of Letheen broth (polysorbate 80, reference BD 268110).

The petri dish covers were removed prior to disinfection with the Nocolyse/Nocospray System which was placed at a height of 40 cm (as seen in the picture below) giving a wide diffusion angle.

The room volume selector was set at seven times the volume of the room since experience has shown this factor to be effective in killing all microorganisms that have been previously evaluated using 350 mL of Nocolyse. Two lots of Nocolyse were tested (2 rooms per lot). One hour after the Nocospray stopped, neutralization was done using 10 ml of Letheen broth. The petri dishes were closed and sent to the Laboratory of 'Ecologie microbienne et d'Epuration des eaux' where the analysis was done. The dishes were kept at 4°C during transportation.
Three control petri dishes were kept at the Lab (non- exposed to the Nocolyse/Nocospray System). The results for the control carriers are illustrated in Tables VII and VIII.

The number of spores on the carriers should be superior to 10^6 in order to prove that a 6 log reduction was obtained.

Immediately at the end of the contact time, 2 mL of sterile normal saline-T was added in the Petri dishes in order to stop the activity (using a suction-delivery apparatus, extremity of a micropipette). 2mL of sterile normal saline-T was used instead of 9.95 mL as stated in the ASTM standard since the carriers were not big enough to carry 9.95mL of solution.

A membrane filtration was done (0.45 µm pore diameter Whatman). After rinsing with 3 X 50 mL sterile distilled water, the membranes were transferred.
Culture plates with the filters were incubated (Refer to table VI). For the control carriers, the suspensions were diluted up to 10^{-6} and 1mL of dilutions 10^{-5} and 10^{-6} were filtered (0.45 µm pore diameter Whatman).

Table VI: The Cultivation of the Various Test Organisms to be Used in the Carrier Test

Microorganisms	**Culture Media**	**Temperature**	**Time**
Clostridium sporogenes	Blood Columbia Agar	30°C	48 h
Bacillus subtilis	Trypticase Soy Agar	35°C	48 h

After the incubation period, the sum was done for the number of colonies counted on the membranes and petri dishes with the carriers (N).
For the controls, the number of colonies counted on the membranes were multiplied by the opposite of the corresponding dilution and by the division of the suspension volume (2 mL) by the filtered dilution in order to obtain the initial population on the carrier. The mean of the 3 carrier population has to be superior to 10^6 for the validity of the test.

The disinfection trial was done in 4 hospital rooms at the Bracops Hospital in Anderlecht (Bruxelles) which is located 50 km from the Laboratory where the carriers were analyzed. In order to prevent any cross contamination or any event that could affect the results during transportation (for example a spill), and in order to accommodate the study, the carriers used were petri dishes (35 mm diameter) instead of metallic disks as used in the ASTM standard.

Calculating Log_{10} Reductions

The log_{10} reduction was calculated for each carrier in relation to the mean of the 3 controls.

Reduction on the Carrier n= $Log_{10}(T) - Log_{10}(N_n)$
T= mean of the number of colonies for the controls
N_n = number of colonies on the carrier n (n= 1 to 5). If $N_n = 0$, we add 1 in order to calculate the log.

The product Nocolyse reached the 6 log kill target and was able to eliminate 99.9999% of the organisms which were tested.

8. Results

Table VII : Log Reduction Results for *Bacillus subtilis* ATCC 19659

Rooms	Lot Numbers	Positions	Testing Date	T	N	Log Reduction
310	060509 OS	1	26/08/2009	1,06E+08	6	7,2
		2	14/09/2009	8,00E+06	1	6,9
		3	26/08/2009	1,06E+08	0	8,0
		4	14/09/2009	8,00E+06	1	6,9
		5	14/09/2009	8,00E+06	1	6,9
311	220709 OS	1	26/08/2009	1,06E+08	2	7,7
		2	26/08/2009	1,06E+08	76	6,1
		3	14/09/2009	8,00E+06	1	6,9
		4	26/08/2009	1,06E+08	6	7,2
		5	14/09/2009	8,00E+06	1	6,9
304	060509 OS	1	14/09/2009	8,00E+06	1	6,9
		2	14/09/2009	8,00E+06	1	6,9
		3	14/09/2009	8,00E+06	1	6,9
		4	26/08/2009	1,06E+08	0	8,0
		5	14/09/2009	8,00E+06	1	6,9
305	220709 OS	1	26/08/2009	1,06E+08	0	8,0
		2	14/09/2009	8,00E+06	1	6,9
		3	26/08/2009	1,06E+08	0	8,0
		4	14/09/2009	8,00E+06	1	6,9
		5	14/09/2009	8,00E+06	1	6,9

T = mean of the spore count on non-exposed carriers (non-exposed to the Nocolyse/Nocospray System)
N = mean of the spore count on exposed carriers placed in different locations in the four rooms
R. Log = Reduction of the strain population by the disinfection system in comparison to the mean of the non exposed carriers (T)

Table VIII: Log Reduction Results for *Clostridium sporogenes* ATCC 3584

Rooms	Lot Numbers	Positions	Testing Date	T	N	LogReduction
310	060509 OS	1	14/09/2009	4,00E+06	0	6,6
		2	14/09/2009	4,00E+06	0	6,6
		3	14/09/2009	4,00E+06	0	6,6
		4	14/09/2009	4,00E+06	0	6,6
		5	14/09/2009	4,00E+06	0	6,6
311	220709 OS	1	14/08/2009	2,97E+07	0	7,5
		2	14/08/2009	2,97E+07	0	7,5
		3	14/08/2009	2,97E+07	0	7,5
		4	14/08/2009	2,97E+07	0	7,5
		5	14/08/2009	2,97E+07	2	7,2
304	060509 OS	1	14/08/2009	2,97E+07	0	7,5
		2	14/08/2009	2,97E+07	0	7,5
		3	14/08/2009	2,97E+07	0	7,5
		4	14/08/2009	2,97E+07	0	7,5
		5	14/08/2009	2,97E+07	26	6,1
305	220709 OS	1	14/08/2009	2,97E+07	0	7,5
		2	14/08/2009	2,97E+07	32	6,0
		3	14/08/2009	2,97E+07	0	7,5
		4	14/08/2009	2,97E+07	2	7,2
		5	14/08/2009	2,97E+07	33	6,0

T = mean of the spore count on non-exposed carriers (non-exposed to the Nocolyse/Nocospray System)
N = mean of the spore count on exposed carriers placed in different locations in the four rooms
R. Log = Reduction of the strain population by the disinfection system in comparison to the mean of the non exposed carriers (T)

9. Conclusions

A 6 log reduction was achieved for all 4 rooms and all 5 tested carriers for each pathogen tested and for the two tested lots (220709 OS and 060509 OS). This proves that the Nocolyse/Nocospray System is effective according to the ASTM E 2197:2002 standard.

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10. ANNEXE 1 : DESCRIPTION OF THE TEST ROOMS (310-311-304-305)

The four test rooms were identical (same dimensions and layout) and all located on the same floor (Bracops Hospital, B Building, 3rd floor):

Standard hospital room with 1 bed, night table, chair and table, small fridge and built in cupboard.
Height of the room: 2.70 m Volume of the room: 48.8 m³

The temperature and humidity of the room were recorded during the trial as well as the distances between the appliances in the room and the carriers.

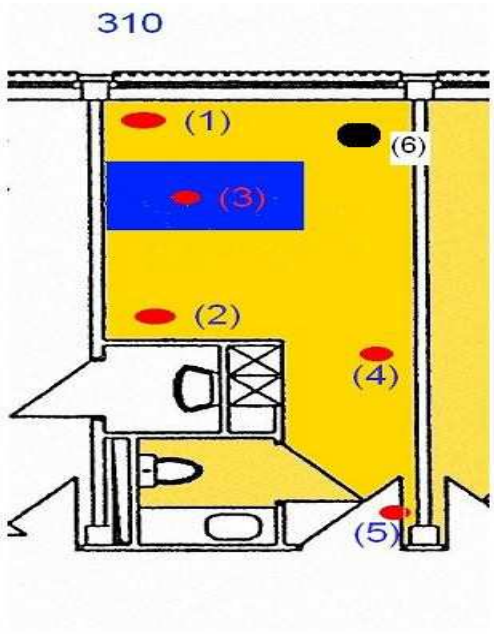
Washroom :

Seperate washroom **:** 6,6 m³

Hallway of the room :

Interior passageway: Durant the trial, the door towards the washroom was left open.

11. ANNEXE 2 : PLAN OF THE TEST ROOMS (310-311-304-305)



Location of the Carriers in the Room
Legend :

(1) left corner of the room beside the bed
(2) right corner of the room beside the bed
(3) in the middle of the room, on the bed
(4) beginning of the corridor in the room
(5) end of the corridor in the room
(6) Nocospray position (at a height of 40 cm)

12. ANNEXE 3 : TESTING PARAMETERS

PARAMETERS	**ROOM 304**	**ROOM 305**	**ROOM 310**	**ROOM 311**
At time 0 (Before the treatment) :				
Time when the parameters were taken	12 :30	12 :40	11 :45	11 :55
Temperature	23.7°C	24.1°C	22°C	23.5°C
Humidity	46%	50%	49%	53%
Volume of the Room	50 m³	50 m³	50 m³	50 m³
Tested Product	Nocolyse	Nocolyse	Nocolyse	Nocolyse
Lot Number	060509 OS	220709 OS	060509 OS	220709 OS
Expiry Date	05/2011	07/2011	05/2011	07/2011
Time at the beginning of the treatment	12 :30	12 :40	11 :45	11 :55
Time at the end of the treatment	12 :52	13 :02	12 :08	12 :17
Programmed Volume	350 m³	350 m³	350 m³	350 m³
Volume of the Product Used	350 ml	350 ml	350 ml	350 ml
At time 1 (After treatment with the Nocolyse/Nocospray System) :				
Temperature	25.2°C	25.4°C	25.6°C	26.5°C
Humidity	47%	46%	48%	47%

REFERENCES

ASTM 2197:2002. Standard quantitative disk carrier test method for determining the bactericidal, virucidal, fungicidal, mycobactericidal and sporocidal activities of liquid chemical germicides. Doc