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ATTACHMENT 12.0

BACTERICIDAL EFFICACY TEST REPORT: *Quantitative suspension test for the evaluation of bactericidal activity against MDR bacteria*

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1. PURPOSE

The following report has the purpose of defining in a clear and detailed way the methods of execution of the analysis in order to guarantee maximum precision and accuracy in compliance with the European standard UNI EN 17272:2020. This standard, employed for the validation of chemical disinfectants and antiseptics, was used for the verification of the bactericidal activity of equipment that uses the Non-Thermal-Plasma technology, or cold plasma. The bactericidal activity was performed using a control strain of *Escherichia coli* and in succession strains of MDR microorganisms (MultiDrug Resistant).

2. TERMS AND DEFINITIONS

Bactericidal activity: the ability of a product to produce a reduction in the number of bacterial colonies through experimental procedures that include precise and defined test conditions.

Colony Forming Units (CFU): number of colonies per mL.

Airborne disinfection contact (ADC) time: time from the first release of the product to the point where the carriers are recovered.

Sensitive microorganism: microorganism in which desiccation causes a log reduction greater than 1.5.

3. INTRODUCTION

The test method to verify the bactericidal activity of the Jonix Cube device (test product) against MDR bacteria was conducted in compliance with the European standard UNI EN 17272:2020 chemical disinfectants and antiseptics- Method for airborne room disinfection using automatic processes. Cold plasma emits light with wavelengths both in the visible and in the ultraviolet part of the spectrum. In addition to the emission of UV radiation, an important property of low-temperature plasma is the presence of highly reactive and high-energy electrons, which cause numerous chemical and physical processes such as oxidation, excitation of atoms and molecules, production of free radicals and other reactive particles.

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4. SAMPLE CHARACTERIZATION

Product: Jonix Cube Non-Thermal-Plasma Device (hereinafter referred to as Jonix Cube)

Product Description: Jonix CUBE is an air purification device; with a design that uses an advanced technology called cold plasma to eliminate bacteria, moulds, viruses, pollutants and odours

Storage conditions: room temperature

Equipment instructions: see attachment

5. EXPERIMENTAL CONDITIONS

Test temperature: 20 ± 2 ° C

Relative humidity (RH): 50-75%

Contact time: 12 h - 14 h - 16 h

Analysis period: test start date: 25-05-2020 to Test end date 28-05-2021

6. MATERIALS AND REAGENTS

Microorganisms used in the experimental phase:

- ***Strain of Escherichia coli* ATCC 10536:** *Escherichia coli* is a Gram-negative bacillus. It is an integral part of the normal intestinal flora of humans and other animals. While most E. coli strains are harmless, some endanger human health.
- ***Acinetobacter baumannii* MDR strain:** OXA-23 type carbapenemase-producing strain. *Acinetobacter baumanni* is a ubiquitous Gram-negative bacillus. It is able to survive about one month on dry surfaces.
- ***Klebsiella pneumoniae* MDR:** KPC type carbapenemase-producing strain. *Klebsiella pneumoniae* is a Gram-negative bacillus capable of causing bacterial pneumonia, although it is more commonly associated with urinary tract infections acquired from hospitals. *Klebsiella pneumoniae* has become a growing nosocomial infection as antibiotic-resistant strains continue to appear.

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- ***Pseudomonas aeruginosa* MDR:** OXA-48 type carbapenemase-producing strain. *Pseudomonas aeruginosa* is a ubiquitous Gram-negative bacillus. It causes numerous types of infections.

* ATCC (American Type Culture Collections)

MEDIUMS OF CULTURE AND REAGENTS

The reagents used are pure for analysis and/or suitable for microbiological applications.

Culture medium for bacterial count

The culture medium used for cell counting is TSA (Tryptone Soya Agar).

Diluent for microbial suspensions

The diluent for microbial suspensions, prepared according to the UNI EN 17272:2020 standard, was used to prepare the microbial suspension and to carry out the dilutions required by the standard indicated above.

7. EQUIPMENT

- Steel carriers
- DensiCheck Plus Biomerieux
- Stopwatch
- Vortex stirrer
- Incubator with a controlled temperature of 36°C ± 1°C.
- "BioHazard" class II vertical laminar flow hood
- pH meter
- Refrigerator with a controlled temperature of 4°C
- Graduated pipettes
- Petri dishes
- Scale
- Beaker
- Scrapers

8. EFFECTIVENESS TEST

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PREPARATION OF THE BACTERIAL SUSPENSION

Bacterial suspension

A TSA subculture was prepared from the mother culture and incubated at 37 ± 1 ° C for 18-24 hours. The step was repeated and incubated for another 24 hours. Finally, a third subculture was generated from the second (subculture II and III are considered working cultures). A loop was taken from the working cultures and transferred into a tube containing the diluent for the microbial suspensions until 0.5 McF was obtained with DensiCheck Plus Biomerieux corresponding to $1x10^8$ cfu/mL.

Interfering substance

The interfering substance (BSA 3g/l) was added to the suspension in order to obtain a 1/10 dilution, and then an adaptation was performed for sensitive microorganisms, adding skimmed milk (100g/l) in concentration 1:20.

DETERMINATION OF THE TITLE

To determine the title of the stock solution, serial dilutions up to 10^{-8} were made. 1 ml of dilution 10^{-6}, 10^{-7} and 10^{-8} was included in TSA agar. In addition, 1 ml of the dilution 10^{-6}, 10^{-7} and 10^{-8} was filtered through the use of filter membranes.
All tests were conducted twice.

EVALUATION OF THE DISINFECTION PROCESS

Survival of the test organism in control carriers

The control carriers were placed inside uncovered Petri dishes and contaminated with 50 µl of microbial suspension, which was then well distributed and dried. At the end of the drying period, the lid of the corresponding plate was applied. Each plate, containing the control carrier, was left in the laboratory for the duration of the exposure period. At the end of the exposure period, the control carriers were transferred into a beaker containing 100 ml of diluent for microbial suspensions. A first phase of scraping, lasting 1 minute, was followed by a second phase of mechanical stirring to allow the detachment of any microorganisms remaining adhered to the carrier. Serial dilutions up to 10^{-3} were carried out from the solution obtained. 1 ml of the 10^{-2} dilution and 1 ml of the 10^{-3} dilution were added in TSA medium with the inclusion technique; the tests carried out were duplicate. The plates were placed in an incubator at 37 ± 1 ° C for 24 hours, for the first count and reincubated for another 24 hours for the second count.

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Preparatory tests to evaluate the absence of residual effects

To evaluate the absence of residual effects, 50 µl of interfering substance were deposited in other carriers. When completely dry, the carriers were placed in the display case to be exposed to the action of the NTP. At the end of the exposure period, the carriers were transferred into a beaker containing 100 ml of diluent for microbial suspensions. A first phase of scraping, lasting 1 minute, was followed by a second phase of mechanical stirring to allow the detachment of any microorganisms adhering to the carrier, thus obtaining a solution, defined as S.
A screening was then performed to evaluate any inhibitory effects in the agar; therefore, 1 ml of solution S was included in the TSA agar together with 1 ml of the known bacterial suspension. The plate was incubated at 37 ± 1 ° C for 24 hours for the first count and reincubated for another 24 hours for the second count. Further screening was performed to assess any inhibitory effects with the filter membranes. To do this, 98 ml of solution S were filtered with 0.45 µm filter membranes, followed by 3 washes with 50 ml of diluent for microbial suspensions. Subsequently, 1 ml of dilution of the known microbial suspension was filtered. The membrane was transferred to a plate containing TSA agar and incubated at 37 ± 1 ° C for 24 hours for the first count, and reincubated for another 24 hours for the second count. Finally, to evaluate a possible carrier-related inhibitory effect, 1 ml of the known microbial suspension was transferred together with the exposed carrier in a Petri dish. Agar was added and the plate was incubated at 37 ± 1 °C for 24 hours for the first count, and reincubated for another 24 hours for the second count.
Preliminary tests were conducted for each microorganism tested.

Effectiveness test

Exposure of carriers to the test product

The carriers were placed inside empty Petri dishes. 50 µl of microbial suspension (contaminated carriers) were deposited, then well distributed on the carrier through the use of a loop, and kept under a hood until the microbial suspension was completely dry.

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When the microbial suspension was completely dry, the carriers to be exposed to the NTP were placed in the case provided with the lid open, in the opposite position to the location of the test device, to allow the carriers to be exposed to the test product. The duration of the exposure was weighted according to the results obtained.

Recovery of carriers

The exposed carriers were deposited inside a beaker containing 100 ml of diluent for microbial suspensions. A first phase of scraping, lasting 1 minute, was followed by a second phase of mechanical stirring to allow the detachment of any microorganisms remaining adhered to the carrier, thus obtaining a solution. 1 ml of solution was used to perform a direct seeding by inclusion in TSA; subsequently, the plate was incubated at 37 ± 1 ° C for 24 hours, for the first count and reincubated for another 24 hours for the second count. 10 ml of solution were filtered with 0.45 µm filter membranes, followed by 3 rinses with recovery liquid. The membrane was then transferred to a TSA plate which was incubated at 37 ± 1 ° C for 24 hours for the first count, and reincubated for another 24 hours for the second count. Finally, the carrier was transferred to a TSA plate to which more TSA agar was then added to completely cover the carrier. The plate was incubated at 37 ± 1 °C for 24 hours for the first count and reincubated for another 24 hours for the second count.

9. RESULTS

***Escherichia coli* ATCC 10536**

In the first experimental phase, the efficacy test was carried out on *E. coli* ATCC 10536 as required by the UNI EN 17272:2020 standard to evaluate the efficacy of the test product on a known microorganism.

Microorganism test	Title of the initial suspension (cfu/mL)	Validation test	Title of the suspension of the carrier of control (cfu/mL)	Log Reduction (R)
E. coli ATCC 10536	4.55 10^8	Valid	1.46 10^6	6.2
12 h				
E. coli ATCC 10536 14 h		Valid	1.45 10^6	5.9
E. coli ATCC 10536 16 h		Valid	1.44 10^6	6.2

Table 1 - E. coli - NTP exposure for 12, 14 and 16 hours.

Having demonstrated that the requirements of the standard with *E. coli* ATCC 10536 are met, the experimental phase continued by testing strains of multidrug-resistant Gram-Negative microorganisms (MDR): *K. pneumoniae* KPC, *A. baumannii* OXA-23 and *P. aeruginosa* OXA- 48.

Klebsiella pneumoniae MDR

Microorganism test	Title of the initial suspension (cfu/mL)	Validation test	Title of the suspension of the carrier of control (cfu/mL)	Log Reduction (R)
K. pneumoniae KPC 12 h	4.45 - 10^8	Valid	1.61 10^6	6.3
K. pneumoniae KPC 14 h		Valid	1.38 10^6	6.3
K. pneumoniae KPC 16 h		Valid	1.83 10^6	6.3

Table 2. - K. pneumoniae - NTP exposure for 12, 14 and 16 hours.

Acinetobacter baumanii MDR

Microorganism test	Title of the initial suspension (cfu/mL)	Validation test	Title of the suspension of the carrier of control (cfu/mL)	Log Reduction (R)
A. baumanni OXA-23 12 h	4.93 - 10^8	Valid	3.93 10^6	3.1

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A. baumanni OXA-23 14 h		Valid	2.45 10^6	4.1
A. baumanni OXA-23 16 h		Valid	2.56 10^6	6.4

Table 3. A. baumannii - NTP exposure for 12, 14 and 16 hours.

Pseudomonas aeruginosa MDR

Microorganism test	Title of the initial suspension (cfu/mL)	Validation test	Title of the suspension of the carrier of control (cfu/mL)	Log Reduction (R)
P. aeruginosa OXA-48 12 h	2.59 - 10^8	Valid	1.17 10^6	4.1
P. aeruginosa OXA-48 14 h		Valid	1.13 10^6	4.8
P. aeruginosa OXA-48 16 h		Valid	1.15 10^6	6.1

Table 4. P. aeruginosa - NTP exposure for 12, 14 and 16 hours.

10. BACTERICIDAL EFFECTIVENESS

The product in question is considered **bactericidal** when, after the contact time, there is a **reduction in the vitality of at least 10^5**, corresponding to a reduction equal to 5 logarithms concerning the bacterial strain test based on the method and the acceptability criteria of UNI EN 17272:2020.

To obtain the reduction of the bacterial load (R), the logarithm of the ratio between the microorganisms presents on the control carrier and the microorganisms surviving in the carrier after exposure to NTP was carried out.

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11. CONCLUSIONS

The results obtained showed that the device is effective from as quick as 12 hours, determining a reduction of the total bacterial load both for the *E. coli* strain ATCC 10536 (R>5) and for the *K. pneumoniae* KPC strain (R>5).

For the strains of *A. baumannii* OXA-23 and *P. aeruginosa* OXA-48, the reduction of the bacterial load is not sufficient to demonstrate the efficacy of the device after 12 hours (respectively R=3.1 and R=4.1) and 14 hours (respectively R=4.1 and R=4.8) of exposure. However, after 16 hours of exposure for both microorganisms, the reduction of the bacterial load is total, demonstrating the effectiveness of the device, with R>5.

The results obtained show that the Jonix Cube device has an effective bacterial activity against various multidrug-resistant microorganisms after a microorganism-related exposure phase.

12. REFERENCES

- EUROPEAN STANDARD UNI EN 17272:2020 Chemical disinfectants and antiseptics - Method for airborne room disinfection using automatic processes
- EUROPEAN STANDARD EN 14476:2019 Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of virucidal activity in the medical area
- ISO 15189: 2012 Medical laboratories - Requirements for quality and competence

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