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ANTIVIRAL - ANTIBACTERIAL CERTIFICATE
B ZERO & B TITANIA SILVER
2020 - 2021

UNIVERSITÀ DEGLI STUDI DI MILANO
FACOLTÀ DI MEDICINA E CHIRURGIA

Final Report: Antiviral activity of specimens treated with “B TITANIA SILVER” and “MULTI-PURPOSE B ZERO”

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ABSTRACT

Test specimens treated with “B TITANIA SILVER” and “Multi-Purpose B ZERO” were assayed to verify for verifying their antiviral effect against SARS-CoV-2, responsible for COVID-19.
The analysis was performed as indicated by the standard method ISO 21702:2019 “Measurement of antiviral activity on plastics and other non-porous surface” [1] with some modifications.
Under the test conditions applied, the test specimens treated with “B TITANIA SILVER” or/and “Multi-Purpose B ZERO” showed antiviral effect against SARS-CoV-2.

Specifically:

1- the combined treatment of "B TITANIA SILVER" and the “Multi-Purpose B ZERO” cleaner (simulation of 15 cleanings) reduced the viral load by more than 99.9%
2- Repeated treatment with the cleaner " Multi-Purpose B ZERO" showed an additive and cumulative antiviral effect, reaching a reduction in viral load of 95% and 98.4% after 15 and 30 applications, respectively.

AIM

The aim of this study was to verify the antiviral activity of low porosity surfaces.
Specifically:
1- Test specimens ("porcelain stoneware") treated with "B TITANIA SILVER"
2- Test specimens ("porcelain stoneware ") treated with the " Multi-Purpose B ZERO" cleaner (15 applications of the product)
3- Test specimens ("porcelain stoneware ") treated with the " Multi-Purpose B ZERO" cleaner (30 applications of the product)
4- Test specimens ("porcelain stoneware ") treated with "B TITANIA SILVER" and then with the " Multi-Purpose B ZERO" cleaner (15 applications of the product)

Briefly, SARS-CoV-2 was added to the samples previously treated with “B TITANIA SILVER” and / or “Multi-Purpose B ZERO” cleaner and, after 18 hours of contact, the residual infectivity of the virus was assessed by Plaque Assay method.

MATERIALS AND METHODS

Test specimens (porcelain stoneware), “B TITANIA SILVER” product and the “Multi-Purpose B ZERO” cleaner were supplied by Bonasystems Italia srl.

Cell culture

Vero cells (Monkey Kidney Epithelial Cells) were maintained in DMEM medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml of penicillin, 100 µg/ml of streptomycin.

Isolation of SARS-CoV-2 from nasal-pharyngeal swabs

SARS-CoV-2 was isolated from 500 µl of nasal-pharyngeal swab, added to Vero cells at 80% confluence; the inoculum was removed after a 3-hour incubation at 37 °C with 5% CO_2 and the cells were incubated at 37 °C, 5% CO_2, for 72 hours, when cytopathic effects (CPE) was evident.
Viral copy numbers in the cell supernatant were quantified via specific quantitative real-time RT-PCR (qRT-PCR).[1] SARS-CoV-2 was precipitated by means of PEG, following the manufacturer's instruction, and viral titer was determined by plaque assay, using dilution factors ranging from 10^1 to 10^9. The complete nucleotide sequence of the SARS-CoV- isolated strain was deposited at Gen Bank, at NCBI (accession number: MT748758)

Preparation of test specimens

Each test specimen is a flat square of (50 ± 2) mm x (50 ± 2) mm.

Test specimens ("porcelain stoneware") treated with "B TITANIA SILVER" and/or "Multi-Purpose B ZERO" cleaner were prepared in laboratory.
"B TITANIA SILVER" product was applied to the sample with a Pasteur pipette in order to cover the entire surface and was air-dried for 60 minutes.
The "Multi-Purpose B ZERO" cleaner was applied 15 or 30 times. Repeated applications simulate daily cleaning with the product, allowing the evaluation of the additive and cumulative effect of the product.

Before use, each test specimen was sterilized by immersion in ethanol 70%, in order to eliminate any bacterial contamination.

Test procedure

Three "B TITANIA SILVER" treated test specimens and 3 untreated test specimens were inoculated with 0.4 ml of virus suspensions (1-5x10^6 PFU/ml). Then, inoculums were covered with a 40 x 40 mm film and incubated for 18 hours at 25 °C and relative humidity > 90%.
At end of the contact time (18 hours), 20 ml of neutralizer SCDLP broth were added to "treated" test specimens and "untreated" test specimens and plaque assay was performed.
Plaque assay was performed in 6 wells plate and six 10-fold serial dilutions of the recovered SCDLP broth in complete medium were tested. Briefly, cells monolayer was inoculated with 0.4 ml of the virus suspension recovered in SCDLP broth and in each dilution, in duplicate. After 2h of inoculum with the virus suspension, the inoculum was removed, the cells were washed and covered with 0.3% agarose dissolved in cell medium and incubated for 72 hours at 37 °C, 5% CO_2. Cells were fixed with 4% formaldehyde solution and, after agarose removal, stained with methylene blue. Plaques were counted and results are expressed as Plaque Forming Unit (PFU)/mL.
At time 0, immediately after virus inoculum, 20 ml of neutralizer SCDLP broth were added to 3 "untreated" test specimens and the residual virus infectivity revealed by plaque assay.

Cytotoxicity and cell sensitivity to virus

For the cytotoxicity assay, cells were seeded into 96-well plates at concentration of 1.3×10^4 cells/well. Twenty ml of neutralizer SCDLP broth were added to 3 "untreated" and 3 "treated" specimens and immediately 0.1 ml was recovered and added to the cells in triplicate. After 2 hours of incubation, SCDLP broth were replaced with complete medium and cells were incubated for 72 hours at 37 °C in 5% CO_2. At the end of incubation, cell viability was measured by MTT assay.[2]

For verification of cell sensitivity to virus, three untreated and three treated test specimens were used.
Twenty ml of neutralizer SCDLP broth were added to the test specimens. Five ml of the SCDLP broth recovered from the test specimen were transferred into new test tubes, and 50 µl of a virus suspension at $4x10^4$ PFU/ml were added. A negative control constituted of SCDLP broth was also used. After 30 min, virus infectivity was measured by plaque assay as previously described. For each test suspension, the infectivity titer of virus was calculated with the following formula:
S= (2.5 x P)
where
S is the infectivity titer of virus per ml per test suspension;
P is the average plaque count for the duplicate wells.

Determination of the infectivity titer of virus

For each test specimen, the infectivity titer of virus recovered was obtained using the formula:
$N = (2.5 \times C \times D \times V) / A$
where
N is the infectivity titer of virus recovered per cm^2 of test specimen;
C is the average number of plaque counted for the duplicate wells;
D is the dilution factor for the wells counted;
V is the volume of the SCDLP added to the specimen, in ml;
A is the surface area of the cover film, in cm^2.

Calculation of the antiviral activity

The antiviral activity was calculated using the formula:
$R = U_t - A_t$
where
R is the antiviral activity;
U_t is the average of the common logarithm of the number of plaques recovered from the three untreated test specimens after 18 h, in PFU/cm^2;
A_t is the average of the common logarithm of the number of plaques recovered from the three treated test specimens after 18 h, in PFU/cm^2.

RESULTS

The results of antiviral tests are summarized below.

1. Test specimens “porcelain stoneware” treated with “B TITANIA SILVER”.

Treatment with "B TITANIA SILVER" induced a viral reduction equal to 0.73 log, that means 81.5 % viral reduction.

Table 1. Results of the antiviral test on " porcelain stoneware " samples treated with the product "B TITANIA SILVER"

Specimen	N* (PFU/cm2)	Log N	R& (Ut-At)	% viral reduction

Untreated	641.9 (214.8)	2.836		
B Titania Silver treated	133.7 (45.7)	2.102	**0.734**	**81.5%**

*N is the infectivity titer of virus recovered per cm2 of test specimen
&R is the antiviral activity
Data are reported as average and standard deviation, in brackets, from three specimens

2. Test specimens "porcelain stoneware" treated with "Multi-purpose B ZERO" cleaner (15 applications of the product).

Treatment with "Multi-Purpose B ZERO" (15 applications) induced a viral reduction equal to 1.304 log, that means 95.0 % viral reduction.

Table 2. Results of the antiviral test on "porcelain stoneware" samples treated with 15 applications of "Multi-Purpose B ZERO" cleaner

Specimen	**N* (PFU/cm2)**	**Log N**	**R& (Ut-At)**	**% viral reduction**
Untreated	1048.6 (578.2)	2.972		
B ZERO treated	86.71 (43.5)	1.668	**1.304**	**95.0 %**

*N is the infectivity titer of virus recovered per cm2 of test specimen
&R is the antiviral activity
Data are reported as average and standard deviation, in brackets, from three specimens

3. Test specimens "porcelain stoneware" treated with "Multi-Purpose B ZERO" cleaner (30 applications of the product).

Treatment with "Multi-Purpose B ZERO" (15 applications) induced a viral reduction equal to 1.955 log, that means 98.4 % viral reduction.

Table 3. Results of the antiviral test on "porcelain stoneware" samples treated with 30 applications of "B ZERO" cleaner

Specimen	**N* (PFU/cm2)**	**Log N**	**R& (Ut-At)**	**% viral reduction**
Untreated	515.3 (101.6)	2.711		
B ZERO treated	6.1 (2.5)	0.756	**1.955**	**98.4 %**

*N is the infectivity titer of virus recovered per cm2 of test specimen
&R is the antiviral activity
Data are reported as average and standard deviation, in brackets, from three specimens

4. Test specimens ("porcelain stoneware") treated with "B TITANIA SILVER" and then with the " Multi-Purpose B ZERO" cleaner (15 applications of the product)

Treatment with "B TITANIA SILVER" and 15 applications of "Multi-Purpose B ZERO" cleaner induced a viral reduction higher than 3.015 log, that means more than 99.9 % viral reduction.

Table 4. Results of the antiviral test on "porcelain stoneware" samples treated "B TITANIA SILVER" and 15 applications of "Multi-Purpose B ZERO" cleaner

Specimen	N* (PFU/cm2)	Log N	R& (Ut-At)	% viral reduction
Untreated	1238.09 (750.84)	3.015		
B Titania Silver and B ZERO treated	0	-	**> 3.015**	**> 99.9 %**

*N is the infectivity titer of virus recovered per cm2 of test specimen
&R is the antiviral activity
Data are reported as average and standard deviation, in brackets, from three specimens

Verification of cytotoxic effect on host cell and sensitivity to virus

Table 5 summarizes the results of cytotoxicity and cells sensitivity to virus.
"B TITANIA SILVER" and "Multi-Purpose B ZERO" cleaner did not induce cytotoxicity and did not decrease sensitivity of cells to virus.

Table 2

	Cytotoxicity	Sensitivity to virus		
	MTT assay	S (Log PFU/ml)	Acceptance criteria	Result
Negative control	1.03 (0.023)*	2.17		
Untreated	1.04 (0.028)	2.07	$\lvert Sn\text{-}Su \rvert \leq 0.5$	0.10 (≤ 0.5, pass)
B Titania Silver treated	1.07 (0.014)	2.04	$\lvert Sn\text{-}St \rvert \leq 0.5$	0.13 (≤ 0.5, pass)
B Titania Silver and B ZERO treated	0.989 (0.031)	1.91	$\lvert Sn\text{-}Su \rvert \leq 0.5$	0.26 (≤ 0.5, pass)

*OD values from MTT assay. Data are the mean and standard deviation from three replicates.

CONCLUSIONS

The test specimens treated with “B TITANIA SILVER” showed antiviral effect against SARS-CoV-2, after 24 hours of contact time.
In details, treatment with “B TITANIA SILVER” induced a viral reduction equal to 0.74 log, that corresponds to a 79.3% viral reduction.
Under the experimental conditions used, the test specimens treated with “B TITANIA SILVER” and / or “Multi-Purpose B ZERO” cleaner showed an antiviral effect against SARS-CoV-2.

Specifically, viral reduction, compared to untreated controls, was:
- 0.734 log on samples treated with “B TITANIA SILVER”;
- 1,304 log on samples treated with 15 applications of " Multi-Purpose B ZERO" cleaner;
- 1,955 log on samples treated with 30 applications of " Multi-Purpose B ZERO" cleaner;
-> 3 log on samples treated with “B TITANIA SILVER” associated with 15 applications of “Multi-Purpose B ZERO” cleaner.

REFERENCES

1. World Health Organization, WHO. Coronavirus disease (COVID-19) technical guidance: Laboratory testing for 2019-nCoV in humans. US CDC Real-time RT-PCR Panel for Detection 2019-Novel Coronavirus (28 January 2020). Available at: https://www.fda.gov/media/134922/download [last access 20 March 2020].

2. D’Alessandro, M. Gelati, N. Basilico, E.A. Parati, R.K. Haynes, D. Taramelli, Differential effects on angiogenesis of two antimalarial compounds, dihydroartemisinin and artemisone: Implications for embryotoxicity, Toxicology. 241 (2007) 66–74. doi:10.1016/j.tox.2007.08.084.

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Certificate n. 2001216-003 Asola, 14/04/2020

Client: BONASYSTEMS ITALIA SRL
Via Borgo S. Chiara, 29 – Torre di Mosto (VE)

Sample number: 2001216-003
Test run: 08/04/2020
Sample recording: TEST B ZERO
Specimen dimension: 50x50 mm
Sterile film used: polyethylene 40 x 40 mm, sp. 0.11 mm
Strain:
Escherichia coli ATCC 8739 (8.4 x 10^5 ufc/ml)
Staphylococcus aureus ATCC 6538 (5.1 x 10^5 ufc/ml)
Samples: by customer
Sampling method: customer's care

Sample arrival data: 13/03/2020
Test report: 14/04/2020

Verification of the effectiveness of the "BACTERI ZERO" sanitizing cleaner after a single application on porcelain stoneware and subsequent measurement of antibacterial activity using the ISO 22196: 2011 method
Measurement of antibacterial activity on plastics and other non-porous surfaces

1. TEST SCOPE

Verification of the procedure for using BZERO, the action of which is achieved through its cumulative and additive capacity to modify the chemical / physical characteristics of the surfaces.

This method is applicable for evaluating the antibacterial activity of antibacterial-treated plastics, and other non-porous, surfaces of products.

Test material is inoculated with a known amount of bacteria suspension inoculum; the amount of bacteria is then measured after 24 hours time contact. The comparison between the two quantities provides a percentage index R of effectiveness of the antimicrobial material.

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2. STAGES OF THE ASSAY

Stages of the treatment of porcelain stoneware tiles with the universal multipurpose cleaner "B ZERO" are:

1. Wet the microfibre cloth with water (impregnation and wringing).
2. Impregnation of the Micron Quick Vileda microfibre cloth n ° 134859 0373 with the universal multipurpose cleaner "b zero".
3. Wringing of excess product.
4. Cleaning of porcelain tiles with light and regular passage.
5. Natural air drying of the humidity released on the surface.

Test method ISO 22196: 2011 involves the following steps:

1. Preparation of bacterial inoculum.
2. Inoculum of bacteria on treated and untreated specimen. Cover with sterile film.
3. Incubation at 35°C for 24 hours.
4. Washing of specimen with Neutralising sample diluent; pour plate technique for bacterial count.
5. Evaluation of test results and calculation of the antibacterial activity of the treated material.

3. MICROORGANISM FOR INOCULATION

The microorganism used for contaminations is:

✶ *Escherichia coli* ATCC 25922

Eschericia coli, also known as E. coli is a Gram-negative, facultative anaerobic, rod-shaped, coliform bacterium of the genus Escherichia that is commonly found in the lower intestine of warm-blooded organisms. Pathogenic strains of E. coli are responsible for urinary tract infections, intestinal disorders such as gastroenteritis and neonatal meningitis. It is the main indicator of faecal contamination.

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✶ *Staphylococcus aureus* ATCC 6538. Staphylococcus aureus is a Gram-positive, round-shaped bacterium. It is a usual member of the microbiota of the body, frequently found in the upper respiratory tract and on the skin. Strains of S. aureus are responsible for skin infections including abscesses, respiratory infections such as sinusitis, and food poisoning.

Standard lyophilized cultures of microorganism were used.
The bacterial inoculum size is between 2.5 x 10^5 and 1.0 x 10^6 cfu / ml.

4. CULTURE MEDIA

To perform the experimental test were used culture media, such as:

- Phosphate-buffered physiological saline (PBS) solution for the preparation of microbial suspensions of standard strains used and serial dilutions;
- Plate Count Agar (PCA) for the method of sowing in inclusion in the Petri dish.
- Specific neutralizing thinner for the final step of testing.

5. TEST PROCEDURE

In order to verify the procedure of use of BZERO, whose action is achieved through its cumulative and additional ability to modify the chemical / physical characteristics of the surfaces, the product is applied one time following the instructions on the back of the product packaging . Once the specimens have been treated, proceed to ISO 22196: 2011.
Antibacterial activity is evaluated by measured the viability of bacteria after contact with a surface treated with antibacterial agents, for 24 hours at 35°C.
The effectiveness of antibacterial agents is measured by comparing the degree of survival of the bacteria put in contact with treated and untreated materials.

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The strains of *Escherichia coli and Staphylococcus aureus* is inoculated into a nutrient broth (PBS). An aliquot of this culture is put on 3 sample of surface treated with antibacterial agents; an other aliquot is put on 3 sample with untreated surface. All samples are then divided in 50x50mm portions and inoculated with bacterial inoculum; the surface is covered with 40x40mm sterile film.
The samples are incubated at 35°C for 24 hours with 90% of humidity.
After incubation, 10 ml of Neutralising sample diluent are added to all the samples (treated and untreated); an aliquot of the Neutralising sample diluent is then plated onto growth medium PCA, for bacterial count.

6. RESULTS

From the microbial count results obtained, anti-bacterial activity R is calculated with the equation given in ISO 22196: 2011.
The analytical results are intended to refer exclusively to the analysed samples received at the laboratory. This document cannot be reproduced even in partial form unless written approval by the Laboratory.

TEST RESULTS WITH ESCHERICHIA COLI

Initial bacterial count (CFU/cm^2) **Uo**	Bacterial count after 24h on untreated samples (CFU/cm^2) **Ut**	Bacterial count after 24h on treated samples (CFU/cm^2) **At**	**Antibacterial activity R= Ut – At**	**Reduction (%)**
5.3×10^4 Log = 4.72	4.8×10^5 Log = 5.68	1.1×10^5 Log = 5.04	**0.64**	**77.08**

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TEST RESULTS WITH STAPHYLOCOCCUS AUREUS

Initial bacterial count (CFU/cm^2) **Uo**	Bacterial count after 24h on untreated samples (CFU/cm^2) **Ut**	Bacterial count after 24h on treated samples (CFU/cm^2) **At**	**Antibacterial activity R= Ut – At**	**Reduction (%)**
9.1×10^4 Log = 4.96	8.6×10^5 Log = 5.93	2.3×10^5 Log = 5.36	**0.57**	**73.26**

7. INTERPRETATION OF RESULTS

R value represented the anti-bacterial activity. The number expressed is the ability to eliminate on a logarithmic basis, in 24 hours, the bacteria that are in contact with the surface treated with antibacterial agent.

The more R factor is high, the more the treated surface has effectiveness to kill bacteria and prevent formation of CFU.

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Certificate n. 2001216-002 Asola, 31/03/2020

Client: BONASYSTEMS ITALIA SRL
Via Borgo S. Chiara, 29 – Torre di Mosto (VE)

Sample number: 2001216-002
Sample arrival data: 13/03/2020
Test run: 25/03/2020
Test report: 31/03/2020
Sample recording: TEST B ZERO
Specimen dimension: 50x50 mm
Sterile film used: polyethylene 40 x 40 mm, sp. 0.11 mm
Strain:
Escherichia coli ATCC 8739 (8.4 x 10^5 ufc/ml)
Staphylococcus aureus ATCC 6538 (5.1 x 10^5 ufc/ml)
Samples: by customer
Sampling method: customer's care

Verification of the effectiveness of the "BACTERI ZERO" sanitizing cleaner after 5 applications on porcelain stoneware and subsequent measurement of antibacterial activity using the ISO 22196: 2011 method
Measurement of antibacterial activity on plastics and other non-porous surfaces

1. TEST SCOPE

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This method is applicable for evaluating the antibacterial activity of antibacterial-treated plastics, and other non-porous, surfaces of products.

Test material is inoculated with a known amount of bacteria suspension inoculum; the amount of bacteria is then measured after 24 hours time contact. The comparison between the two quantities provides a percentage index R of effectiveness of the antimicrobial material.

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1. Wet the microfibre cloth with water (impregnation and wringing).
2. Impregnation of the Micron Quick Vileda microfibre cloth n ° 134859 0373 with the universal multipurpose cleaner "b zero".
3. Wringing of excess product.
4. Cleaning of porcelain tiles with light and regular passage.
5. Natural air drying of the humidity released on the surface.

This procedure was repeated 5 times.

Test method ISO 22196: 2011 involves the following steps:

1. Preparation of bacterial inoculum.
2. Inoculum of bacteria on treated and untreated specimen. Cover with sterile film.
3. Incubation at 35°C for 24 hours.
4. Washing of specimen with Neutralising sample diluent; pour plate technique for bacterial count.
5. Evaluation of test results and calculation of the antibacterial activity of the treated material.

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Standard lyophilized cultures of microorganism were used.
The bacterial inoculum size is between 2.5 x 10^5 and 1.0 x 10^6 cfu / ml.

4. CULTURE MEDIA

To perform the experimental test were used culture media, such as:

- Phosphate-buffered physiological saline (PBS) solution for the preparation of microbial suspensions of standard strains used and serial dilutions;
- Plate Count Agar (PCA) for the method of sowing in inclusion in the Petri dish.
- Specific neutralizing thinner for the final step of testing.

5. TEST PROCEDURE

In order to verify the procedure of use of BZERO, whose action is achieved through its cumulative and additional ability to modify the chemical / physical characteristics of the surfaces, the product is applied for 5 consecutive times following the instructions on the back of the product packaging . Once the specimens have been treated, proceed to ISO 22196: 2011.
Antibacterial activity is evaluated by measured the viability of bacteria after contact with a surface treated with antibacterial agents, for 24 hours at 35°C.
The effectiveness of antibacterial agents is measured by comparing the degree of survival of the bacteria put in contact with treated and untreated materials.

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The strains of *Escherichia coli and Staphylococcus aureus* is inoculated into a nutrient broth (PBS). An aliquot of this culture is put on 3 sample of surface treated with antibacterial agents; an other aliquot is put on 3 sample with untreated surface. All samples are then divided in 50x50mm portions and inoculated with bacterial inoculum; the surface is covered with 40x40mm sterile film.
The samples are incubated at 35°C for 24 hours with 90% of humidity.
After incubation, 10 ml of Neutralising sample diluent are added to all the samples (treated and untreated); an aliquot of the Neutralising sample diluent is then plated onto growth medium PCA, for bacterial count.

6. RESULTS

From the microbial count results obtained, anti-bacterial activity R is calculated with the equation given in ISO 22196: 2011.
The analytical results are intended to refer exclusively to the analysed samples received at the laboratory. This document cannot be reproduced even in partial form unless written approval by the Laboratory.

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8.6×10^4 Log = 4.93	8.1×10^5 Log = 5.91	2.3×10^4 Log = 4.36	**1.55**	**97.16**

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TEST RESULTS WITH STAPHYLOCOCCUS AUREUS

Initial bacterial count (CFU/cm^2) **Uo**	Bacterial count after 24h on untreated samples (CFU/cm^2) **Ut**	Bacterial count after 24h on treated samples (CFU/cm^2) **At**	**Antibacterial activity R= Ut – At**	**Reduction (%)**
4.8×10^4 Log = 4.68	6.9×10^5 Log = 5.84	3.1×10^4 Log = 4.49	**1.35**	**95.51**

7. INTERPRETATION OF RESULTS

R value represented the anti-bacterial activity. The number expressed is the ability to eliminate on a logarithmic basis, in 24 hours, the bacteria that are in contact with the surface treated with antibacterial agent.

The more R factor is high, the more the treated surface has effectiveness to kill bacteria and prevent formation of CFU.

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Technical Manager
Dott. Matteo Sarzi Amade'

Micro-B
LABORATORIO ANALISI CHIMICHE E MICROBIOLOGICHE

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Certificate n. 2001216-003 Asola, 14/04/2020

Client: BONASYSTEMS ITALIA SRL
Via Borgo S. Chiara, 29 – Torre di Mosto (VE)

Sample number: 2001216-003
Test run: 08/04/2020
Sample recording: TEST B ZERO
Specimen dimension: 50x50 mm
Sterile film used: polyethylene 40 x 40 mm, sp. 0.11 mm
Strain:
Escherichia coli ATCC 8739 (8.4 x 10^5 ufc/ml)
Staphylococcus aureus ATCC 6538 (5.1 x 10^5 ufc/ml)
Samples: by customer
Sampling method: customer's care

Sample arrival data: 13/03/2020
Test report: 14/04/2020

Verification of the effectiveness of the "BACTERI ZERO" sanitizing cleaner after 10 applications on porcelain stoneware and subsequent measurement of antibacterial activity using the ISO 22196: 2011 method
Measurement of antibacterial activity on plastics and other non-porous surfaces

1. TEST SCOPE

Verification of the procedure for using BZERO, the action of which is achieved through its cumulative and additive capacity to modify the chemical / physical characteristics of the surfaces.

This method is applicable for evaluating the antibacterial activity of antibacterial-treated plastics, and other non-porous, surfaces of products.

Test material is inoculated with a known amount of bacteria suspension inoculum; the amount of bacteria is then measured after 24 hours time contact. The comparison between the two quantities provides a percentage index R of effectiveness of the antimicrobial material.

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2. STAGES OF THE ASSAY

Stages of the treatment of porcelain stoneware tiles with the universal multipurpose cleaner "B ZERO" are:

1. Wet the microfibre cloth with water (impregnation and wringing).
2. Impregnation of the Micron Quick Vileda microfibre cloth n ° 134859 0373 with the universal multipurpose cleaner "b zero".
3. Wringing of excess product.
4. Cleaning of porcelain tiles with light and regular passage.
5. Natural air drying of the humidity released on the surface.

This procedure was repeated 10 times.

Test method ISO 22196: 2011 involves the following steps:

1. Preparation of bacterial inoculum.
2. Inoculum of bacteria on treated and untreated specimen. Cover with sterile film.
3. Incubation at 35°C for 24 hours.
4. Washing of specimen with Neutralising sample diluent; pour plate technique for bacterial count.
5. Evaluation of test results and calculation of the antibacterial activity of the treated material.

3. MICROORGANISM FOR INOCULATION

The microorganism used for contaminations is:

✶ *Escherichia coli* ATCC 25922

Eschericia coli, also known as E. coli is a Gram-negative, facultative anaerobic, rod-shaped, coliform bacterium of the genus Escherichia that is commonly found in the lower intestine of warm-blooded organisms. Pathogenic strains of E. coli are responsible for urinary tract infections, intestinal disorders such as gastroenteritis and neonatal meningitis. It is the main indicator of faecal contamination.

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✶ *Staphylococcus aureus* ATCC 6538. Staphylococcus aureus is a Gram-positive, round-shaped bacterium. It is a usual member of the microbiota of the body, frequently found in the upper respiratory tract and on the skin. Strains of S. aureus are responsible for skin infections including abscesses, respiratory infections such as sinusitis, and food poisoning.

Standard lyophilized cultures of microorganism were used.
The bacterial inoculum size is between 2.5 x 10^5 and 1.0 x 10^6 cfu / ml.

4. CULTURE MEDIA

To perform the experimental test were used culture media, such as:

- Phosphate-buffered physiological saline (PBS) solution for the preparation of microbial suspensions of standard strains used and serial dilutions;
- Plate Count Agar (PCA) for the method of sowing in inclusion in the Petri dish.
- Specific neutralizing thinner for the final step of testing.

5. TEST PROCEDURE

In order to verify the procedure of use of BZERO, whose action is achieved through its cumulative and additional ability to modify the chemical / physical characteristics of the surfaces, the product is applied for 10 consecutive times following the instructions on the back of the product packaging . Once the specimens have been treated, proceed to ISO 22196: 2011.
Antibacterial activity is evaluated by measured the viability of bacteria after contact with a surface treated with antibacterial agents, for 24 hours at 35°C.
The effectiveness of antibacterial agents is measured by comparing the degree of survival of the bacteria put in contact with treated and untreated materials.

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The strains of *Escherichia coli and Staphylococcus aureus* is inoculated into a nutrient broth (PBS). An aliquot of this culture is put on 3 sample of surface treated with antibacterial agents; an other aliquot is put on 3 sample with untreated surface. All samples are then divided in 50x50mm portions and inoculated with bacterial inoculum; the surface is covered with 40x40mm sterile film.

The samples are incubated at 35°C for 24 hours with 90% of humidity.

After incubation, 10 ml of Neutralising sample diluent are added to all the samples (treated and untreated); an aliquot of the Neutralising sample diluent is then plated onto growth medium PCA, for bacterial count.

6. RESULTS

From the microbial count results obtained, anti-bacterial activity R is calculated with the equation given in ISO 22196: 2011.

The analytical results are intended to refer exclusively to the analysed samples received at the laboratory. This document cannot be reproduced even in partial form unless written approval by the Laboratory.

TEST RESULTS WITH ESCHERICHIA COLI

Initial bacterial count (CFU/cm^2) **Uo**	Bacterial count after 24h on untreated samples (CFU/cm^2) **Ut**	Bacterial count after 24h on treated samples (CFU/cm^2) **At**	**Antibacterial activity R= Ut – At**	**Reduction (%)**
5.3×10^4 Log = 4.72	4.8×10^5 Log = 5.68	1.1×10^5 Log = 5.04	**0.64**	**77.08**

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TEST RESULTS WITH STAPHYLOCOCCUS AUREUS

Initial bacterial count (CFU/cm^2) **Uo**	Bacterial count after 24h on untreated samples (CFU/cm^2) **Ut**	Bacterial count after 24h on treated samples (CFU/cm^2) **At**	**Antibacterial activity R= Ut – At**	**Reduction (%)**
9.1×10^4 Log = 4.96	8.6×10^5 Log = 5.93	2.3×10^5 Log = 5.36	**0.57**	**73.26**

7. INTERPRETATION OF RESULTS

R value represented the anti-bacterial activity. The number expressed is the ability to eliminate on a logarithmic basis, in 24 hours, the bacteria that are in contact with the surface treated with antibacterial agent.

The more R factor is high, the more the treated surface has effectiveness to kill bacteria and prevent formation of CFU.

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Certificate n. 1704660-001 Asola, 23/11/2017

Client: BONASYSTEMS ITALIA SRL
Via Borgo S. Chiara, 29 – Torre di Mosto (VE)

Sample: 1704660-001
Test run: 17/11/2017
Sample recording: B ZERO
Samples: by customer
Sampling method: customer's care

Sample arrival data: 17/11/2017
Test report: 22/11/2017

Measurement of antibacterial activity of antibacterial solution by bacterial strains contamination and superficial swab. Measurement of heavy metals.

1. TEST SCOPE

This method is applicable to many different materials that are supposed to have antibacterial activity and provides a quantitative measure of the effectiveness of such activities.

2. MICROORGANISM FOR INOCULATION

The microorganisms used for contaminations are:

✶ *Escherichia coli* ATCC 25922
✶ *Staphylococcus aureus* ATCC 6538

Standard lyophilized cultures of microorganism were used.

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The bacterial inoculum size is about 10^6 cfu / ml.

3. CULTURE MEDIA

To perform the experimental test were used culture media, such as:

- sterile distilled water:
- Phosphate-buffered physiological saline (PBS) solution for the preparation of microbial suspensions of standard strains used and serial dilutions;
- Plate Count Agar (PCA) for the method of sowing in inclusion in the Petri dish.
- Specific neutralizing thinner for the final step of testing.
- Superficial swabs and 10x10cm sterile mask.

4. SAMPLE

- **1704660-001** B ZERO

5. TEST PERFORMANCE

Ten times surface treatment with B ZERO
as explained in the technical data sheet:

1- Shake vigorously before the use.
2- Moisten the rag with a little amount of product.
3- Treat the surface by revolving movements, maintaining a little pressure.
4- Finally allow surface to dry.

↓

Surface contamination by bacterial strains

↓

B ZERO once surface treatment as explained in the technical data sheet.

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Swab the 10x10 cm surface 10 minutes after the treatment.

Inclusion in agar (PCA for bacterial cultures) by seeding the suspension buffer and subsequent decimal dilutions made in diluent.

Incubation of the plates at 30-35 ° C for 5 days.

Counting plate colonies.

NB: each of the steps prior to inclusion in the agar was carried out in the dark.

6. RESULTS

From the microbial count results obtained, the percentage of reduction with respect to the inoculum is calculated, and the related logarithmic reductions. Table 1 shows the values of the inoculations carried out at time T0. Table 2 shows the results obtained in the sample under analysis and the relative reduction percentages with respect to the inoculum. Table 3 instead shows the results obtained in the sample under analysis and the related logarithmic reductions with respect to the inoculum. The analytical results are intended to refer exclusively to the analysed samples received at the laboratory. This document cannot be reproduced even in partial form unless written approval by the Laboratory.

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Table 1. TEST BLANK

Microrganism	Inoculoum (CFU/ml)
S.aureus	7.2 x 10^6 Log = 5.86
E. coli	3.4 x 10^6 Log = 5.53

• REDUCTION PERCENTAGE TABLE:

Table 2. SAMPLE 1704660-001 B ZERO

Microrganism	CFU/ml	REDUCTION (%)
S.aureus	6.2x10^3	99.14
E.coli	7.9x10^3	97.68

• RELATED LOGARITHMIC REDUCTION TABLE:

Table 3. SAMPLE 1701307-001 B ZERO

Microrganism	CFU/ml	REDUCTION (Log)
S.aureus	6.2x10^3 Log=3.79	2.07
E.coli	7.9x10^3 Log=3.89	1.64

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6. DETERMINATION OF HEAVY METALS

In addition to verifying the antibacterial activity, an analysis was carried out in order to search for heavy metals, as can be seen from the following table:

Table 4. SAMPLE 1704660-001 B ZERO

Test	Results	Unit of measure
Arsenic	<1	mg/Kg
Cadmium	<0.1	mg/Kg
Cobalt	<0.2	mg/Kg
Chromium (total)	<0.1	mg/Kg
Chromium VI	<0.1	mg/Kg
Nickel	<0.2	mg/Kg
Lead	<1.5	mg/Kg
Copper	<0.2	mg/Kg
Zinc	1.1	mg/Kg

7. CONCLUSIONS

As can be seen from the results obtained, pathogens were found in the swabs carried out on the surface treated for 10 consecutive times with the product under analysis. Consequently, the concerned product cannot be considered as an effective bactericide considering that the acceptability criterion foresees a decrease of 5 Log powers.

The values found by the heavy metal analysis are lower than the limit of quantification foreseen by the method in use by our Laboratory, except as regards Zinc which is present in very low concentration.

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ISO 21702:2019

MEASUREMENT OF ANTIVIRAL ACTIVITY ON PLASTICS AND OTHER NON-POROUS SURFACES

Sample ID:	*20.512718.0001*
Description:	sample 1: B TITANIA SILVER - PIASTRINE “GRES PORCELLANATO” TRATTATE E NON TRATTATE sample 2: B-ZERO - PIASTRINE “GRES PORCELLANATO” DA TRATTARE
Ref. AR:	*AR 2020/128/C cap. 1*
Starting date:	*07/05/2020*
Report emission date:	*15/06/2020*
Prepared by:	*Federica Licitra*

INDEX

1 ABSTRACT OF RESULTS

Test specimens treated respectively with products “B TITANIA SILVER” and multipurpose cleaner “B-ZERO” were assayed according to ISO 21702:2019 for verifying their antiviral effect against vacciniavirus (surrogate virus for viruses with envelope, i.e. coronavirus like SARS-CoV-2).

Under the test conditions applied, both the test specimens treated respectively with “B TITANIA SILVER” and multipurpose cleaner “B-ZERO” resulted to have an antiviral effect against vacciniavirus after 24 hours of contact time, as reported in the scheme below:

Test Specimen	Contact time	Reduction	Result
UNTREATED	0 min	/	/
	24 hours	/	/
B TITANIA SILVER TREATED	24 hours	1.75	PRESENCE OF ANTIVIRAL EFFECT (viral reduction 98%)
B ZERO TREATED	24 hours	1.00	PRESENCE OF ANTIVIRAL EFFECT (viral reduction 90%)

2 AIM OF THE METHOD

The purpose of this study is to verify the antiviral activity of the test material “gres porcellanato” treated respectively with the test products “TITANIA SILVER” or the multipurpose cleaner “B-ZERO”.

The analysis is performed as indicated by the standard method ISO 21702:2019 “Measurement of antiviral activity on plastics and other non-porous surface”.

Briefly, the treated and untreated test material are added with vacciniavirus, used as surrogate virus to demonstrate efficacy against enveloped viruses (i.e. coronavirus, like SARS-CoV-2 responsible for COVID19). After the specific contact time of 24 hours, the remaining infectious virus is evaluated by applying the Spearman-Kärber method. Reduction rate is calculated by comparison between the antiviral product test specimen (treated test specimen) and the control specimen (untreated test specimen).

3 MATERIALS AND EQUIPMENT

3.1 Materials

- PBS (Phosphate Buffer Saline)
- FCS (Fetal Calf Serum)
- SDCLP medium
- Medium for cell culture: EMEM (Eagle’s minimal essential medium)

- Growth medium (for cell multiplication): EMEM, 10% FCS, 2 mM Glutamine, 1 mM Sodium Pyruvate and 1% Penicillin-Streptomycin mix.
- Maintenance medium (to maintain the cell culture metabolism without stimulation of cell proliferation): EMEM, 2% FCS
- MICROSPIN S400HR

3.2 Equipment

- Timer
- Graduated pipettes of nominal capacities 10 ml, 5 ml, 2 ml, 1 ml
- Automatic pipette 2-20 µl; 20-200 µl; 100-1000 µl
- CO2 Incubator (37 °C ± 1°C, 95% humidity, 5 % CO2)
- Inverted microscope
- 96-well plates
- Container: test tubes or flasks of suitable capacity
- Biological Hood (Laminar Flow)

4 TEST SAMPLES

Sample 1 identification

- Product Name: B TITANIA SILVER
- Batch n°: n.a
- Manufacturing date: n.a.
- Expiry date: n.a.
- Receiving date: 29/04/2020
- Storage condition: RT, darkness

Sample 2 identification

- Product Name: B-ZERO
- Batch n°: L0919
- Manufacturing date: n.a.
- Expiry date: n.a.
- Receiving date: 29/04/2020
- Storage condition: RT, darkness

5 EXPERIMENTAL CONDITIONS

- Test virus and number of passages:
  Vaccinia virus, strain Ankara, ATCC-VR-1508 (passage n° 4), surrogate virus to demonstrate efficacy against enveloped viruses (i.e. coronavirus, like SARS-nCOv-2 responsible for COVID19)
- Cell line and number of passages:

BHK-21-cl 13, IZS-Brescia for the propagation of *Vaccinia virus* (passage n° 93+2)

- Test specimen type and size: “gres porcellanato”, flat size (50 ± 2) mm x (50 ± 2) mm, thickness 6 mm (supplied by the client)
- Cover film: Low-density polyethylene, thickness 0.10 mm, square shaped, size 40 x 40 mm
- Contact times: 24 hours
- Test conditions: temperature 25 ± 1 °C, > 90 % humidity
- Neutralization method: SDCLP broth and filtration with columns MICROSPIN S400 HR
- Growth medium: EMEM, 10% FCS, 1 mM L-Glutamine, 1 mM Sodium Pyruvate, 1% Pen-Strep mix
- Maintenance medium: EMEM, 2% FCS
- Viral titration method: Spearmen-Karber
- Cleaning method: immersion in ethanol 70 % and drying

6 PROCEDURE

6.1 Preparation of test specimens

A total of 12 untreated test specimens and 9 test specimens treated respectively with the test products “B TITANIA SILVER” or “B-ZERO” are necessary to perform the test.
Each test specimen is a flat square of (50 ± 2) mm x (50 ± 2) mm.
Test specimens treated with “TITANIA SILVER” were supplied by the client.
Test specimens treated with “B-ZERO” were prepared in laboratory, by applying the product 10 times on the specimen surface. The repeated application, mimicking every day use conditions, allow to evaluate also the cumulative effect of the product multipurpose cleaner “B-ZERO”. The application procedure is reported below:

- Moistening of the microfiber cloth (Vileda MicronQuick BSystems 134859 0373) with water
- Impregnation of the microfiber cloth with the product “B-ZERO”
- Wringing the excess of product
- Gentle cleaning of the surface
- Natural air drying for 30 minutes

Before the use, each test specimen was also sterilized by immersion in ethanol 70%, for eliminating any bacterial contamination.

6.2 Test Procedure

3 “treated” test specimens and 6 “untreated” test specimens were inoculated with 0.4 ml of virus suspensions (viral titer $TCID_{50}$ 10^7/ml). Then, inoculums were covered with a 40 x 40 mm film and incubated for 24 hours at 25 °C and relative humidity > 90%.

Immediately after inoculation, 3 “untreated” test specimens were added with 10 ml of neutralizer SCDLP broth and the residual virus infectivity revealed by titration.
At end of the contact time, the procedure described above was repeated for the “treated” test specimens and the remaining “untreated” test specimens.

6.3 Virus Titration

Titration was performed by means of the Spearman-Kärber method.
In details, for each treatment serial dilution till 10^{-10} were prepared in maintenance medium.
Then, 100 µl of each dilution were transferred into eight wells of a 96-well plate containing a confluent (> 90%) cell monolayer prepared the previous day. After 1 h of incubation at 37 °C and 5 % CO_2, 100 µl of maintenance medium were added to each well and plates placed in the incubator at 37 °C and 5 % CO_2 for 7 days, time needed for virus infection.
After incubation, cells were observed for evaluating the viral presence and viral titre was calculated using Spearman-Karber method. The following formula was applied:
$TCID_{50}$ = log of the highest virus concentration used - [(log of dilutions)×(X-0.5)]
Where: X is the sum of the ratio between infected wells on total wells per each dilution tested.

6.3.1 Determination of the infectivity titre of virus

For each test specimen, the infectivity titre of virus recovered was obtained as follows:
$N = (10 \times TCID_{50} \times V) / A$
Where:
N is the infectivity titre of virus recovered per cm^2 of test specimen
V is the volume of the SCDLP broth added to the specimen, in ml
A is the surface area of the cover film, in cm^2

6.4 Verification of methodology

The test is valid if the following criteria are fulfilled:

- The average $TCID_{50}$ recovered immediately after inoculation from the untreated test specimens shall be within the range of 2.5×10^5 $TCID_{50}/cm^2$ and 1.2×10^6 $TCID_{50}/cm^2$
- The average $TCID_{50}$ recovered from the untreated test specimens after 24 hours of contact time shall be not less than 6.2×10^2 $TCID_{50}/cm^2$
- The suppressive efficiency of the agent’s activity (neutralization) is to be confirmed

6.5 Calculation of the antiviral activity

The antiviral activity is calculated as follows:

R= Ut - At

Where:

R is the antiviral activity

Ut is the average log of the $TCID_{50}/cm^2$ from the 3 untreated test specimens after 24 hours

At is the average log of the $TCID_{50}/cm^2$ from the 3 treated test specimens after 24 hours

6.6 Controls

6.6.1 Verification of cytotoxic effects on host cells

3 “treated” test specimens and 3 “untreated” test specimens were added with 10 ml of neutralizer SCDLP broth. Then, the wash-out solution was added to cells mimicking procedure for titration. Test specimen need to be non cytotoxic.

6.6.2 Verification of cell sensitivity to virus and the inactivation of antiviral activity

3 “treated” test specimens and 3 “untreated” test specimens were added with 10 ml of neutralizer SCDLP broth. Then, 5 ml of surnatant from each test specimen, as well as 5 ml of SCDLP broth to be used as negative control, were transferred into new tubes and added with 50 µl of virus suspension at concentration $TCID_{50}$ 10^4/ml. The mix was incubated at 25 °C for 30 min. Finally, the solution was titrated as described above.

Infectivity titre was calculated by applying the formula:

S = (10 x P)

Where:

S is the infectivity titre of virus per ml per test suspension

P is the average titre $TCID_{50}$

Acceptance criteria: $|Sn - Su| \leq 0,5$ and $|Sn - St| \leq 0,5$

Where:

Sn is the average log of the infectivity titre of virus in TCID50/ml for the negative control

Su is the average log of the infectivity titre of virus in TCID50/ml for the untreated test specimen

St is the average log of the infectivity titre of virus in TCID50/ml for the treated test specimen

7 RESULTS

Results are summarized in the tables below n° 1 and 2:

Table 1: Results of the antiviral test, ISO 21702:2019

Vacciniavirus							
Test Specimen	**Contact time**	**Mean virus Titration (Log $TCID_{50}$)**	**Mean virus Titration ($TCID_{50}$ /100 µl)**	**N ($TCID_{50}/cm^2$)**	**Reduction (Ut – At)**	**Acceptance criteria**	**Result**
UNTREATED	0 min	5.00	10^5	6.25×10^5	/	2.5×10^5 - 1.2×10^6 ($TCID_{50}/cm^2$)	PASS
	24 hours	4.625	$10^{4.625}$	2.64×10^5	/	> 6.2×10^2 ($TCID_{50}/cm^2$)	PASS
B TITANIA SILVER TREATED	24 hours	2.875	$10^{2.875}$	4.69×10^3	1.75	/	PRESENCE OF ANTIVIRAL EFFECT (viral reduction 98%)
B ZERO TREATED	24 hours	3.625	$10^{3.625}$	2.64×10^4	1.00	/	PRESENCE OF ANTIVIRAL EFFECT (viral reduction 90%)

Table 2: Results of controls

Vacciniavirus					
Sensitivity test	**Mean virus Titration (Log $TCID_{50}$)**	**Mean virus Titration ($TCID_{50}$ /100 µl)**	**S (TCID50/ml)**	**Acceptance criteria**	**Result**
Negative control	2.875	$10^{2.875}$	$Sn = 10^{3.875}$	/	/
UNTREATED	2.875	$10^{2.875}$	$Su = 10^{3.875}$	$\|Sn - Su\| \leq 0.5$	0 (PASS)
B TITANIA SILVER TREATED	2.750	$10^{2.750}$	$St = 10^{3.750}$	$\|Sn - St\| \leq 0.5$	0.125 (PASS)
B ZERO TREATED	2.625	$10^{2.625}$	$St = 10^{3.625}$	$\|Sn - St\| \leq 0.5$	0.25 (PASS)

8 CONCLUSIONS

According to ISO 21702:2019, under the test conditions applied, both the test specimens treated respectively with “B TITANIA SILVER” and “B-ZERO” resulted to have an antiviral effect against vacciniavirus (surrogate virus for viruses with envelope, i.e. coronavirus like SARS-CoV-2), after 24 hours of contact time.

In details, treatment with “B TITANIA SILVER” showed to produce a viral reduction equal to 1.75 log, that means a 98% viral reduction. On the other hand treatment with the multipurpose cleaner “B-ZERO” demonstrated to produce a viral reduction equal to 1.00 log, i.e a 90 % viral reduction.

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ISO 21702:2019

MEASUREMENT OF ANTIVIRAL ACTIVITY ON PLASTICS AND OTHER NON-POROUS SURFACES

Sample ID:	*20.512718.0001*
Description:	sample 1: B TITANIA SILVER - PIASTRINE “GRES PORCELLANATO” TRATTATE E NON TRATTATE sample 2: B-ZERO PULITORE MULTIUSO - PIASTRINE “GRES PORCELLANATO” DA TRATTARE
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1 ABSTRACT OF RESULTS

Test specimens treated respectively with products “B TITANIA SILVER” and multipurpose cleaner “B-ZERO” were assayed according to ISO 21702:2019 for verifying their antiviral effect against vacciniavirus (surrogate virus for viruses with envelope, i.e. coronavirus like SARS-CoV-2).

Under the test conditions applied, both the test specimens treated respectively with “B TITANIA SILVER” and multipurpose cleaner “B-ZERO” resulted not to have an antiviral effect against vacciniavirus after 3 hours of contact time, as reported in the scheme below:

Test Specimen	Contact time	Reduction	Result
UNTREATED	0 min	/	/
	3 hours	/	/
B TITANIA SILVER TREATED	3 hours	0.875	ABSENCE OF ANTIVIRAL EFFECT
B ZERO TREATED	3 hours	0.125	ABSENCE OF ANTIVIRAL EFFECT

2 AIM OF THE METHOD

The purpose of this study is to verify the antiviral activity of the test material “gres porcellanato” treated respectively with the test products “TITANIA SILVER” or the multipurpose cleaner “B-ZERO”.

The analysis is performed as indicated by the standard method ISO 21702:2019 “Measurement of antiviral activity on plastics and other non-porous surface”.

Briefly, the treated and untreated test material are added with vacciniavirus, used as surrogate virus to demonstrate efficacy against enveloped viruses (i.e. coronavirus, like SARS-CoV-2 responsible for COVID19). After the specific contact time of 3 hours, the remaining infectious virus is evaluated by applying the Spearman-Kärber method. Reduction rate is calculated by comparison between the antiviral product test specimen (treated test specimen) and the control specimen (untreated test specimen).

3 MATERIALS AND EQUIPMENT

3.1 Materials

- PBS (Phosphate Buffer Saline)
- FCS (Fetal Calf Serum)
- SDCLP medium
- Medium for cell culture: EMEM (Eagle’s minimal essential medium)

- Growth medium (for cell multiplication): EMEM, 10% FCS, 2 mM Glutamine, 1 mM Sodium Pyruvate and 1% Penicillin-Streptomycin mix.
- Maintenance medium (to maintain the cell culture metabolism without stimulation of cell proliferation): EMEM, 2% FCS
- MICROSPIN S400HR

3.2 Equipment

- Timer
- Graduated pipettes of nominal capacities 10 ml, 5 ml, 2 ml, 1 ml
- Automatic pipette 2-20 µl; 20-200 µl; 100-1000 µl
- CO2 Incubator (37 °C ± 1°C, 95% humidity, 5 % CO2)
- Inverted microscope
- 96-well plates
- Container: test tubes or flasks of suitable capacity
- Biological Hood (Laminar Flow)

4 TEST SAMPLES

Sample 1 identification

- Product Name: B TITANIA SILVER
- Batch n°: n.a
- Manufacturing date: n.a.
- Expiry date: n.a.
- Receiving date: 29/04/2020
- Storage condition: RT, darkness

Sample 2 identification

- Product Name: B-ZERO MULTIPURPOSE CLEANER
- Batch n°: L0919
- Manufacturing date: n.a.
- Expiry date: n.a.
- Receiving date: 29/04/2020
- Storage condition: RT, darkness

5 EXPERIMENTAL CONDITIONS

- Test virus and number of passages:
  Vaccinia virus, strain Ankara, ATCC-VR-1508 (passage n° 4), surrogate virus to demonstrate efficacy against enveloped viruses (i.e. coronavirus, like SARS-nCOv-2 responsible for COVID19)
- Cell line and number of passages:

BHK-21-cl 13, IZS-Brescia for the propagation of *Vaccinia virus* (passage n° 93+2)

- Test specimen type and size: “gres porcellanato”, flat size (50 ± 2) mm x (50 ± 2) mm, thickness 6 mm (supplied by the client)
- Cover film: Low-density polyethylene, thickness 0.10 mm, square shaped, size 40 x 40 mm
- Contact times: 3 hours
- Test conditions: temperature 25 ± 1 °C, > 90 % humidity
- Neutralization method: SDCLP broth and filtration with columns MICROSPIN S400 HR
- Growth medium: EMEM, 10% FCS, 1 mM L-Glutamine, 1 mM Sodium Pyruvate, 1% Pen-Strep mix
- Maintenance medium: EMEM, 2% FCS
- Viral titration method: Spearmen-Karber
- Cleaning method: immersion in ethanol 70 % and drying

6 PROCEDURE

6.1 Preparation of test specimens

A total of 12 untreated test specimens and 9 test specimens treated respectively with the test products “B TITANIA SILVER” or “B-ZERO” are necessary to perform the test.
Each test specimen is a flat square of (50 ± 2) mm x (50 ± 2) mm.
Test specimens treated with “TITANIA SILVER” were supplied by the client.
Test specimens treated with “B-ZERO” were prepared in laboratory, by applying the product 10 times on the specimen surface. The repeated application, mimicking every day use conditions, allow to evaluate also the cumulative effect of the product multipurpose cleaner “B-ZERO”. The application procedure is reported below:

- Moistening of the microfiber cloth (Vileda MicronQuick BSystems 134859 0373) with water
- Impregnation of the microfiber cloth with the product “B-ZERO”
- Wringing the excess of product
- Gentle cleaning of the surface
- Natural air drying for 30 minutes

Before the use, each test specimen was also sterilized by immersion in ethanol 70%, for eliminating any bacterial contamination.

6.2 Test Procedure

3 “treated” test specimens and 6 “untreated” test specimens were inoculated with 0.4 ml of virus suspensions (viral titer $TCID_{50}$ 10^7/ml). Then, inoculums were covered with a 40 x 40 mm film and incubated for 3 hours at 25 °C and relative humidity > 90%.

Immediately after inoculation, 3 “untreated” test specimens were added with 10 ml of neutralizer SCDLP broth and the residual virus infectivity revealed by titration.
At end of the contact time, the procedure described above was repeated for the “treated” test specimens and the remaining “untreated” test specimens.

6.3 Virus Titration

Titration was performed by means of the Spearman-Kärber method.
In details, for each treatment serial dilution till 10^{-10} were prepared in maintenance medium.
Then, 100 µl of each dilution were transferred into eight wells of a 96-well plate containing a confluent (> 90%) cell monolayer prepared the previous day. After 1 h of incubation at 37 °C and 5 % CO_2, 100 µl of maintenance medium were added to each well and plates placed in the incubator at 37 °C and 5 % CO_2 for 7 days, time needed for virus infection.
After incubation, cells were observed for evaluating the viral presence and viral titre was calculated using Spearman-Karber method. The following formula was applied:
$TCID_{50}$ = log of the highest virus concentration used - [(log of dilutions)×(X-0.5)]
Where: X is the sum of the ratio between infected wells on total wells per each dilution tested.

6.3.1 Determination of the infectivity titre of virus

For each test specimen, the infectivity titre of virus recovered was obtained as follows:
$N = (10 \times TCID_{50} \times V) / A$
Where:
N is the infectivity titre of virus recovered per cm^2 of test specimen
V is the volume of the SCDLP broth added to the specimen, in ml
A is the surface area of the cover film, in cm^2

6.4 Verification of methodology

The test is valid if the following criteria are fulfilled:

- The average $TCID_{50}$ recovered immediately after inoculation from the untreated test specimens shall be within the range of 2.5×10^5 $TCID_{50}/cm^2$ and 1.2×10^6 $TCID_{50}/cm^2$
- The suppressive efficiency of the agent’s activity (neutralization) is to be confirmed

6.5 Calculation of the antiviral activity

The antiviral activity is calculated as follows:
R= Ut - At
Where:

R is the antiviral activity
Ut is the average log of the $TCID_{50}/cm^2$ from the 3 untreated test specimens after 3 hours
At is the average log of the $TCID_{50}/cm^2$ from the 3 treated test specimens after 3 hours

6.6 Controls

6.6.1 Verification of cytotoxic effects on host cells

3 “treated” test specimens and 3 “untreated” test specimens were added with 10 ml of neutralizer SCDLP broth. Then, the wash-out solution was added to cells mimicking procedure for titration. Test specimen need to be non cytotoxic.

6.6.2 Verification of cell sensitivity to virus and the inactivation of antiviral activity

3 “treated” test specimens and 3 “untreated” test specimens were added with 10 ml of neutralizer SCDLP broth. Then, 5 ml of surnatant from each test specimen, as well as 5 ml of SCDLP broth to be used as negative control, were transferred into new tubes and added with 50 µl of virus suspension at concentration $TCID_{50}$ 10^4/ml. The mix was incubated at 25 °C for 30 min. Finally, the solution was titrated as described above.
Infectivity titre was calculated by applying the formula:
S = (10 x P)

Where:
S is the infectivity titre of virus per ml per test suspension
P is the average titre $TCID_{50}$

Acceptance criteria: $|Sn - Su| \leq 0,5$ and $|Sn - St| \leq 0,5$

Where:
Sn is the average log of the infectivity titre of virus in TCID50/ml for the negative control
Su is the average log of the infectivity titre of virus in TCID50/ml for the untreated test specimen
St is the average log of the infectivity titre of virus in TCID50/ml for the treated test specimen

7 RESULTS

Results are summarized in the tables below n° 1 and 2:

Table 1: Results of the antiviral test, ISO 21702:2019

Vacciniavirus							
Test Specimen	**Contact time**	**Mean virus Titration (Log $TCID_{50}$)**	**Mean virus Titration ($TCID_{50}$ /100 µl)**	**N ($TCID_{50}/cm^2$)**	**Reduction (Ut – At)**	**Acceptance criteria**	**Result**
UNTREATED	0 min	5.00	10^5	6.25×10^5	/	2.5×10^5 - 1.2×10^6 ($TCID_{50}/cm^2$)	PASS
	3 hours	4.875	$10^{4.875}$	4.69×10^5	/	/	/
B TITANIA SILVER TREATED	3 hours	4.00	$10^{4.00}$	6.25×10^4	0.875	/	ABSENCE OF ANTIVIRAL EFFECT
B ZERO TREATED	3 hours	4.75	$10^{4.75}$	3.51×10^5	0.125	/	ABSENCE OF ANTIVIRAL EFFECT

Table 2: Results of controls

Vacciniavirus					
Sensitivity test	**Mean virus Titration (Log $TCID_{50}$)**	**Mean virus Titration ($TCID_{50}$ /100 µl)**	**S (TCID50/ml)**	**Acceptance criteria**	**Result**
Negative control	2.875	$10^{2.875}$	$Sn = 10^{3.875}$	/	/
UNTREATED	2.875	$10^{2.875}$	$Su = 10^{3.875}$	$\|Sn - Su\| \leq 0.5$	0 (PASS)
B TITANIA SILVER TREATED	2.750	$10^{2.750}$	$St = 10^{3.750}$	$\|Sn - St\| \leq 0.5$	0.125 (PASS)
B ZERO TREATED	2.625	$10^{2.625}$	$St = 10^{3.625}$	$\|Sn - St\| \leq 0.5$	0.25 (PASS)

8 CONCLUSIONS

According to ISO 21702:2019, under the test conditions applied, both the test specimens treated respectively with “B TITANIA SILVER” and “B-ZERO” resulted not to have an antiviral effect against vacciniavirus (surrogate virus for viruses with envelope, i.e. coronavirus like SARS-CoV-2), after 3 hours of contact time.

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