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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

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