DR. BRILL + DR. STEINMANN
INSTITUTE FOR HYGIENE AND MICROBIOLOGY

TEST REPORT

01/04/2020

Test report

Efficacy of

STERISAFE Pro Version 1.0

Test virus:	bovine coronavirus (BCoV) (surrogate of human coronaviruses)
Method:	based on NF T 72-281:2011 (Phase 2/Step 2) Quantitative Non-Porous Surface Test for Evaluation of Bactericidal and/or Fungicidal Activity of Chemical Disinfectants and Antiseptics Used in Food, Industrial, Domestic, and Institutional Areas

Sponsor:
STERISAFE ApS
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1. Introduction

It was the aim of our study to evaluate the virus-inactivating properties of ozone generated by **STERISAFE Pro Version 1.0** for room disinfection. The bovine coronavirus (BCoV) (surrogate of human coronaviruses) was chosen as test virus. These experiments were performed based on the NF T 72-281:2011.

Carries (5x5 cm) of stainless steel, solid core furniture board and ceramic tiles are contaminated with a virus inoculum (test virus suspension + soil load) and placed in a suited room at a defined place. Then the inactivation of the test virus as mentioned above by ozone (80 ppm) generated by **STERISAFE Pro Version 1.0** was performed with a holding time of 60 minutes. The treated carriers were checked after elution for residual virus at the end of the experiment. The virus-inactivating properties of this procedure under the chosen conditions can be calculated by comparing the virus titres with the controls (carriers in a different room without **STERISAFE Pro Version 1.0** treatment).

2. Test laboratory

University Medical Center Hamburg-Eppendorf, Institute for Medical Microbiology, Virology and Hygiene, Martinistraße 52, DE – 20246 Hamburg

Dr. Brill + Partner GmbH Institute for Hygiene and Microbiology, Norderoog 2, DE - 28259 Bremen

3. Identification of the device

Manufacturer	STERISAFE ApS
Name of device	STERISAFE Pro Version 1.0 device no 006
Serial number	not specified
System	in-situ generation of ozone
Output	80.4 ppm ozone (mean value) (1st cycle) 79.5 ppm ozone (mean value) (2nd cycle)
Exit	outlet duct which extends vertically to the top

4. Material

4.1 Culture medium and reagents

- Eagle`s Minimum Essential Medium with Earle`s BSS (EMEM, Biozym Scientific GmbH, catalogue no. 880121)
- fetal calf serum (Biochrom AG, article no. S 0115)
- Aqua bidest. (SG ultrapure water system, type ultra Clear; serial no. 86996-1)
- PBS (Invitrogen, article no. 18912-014)
- BSA (Sigma-Aldrich-Chemie GmbH, article no. CA-2153).

4.2 Virus and cells

The BCoV strain L9 was obtained by Dr. G. Zimmer, Institute of Virology at the School of Veterinary Medicine Hannover (Tierärztliche Hochschule, DE - 30559 Hannover).

The *U373 cells* (passage 8) were as well obtained by Dr. G. Zimmer, Institute of Virology at the School of Veterinary Medicine Hannover (Tierärztliche Hochschule, DE - 30559 Hannover).

The cells were inspected regularly for morphological alterations and for contamination by mycoplasmas. No morphological alterations of cells and no contamination by mycoplasmas could be detected.

4.3 Ozone application unit

The ozone application unit **STERISAFE Pro version 1.0** device no 006 was supplied by STERISAFE ApS, Ole Maaløe's vej 5, DK – 2200 Copenhagen.

During the build-up phase the ozone and humidity levels are build-up and actively circulated in the room. The humidity is increased through a fine water mist. In the decontamination phase the ozone concentration in the air is actively regulated to the desired level. During the cleaning phase, the ozone gas is catalytically neutralized and particles are electrostatically precipitated.

4.4 Apparatus, glassware and small items of equipment

- CO_2 incubator, Nunc GmbH & Co. KG, model QWJ 350
- Agitator (Vortex Genie Mixer, type G 560E)
- pH measurement 315i (WTW, article no. 2A10-100)
- Centrifuge (Sigma-Aldrich-Chemie GmbH, type 113)

- Microscope (Olympus, type CK 30)
- Centrifuge 5804 R (Eppendorf AG)
- Water bath (JULABO, Julabo U 3)
- Adjustable volume automatic pipettes (Eppendorf AG)
- Polysterol 96-well microtitre plate (Nunc GmbH & Co. KG, Wiesbaden)
- Cell culture flask (Nunc GmbH & Co. KG, Wiesbaden)
- Sealed test tubes (Sarstedt AG & Co., Nümbrecht)
- 5x5cm square stainless steel carriers (#0344818, Modulor GmbH, Berlin)
- 5x5cm ceramic tiles with white matt glaze (#3709PN00, Villeroy&Boch, Mettlach, Germany)
- 5x5cm solid core furniture board (pre-used in a children hospital)

5. Experimental conditions

			cycle 1	cycle 2
Test temperature	(test room)		18.8 °C	19.2 °C
	(control room)		19.4 °C	20.4 °C
Relative humidity	(test room)	Start:	41.6 %	34.2 %
		Average:	91.6 %	89.8 %
		Maximum:	98.5 %	97.9 %
	(control room)		36.2 %	38.6 %
Concentration of test product in the decontamination phase (mean value)			80.4 ppm ozone	79.5 ppm ozone
Exposure times		Build-up:	3 min	5 min
		Decontamination:	60 min	60 min
		Cleaning:	7 min	7 min
Position of the carriers			horizontal	
Distance: Ozone application unit / carriers			Distance to shelf: 0.65 m Highest position on shelf: 1.6 m Lowest position on shelf: 0.05 m	
Test room ground area			1.8 x 1.8 m	
Test room height			1.8 m	
Test room volume			5,832 m^3	
Total quantity used (test product)			not applicable	
Total quantity m^3			not applicable	
Total quantity m^2			not applicable	
Interfering substance (s)			0.3 g/l bovine serum albumin	
Procedure to stop action of product			immediate dilution	
Test virus			bovine coronavirus strain L9	
Period of analysis			04/03/2020 - 01/04/2020	
End of testing			01/04/2020	

6. Method

The tests were carried out based on NF T 72-281 "Methods of airborne disinfection of surfaces – Determination of bactericidal, fungicidal, yeasticidal and sporicidal activity (Phase 2/Step 2)".

6.1 Preparation of test virus suspension

For preparation of test virus solution, *U373 cells* were cultivated in a 75 cm^2 flask with in EMEM supplemented with L-glutamine, non-essential amino acids and sodium pyruvate and 10 % fetal calf serum. Before virus infection, cells were washed two times with phosphate buffered saline (PBS), incubated for 3 h with EMEM without FCS and were washed once with EMEM supplemented with trypsin. For virus production, BCoV strain L9 was added to the prepared monolayer. After an incubation period of 24 to 48 hours cells were lysed by a rapid freeze/thaw cycle. Cellular debris was removed by low speed centrifugation and the supernatant was directly used as the test virus suspension.

6.2 Preparation of virus inoculum

For the preparation of virus inoculum 9 parts of the test virus suspension were mixed with 1 part of a 3.0 g/l BSA solution (final concentration: 0.3 g/l BSA).

6.3 Preparation of carriers

Prior to use, all carriers, stainless steel, solid core furniture board, ceramic tile were sterilized (steam sterilization).

6.4 Experimental conditions

50 µl of the virus inoculum (suspension of test virus with interfering substance) were applied to the carriers and dried afterwards.

Five carrier per material were positioned in a shelfboard (horizontal), three of each carrier at the highest position and two of each carrier at the lowest. The distance to the ozone application unit was 0.65 m with a height of 0.05 to 1.6 m.

The ozone application unit was prepared according to the instruction of the manufacturer and started. The build-up phase (increasing of ozone and humidity with active circulation) took place over 3 minutes in cycle 1 and 5 minutes in cycle 2. The virus-inactivating properties of a treatment with the STERISAFE Pro Version 1.0 were examined for 60 min at 80.4 ppm ozone and average 91.6 % relative humidity such as in cycle 2 79.5 ppm ozone and average 89.8 % relative humidity. After a cleaning phase the carriers were transferred for elution in a 15 ml tube with 1 ml medium (EMEM).

For further processing the eluates were transported to Dr. Brill + Partner GmbH. Series of ten-fold dilutions of the eluate in ice-cold maintenance medium were prepared and inoculated on cell culture.

6.5 Controls

All controls were performed as described in 6.4. Determination of VC was done in another room without treatment. Preparations exactly followed the procedure as described in 6.4.

6.5.1 Virus controls

For the control of the initial virus titre in the test assay and for determination of the stability after drying a virus control before drying is needed (VC before). For this control 50 µl virus inoculum was given into 950 µl medium without FCS (elution).

In addition, three virus controls for each tested material ($VC_{stainless\ steel}$, $VC_{furniture\ board}$, $VC_{ceramic\ tile}$) were incorporated. The elution for the virus controls was run in parallel to the room disinfection after incubation of the carriers in a separate room without surface and air disinfection. The virus controls are needed as references for the calculation of the reduction factor after treatment with the test product.

6.5.2 Control of cytotoxicity

The cytotoxicity control is needed to make a differentiation between cytopathic and cell toxic effects.
For the determination of cytotoxicity 50 µl medium instead of virus inoculum without FCS was deposited onto one carrier. After drying, room disinfection and further dwelling time an elution with 1 ml medium was performed. The cytotoxicity control is needed for definition of the lower detection limit.

6.5.3 Cell control

The cells were only treated with cell culture medium.

6.6 Determination of infectivity

Infectivity was determined as endpoint titration transferring 0.1 ml of each dilution into eight wells of a microtitre plate with a preformed *U373* monolayer. Before addition of virus, cells were washed twice with EMEM and incubated for 3 h with 100 µl EMEM with trypsin. Incubation was at 37 °C in a CO2-atmosphere (5.0 % CO_2 - content). Finally,

cultures were observed for cytopathic effects for six days of inoculation. Calculation of the infective dose $TCID_{50}$/ml was calculated with the method of Spearman (3) and Kärber (4).

7. Calculation of virus-inactivating properties

The virus-inactivating properties of a treatment with the STERISAFE Pro Version 1.0 were measured by subtracting the mean virus titres (after treatment) from the virus titres resulted in the parallel without surface and air disinfection. The difference is given as reduction factor (RF) and shown in tables 1 and 2.

8. Results

In parallel to the inactivation experiments the temperature and humidity were measured. In the test room the temperature was 18.8 °C and the average humidity was 91.6 % (1st cycle) and 19.2 °C and the average humidity was 89.8 % (2nd cycle).

The results show a loss of virus titre of 0.83 log_{10}-on stainless steel carriers, 0.88 log_{10}-on solid core furniture and 1.42 log_{10}-on ceramic tiles in comparison to the virus titre on the carrier without drying (VC before) in the 1st cycle. In the 2nd cycle the results show a loss of virus titre of 1.00 log_{10}-on stainless steel carriers, 1.50 log_{10}-on solid core furniture boards and 1.25 log_{10}-on ceramic tiles in comparison to the virus titre on the carrier without drying (VC before).

The cytotoxicity was 0.50 CD_{50}/ml on *U373 cells* calculated in parallel to the infective dose $TCID_{50}$/ml showing the lower detection limit.

Our experiments show that after decontamination with ozone no residual BCoV could be detected on each tested material in both test runs. The calculated reduction factor (RF) in the 1st cycle after a decontamination time of 60 minutes at 80.4 ppm and average 91.6 % of humidity was ≥ 5.29 ± 0.38 on stainless steel discs (table 1), ≥ 5.25 ± 0.43 on solid core furniture boards (table 2) and ≥ 4.71 ± 0.52 on ceramic tiles (table 3).
The calculated reduction factor (RF) in the 2nd cycle after a decontamination time of 60 minutes at 79.5 ppm and average 89.8 % of humidity was ≥ 5.25 ± 0.50 on stainless steel discs (table 1), ≥ 4.75 ± 0.25 on solid core furniture boards (table 2) and ≥ 5.00 ± 0.25 on ceramic tiles (table 3).

9. Conclusions

Under the defined conditions a sufficient activity (4 $\log_{10}$ reduction) of ozone generated by **STERISAFE Pro Version 1.0** against BCoV was found on different carrier material in two independent cycles. Therefore, the room disinfectant device **STERISAFE 1.0** can be declared as active against BCoV as follows:

60 minutes of decontamination with 80.4 ppm and average 91.6 % humidity
60 minutes of decontamination with 79.5 ppm and average 89.8 % humidity

Bremen, 01/04/2020

- Dr. Britta Becker -
Head of Laboratory

- Dr. Dajana Paulmann -
Scientific Project Manager

10. Quality control

The Quality Assurance of the results was maintained by performing the determination of the virus-inactivating properties of the disinfectant in accordance with Good Laboratory Practice regulations:

1) Chemicals Act of Germany, Appendix 1, dating of 01.08 1994 (BGBI. I, 1994, page 1703). Appendix revised at 14. 05. 1997 (BGBI. I, 1997, page 1060)
2) OECD Principles of Good Laboratory Practice (revised 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring – Number 1. Environment Directorate, Organization for Economic Co-operation and Development, Paris 1998.

The plausibility of the results was additionally confirmed by different controls incorporated in the inactivation assays.

11. Records to be maintained

All testing data, protocol, protocol modifications, the final report, and correspondence between Dr. Brill + Partner GmbH and the sponsor will be stored in the archives at Dr. Brill + Partner GmbH.

12. Literature

1) NF T 72-281:2011: Methods of airborne disinfection of surfaces – Determination of bactericidal, fungicidal, yeasticidal and sporicidal activity (English version of French standard NF T 72-281:2009 : Procédés de désinfection des surfaces par voie aérienne – Détermination de l'activité bactéride, fongicide, levuricide et sporicide)

2) Spearman, C.: The method of `right or wrong cases` (constant stimuli) without Gauss's formulae.Brit J Psychol; 2 1908, 227-242

3) Kärber, G.: Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. Arch Exp Path Pharmak; 162, 1931, 480-487

Appendix:

Legend to the tables

Table 1: Results with BCoV on stainless steel discs

Table 2: Results with BCoV on solid core furniture boards

Table 3: Results with BCoV on ceramic tiles

Table 1: Results with BCoV on stainless steel discs and decontamination for 60 minutes at 80.4 ppm ozone and 91.6 % humidity (#6423 (1st cycle)), and at 79.5 ppm ozone and 89.8 % humidity (#6461 (2nd cycle))

Cycle	Assay	Interfering substance in virus inoculum	log_{10} $TCID_{50}$/ml with 95% CI	log_{10} $TCID_{50}$/ml with 95% CI after drying							reduction	
			before drying	carrier 1	carrier 2	carrier 3	carrier 4	carrier 5	MV	2xSD	RF	95 % CI
1	VC_{before} (virus inoculum)	clean	6.63±0.41	n.a.	n.a.	n.a.	n.a.	n.a.	**6.63**	**n.a.**	**n.a.**	**n.a.**
	$VC_{stainless\ steel}$	clean	n.a.	6.00±0.38	5.63±0.25	5.75±0.33	n.d.	n.d.	**5.79**	**0.38**	**0.83**	**n.a.**
	ozone	clean	n.a.	≤0.50±0.00	≤0.50±0.00	≤0.50±0.00	≤0.50±0.00	≤0.50±0.00	**≤0.50**	**0.00**	**≥5.29**	**0.38**
2	VC_{before} (virus inoculum)	clean	6.75±0.33	n.a.	n.a.	n.a.	n.a.	n.a.	**6.75**	**n.a.**	**n.a.**	**n.a.**
	$VC_{stainless\ steel}$	clean	n.a.	6.00±0.38	5.75±0.33	5.50±0.35	n.d.	n.d.	**5.75**	**0.50**	**1.00**	**n.a.**
	ozone	clean	n.a.	≤0.50±0.00	≤0.50±0.00	≤0.50±0.00	≤0.50±0.00	≤0.50±0.00	**≤0.50**	**0.00**	**≥5.25**	**0.50**

n.a. = not applicable n.d. = not done

Table 2: Results with BCoV on solid core furniture board and decontamination for 60 minutes at 80.4 ppm ozone and 91.6 % humidity (#6423 (1st cycle)), and at 79.5 ppm ozone and 89.8 % humidity (#6461 (2nd cycle))

Cycle	Assay	Interfering substance in virus inoculum	log_{10} $TCID_{50}$/ml with 95% CI	log_{10} $TCID_{50}$/ml with 95% CI after drying							reduction	
			before drying	carrier 1	carrier 2	carrier 3	carrier 4	carrier 5	MV	2xSD	RF	95 % CI
1	VC_{before} (virus inoculum)	clean	6.63±0.41	n.a.	n.a.	n.a.	n.a.	n.a.	**6.63**	**n.a.**	**n.a.**	**n.a.**
	$VC_{solid\ core\ furniture\ board}$	clean	n.a.	5.63±0.43	6.00±0.38	5.63±0.41	n.d.	n.d.	**5.75**	**0.43**	**0.88**	**n.a.**
	ozone	clean	n.a.	≤0.50±0.00	≤0.50±0.00	≤0.50±0.00	≤0.50±0.00	≤0.50±0.00	**≤0.50**	**0.00**	**≥5.25**	**0.43**
2	VC_{before} (virus inoculum)	clean	6.75±0.33	n.a.	n.a.	n.a.	n.a.	n.a.	**6.75**	**n.a.**	**n.a.**	**n.a.**
	$VC_{solid\ core\ furniture\ board}$	clean	n.a.	5.25±0.33	5.38±0.41	5.13±0.45	n.d.	n.d.	**5.25**	**0.25**	**1.50**	**n.a.**
	ozone	clean	n.a.	≤0.50±0.00	≤0.50±0.00	≤0.50±0.00	≤0.50±0.00	≤0.50±0.00	**≤0.50**	**0.00**	**≥4.75**	**0.25**

n.a. = not applicable n.d. = not done

Table 3: Results with BCoV on ceramic tiles and decontamination for 60 minutes at 80.4 ppm ozone and 91.6 % humidity (#6423 (1st cycle)), and at 79.5 ppm ozone and 89.8 % humidity (#6461 (2nd cycle))

Cycle	Assay	Interfering substance in virus inoculum	log_{10} $TCID_{50}$/ml with 95% CI	log_{10} $TCID_{50}$/ml with 95% CI after drying							reduction	
			before drying	carrier 1	carrier 2	carrier 3	carrier 4	carrier 5	MV	2xSD	RF	95 % CI
1	VC_{before} (virus inoculum)	clean	6.63±0.41	n.a.	n.a.	n.a.	n.a.	n.a.	**6.63**	**n.a.**	**n.a.**	**n.a.**
	$VC_{ceramic\ tiles}$	clean	n.a.	5.00±0.49	5.50±0.52	5.13±0.37	n.d.	n.d.	**5.21**	**0.52**	**1.42**	**n.a.**
	ozone	clean	n.a.	≤0.50±0.00	≤0.50±0.00	≤0.50±0.00	≤0.50±0.00	≤0.50±0.00	**≤0.50**	**0.00**	**≥4.71**	**0.52**
2	VC_{before} (virus inoculum)	clean	6.75±0.33	n.a.	n.a.	n.a.	n.a.	n.a.	**6.75**	**n.a.**	**n.a.**	**n.a.**
	$VC_{ceramic\ tiles}$	clean	n.a.	5.38±0.25	5.63±0.25	5.50±0.46	n.d.	n.d.	**5.50**	**0.25**	**1.25**	**n.a.**
	ozone	clean	n.a.	≤0.50±0.00	≤0.50±0.00	≤0.50±0.00	≤0.50±0.00	≤0.50±0.00	**≤0.50**	**0.00**	**≥5.00**	**0.25**

n.a. = not applicable n.d. = not done