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TEST REPORT

05/03/2018

Test report L18/0070M.1

Efficacy of

STERISAFE Pro Version 1.0

Test virus: murine norovirus (as surrogate of human norovirus)

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:
Infuser Denmark
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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 murine norovirus (MNV) was chosen as surrogate for human noroviruses. These experiments were performed based on the NF T 72-281.

Stainless steel discs 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 generated by **STERISAFE Pro Version 1.0** was studied. 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

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

3. Identification of the device

Manufacturer	Infuser Denmark
Confirmation no.	204212
Name of device	STERISAFE Pro Version 1.0 device no 006
Serial number	not specified
System	in-situ generation of ozone
Output	79.05 ppm ozone (mean value)
Diffusion rate of the test device (CT value per hour)	active circulation of ozone by build-in fan 165 mg/m^3
Exit	outlet duct which extends vertically to the top

4. Material

4.1 Culture medium and reagents

- Dulbecco´s Modified Eagle´s Medium (DMEM, Biozym Scientific GmbH, catalogue no. 880006)
- fetal calf serum (Thermo Fisher, article no. CH30160.02)
- 1.4 % formaldehyde solution (dilution of Roti®-Histofix 4 %, Carl Roth GmbH)
- 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)
- skim milk powder (Fluka Analytical; article no. 70166-500).

4.2 Virus and cells

The murine norovirus (MNV) was obtained from PD. Dr. E. Schreier, Head of FG15 Molecular Epidemiology of Viral Pathogens at the Robert Koch-Institute (RKI) in Berlin. Prior to inactivation, MNV was passaged three times in *RAW 264.7 cells* (a macrophage-like, Abelson leukemia virus transformed cell line derived from BALB/c mice, ATCC TIB-71). *RAW 264.7 cells* were cultured with Dulbecco's Modified Eagle's Medium with 4.5 g/l glucose and fetal calf serum with low endotoxin. Furthermore, cells (passage 31) 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 (figure 1) was supplied by Infuser Denmark, Ole Maaløe's vej 5, DK – 2200 Copenhagen.

Figure 1: STERISAFE Pro Version 1.0

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)
- Container, flat bottom, 25 cm, with cap (Sarstedt AG & Co., Nümbrecht)
- Stainless steel discs (2 cm diameter discs) with Grade 2 B finish on both sides (article no. 4174-3000, GK Formblech GmbH, Berlin).

5. Experimental conditions

		cycle
Test temperature	(test room)	21.72 °C
	(control room)	23.8 °C
Relative humidity	(test room) Start:	37.37 %
	(test room) Average:	85.30 %
	(test room) Maximum:	92.82 %
	(control room)	42.3 %
Concentration of test product in the decontamination phase (mean value)		79.05 ppm ozone
Exposure times	Build-up:	24 min
	Decontamination:	435 min
	Cleaning:	60 min
Position of the carriers		vertical
Distance: Ozone application unit / carriers		3.60 m (height: 1.0 m from ground) (see figure 2)
Test room ground area		4.95 x 4.95 m
Test room height		2.55 m
Test room volume		62.48 m^3
Total quantity used (test product) in 62.48 m^3		not applicable
Total quantity m^3		not applicable
Total quantity m^2		not applicable
Interfering substance (s)		0.5 % skimmed milk
Procedure to stop action of product		immediate dilution
Test virus		murine norovirus (Berlin 06 / 06 / DE Isolate S99)
Period of analysis		07/02/2018 - 05/03/2018
End of testing		05/03/2018

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

To prepare the test virus suspension, *RAW 264.7 cells* which have been cultured with Dulbecco's Modified Eagle's Medium with 4.5 g/l glucose and 10 % fetal calf serum with low endotoxin were inoculated with MNV (stock virus solution) in a 175 cm^2 cell culture flask. Once a cytopathic effect had been induced (approx. 1-3 days), freezing and thawing was carried out two times. The cell debris was removed by low speed centrifugation and the supernatant was recovered as test viral suspension, aliquoted and stored at -80 °C.

6.2 Preparation of virus inoculum

For the preparation of virus inoculum 19 parts of the test virus suspension were mixed with 1 part of a 10 % skimmed milk solution (final concentration: 0.5 %).

6.3 Preparation of carriers

Prior to use, the carriers (stainless steel discs) were placed in a container with an appropriate quantity of 5 % (v/v) Decon 90® for 60 minutes (at room temperature), in a manner that they do not stick together and the surface gets no damage. Following this, the discs were immediately rinsed off thoroughly with aqua dest. for no less than 10 seconds each. This procedure was repeated once more to remove all surfactants. Afterwards, without drying the carriers, the discs were submerged in 70 % (v/v) isopropyl alcohol for 15 minutes, air-dried by evaporation under the laminar air flow and finally sterilized (steam sterilization). Carriers were only being handled with forceps and were supposed for single use only.

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.

The carriers (in triplicate) were deposited in slat (see figures 2 and 3) and transported in the room chosen for surface and air disinfection (vertical position).

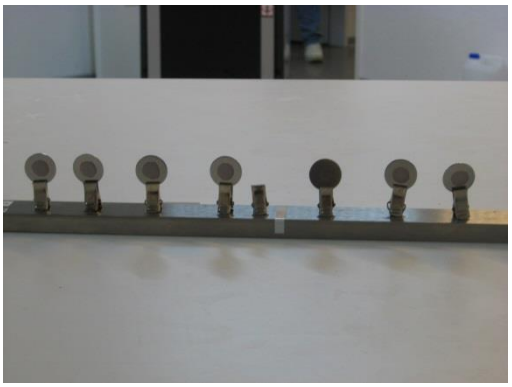

Figure 2: Slats with the inoculated carrier in vertical position

In this room the carriers were placed in a distance of 3.6 m from the ozone application unit (with the contaminated side turned away from device) with a height of 1 to 1.5 m (here 1.0 m).

Figure 3: Position of the slats with the inoculated carrier in the test room

The ozone application unit was prepared according to the instruction of the manufacturer and started (see figure 4).

Figure 4: Position of the STERISAFE Pro Version 1.0 in the test room

The build-up phase (increasing of ozone and humidity with active circulation) took place over 24 minutes. The virus-inactivating properties of a treatment with the STERISAFE Pro Version 1.0 were examined for 435 min at 79.05 ppm ozone and average 85.30 % relative humidity. After a cleaning phase of 60 min the carriers were transferred for elution in a 25 ml vial with 10 ml medium without FCS and vortexed for 60 seconds. Directly after elution, 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, for determination of the stability after drying and for evaluation of the neutralization of the disinfectant a virus control before drying is needed (VC before). For this control 50 µl virus inoculum was given into 9.950 ml medium without FCS (elution).

In addition, two virus controls directly after drying (VCt0) and three carriers for each exposure time tested (VC t_{cycle} – 435 min) were incorporated. For the VC t0 the elution took place immediately after drying of virus inoculum in 10 ml medium without FCS. The elution for VC t_{cycle} was run in parallel to the room disinfection after incubation of the carriers in a separate room without surface and air disinfection. VC t_{cycle} is needed as reference for the calculation of the reduction factor after treatment with the test product.

For the formaldehyde control (see 6.5.5) a virus control before drying with phosphate buffer is needed (VC PBS). For this control 100 µl of the test virus suspension were mixed with 100 µl PBS and 800 µl WSH and incubated for 60 min at 20 °C.

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 10 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.

6.5.4 Control of efficacy for suppression of disinfectant's activity (neutralization control)

For demonstration that the addition of medium without FCS will contribute to a sufficient neutralization of the activity of the test product 50 µl test virus suspension were added to a second cytotoxicity control and incubated for 30 min on ice. Finally a virus titration was performed.

6.5.5 Reference control

As reference for test validation a 0.7 % formaldehyde (v/v) solution was included. Therefore, 100 µl of test virus suspension were mixed with 400 µl phosphate buffer and 500 µl of a 1.4 % formaldehyde solution. 5, 15, 30 and 60 minutes were chosen as contact times. In addition, cytotoxicity of formaldehyde test solution was determined with dilutions up to 10^{-5}.

The difference of the logarithmic titre of the virus control (VC PBS) minus the logarithmic titre of the test virus in the reference inactivation test had to be in the range of the values from different other tests in our lab (mean value), respectively (data not shown).

6.6 Determination of infectivity

Infectivity was determined as endpoint titration according to WI 5.7.1 transferring 0.1 ml of each dilution into eight wells of a microtitre plate, beginning with the highest dilution. This was followed by the addition of 0.1 ml of freshly scraped cells. This cell suspension was adjusted to reach 10-15 x 10^3 cells per well. Microtitre plates were incubated at 37 °C in a 5 % CO_2-atmosphere. The cytopathic effect was read by using an inverted microscope after five days. Calculation of the infective dose $TCID_{50}$/ml was calculated with the method of Spearman (2) and Kärber (3) with the following formula:

$$- \log_{10} TCID_{50} = X_0 - 0.5 + \sum r/n$$

meaning

X_0 = $\log_{10}$ of the lowest dilution with 100 % positive reaction

r = number of pos. determinations of lowest dilution step with 100 % positive and all higher positive dilution steps

n = number of determinations for each dilution step.

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 table 1.

8. Verification of the methodology

Since all the following criteria were fulfilled, examination with MNV is valid.

a) The titre of the test virus suspension allowed the determination of a ≥ 4 $\log_{10}$ reduction

b) The test product showed no cytotoxicity in the 1:10 dilutions thus allowing the detection of a 4 $\log_{10}$ reduction of virus titre.

c) The control of efficacy for suppression of disinfectant's activity showed no decrease (≤ 0.5 $\log_{10}$) in virus titre.

d) The difference of the logarithmic titre of the virus control minus the logarithmic titre of the test virus in the reference inactivation test (see 6.6.5) was in the range of the values from different test in our lab with the MNV (between 0.00 – 1.58 after 5 min and 0.50 – 2.62 after 30 min, data not shown).

9. Results

In parallel to the inactivation experiments the temperature and humidity were measured. In the test room the temperature was 21.72 °C and the average humidity was 85.30 %.

The results show a loss of virus titre of the control carriers of 0.38 $\log_{10}$-steps in comparison to the virus titre on the carrier without drying (VC before).

The cytotoxicity was 1.50 CD_{50}/ml on *RAW 264.7 cells* calculated in parallel to the infective dose $TCID_{50}$/ml showing the lower detection limit.

Our experiments show that after the decontamination cycle residual MNV could be detected. The calculated reduction factor (RF) after a decontamination time of 435 minutes at 79.05 ppm and average 85.30 % of humidity was ≥ 4.96 (table 1).

10. Conclusions

Under the defined conditions a sufficient activity (4 log_{10} reduction) of ozone generated by **STERISAFE Pro Version 1.0** against MNV was found. Therefore, the room disinfectant device **STERISAFE 1.0** can be declared as active against MNV as follows:

435 minutes of decontamination with 79.05 ppm and average 85.30 % humidity

Bremen, 05/03/2018

- Dr. Britta Becker -
Head of Laboratory

- Dr. Dajana Paulmann -
Scientific Project Manager

11. 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.

12. 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.

13. 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 MNV, decontamination (435 minutes at 79.05 ppm ozone and 85.30 % humidity)

Table 2: Results with formaldehyde solution (0.7 %) (quantal test; 8 wells)

Table 1: Results with MNV, decontamination 435 minutes at 79.05 ppm ozone and 85.3 % humidity) (#5400)

virus control	carrier	log_{10} $TCID_{50}$/ml	average log (geometric)	RF
VC $_{before\ drying}$	carrier - 1	7.75	7.75	**n.a.**
	carrier - 2	n.d.		
	carrier - 3	n.d.		
VC t0	carrier - 1	7.88	7.75	**0.00**
	carrier - 2	7.63		
	carrier - 3	n.d.		
VC t_{cycle}	carrier - 1	7.25	7.38	**0.38**
	carrier - 2	7.50		
	carrier - 3	n.d.		

neutralization control	log_{10} $TCID_{50}$/ml	RF
VC $_{before\ drying}$	7.75	**n.a.**
disinfectant	7.63	**0.13**

decontamination time	disinfectant	concentration	carrier	log_{10} $TCID_{50}$/ml	average log (geometric)	RF
cycle 435 min	**ozone**	**79.05 ppm**	carrier - 1	≤2.75	≤2.42	**≥4.96**
			carrier - 2	≤2.25		
			carrier - 3	≤2.25		

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

Table 2: Results for formaldehyde solution (0.7 %) tested against MNV at 20 °C (quantal test; 8 wells) (#5400)

Product	Con-centration	Level of cytotoxicity	log_{10} $TCID_{50}$/ml after ....min				
			0	5	15	30	60
formaldehyde	0.7 % (w/v)	4.50	n.d.	8.13	7.13	6.88	5.88
virus control (VC PBS)	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	7.88

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