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TEST REPORT

19/05/2020

Test report L20/0361aMV.3

Efficacy of

FLO-D MINI - Mark 2

Test virus: modified vaccinia virus Ankara (MVA)

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:
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1. Introduction

It was the aim of our study to evaluate the virus-inactivating properties of ozone generated by **FLO-D MINI - Mark 2** for room disinfection. The modified vaccinia virus Ankara (MVA) was chosen as test virus because in Europe, MVA represents the official model virus for all enveloped viruses, including members of the virus family *coronaviridae* (like MERS-CoV, SARS-CoV-1 and SARS-CoV-2). 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 **FLO-D MINI - Mark 2** was studied over a period of three hours. During the complete test, the power-humidifier ECA of Stadler Form (serial no. I1971 1608) was used to enhance the humidity to 75 % in the test room. 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 **FLO-D MINI - Mark 2** treatment).

2. Test laboratory

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

3. Identification of the device

Manufacturer	JIMCO A/S
Name of device	FLO-D MINI - Mark 2
Corfirmation no.	214205 / 214909
Serial number	-
System	generation of ozone
Output	10 ppm ozone (max. value)

4. Material

4.1 Culture medium and reagents

- Eagle`s Minimum Essential Medium with Hank`s BSS (MEM, Biozym Scientific GmbH, catalogue no. 880144)
- 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).

4.2 Virus and cells

The modified vaccinia virus Ankara (MVA) originated from Dr. Manteufel, Institut für Tierhygiene und Öffentliches Veterinärwesen, DE - 04103 Leipzig. Before inactivation assays, virus had been passaged three times in *BHK 21-cells* (Baby Hamster Kidney).

BHK 21-cells (passage 10) originated from the Friedrich-Löffler-Institut, Bundesforschungsinstitut für Tiergesundheit (formerly Bundesforschungsanstalt für Viruskrankheiten der Tiere, isle of Riems).

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 **FLO-D MINI - Mark 2** (figure 1) was supplied by JIMCO A/S, Mjølbyvej 7, DK - 5900 Rudkøbing. The FLO-D mini produces Ozone by draining the air in the room through the system's UV-C chamber where oxygen O_2 contained in the air is converted to ozone O_3. The ozone then blows out and spreads into the room.

Figure 1: FLO-D MINI - Mark 2

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 (3 cm diameter discs (4)) with Grade 2 B finish on both sides (article no. 1000-3072, GK Formblech GmbH, Berlin).

5. Experimental conditions

Test temperature	22.0 °C (beginning) – 20.5 °C (end)
Relative humidity	44.5 % (beginning) – 75.5 % (maximum) – 73.5 % (end)
Exposure time	180 minutes
Diffusion rate of the system	-
Position of the carriers	vertical
Distance: device / carriers	3.60 m (height: 1.0 m from ground)
Test room ground area	4.95 x 4.95 m
Test room height	2.55 m
Test room volume	62.48 m^3
Interfering substance (s)	clean conditions: 0.3 g/l BSA
Procedure to stop action of product	immediate dilution
Test virus	modified vaccinia virus Ankara (MVA) (ATCC VR-1508)
Period of analysis	07/05/2020 - 19/05/2020
End of testing	19/05/2020

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

To prepare the test virus suspension, *BHK 21-cells* were cultivated with EMEM and 10 % or 2 % fetal calf serum. Cells were infected with a multiplicity of infection of 0.1. After cells showed a cytopathic effect, they were subjected to a freeze/thaw procedure followed by a low speed centrifugation in order to sediment cell debris. After aliquotation, test virus suspension was stored at – 80 °C.

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 g/l BSA solution (final concentration: 0.3 g/l).

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, distributed over an area with a diameter of 2 cm approximately (3.0 ± 0.5 cm^2) 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).

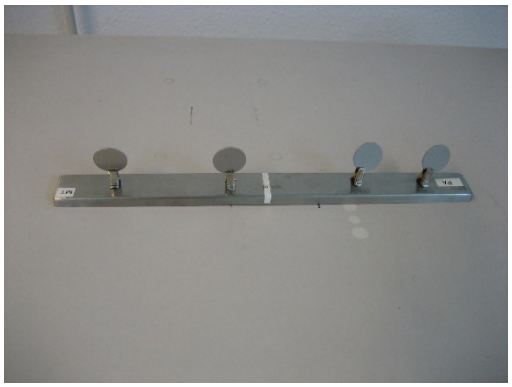

Figure 2: Slats with the inoculated carrier in vertical position

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 by the manufacturer and started (see figure 4).

Figure 4: Position of the FLO-D MINI - Mark 2 and the power-humidifier EVA in the test room

The virus-inactivating properties of a treatment with the FLO-D MINI - Mark 2 were examined over a period of three hours. Immediately at the end of the exposure time carriers (in triplicate for the inactivation assays and in duplicate for the virus control (VC)) 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 t180) 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 t180 was run in parallel to the room disinfection after incubation of the carriers in a separate room without surface and air disinfection. VC t180 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 and room disinfection 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 Cell susceptibility

For the control of cell susceptibility one volume of the lowest apparently non-cytotoxic dilution of the eluate (or PBS as control) was added to one volume of double concentrated cell suspension. After 1 h at 37 °C the cells were centrifuged and re-suspended in cell culture medium.

Finally, a comparative titration of the test virus suspension with the virus inoculums was performed on the pre-treated (disinfectant) and non-pre-treated (PBS) cells as described above. The comparative titration on pre-treated (disinfectant) and non-pre-treated (PBS) cells should show no significant difference (< 1 log_{10}) of virus titre.

6.5.6 Reference control

As reference for test validation a 0.7 % formaldehyde (v/v) solution according to EN 14476 (5) 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 by transferring 0.1 ml of each dilution into eight wells of a microtitre plate to 0.1 ml of freshly trypsinised *BHK 21-cells* (10-15 x 10^3 cells per well), beginning with the highest dilution. 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).

7. Calculation of virus-inactivating properties

The virus-inactivating properties of a treatment with the FLO-D MINI - Mark 2 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 MVA 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 MVA (between 0.60 – 2.72 after 5 min and 1.18 – 3.30 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 22.5 °C in the beginning and 20.5 °C in the end. The humidity was 44.5 % in the beginning and 73.5 % in the end (maximum: 75.5 %).

The results show a loss of virus titre of the control carriers of 0.34 $\log_{10}$-steps in comparison to the virus titre on the carrier without drying (VC before).

The cytotoxicity was 0.50 CD_{50}/ml on *BHK 21-cells* calculated in parallel to the infective dose $TCID_{50}$/ml showing the lower detection limit.

Our experiments show that after a decontamination with 10 ppm ozone for 180 minutes with a humidity of 44.5 % to 73.5 %, produced by an additional humidifier, a sufficient reduction of MVA could be detected. The calculated reduction factor (RF) was ≥ 4.03 (table 1). This corresponded to an inactivation of ≥ 99.99 %.

10. Conclusions

Under the defined conditions a sufficient reduction of MVA could be demonstrated with ozone generated by **FLO-D MINI - Mark 2.** Therefore, ozone generated by **FLO-D MINI - Mark 2** can be declared as active against MVA for room disinfection as follows:

10 ppm ozone for 180 minutes with a humidity of 44.5 % to 73.5 % under clean conditions

Bremen, 19/05/2020

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- Dr. Dajana Paulmann -
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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

4) NF T 72-281:2014: Procédés de désinfection des surfaces par voie aérienne — Détermination de l'activité bactéricide, fongicide, levuricide, mycobactéricide, tuberculocide sporicide et virucide incluant les bactériophages

5) EN 14476:2013+A2:2019: Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of virucidal activity of chemicals disinfectants and antiseptics in human medicine test - Test method and requirements (phase 2, step 1)

Appendix:

Legend to the Tables

Table 1: Results with MVA (180 minutes at 10.0 ppm ozone and 44.5 – 73.5 % humidity)

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

Table 1: Results with MVA (180 minutes at 10.0 ppm ozone and 44.5 – 73.5 % humidity) (#6550)

virus control	carrier	log_{10} $TCID_{50}$/ml	average log (geometric)	RF
VC before drying	carrier - 1	5.00	5.00	**n.a.**
	carrier - 2	n.d.		
	carrier - 3	n.d.		
VC t0	carrier - 1	4.75	4.81	**0.19**
	carrier - 2	4.88		
	carrier - 3	n.d.		
VC t180	carrier - 1	4.50	4.66	**0.34**
	carrier - 2	4.88		
	carrier - 3	4.63		

decontamination time	disinfectant	Concentration	carrier	log_{10} $TCID_{50}$/ml	average log (geometric)	RF
180 min	**ozone**	**10.0 ppm**	carrier - 1	≤0.50	≤0.63	**≥4.03**
			carrier - 2	≤0.75		
			carrier - 3	≤0.63		

neutralization control	log_{10} $TCID_{50}$/ml	RF
VC before drying	5.00	**n.a.**
disinfectant	4.88	**0.13**

cell susceptibility control	log_{10} $TCID_{50}$/ml	RF
PBS	4.75	**n.a.**
disinfectant	4.75	**0.00**

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

Table 2: Results for formaldehyde solution (0.7 %) tested against MVA at 20 °C (quantal test; 8 wells) (#6550)

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.	≤4.88	≤4.50	≤4.50	≤4.50
virus control (VC PBS)	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	6.63

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