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

DAkkS Deutsche Akkreditierungsstelle D-PL-13412-01-01 D-PL-13412-01-02

TEST REPORT

19/08/2020

Test report L20/1055MV.1

Efficacy of

OZ2000

Test virus: modified vaccinia virus Ankara (MVA)

Method: based on EN 17272

Chemical disinfectants and antiseptics – Methods of airborne room disinfection by automated process – Determination of bactericidal, mycobactericidal, sporicidal, fungicidal, yeasticidal, virucidal and phagocidal activities

Sponsor:
JIMCO A/S
Mjølbyvej 7
DK - 5900 Rudkøbing

Norderoog 2, DE - 28259 Bremen
Tel.: +49 40-557631-0, Fax: +49 40-557631-11
info@brillhygiene.com, http://www.brillhygiene.com

1. Introduction

It was the aim of our study to evaluate the virus-inactivating properties of ozone generated by **OZ2000** for room disinfection. The modified vaccinia virus Ankara (MVA) was chosen as test virus. These experiments were performed based on the EN 17272.

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 two devices **OZ2000** was studied over a period of four 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. In addition, a fan was placed in front of the devices to allow a better distribution of the ozone. 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 **OZ2000** 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	OZ2000
Corfirmation no.	215575
Serial number	-
System	generation of ozone
Output	1.68 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 30) 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 Reference organism (distribution test)

Staphylococcus aureus ATCC 6538 was used to determine the reduction in the number of viable bacterial cells in the enclosed space to achieve a reduction of 5 lg on each carrier.

4.4 Ozone application unit

The two ozone application units **OZ2000** (figure 1) were supplied by JIMCO A/S, Mjølbyvej 7, DK - 5900 Rudkøbing. The OZ2000 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: The two OZ2000 devices

4.5 Apparatus, glassware and small items of equipment

- CO_2 incubator
- 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	23.0 °C (beginning) – 24.0 °C (end)
Relative humidity	75.5 % (beginning) – 84.0 % (maximum) – 84.0 % (end)
Exposure time	240 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	09/07/2020 - 19/08/2020
End of testing	19/08/2020

6. Method

The tests were carried out based on EN 17272 "Chemical disinfectants and antiseptics – Methods of airborne room disinfection by automated process – Determination of bactericidal, mycobactericidal, sporicidal, fungicidal, yeasticidal, virucidal and phagocidal activities ".

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 bacteria inoculum (Reference)

Staphylococcus aureus ATCC 6538 was sub cultivated for approximately 18 – 24 hours on CSA at 37 °C. A test suspension with 5.0 x 10^7 – 5.0 x 10^9 CFU/ml was prepared and used within two hours.

6.4 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.

6.5 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).

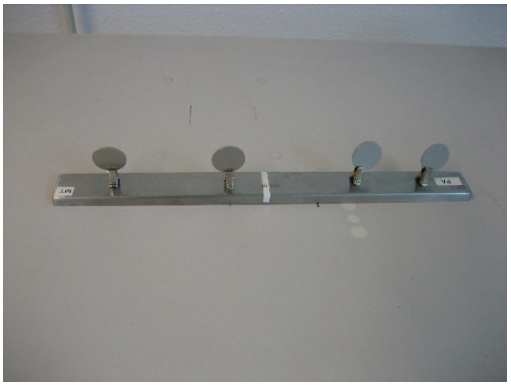

Figure 2: Slats with the inoculated carrier in vertical position

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). The ozone application unit, the humidifier and the fan were placed in the room (see figure 3) and prepared by the manufacturer and started.

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

The virus-inactivating properties of a treatment with the OZ2000 were examined over a period of four 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.

6.6 Controls

All controls were performed as described in 6.5. Determination of VC was done in another room without treatment. Preparations exactly followed the procedure as described in 6.5.

6.6.1 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 t240) 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 t240 was run in parallel to the room disinfection after incubation of the carriers in a separate room without surface and air disinfection. VC t240 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.

6.6.2 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.

6.6.3 Cell control

The cells were only treated with cell culture medium.

6.6.4 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.

6.6.5 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.

6.6.6 Reference control

As reference for test validation a 0.7 % formaldehyde (v/v) solution according to EN 14476 (4) 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).

6.6.7 Distribution test

In the distribution test carried out in parallel to the effectiveness test, the reference strain *Staphylococcus aureus* is distributed at four sampling points in the room (two (one horizontal, one vertical) carriers in two opposite corners at a distance of 0.5 m to the ground and the wall (meaning four in total) and two carriers (one horizontal, one verticl) carriers in two opposite corners at a distance of 0.5 m to the ceiling and the wall (meaning four in total).

The test is valid if a reduction of 5 log steps can be demonstrated on each of the carriers.

6.7 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 OZ2000 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 not all of the following criteria according to EN 17272 5.5.2.4 were fulfilled, examination with modified vaccinia virus Ankara (MVA) is not valid.

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

b) The detectable reduction is at least 4 lg → valid

c) Cell susceptibility test: viral titration reduction < 1 → valid

d) Disinfectant neutralisation test: difference between tests and controls < 0.5 lg → valid

e) The difference of the logarithmic titre of the virus control minus the logarithmic titre of the test virus in the reference inactivation test (see 6.7.6) 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) → valid

f) A reduction of 5 log steps of the reference strain *Staphylococcus aureus* can be demonstrated on each of the carriers. → not valid

9. Results

In parallel to the inactivation experiments the temperature and humidity were measured. In the test room the temperature was 23.0 °C in the beginning and 24.0 °C in the end. The humidity was 75.5 % in the beginning and 84.0 % in the end (maximum: 84.0 %).

The results show a loss of virus titre of the control carriers of 0.58 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 a maximum of 1.68 ppm ozone for 240 minutes with a humidity of 75.5 % to 84.0 %, produced by an additional humidifier, no sufficient reduction of MVA could be detected. The calculated reduction factor (RF) was 2.63 (table 1).

In the distribution test according to table 3 no sufficient reduction of *Staphylococcus aureus* was measured.

10. Conclusions

No sufficient reduction of MVA could be demonstrated with of ozone generated by **OZ2000,** in addition this test is not valid because no sufficient activity against *Staphylococcus aureus* was measured.

Bremen, 19/08/2020

- Dr. Britta Becker -	**- Dr. Dajana Paulmann -**
Head of Laboratory	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) EN 17272:2020 Chemical disinfectants and antiseptics – Methods of airborne room disinfection by automated process – Determination of bactericidal, mycobactericidal, sporicidal, fungicidal, yeasticidal, virucidal and phagocidal activities

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) 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 (240 minutes with ozone and 75.5 – 84.0 % humidity)

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

Table 3: Results of ozone for 240 minutes against *Staphylococcus aureus* (distribution test)

Table 1: Results with MVA (240 minutes at max 1.68 ppm ozone and 75.5 – 84.5 % humidity) (#5715)

virus control	carrier	log_{10} $TCID_{50}$/ml	average log (geometric)	RF
VC before drying	carrier - 1	6.00	6.00	**n.a.**
	carrier - 2	n.d.		
	carrier - 3	n.d.		
VC t0	carrier - 1	5.88	5.69	**0.31**
	carrier - 2	5.50		
	carrier - 3	n.d.		
VC t240	carrier - 1	5.63	5.42	**0.58**
	carrier - 2	5.38		
	carrier - 3	5.25		

decontamination time	disinfectant	Concentration	carrier	log_{10} $TCID_{50}$/ml	average log (geometric)	RF
240 min	**ozone**	**max 1.68 ppm**	carrier - 1	2.75	2.79	**2.63**
			carrier - 2	2.75		
			carrier - 3	2.88		

neutralization control	log_{10} $TCID_{50}$/ml	RF
VC before drying	6.00	**n.a.**
disinfectant	5.88	**0.13**

cell susceptibility control	log_{10} $TCID_{50}$/ml	RF
PBS	5.88	**n.a.**
disinfectant	5.63	**0.25**

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) (#6715)

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.	≤5.13	≤4.50	≤4.50	≤4.50
virus control (VC PBS)	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	6.88

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

Table 3: Results of max 1.68 ppm ozone against *Staphylococcus aureus*, contact time 240 minutes

Water control

Water control (T):	T	a		a'		$\bar{x}$ =	4,43E+06
	1,00E-04	31	38	51	57	lgT =	**6,65**
	1,00E-05	6	8	3	8	lgT > 6 lg ?	**Yes**

Test

Concentration of product in test solution [ppm]	Dilution step	n'1	n'1	N_d ($\bar{x}$ v $\bar{x}$wm) cfu/ml	lg N_d = lg ($\bar{x}$ v $\bar{x}$wm)	d (lgT =6,65)	Exposure time (h)
max. 1.68 ppm	1,00E+00	82	64	7,30E+02	2,86	3,78	4
	1,00E-01	16	20				
	1,00E-02	4	9				
	1,00E+00	>300	>300	2,16E+04	4,33	2,31	4
	1,00E-01	236	196				
	1,00E-02	17	20				
	1,00E+00	>300	>300	4,80E+03	3,68	2,96	4
	1,00E-01	45	51				
	1,00E-02	10	13				
	1,00E+00	202	234	2,18E+03	3,34	3,31	4
	1,00E-01	31	38				
	1,00E-02	3	10				
	1,00E+00	>300	>300	1,70E+04	4,23	2,42	4
	1,00E-01	166	174				
	1,00E-02	22	26				
	1,00E+00	134	144	1,39E+03	3,14	3,50	4
	1,00E-01	33	37				
	1,00E-02	4	5				
	1,00E+00	>300	>300	2,06E+04	4,31	2,33	4
	1,00E-01	192	220				
	1,00E-02	34	37				
	1,00E+00	>300	>300	1,26E+04	4,10	2,55	4
	1,00E-01	114	137				
	1,00E-02	20	29				