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TEST REPORT

14/04/2020

Test report L20/0143MV.1

Evaluation of the effectiveness of

Bacoban WB

Test virus: modified vaccinia virus Ankara (MVA)

Method: based on **ASTM E2180** (Standard Test Methods for Determining the Activity of Incorporated Antimicrobial Agent(s) In Polymeric or Hydrophobic Materials)

Sponsor:
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1. Introduction

The objective of this study was to evaluate the virus-inactivating efficacy of Bacoban WB against modified vaccinia virus Ankara (MVA) using a quantitative carrier test based on the ASTM E2180 (1).

Ceramic tiles treated with Bacoban WB (and untreated controls) are contaminated with test virus suspension in an agar slurry. The ceramic tiles were incubated at room temperature for 5, 15 and 30 minutes. The inactivation of the test virus was studied in one run with three parallels for each exposure time. The ceramic tiles were checked after eluation for residual virus at the end of the experiment. The virus-inactivating properties of Bacoban WB under the chosen conditions can be calculated by comparing the virus titres of treated ceramic tiles with the controls (non-treated carriers).

2. Identification of test laboratory

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

3. Identification of sample

Manufacturer	ROPIMEX R. OPEL GmbH
Name of product	Bacoban WB
Confirmation no.	212558
Product diluent recommended by the manufacturer	-
Batch number	2002130
Application	surface disinfection
Production date	-
Expiry date	02/2022
Active compound (s) (100 g)	QAV
Appearance, odour	clear, yellow, viscous liquid product specific
pH-values	undiluted: 5.70 (20 °C) 1.0 %: 6.47 (20 °C)
Storage conditions	room temperature in the dark (area with restricted access)
Date of arrival in the laboratory	19/02/2020

4. Materials

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 dest. (SG ultrapure water system, type Ultra Clear; serial no. 86996-1)
- PBS (Invitrogen, article no. 18912-014)
- Penicillin/ streptomycin (Sigma-Aldrich, article no. P-0781)
- BSA (Sigma-Aldrich-Chemie GmbH, article no. CA-2153)
- Agar-Agar (Carl Roth GmbH, article no. 5210.2)
- NaCl (Carl Roth GmbH, article no. 3957.1)
- Propan-2-ol (Carl Roth GmbH, article no. 6752.1)

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 104) 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 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 and fixed-volume pipettes (Eppendorf AG)

- Pipette ErgoOne (STARLAB)
- Polysterol 96-well microtitre plate (Nunc GmbH & Co. KG, Wiesbaden)
- Cell culture flask (Nunc GmbH & Co. KG, Wiesbaden)
- Container, flat bottom, 25 cm, with cap (Sarstedt AG & Co., Nümbrecht)
- Glass petri dishes (Nunc GmbH & Co. KG, Wiesbaden)
- Ceramic tiles Ø 2 cm (provided by the sponsor)

5. Experimental conditions

Test temperature	room temperature (21.0 ± 2.0 °C)
Test product concentration (Bacoban WB)	1.0 %
Diluent of the test product solution	water of standardised hardness (WSH)
Size of test samples	2.0 cm in diameter
Coating of the ceramic tiles	10 days before inactivation test
Contact time of the Bacoban WB-coated ceramic tiles to the virus inoculum	5, 15 and 30 minutes
Volume of virus inoculum	50 µl (40 µl virus suspension + 10 µl interfering substance)
Interfering substances in the virus inoculum	0.3 g/l bovine serum albumin (clean conditions, EN 14476)
Procedure to stop action of disinfectant	immediate dilution
Test virus	modified vaccinia virus Ankara (MVA) (ATCC VR-1508)
Period of analysis	23/03/2020 – 14/04/2020
End of testing	14/04/2020

6. Methods

6.1 Preparation of test virus suspension

For preparation of test virus suspension *BHK 21-cells* were cultivated with MEM 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 the test product solution

Bacoban WB was used as 1.0 % solution. This solution was prepared with water of standardiser hardness immediately before coating of the carriers.

6.3 Preparation and coating of ceramic tiles

The ceramic tiles were provided by the sponsor. These were carriers with a diameter of 2 cm, which had been briefly immersed in 100 % isopropanol before coating. After removal, they were wiped off with a cloth and placed in square 8-well plates for air drying (4 carriers per plate in the inner 4 wells). The coating was then carried out with 50 µL Bacoban WB (1.0 % solution), the surface disinfectant being distributed to the edge by swirling, until they were visible dry. The untreated carriers for the control assays were also placed in square 8-well plates. The storage time in the covered plates at room temperature was 10 days until used in the experiments.

6.4 Preparation of agar slurry

0.3 g Agar-Agar were solved in 100 ml of a 0.85 % saline solution by stirring on a heat plate. Afterwards, the agar slurry was autoclaved (121 °C, 15 min) and cooled down to 37 – 45 °C.

6.5 Preparation of the virus inoculum

Four volumes of test virus suspension were mixed with one volume of interfering substance solution and five volumes of the agar slurry (see 6.4).

6.6 Inactivation assays and controls

Tests were carried out at room temperature (21.0 ± 2.0 °C). For each exposure time three ceramic tiles treated with Bacoban WB (non-treated ceramic tiles as controls) were prepared. The inactivation experiments were run in one assay.

Two of the prepared carriers (treated with Bacoban WB or untreated controls) are placed in the inner wells of a 6-well plate. The outer wells are filled with Aqua bidest (moist chamber). The carriers are each inoculated with 100 µl virus inoculum and incubated in the closed plate for the respective exposure time. At the end of the respective exposure time, 2 ml ice-cold cell culture medium (without FCS) are removed from the prepared eluate container with 9.9 ml cell culture medium and added to the carrier. The carrier is then rinsed 14 times with 1 ml of the medium and then the complete eluate is returned to the eluate container (total volume 9.9 ml). Directly after elution, series of ten-fold dilutions of the eluate in ice-cold maintenance medium were prepared and inoculated on cell culture.

The non-treated ceramic tiles were processed as described above. Three test pieces were incubated for each respective exposure time.

To determine the initial virus titre a virus input control (VIC) at the beginning and the end of testing were included. Therefore, 100 µl of the virus inoculum were added to 9.9 ml cell culture.

Determination of cytotoxicity was performed as follows: Ceramic tiles treated with Bacoban WB as described above were inoculated with a mixture of 40 µl cell culture medium, 10 µl interfering substance and 50 µl agar slurry instead of the virus inoculum. This assay was incubated for the longest exposure time (30 minutes) in a moist chamber at room temperature and afterwards eluted as described above. The cytotoxicity control is needed for definition of the lower detection limit.

In addition, a control of efficiency for suppression of disinfectant's activity was included. Therefore, 4.95 ml of the undiluted eluate from the cytotoxicity controle (see above) were mixed with 50 µl virus inoculum and incubated on ice for 30 minutes. Afterwards this assay was diluted, and the infectivity was determined. The result is compared with the mean of the virus input control and the difference should be ≤ 0.5 (based on EN 14476 (4)).

Furthermore, a cell control (only addition of medium) was incorporated.

6.7 Determination of infectivity

Infectivity was determined by means of end point dilution method by transferring 0.1 ml of each dilution into eight wells of a microtitre plate with 0.1 ml of freshly trypsinized *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 after six days by using an inverted microscope. Calculation of the infective dose $TCID_{50}$/ml was calculated with the method of Spearman (2) and Kärber (3).

6.8 Calculation of virucidal activity

The virucidal activity of Bacoban WB as coating on ceramic tiles was evaluated by calculating the difference in the logarithmic virus titres between treated ond non-treated ceramic tiles after inoculation and incubation, giving the reduction factor.

7. Verification of the methodology

The following criteria were fulfilled:

a) The mean virus titre of the virus input controls (VIC) at the beginning (6.50 ± 0.35) and the end of testing (6.25 ± 0.44) has a similarity of > 90 %.
b) The recovery of the virus on the non-treated carriers immediately after addition of the virus inoculum (VCt5, 6,25 ± 0.25) is ≥ 80 % compared to the mean VIC (6.38 ± 0.28).
c) The initial virus titre allows a significant reduction in virus titre.
d) The cytotoxicity of the test sample did not influence cell morphology or growth in no way that a significant reduction of virus titre could not be shown.
e) The difference of the virus titres of the control for suppression of disinfectant's activity and the VIC (based on EN 14476) is ≤ 0.5 log (6.50 ± 0.46 (test sample) versus 6.38 ± 0.28 (mean VIC)).

Since all criteria were fulfilled, examination with MVA based on ASTM E2180 was valid.

8. Results

The ceramic tiles coated with Bacoban WB were examined for 5, 15 and 30 minutes at room temperature. The results are shown in table 1.

The mean virus titres on the ceramic tiles coated with a 1.0 % solution of Bacoban WB ten days before the inactivation tests were ≤ 4.00 ± 0.87 $\log_{10}$ $TCID_{50}$/ml after 5 minutes, ≤ 3.96 ± 0.52 $\log_{10}$ $TCID_{50}$/ml after 15 minutes and ≤ 3.54 ± 0.14 $\log_{10}$ $TCID_{50}$/ml after 30 minutes. The reduction factors were therefore ≥ 2.25 ± 0.90 after 5 minutes, ≥ 2.21 ± 0.74 after 15 minutes and ≥ 2.58 ± 0.29 after 30 minutes.

9. Conclusion

The ceramic tiles coated with Bacoban WB were able to demonstrate a significant ($P < 0,01$) log_{10} reduction of MVA after an exposure time of 5, 15 and 30 minutes.

Bremen, 14/04/2020

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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 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. Standard Test Method for Determining the Activity of Incorporated Antimicrobial Agent(s) In Polymeric or Hydrophobic Materials ; ASTM E2180-18.

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 of ceramic tiles treated wit a 1.0 % solution of Bacoban WB and untreated ceramic tiles as controls against MVA

Table 1: Results of ceramic tiles treated wit a 1.0 % solution of Bacoban WB and untreated ceramic tiles as controls against MVA (#6487)

	Conc.	Exposure time	Interfering substance in virus inoculum	Cytotoxicity log_{10} CD_{50}/ml	log_{10} $TCID_{50}$/ml before drying		log_{10} $TCID_{50}$/ml after drying					reduction	
							Carrier 1	Carrier 2	Carrier 3	MV	2xSD	RF	95 % CI
VIC (virus inoculum)	n.a.	n.a.	clean	n.a.	6.50	6.25	n.a.	n.a.	n.a.	**6.38**	**n.d.**	**n.a.**	**n.a.**
VC	n.a.	5 min	clean	n.a.	n.a.	n.a.	6.13	6.25	6.38	**6.25**	**0.25**	**0.13**	**n.a.**
VC	n.a.	15 min	clean	n.a.	n.a.	n.a.	6.25	5.88	6.38	**6.17**	**0.52**	**0.21**	**n.a.**
VC	n.a.	30 min	clean	n.a.	n.a.	n.a.	6.13	6.00	6.25	**6.13**	**0.25**	**0.25**	**n.a.**
Bacoban WB	1,0%	5 min	clean	3.50	n.a.	n.a.	≤ 3.75	≤ 4.50	≤ 3.75	**≤ 4.00**	**0.87**	**≥ 2.25**	**0.90**
Bacoban WB	1,0%	15 min	clean	3.50	n.a.	n.a.	≤ 4.25	≤ 3.88	≤ 3.75	**≤ 3.96**	**0.52**	**≥ 2.21**	**0.74**
Bacoban WB	1,0%	30 min	clean	3.50	n.a.	n.a.	≤ 3.50	≤ 3.63	≤ 3.50	**≤ 3.54**	**0.14**	**≥ 2.58**	**0.29**

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

VIC = virus input control (virus control)
MV = mean value
SD = standard deviation

VC = virus control
CI = confidence

RF = reduction factor