Final report submitted to

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Evaluation of the effectiveness of

KÄRCHER RM 735 Desinfektionsmittel

against

vaccinia virus

in
a quantitative suspension test

Test method according to the guideline of BGA and DVV

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Test report # S05185

1. Identification of test laboratory

MikroLab GmbH, Norderoog 2, D-28259 Bremen

2. Identification of sample

Sponsor	ALFRED KÄRCHER GMBH & Co. KG
Name of product	Kärcher RM 735 Desinfektionsmittel
Application	surface disinfection
Lot no.	023038
Expiry date	-
Active substance(s) and concentration(s) in 100 g	6.66 g alkylbenzyldiammonium chloride 3.33 g dialkyldimethylammonium chloride
Appearance and smell	clear, colourless liquid product specific;
pH-value(s)	undiluted: 6.76 (20°C) 7.5 %: 6.28 (20°C)
Conditions of storage	room temperature in the dark (area with limited access)
Date of receipt at laboratory	2005-03-21

3. Materials

3.1 Culture medium and reagents

- Eagle`s Minimum Essential Medium with Earle`s BSS (EMEM, Cambrex Bio Science Verviers s.p.r.l., catalogue no. 12-125F)
- fetal calf serum (Biochrom AG, article no. S 0115)
- formaldehyde (Riedel-de-Häen, article no. 33220)
- Aqua bidest. (Fresenius Kabi Deutschland, article no. P2N 1636071)
- PBS (Invitrogen, article no. 18912-014)

3.2 Virus and cells

Vaccinia virus strain Elstree originated from the Institute of Medical Virology and Immunology of the University of Essen, D-45122 Essen. Before inactivation assays, virus had been passaged 10 times in *GMK AH-1 cells* (green monkey kidney cell line), three times in *HeLa cells* and five times in *Vero cells*.

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.

3.3 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 volume automatic pipettes (Eppendorf AG)

4. Experimental conditions

Test temperature	20°C ± 1.0°C
Concentration of test product	1.0 %, 3.0 % and 7.5 %
Contact times	1, 5, 15, 30 and 60 minutes
Interfering substance	0.2 % bovine serum albumin 10 % fetal calf serum (FCS)
Procedure to stop action of disinfectant	immediate dilution
Diluent	Aqua bidest.
Virus strain	vaccinia virus strain Elstree
Date of testing	2005-04-18 – 2005-08-25

5. Methods

5.1 Preparation of virus suspension

For preparation of virus suspension, *Vero cells* (ATCC CC81; permanent monkey kidney cells) were cultivated with Eagle's Minimum Essential Medium (EMEM, Cambrex Bio Science Verviers s.p.r.l., B-4800 Verviers, Belgium) and 10 % or 2 % fetal calf serum (FCS, Biochrom AG, D-12247 Berlin, Germany).

Vero cells were infected with a multiplicity of infection of 0.1. After cells showed a cytopathic effect, they were treated with ultrasound (HD 2200, Bandelin electronic GmbH & Co. KG, D-

12207 Berlin) followed by a low speed centrifugation (10 min and 1000 x g) in order to sediment cell debris. After aliquotation, test virus suspension was stored at –80°C.

5.2 Inactivation assays and controls

Tests were carried out in accordance with the BGA and DVV guideline (1). Eight parts by volume of the disinfectant (1.25x of the desired concentration) were mixed with one part by volume of virus suspension and one part by volume of Aqua bidest. In tests with interfering substance, instead of Aqua bidest., one part by volume of a 2.0% solution of BSA or one part by volume of fetal calf serum was added. Immediately at the end of the chosen exposure time, activity of the disinfectant was stopped by serial dilutions.

Due to a more convenient handling and due to a limited amount of test virus suspension, the volumes in the inactivation assay were 0.1 mL test virus suspension, 0.1 mL interfering substance and 0.8 mL test product.

Virus controls were incorporated after the longest exposure time. One part by volume of test virus suspension solution was mixed with nine parts by volume of Aqua bidest. or with one part by volume of FCS and BSA, respectively and eight parts by volume of Aqua bidest.

A control was one part by volume of test virus suspension, four parts by volume of PBS (0.1M, pH value 7.0) and five parts by volume of 1.4 % formaldehyde solution. 5, 15, 30 und 60 minutes were chosen as contact times.

For determination of cytotoxicity of the disinfectant, two parts by volume of Aqua bidest. were mixed with eight parts by volume of the disinfectant, diluted with ice-cold EMEM and inoculated onto permissive cells. Values are given as $\log_{10}CD_{50}$/mL (in analogy to $\log_{10}TCID_{50}$/mL).

Inactivation tests were carried out in sealed test tubes (Sarstedt AG & Co., D-51588 Nümbrecht, Germany) in a water bath at 20°C ± 1.0°C. Aliquots were retained after appropriate exposure times, and the residual infectivity was determined.

A control of efficiency for suppression of disinfectant activity was not included since at the end of the exposure time dilutions were done immediately.
Furthermore, a cell control was incorporated.

5.3 Determination of infectivity

Infectivity was determined by means of end point dilution titration in a micro-procedure. For this, samples were diluted with ice-cold EMEM and 100 µL of each dilution were placed in 8 wells of a sterile polystyrene flat bottomed microtitre plate (Nunc A/S, DK-4000 Roskilde, Denmark). 100 µL of a fresh trypsinized Vero cells were added. Suspension was adjusted to reach approximately 10-15 x 10^3 cells per well. Incubation was at 37°C in a CO_2-atmosphere (5.0 % CO_2 - content). Finally, cultures were observed for cytopathic effects for ten days of inoculation. The infective dose ($TCID_{50}$) was calculated according to 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 positive determinations of lowest dilution step with 100% positive and all higher positive dilution steps

n = number of determinations for each dilution step.

5.4 Calculation and verification of virucidal activity

The virucidal activity of the test disinfectant was evaluated by calculating the decrease in titre in comparison with the control titration without disinfectant (virus control). The difference is given as reduction factor (RF).

According to the guideline of BGA and DVV, a disinfectant or a disinfectant solution at a particular concentration is having virus-inactivating efficacy if within the recommended exposure period the titre is reduced at least by four $\log_{10}$ steps within the recommended exposure period.

6. Results

6.1 Determination of cytotoxicity

In parallel with the inactivation tests, the cytotoxicity of Kärcher RM 735 Desinfektionsmittel (1.0 %, 3.0 % and 7.5 %) and 0.7 % formaldehyde was measured. The formaldehyde

solution was toxic for the cells in the 1:1000 dilutions. This corresponded to a $\log_{10}CD_{50}$/mL of 4.50 (Table 1).
Examinations also showed that the surface disinfectant Kärcher RM 735 Desinfektionsmittel (1.0 %, 3.0 % and 7.5 %) had a $\log_{10}CD_{50}$/mL of 3.50 (cytotoxicity in the 1:100 dilutions) (Tables 1 to 3).

These tests to measure cytotoxicity are imperative, because in this manner the lower detection threshold for non-inactivated vaccinia virus could be determined.

6.2 Virus-inactivating properties of formaldehyde control

Results of inactivation tests are found in table 4. Formaldehyde (0.7 %) reduced the vaccinia virus titre after 5 and 15 minutes by ≥ 2.75 and ≥ 3.38 $\log_{10}$ steps. After 30 and 60 minutes reduction factors of ≥ 3.38 were measured (Table 4).

6.3 Virus-inactivating properties of disinfectant

Results of inactivation assays are demonstrated in tables 4 to 6.
The surface disinfectant Kärcher RM 735 Desinfektionsmittel was examined as 1.0 %, 3.0 % and 7.5 % solutions. Exposure times were 1, 5, 15 and 30 minutes.

Kärcher RM 735 Desinfektionsmittel was active against vaccinia virus as 1.0 % solution in the assay without interfering substance and with 0.2% BSA after 15 minutes exposure time. The reduction factors were ≥ 4.38 (assay without soil load) and ≥ 4.38 (assays with BSA).
In the presence of FCS the concentration was 3.0 % and the exposure time 15 minutes.

Finally, Kärcher RM 735 Desinfektionsmittel as 7.5 % solution was chosen for testing the disinfectant with short exposure times. Table 6 shows that in all assays the surface disinfectant was able to reduce the virus titre by ≥ four $\log_{10}$ steps after 1 minute.

Due to the lack of virological guidelines simulating practical conditions in Europe (phase 2, step 2 tests) the data of this quantitative suspension test lead to the recommendation to use the surface disinfectant Kärcher RM 735 Desinfektionsmittel for inactivation of vaccinia virus as follows:

without soil load	**1.0 %**	**15 minutes**
with soil load	**3.0 %**	**15 minutes**
with soil load	**7.5 %**	**1 minute**

- Dr. J. Steinmann -

7. 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 (BGBl. I, 1994, page 1703). Appendix revised at 14. 05. 1997 (BGBl. 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 were additionally confirmed by controls incorporated in the inactivation assays.

8. Recorders to be maintained

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

9. Literature

1. Richtlinien des Bundesgesundheitsamtes und der Deutschen Vereinigung zur Bekämpfung der Viruskrankheiten e.V. zur Prüfung von chemischen Desinfektionsmitteln auf Wirksamkeit gegen Viren.
Bundesgesundheitsblatt 25, 1982, 397-398

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

Table 1: cytotoxicity of KÄRCHER RM 735 Desinfektionsmittel (1.0%) before and after treatment with MicroSpin™ S-400 HR columns

before treatment	conc.	soil load	dilutions 10^{-1}	10^{-2}	10^{-3}	10^{-4}
test product	1.0%	Aqua bid.	+	+	-	-
test product	1.0%	0.2% BSA	+	+	-	-
test product	1.0%	10.0% FKS	+	+	-	-
formaldehyde	0.7%	PBS	+	+	+	n.d.
after treatment	**conc.**	**soil load**	**dilutions 10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**
test product	1.0%	Aqua bid.	n.d.	n.d.	n.d.	n.d.
test product	1.0%	0.2% BSA	n.d.	n.d.	n.d.	n.d.
test product	1.0%	10.0% FKS	n.d.	n.d.	n.d.	n.d.
formaldehyde	0.7%	PBS	n.d.	n.d.	n.d.	n.d.

n.d. = not done

Table 2: cytotoxicity of KÄRCHER RM 735 Desinfektionsmittel (3.0%) before and after treatment with MicroSpin™ S-400 HR columns

before treatment	conc.	soil load	dilutions			
			10^{-1}	10^{-2}	10^{-3}	10^{-4}
test product	3.0%	Aqua bidest.	+	+	-	-
test product	3.0%	0.2% BSA	+	+	-	-
test product	3.0%	10.0% FKS	+	+	-	-
formaldehyde	0.7%	PBS	+	+	+	n.d.
after treatment	**conc.**	**soil load**	**dilutions**			
			10^{-1}	10^{-2}	10^{-3}	10^{-4}
test product	3.0%	Aqua bidest.	n.d.	n.d.	n.d.	n.d.
test product	3.0%	0.2% BSA	n.d.	n.d.	n.d.	n.d.
test product	3.0%	10.0% FKS	n.d.	n.d.	n.d.	n.d.
formaldehyde	0.7%	PBS	n.d.	n.d.	n.d.	n.d.

n.d. = not done

Table 3: cytotoxicity of KÄRCHER RM 735 Desinfektionsmittel (7.5%) before and after treatment with MicroSpin™ S-400 HR columns

before treatment	conc.	soil load	dilutions 10^{-1}	10^{-2}	10^{-3}	10^{-4}
test product	7.5%	Aqua bidest.	+	+	-	-
test product	7.5%	0.2% BSA	+	+	-	-
test product	7.5%	10.0% FKS	+	+	-	-
formaldehyde	0.7%	PBS	+	+	+	n.d.
after treatment	**conc.**	**soil load**	**dilutions 10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**
test product	7.5%	Aqua bidest.	n.d.	n.d.	n.d.	n.d.
test product	7.5%	0.2% BSA	n.d.	n.d.	n.d.	n.d.
test product	7.5%	10.0% FKS	n.d.	n.d.	n.d.	n.d.
formaldehyde	0.7%	PBS	n.d.	n.d.	n.d.	n.d.

n.d. = not done

Table 4: inactivation of vaccinia virus by KÄRCHER RM 735 Desinfektionsmittel (1.0%) in a quantitative suspension test at 20°C

product	conc.	soil load	$log_{10}TCID_{50}$/mL after 15 min.	30 min.	60 min.	120 min.	≥ 4 log_{10} reduction after
test product	1.0%	Aqua bidest.	≤ 3.50	≤ 3.50	≤ 3.50	n.d.	15 min.
test product	1.0%	0.2% BSA	≤ 3.50	≤ 3.50	≤ 3.50	n.d.	15 min.
test product	1.0%	10.0% FKS	6.63	6.63	6.13	n.d.	> 60 min.
controls	**conc.**	**soil load**	**$log_{10}TCID_{50}$/mL after 5 min.**	**15 min.**	**30 min.**	**60 min.**	**≥ 4 log_{10} reduction after**
formaldehyde	0.7%	PBS	≤ 5.13	≤ 4.50	≤ 4.50	≤ 4.50	≥ 15 min
virus control	n.a.	Aqua bidest.	n.d.	n.d.	n.d.	7.88	n.a.
virus control	n.a.	0.2% BSA	n.d.	n.d.	n.d.	7.88	n.a.
virus control	n.a.	10.0% FKS	n.d.	n.d.	n.d.	7.88	n.a.

n.d. = not done

n.a. = not applicable

Table 5: inactivation of vaccinia virus by KÄRCHER RM 735 Desinfektionsmittel (3.0%) in a quantitative suspension test at 20°C

product	conc.	soil load	$log_{10}TCID_{50}$/mL after 15 min.	30 min.	60 min.	120 min.	≥ 4 log_{10} reduction after
test product	3.0%	Aqua bidest.	n.d.	n.d.	n.d.	n.d.	n.d.
test product	3.0%	0.2% BSA	n.d.	n.d.	n.d.	n.d.	n.d.
test product	3.0%	10.0% FKS	≤ 3.50	≤ 3.50	≤ 3.50	n.d.	15 min.
controls	**conc.**	**soil load**	**5 min.**	**15 min.**	**30 min.**	**60 min.**	**≥ 4 log_{10} reduction after**
formaldehyde	0.7%	PBS	≤ 5.13	≤ 4.50	≤ 4.50	≤ 4.50	≥ 15 min
virus control	n.a.	Aqua bidest.	n.d.	n.d.	n.d.	7.88	n.a.
virus control	n.a.	0.2% BSA	n.d.	n.d.	n.d.	7.88	n.a.
virus control	n.a.	10.0% FKS	n.d.	n.d.	n.d.	7.88	n.a.

n.d. = not done

n.a. = not applicable

Table 6: inactivation of vaccinia virus by KÄRCHER RM 735 Desinfektionsmittel (7.5%) in a quantitative suspension test at 20°C

product	conc.	soil load	$log_{10}TCID_{50}$/mL after 1 min.	5 min.	10 min.	15 min.	≥ 4 log_{10} reduction after
test product	7.5%	without	≤ 3.50	≤ 3.50	n.d.	n.d.	1 min.
test product	7.5%	0.2% BSA	≤ 3.50	≤ 3.50	n.d.	n.d.	1 min.
test product	7.5%	10.0% FKS	≤ 3.50	≤ 3.50	n.d.	n.d.	1 min.
controls	**conc.**	**soil load**	$log_{10}TCID_{50}$/mL after 5 min.	15 min.	30 min.	60 min.	≥ 4 log_{10} reduction after
formaldehyde	0.7%	PBS	≤ 5.13	≤ 4.50	≤ 4.50	≤ 4.50	≥ 15 min
virus control	n.a.	without	n.d.	n.d.	n.d.	7.88	n.a.
virus control	n.a.	0.2% BSA	n.d.	n.d.	n.d.	7.88	n.a.
virus control	n.a.	10.0% FKS	n.d.	n.d.	n.d.	7.88	n.a.

n.d. = not done

n.a. = not applicable