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Final report submitted to

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Evaluation of the effectiveness of

CHEMISEPT G

against Vaccinia virus strain Elstree

Test method according to guideline of BGA and DVV

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1. Introduction

As requested, the hand disinfectant CHEMISEPT G of CHEMI – PHARM AS was tested for its virucidal properties against vaccinia virus strain Elstree. Investigations were carried out in accordance with the guideline on testing chemical disinfectants for effectiveness against viruses published by the Federal Office of Health (Bundesgesundheitsamt, BGA now Robert Koch-Institute, Berlin) and the German Association for the Control of Virus Diseases (Deutsche Vereinigung zur Bekämpfung der Viruskrankheiten e. V., DVV) (1,2).

2. Identification of test laboratory

MikroLab GmbH, Norderoog 2, D-28259 Bremen

3. Identification of sample

Name of the product	CHEMISEPT G
Manufacturer	CHEMI – PHARM AS
Lot no.	-
Appearance and smell of the product	clear, colourless solution, product specific
pH-value	undiluted: 6.68 (20°C)
Date of receipt at laboratory	2005-12-05
Conditions of storage	room temperature in the dark (area with restricted access)
Active substance(s) and concentration(s)	ethanol 75 g; blend of N-alkylbenzyldimethyl-ammonium chloride and N-alkyldimetyl-ammonium chloride 0.1 g

4. Experimental conditions

Date of examinations	2005-12-06 – 2006-02-18
Test temperature	20°C ± 1°C
Diluent of product	80.0%
Contact times	0.5, 1.0, 2.0 and 5.0 minutes
Interfering substances	2.0% solution of bovine serum albumin (BSA) fetal calf serum (FCS)
Diluent	Aqua bidest.
Procedure to stop action of disinfectant	immediate dilution
Test virus	vaccinia virus strain Elstree

5. Material and methods

5.1. Preparation of test virus suspension

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) and three times in HeLa cells.

For preparation of virus suspension, Vero cells (ATCC CC81; permanent monkey kidney cells) were cultivated with Dulbecco`s Modified Eagle`s Medium (DMEM, 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 tests

Tests were carried out in accordance to BGA and DVV guideline. Eight parts by volume of the disinfectant were mixed with one part by volume of virus suspension and one part by volume of Aqua bidest. In tests with interfering substances, instead of double distilled water, one part by volume of fetal calf serum or of a 2% serum albumin solution (bovine serum albumin, BSA, Cohn fraction V, Sigma-Aldrich Chemie GmbH, D-82018 Taufkirchen, Germany) was added.

Inactivation tests were carried out in sealed glass test-tubes in a water bath at 20°C ± 1°C. Aliquots were removed after appropriate times, and residual infectivity was determined.
A control was one part by volume of virus suspension, four parts by volume of PBS and five parts by volume of 1.4% formaldehyde. The concentration of formaldehyde was determined by the hydroxylammonium chloride method.

In addition, in accordance with the guideline, virus controls were carried out.

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 DMEM 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 cell culture 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 five days of inoculation. Infective dose ($TCID_{50}$/mL) was calculated according to the method of Spearman (3) and Kärber (4) 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.

5.4. Determination of cytotoxicity

For determination of cytotoxicity of the disinfectant, two parts by volume of PBS were mixed with eight parts by volume of the disinfectant, diluted with ice-cold DMEM and a suspension of Vero cells were added as described above.

5.5. Calculation of virucidal effect

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

6. Results

In parallel with the inactivation tests, cytotoxicity of the 0.7% formaldehyde solution and of the hand disinfectant was measured (Table 1). The formaldehyde solution was toxic for the Vero cells in the 1:100 dilutions. This corresponded to a $\log_{10}CD_{50}$/mL of 3.50. Examinations showed that the hand disinfectant (80.0%) also had a $\log_{10}CD_{50}$/mL of 3.50 (cytotoxicity in the 1:100 dilutions).
These tests to measure the cytotoxicity are imperative, because in this way the lower detection threshold for non-inactivated vaccinia virus is determined.
Formaldehyde (0.7%) reduced the vaccinia virus titre after 5 and 15 minutes by 1.12 and 1.50 $\log_{10}$ steps. Reduction factors of 1.75 and 2.75 were measured after 30 and 60 minutes contact time (table 2).

Results of inactivation tests are found in table 2. CHEMISEPT G was tested undiluted. Due to the addition of virus suspension and interfering substances a test concentration of 80.0% resulted. The exposure times were 0.5, 1.0, 2.0 and 5.0 minutes.

The hand disinfectant CHEMISEPT G exhibited a strong virucidal efficacy against the test virus. After an exposure time of 30 seconds no vaccinia virus was detectable any longer. The virus titres were reduced by ≥ 4.25 (assay without interfering substances), ≥ 4.00 (assay with BSA) and ≥ 4.25 $\log_{10}$-steps (assay with FCS). This corresponds in all cases to an inactivation of ≥ 99.99%. According to the guideline of BGA/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.

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 hand disinfectant CHEMISEPT G for inactivation of vaccinia virus as follows:

undiluted **30 s**

Bremen, 2006-02-18

- Dr. J. Steinmann -

Literature

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

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3. Spearman, C.: The method of ´right or wrong cases` (constant stimuli) without Gauss`s formulae.
Brit J Psychol 1908; 2: 227-242

4. Kärber, G.: Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche.
Arch Exp Path Pharmak 1931; 162: 480-487

Literature

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Table 1: Cytotoxicity of Chemisept G (80.0%) and 0.7% formaldehyde before and after treatment with MicroSpin™ S-400 HR columns.

before treatment	conc.	soil load	dilutions				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
product	80.0%	without	+	+	-	-	-
product	80.0%	0.2% BSA	+	+	-	-	-
product	80.0%	10.0% FCS	+	+	-	-	-
formaldehyde	0.7%	without	+	+	-	-	-
after treatment	**conc.**	**soil load**	**dilutions**				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
product	80.0%	without	n.d.	n.d.	n.d.	n.d.	n.d.
product	80.0%	0.2% BSA	n.d.	n.d.	n.d.	n.d.	n.d.
product	80.0%	10.0% FCS	n.d.	n.d.	n.d.	n.d.	n.d.
formaldehyde	0.7%	without	n.d.	n.d.	n.d.	n.d.	n.d.

n.d = not done

Table 2: inactivation of vaccinia virus by Chemisept G (80.0%) and formaldehyde (0.7%) in quantitative suspension test at 20°C.

product	conc.	soil load	virus titre ($log_{10}TCID_{50}$/mL) 0.5 min.	1.0 min.	2.0 min.	5.0 min.	≥ 4 log_{10} reduction after
test product	80.0%	without	≤ 3.50	≤ 3.50	≤ 3.50	≤ 3.50	0.5 min.
test product	80.0%	0.2% BSA	≤ 3.50	≤ 3.50	≤ 3.50	≤ 3.50	0.5 min.
test product	80.0%	10.0% FCS	≤ 3.50	≤ 3.50	≤ 3.50	≤ 3.50	0.5 min.
controls	**conc.**	**soil load**	**RF 5 min.**	**15 min.**	**30 min.**	**60 min.**	**≥ 4 log_{10} reduction after**
formaldehyde	0.7%	without	6.63	6.25	6.00	5.00	≥ 60 min.
virus control	n.a.	without	n.d.	n.d.	n.d.	7.75	n.a.
virus control	n.a.	0.2% BSA	n.d.	n.d.	n.d.	7.50	n.a.
virus control	n.a.	10.0% FCS	n.d.	n.d.	n.d.	7.75	n.a.

n.d. = not done

n.a. = not applicable

Appendix table 1: raw data (vaccinia virus) of Chemisept G (BGA/DVV)

product	concentration	interfering substances	exposure time (sec)	dilutions (log_{10})								
				1	2	3	4	5	6	7	8	9
Chemisept G	80.0%	Aqua bidest.	30	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			60	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			120	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			300	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
		0.2% BSA	30	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			60	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			120	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			300	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
		10.0% FCS	30	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			60	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			120	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			300	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
Chemisept G cytotoxicity	80.0%	PBS	n.a.	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.
		0.2% BSA	n.a.	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.
		10.0% FCS	n.a.	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.
virus control	n.a.	Aqua bidest.	n.a.	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4344 4444	0000 0420	0000 0000	0000 0000
		0.2% BSA	n.a.	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	1443 3324	0000 0000	0000 0000	0000 0000
		10.0% FCS	n.a.	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4443 2333	0000 2003	0000 0000	0000 0000

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

t = cytotoxic

0 = no virus detectable
1 to 4 = detection of virus (degree of CPE in 8 wells of a microtitre plate)

Appendix Table 2: raw data (vaccinia virus) of formaldehyde control (20°C)

product	concentration	interfering substance	exposure time (min)	dilutions (log_{10})								
				1	2	3	4	5	6	7	8	9
formaldehyde	0.7% (m/V)	PBS	5	tttt tttt	tttt tttt	4444 4444	4444 4444	3323 3233	0000 0001	0000 0000	0000 0000	n.d.
			15	tttt tttt	tttt tttt	4444 4444	4333 2333	3220 0112	0000 0000	0000 0000	0000 0000	n.d.
			30	tttt tttt	tttt tttt	4444 4444	1122 3223	0012 1002	0000 0000	0000 0000	0000 0000	n.d.
			60	tttt tttt	tttt tttt	4444 4444	0022 1100	0000 0000	0000 0000	0000 0000	0000 0000	n.d.
formaldehyde cytotoxicity	0.7% (m/V)	PBS	n.a.	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.

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

t = cytotoxic

0 = no virus detectable
1 to 4 = detection of virus (degree of CPE in 8 wells of a microtitre plate)