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Evaluation of the effectiveness of

CHEMISEPT G

against Bovine Viral Diarrhea Virus (Surrogate of Hepatitis C Virus)

Test method according to guideline of BGA and DVV

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1. Introduction

Examinations of virucidal activity of chemical disinfectants against hepatitis C virus (HCV) are not possible, since there are no in vitro tests available. Therefore, bovine viral diarrhea virus (BVDV) serves as a model virus, because this pestivirus also belongs to the family Flaviviridae and some of its properties are similar to HCV (1,2). BVDV is a small ssRNA virus with a non-segmented positive strand and it is frequently used in blood product industry as a surrogate of HCV.

This study was 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, Germany) and the German Association for the Control of Viruses Diseases (Deutsche Vereinigung zur Bekämpfung der Viruskrankheiten e.V., DVV) (3,4).

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.	
Application	hand disinfection
Appearance and smell of product	clear, colourless solution, product specific
pH-value	undiluted: 6.68 (20°C)
Expiry date	-
Date of receipt at laboratory	2005-12-05
Conditions of storage	room temperature in the dark (area with limited 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

Period of analysis	2005-12-02 – 2005-12-30
Test temperature	20°C ± 1°C
Concentration of test product	80.0%
Contact times	30, 60 and 120 s
Interfering substances	2.0% solution of bovine serum albumin (BSA); fetal calf serum (FCS)
Diluent of product	-
Procedure to stop action of disinfectant	gel filtration
Test virus	BVDV strain NADL

5. Material and methods

5.1 Preparation of virus suspension

BVDV strain NADL (VR-534) was obtained from Dr. Stephanie Bendtfeld, Institute of Virology at the School of Veterinary Medicine Hannover (Tierärztliche Hochschule, D-30559 Hannover). Prior to inactivation assays, the virus was passaged 5 times in KOP-R (primary cells from bovine oropharyngeal tissue) and once in primary bovine kidney cells. KOP-R cells originated from the Bundesforschungsanstalt für Viruskrankheiten der Tiere, isle of Riems (Dr. R. Riebe, catalog No. RIE 244).

For the preparation of the virus suspension, KOP-R cells, which were cultivated with Eagle's Minimum Essential Medium (EMEM; Cambrex Bio Science Verviers s.p.r.l., B-4800 Verviers, Belgium) supplemented with L-glutamine, sodium pyruvate and 10% or 2% fetal calf serum (FCS, Biochrom AG, D-12247 Berlin, Germany; antibodies against BVDV not detectable), were infected with BVDV. As soon as cells showed a constant cytopathic effect, they were subjected to a rapid freeze/thawing procedure. This was followed by 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 with the 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 double distilled water. In tests with interfering substances, instead of

double distilled water, one part by volume of a 2.0% solution of bovine serum albumin (BSA, Sigma-Aldrich Chemie GmbH, D-82018 Taufkirchen) or one part by volume of fetal calf serum was added.

Inactivation tests were carried out in sealed test tubes (Sarstedt AG & Co., D-51588 Nümbrecht, Germany) in a water bath at 20°C ± 1°C. Aliquots were retained after appropriate exposure times, and the 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 hydroxyl-ammonium chloride method.

To reduce cytotoxicity, immediately at the end of the exposure time the mixture was added to a MicroSpin™ S-400 HR column (see 5.5 elimination of cytotoxicity) and centrifuged.

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

5.3 Determination of infectivity

Infectivity was determined by 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 plate (Nunc A/S, DK-4000 Roskilde, Denmark). 100 µL of a freshly trypsinized KOP-R cell culture (passage 28th-35 th) were added. The 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. The infective dose ($TCID_{50}$) was calculated according to the method of Spearman (5) and Kärber (6) 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 EMEM and inoculated into the cell culture. Values are given as $\log_{10}CD_{50}$/mL (in analogy to $\log_{10}TCID_{50}$/mL). These

tests were also performed with the interfering substances and with MicroSpin™ S-400 HR columns.

5.5 Elimination of cytotoxicity

Since the cytotoxicity did not allow following the reduction of residual infectivity titre over the range of four log_{10} steps, ready to use MicroSpin™ S-400 HR columns (Amersham Biosciences Europe GmbH, D-79021 Freiburg, Germany) were used according to the instructions of the manufacturer with addition of a 0.5 % BSA solution before loading.

5.6 Calculation of the 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. The difference is given as reduction factor (RF).

6. Results

In parallel with the inactivation tests, the cytotoxicity of the hand disinfectant CHEMISEPT G (80.0%) and 0.7% formaldehyde was measured. The formaldehyde solution was toxic for the KOP-R cells in the 1:1000 dilutions. This corresponds to a $log_{10}CD_{50}$/mL of 4.50 (Table 1).
Examinations also showed that without treatment the hand disinfectant CHEMISEPT G (80.0%) had a $log_{10}CD_{50}$/mL of 2.50 (cytotoxicity in the 1:10 dilution), where-as no cytotoxic effect after treatment with the columns was measured resulting in a $log_{10}CD_{50}$/mL of ≤ 1.50 (Table 1).
These tests to measure cytotoxicity are imperative, because in this way the lower detection threshold for non-inactivated BVDV is determined.
Virus titres without treatment with MicroSpin™ S-400 HR columns were 5.50 (assay without protein load), 5.88 (assay with BSA) and 5.75 $log_{10}TCID_{50}$/mL (assay with FCS) (data not shown in table).

Results of inactivation tests are found in table 2. Formaldehyde (0.7%) reduced the BVDV titre after 5 minutes by ≥ 0.25 log_{10} steps. After 15, 30 and 60 minutes reduction factors were ≥ 1.25 (Table 2).

The hand disinfectant CHEMISEPT G was examined undiluted. Due to the addition of virus suspension and interfering substances a test concentration of 80.0% resulted. Exposure times were 30, 60 and 120 seconds.

Testing CHEMISEPT G undiluted, an efficacy was measured after an exposure time of 30 s (Table 2). At this time, no BVDV was detectable any longer. The reduction factors were ≥ 4.25 (assay without soil load), ≥ 4.25 (assay with BSA) and ≥ 4.38 (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 BVDV (surrogate of hepatitis C virus) as follows:

undiluted **30 s**

Dr. J. Steinmann

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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	-	-	-	-	-
product	80.0%	0.2% BSA	-	-	-	-	-
product	80.0%	10.0% FCS	-	-	-	-	-
formaldehyde	0.7%	without	n.d.	n.d.	n.d.	n.d.	n.d.

n.d = not done

Table 2: Inactivation of BVDV by CHEMISEPT G (80.0%) und formaldehyde (0.7%) in a quantitative suspension test at 20°C ± 1°C after treatment with MicroSpinTM S-400 HR columns (Virus titres of formaldehyde controls without treatment).

product	conc.	soil load	$log_{10}TCID_{50}$/mL after				≥ 4 log_{10} reduction after
			30 s	60 s	120 s	240 s	
test product	80.0%	without	≤ 1.50	≤ 1.50	≤ 1.50	n.d.	30 s
test product	80.0%	0.2% BSA	≤ 1.50	≤ 1.50	≤ 1.50	n.d.	30 s
test product	80.0%	10.0% FCS	≤ 1.50	≤ 1.50	≤ 1.50	n.d.	30 s
			5 min	**15 min**	**30 min**	**60 min**	
formaldehyde	0.7%	without	≤ 5.50	≤ 4.50	≤ 4.50	≤ 4.50	≥ 15 min
virus control	n.a.	without	n.d.	n.d.	n.d.	5.75	n.a.
virus control	n.a.	0.2% BSA	n.d.	n.d.	n.d.	5.75	n.a.
virus control	n.a.	10.0% FCS	n.d.	n.d.	n.d.	5.88	n.a.

n.d. = not done

n.a. = not applicable

Appendix table 1: raw data (BVDV) of CHEMISEPT G (BGA/DVV) after treatment with MicroSpin S-400 HR columns

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	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			60	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			120	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			240	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		0.2% BSA	30	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			60	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			120	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			240	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		10.0% FCS	30	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			60	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			120	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			240	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Chemisept G cytotoxicity	80.0%	PBS	n.a.	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.
		0.2% BSA	n.a.	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.
		10.0% FCS	n.a.	0000 0000	0000 0000	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 4344	0400 0300	0000 0000	0000 0000	0000 0000	0000 0000
		0.2% BSA	n.a.	4444 4444	4444 4444	4444 4444	4444 4444	0000 2300	0000 0000	0000 0000	0000 0000	0000 0000
		10.0% FCS	n.a.	4444 4444	4444 4444	4444 4444	4444 4444	0003 2200	0000 0000	0000 0000	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 (BVDV) 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	tttt tttt	2200 2343	2000 0300	0000 0000	0000 0000	0000 0000	n.d.
			15	tttt tttt	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.
			30	tttt tttt	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.
			60	tttt tttt	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.
formaldehyde cytotoxicity	0.7% (m/V)	PBS	n.a.	tttt tttt	tttt tttt	tttt tttt	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)