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TEST REPORT

09.07.2014

Test report Z14ML1726BC

Evaluation of the effectiveness of

Zoono Z-71 Microbe Shield

Test virus:	bovine corona virus (BCV) (as surrogate of other members of coronaviridae family including MERS-CoV)
Method:	following EN 14476:2013
	quantitative suspension test for the evaluation of virucidal activity of chemical disinfectants and antiseptics used in human medicine

Sponsor:
Zoono Group of Companies
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1. Identification of test laboratory

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

2. Identification of sample

Manufacturer	Zoono Group of Companies
Name of product	Zoono Z-71 Microbe Shield (Form. 90023-00)
Product diluent recommended by the manufacturer	-
Batch number	30947-A
Application	surface disinfection
Production date	-
Expiry date	05/2017
Active compound (s) (100 g)	-
Appearance, odour	clear, colorless liquid product specific
pH-values (in WSH)	undiluted: 4.38 (20 °C) 50.0 %: 4.46 (20 °C)
Storage conditions	20 °C in the dark (area with restricted access)
Date of arrival in the laboratory	10.06.2014

3. Materials

3.1 Culture medium and reagents

- Dulbecco`s Modified Eagle`s Medium (DMEM, Biozym Scientific GmbH, catalogue no. 880021)
- fetal calf serum (Biochrom AG, article no. S 0115)
- 1.4 % formaldehyde solution
- Aqua bidest. (Fresenius Kabi Deutschland, article no. P2N 1636071)
- PBS (Invitrogen, article no. 18912-014)
- BSA (Sigma-Aldrich-Chemie GmbH, article no. CA-2153)
- Trypsin (SERVA Electrophoresis GmbH, article no. 37290)

3.2 Virus and cells

The BCV strain L9 was obtained by Dr. G. Zimmer, Institute of Virology at the School of Veterinary Medicine Hannover (Tierärztliche Hochschule, D-30559 Hannover).

The *U373 cells* (passage 12) were as well obtained by Dr. G. Zimmer, Institute of Virology at the School of Veterinary Medicine Hannover (Tierärztliche Hochschule, D-30559 Hannover).

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 and fixed-volume pipettes (Eppendorf AG)
- Polysterol 96-well microtitre plate (Nunc GmbH & Co. KG, Wiesbaden, Germany)
- Cell culture flask (Nunc GmbH & Co. KG, Wiesbaden, Germany)
- Sealed test tubes (Sarstedt AG & Co., Nümbrecht, Germany).

4. Experimental conditions

Test temperature	20 °C ± 0.5 °C
Concentration of test product	undiluted (80.0 %) and as 50.0 % and 10.0 % (non-active range) solutions
Appearance of product dilutions	no precipitation
Contact times	1, 5, 10 and 30 minutes
Interfering substance	0.3 g/l bovine serum albumin (BSA, clean conditions EN 14476:2013)
Stability of product in the mix with virus and interfering substance	no flocculation
Procedure to stop action of disinfectant	immediate dilution
Diluent	water
Virus strain	bovine corona virus strain L9
Date of testing	10.06.2014 – 09.07.2014
End of testing	09.07.2014

5. Methods

5.1 Preparation of test virus suspension

For preparation of test virus solution, *U373* cells were cultivated in a 75 cm^2 flask with in EMEM supplemented with L-glutamine, non-essential amino acids and sodium pyruvate and 10 % fetal calf serum. Before virus infection, cells were washed three times with phosphate buffered saline (PBS), incubated for 3 h with EMEM without FCS and were washed once with EMEM supplemented with trypsin. For virus production, BCV strain L9 was added to the prepared monolayer. After an incubation period of 24 to 48 hours cells were lysed by a rapid freeze/thaw cycle. Cellular debris was removed by low speed centrifugation and the supernatant was directly used as the test virus suspension.

5.2 Preparation of disinfectant (dilutions)

The test product was evaluated undiluted. Due to the addition of test virus suspension and interfering substance an 80.0 % solution resulted.

Furthermore, the test product was evaluated as 50.0 % and 10.0 % solutions (demonstration of non-active range). These solutions were prepared with water immediately before the inactivation tests.

5.3 Infectivity assay

Infectivity was determined by means of end point dilution titration using the microtitre process. For this, samples were immediately diluted at the end of the exposure time with ice-cold EMEM with trypsin and 100 µl of each dilution were placed in eight wells of a sterile polystyrene flat bottomed plate with a preformed *U373* monolayer. Before addition of virus, cells were washed once with EMEM and incubated for 3 h with 100 µl EMEM with trypsin. Incubation was at 37 °C in a CO_2-atmosphere (5.0 % CO_2 - content). Finally, cultures were observed for cytopathic effects for six days of inoculation. The infectious 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 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 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. The difference is given as reduction factor (RF).

According to the EN 14476:2013, a disinfectant or a disinfectant solution at a particular concentration is having virus-inactivating efficacy if the titre is reduced at least by four $\log_{10}$ steps within the recommended exposure period. This corresponds to an inactivation of ≥ 99.99 %.

5.5 Inactivation assay

Determination of virucidal activity has been carried out in accordance to EN 5.5. The test product was examined with undiluted (80.0 %) and as 50.0 % and 10.0 % (demonstration of non-active range) solutions in water at 20 °C following EN 14476:2013. 1, 5, 10 and 30 minutes were chosen as contact times.

Immediately at the end of a chosen contact time, activity of the disinfectant was stopped by dilution to 10^{-8}.

Titrations of the virus control were performed after the longest exposure time (EN 5.5.7).

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

Inactivation tests were carried out in sealed test tubes in a water bath at 20 °C ± 1.0 °C. Aliquots were retained after appropriate exposure times and residual infectivity was determined.

5.6 Determination of cytotoxicity

Determination of cytotoxicity was performed according to EN 5.5.4.1.

5.7 Cell sensitivity to virus

For the control of cell sensitivity to virus two parts by volume water were mixed with eight parts by volume of the lowest apparently non-cytotoxic dilution of the product. This mixture or PBS as control was added to a volume of double concentrated cell suspension. After 1 h at 37 °C the cells were centrifuged and re-suspended in cell culture medium (EN 5.5.4.2b).

Finally, a comparative titration of the test virus suspension was performed on the pre-treated (disinfectant) and non-pre-treated (PBS) cells as described above.

5.8 Control of efficacy for suppression of disinfectant's activity

Furthermore, a control of efficiency for suppression of disinfectant's activity was included (EN 5.5.5).

5.9 Reference virus inactivation test

As reference for test validation a 0.7 % formaldehyde solution according to EN 5.5.6 was included. 5, 15, 30 and 60 minutes were chosen as contact times. In addition, cytotoxicity of formaldehyde test solution was determined following EN 5.5.6.2 with dilutions up to 10^{-5}.

6. Verification of the methodology

The following criteria as mentioned in EN 5.7 were fulfilled:

a) The titre of the test virus suspension allowed the determination of a ≥ 4 log_{10} reduction (maximal virus reduction ≥ 4.25 ± 0.29).

b) The test product (undiluted) showed cytotoxicity in the 1:100 dilutions thus allowing the detection of a four log_{10} reduction of virus titre.

c) The comparative titration on pre-treated (disinfectant) and non-pre-treated (PBS) *BGM cells* showed no significant difference (< 1 log_{10}; EN 5.7) of virus titre: 6.50 ± 0.00 (PBS) versus 5.75 ± 0.33 (1:1,000 dilutions of disinfectant, 80.0 %) log_{10} $TCID_{50}$/ml.

d) The control of efficacy for suppression of disinfectant's activity (80.0 %) showed no decrease in virus titre (7.88 ± 0.37 versus 8.38 ± 0.41).

Since all criteria according EN 5.7 were fulfilled, examination with bovine corona virus following EN 14476:2013 is valid.

7. Results

Results of examination are shown in tables 1 to 7. Tables 1 to 6 demonstrate the raw data, whereas table 7 (a+b) gives a summary of results.

The test product undiluted (80.0 %) was able to inactivate bovine corona virus after one minute in this quantitative suspension test. The reduction factor was ≥ 4.25 ± 0.29 (Table 1).

Furthermore, the test product as 50.0 % solution was able to inactivate inactivate bovine coronavirus after 5 minutes in this quantitative suspension test. The reduction factor was ≥ 4.25 ± 0.29 (Table 2). This corresponded to an inactivation of ≥ 99.99 %.

Tested as 10.0 % solution an activity was found after 30 minutes (shorter times not tested) (Table 3).

8. Conclusion

The surface disinfectant Zoono Z-71 Microbe Shield tested undiluted (80.0 %) demonstrated effectiveness against inactivate bovine corona after an exposure time of one minute under clean conditions.

Therefore, the surface disinfectant Zoono Z-71 Microbe Shield can be declared as active against bovine corona virus as surrogate of other members of coronaviridae family including MERS-CoV as follows:

undiluted 1 minute

Bremen, 09.07.2014

- Dr. Jochen Steinmann
Scientific Director

9. 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.

10. Records 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 Dr. Brill + Partner GmbH.

11. Literature

1. EN 14476:2013: 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)

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

Appendix:

Legend to the Tables

Table 1: Raw data for Zoono Z-71 Microbe Shield (80.0 %) tested against bovine corona virus

Table 2: Raw data for Zoono Z-71 Microbe Shield (50.0 %) tested against bovine corona virus

Table 3: Raw data for Zoono Z-71 Microbe Shield (10.0 %) tested against bovine corona virus

Table 4: Raw data for formaldehyde solution (0.7 %) tested against bovine corona virus

Table 5: Raw data for control of efficacy for suppression of disinfectant's activity

Table 6: Raw data (bovine corona virus) for cell sensitivity

Table 7: Summary of results with Zoono Z-71 Microbe Shield and bovine corona virus

Legend to the Figures

Figure 1: Virus-inactivating properties of Zoono Z-71 Microbe Shield (80.0 %)

Figure 2: Virus-inactivating properties of formaldehyde (0.7 %)

Table 1: Raw data for Zoono Z-71 Microbe Shield (80.0 %) tested against bovine corona virus at 20 °C (quantal test; 8 wells) (3582)

Product	Concentration	Interfering substance	Contact time (min)	Dilutions (log_{10})								
				1	2	3	4	5	6	7	8	9
test product	80.0 %	clean conditions	1	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.
			2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			5	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.
			10	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.
test product cytotoxicity	80.0 %	clean conditions	n.a.	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.
virus control	n.a.	clean conditions	0	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4404 0434	0000 0000	0000 0000
			60	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	3444 4444	0040 0004	0000 0000	0000 0000

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

0 = no virus present; t = cytotoxic
1 to 4 = virus present (degree of CPE in 8 cell culture units) (wells of microtitre plates)

Table 2: Raw data for Zoono Z-71 Microbe Shield (50.0 %) tested against bovine corona virus at 20 °C (quantal test; 8 wells) (3582)

Product	Concentration	Interfering substance	Contact time (min)	Dilutions (log_{10})								
				1	2	3	4	5	6	7	8	9
test product	50.0 %	clean conditions	1	tttt tttt	tttt tttt	2000 3000	4000 0040	0000 0000	0000 0000	0000 0000	0000 0000	n.d.
			2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			5	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.
			10	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.
test product cytotoxicity	50.0 %	clean conditions	n.a.	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.
virus control	n.a.	clean conditions	0	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4404 0434	0000 0000	0000 0000
			60	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	3444 4444	0040 0004	0000 0000	0000 0000

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

0 = no virus present; t = cytotoxic
1 to 4 = virus present (degree of CPE in 8 cell culture units) (wells of microtitre plates)

Table 3: Raw data for Zoono Z-71 Microbe Shield (10.0 %) tested against bovine corona virus at 20 °C (quantal test; 8 wells) (3582)

Product	Concentration	Interfering substance	Contact time (min)	Dilutions (log_{10})								
				1	2	3	4	5	6	7	8	9
test product	10.0 %	clean conditions	1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			30	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.
test product cytotoxicity	10.0 %	clean conditions	n.a.	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.
virus control	n.a.	clean conditions	0	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4404 0434	0000 0000	0000 0000
			60	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	3444 4444	0040 0004	0000 0000	0000 0000

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

0 = no virus present; t = cytotoxic
1 to 4 = virus present (degree of CPE in 8 cell culture units) (wells of microtitre plates)

Table 4: Raw data for formaldehyde solution (0.7 %) tested against bovine corona virus at 20 °C (quantal test; 8 wells) (3582)

Product	Concentration	Interfering substance	Contact 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	0000 3330	0000 0000	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.
virus control	n.a.	PBS	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			60	4444 4444	4444 4444	4444 4444	4444 4444	4404 4444	0040 0400	0000 0000	0000 0000	0000 0000

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

0 = no virus present; t = cytotoxic
1 to 4 = virus present (degree of CPE in 8 cell culture units) (wells of microtitre plates)

Table 5: Raw data for control of efficacy for suppression of disinfectant's activity (3582)

Product	Interfering substance	dilutions (log_{10})								
		1	2	3	4	5	6	7	8	9
test product	PBS	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
test product	clean conditions	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	0004 4400	0000 0000	n.d.
test product	dirty conditions	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

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

0 = no virus present; t = cytotoxic
1 to 4 = virus present (degree of CPE in 8 cell culture units) (wells of microtitre plates)

Table 6: Raw data (bovine corona virus) for cell sensitivity (3582)

Product	Dilution	Dilutions (log_{10})								
		1	2	3	4	5	6	7	8	9
PBS	-	4444 4444	4444 4444	4444 4444	4444 4444	4443 4444	0000 0000	0000 0000	0000 0000	n.d.
test product	1:10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
test product	1:100	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
test product	1:1,000	4444 4444	4444 4444	4444 4444	2331 1222	0010 0010	0000 0000	0000 0000	0000 0000	n.d.

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

0 = no virus present; t = cytotoxic
1 to 4 = virus present (degree of CPE in 8 cell culture units) (wells of microtitre plates)

Table 7a: Summary of results with Zoono Z-71 Microbe Shield and bovine corona virus

Product	Con-centration	Interfering substance	Level of cytotoxicity	log_{10} $TCID_{50}$/ml after ....min					> 4 log_{10} reduction after ... min
				1	2	5	10	30	
test product	80.0 %	clean	3.50	≤3.50±0.00	n.d.	≤3.50±0.00	≤3.50±0.00	n.d.	1 (RF ≥4.25±0.29)
test product	50.0 %	clean	3.50	≤4.00±0.46	n.d.	≤3.50±0.00	≤3.50±0.00	n.d.	5 (RF ≥4.25±0.29)
test product	10.0 %	clean	3.50	n.d.	n.d.	n.d.	n.d.	≤3.50±0.00	30 (RF ≥4.25±0.29)

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

Table 8b: Summary of results with Zoono Z-71 Microbe Shield and bovine corona virus

Product	Con-centration	Interfering substance	Level of cytotoxicity	log_{10} $TCID_{50}$/ml after ....min					> 4 log_{10} reduction after ... min
				0	5	15	30	60	
formaldehyde	0.7% (w/v)	PBS	4.50	n.d.	≤4.88±0.37	≤4.50±0.00	≤4.50±0.00	≤4.50±0.00	≥ 15 (≥2.13±0.41)
virus contr.	n.a.	PBS	n.a.	n.d.	n.d.	n.d.	n.d.	6.63±0.41	n.a.
virus contr.	n.a.	clean	n.a.	n.d.	n.d.	n.d.	n.d.	8.38±0.41	n.a.
virus contr.	n.a.	clean	n.a.	8.25±0.33	n.d.	n.d.	n.d.	7.75±0.33	n.a.
suppression control	80.0 %	clean	3.50	n.d.	n.d.	n.d.	7.88±0.37	n.d.	n.a.
sens.control PBS	n.a.	clean	n.a.	n.d.	n.d.	n.d.	n.d.	6.50±0.00	n.a.
sens. control test product	n.a.	clean	n.a.	n.d.	n.d.	n.d.	n.d.	5.75±0.33	n.a.

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

Figure 1: Virus-inactivating properties of Zoono Z-71 Microbe Shield (80.0 %)

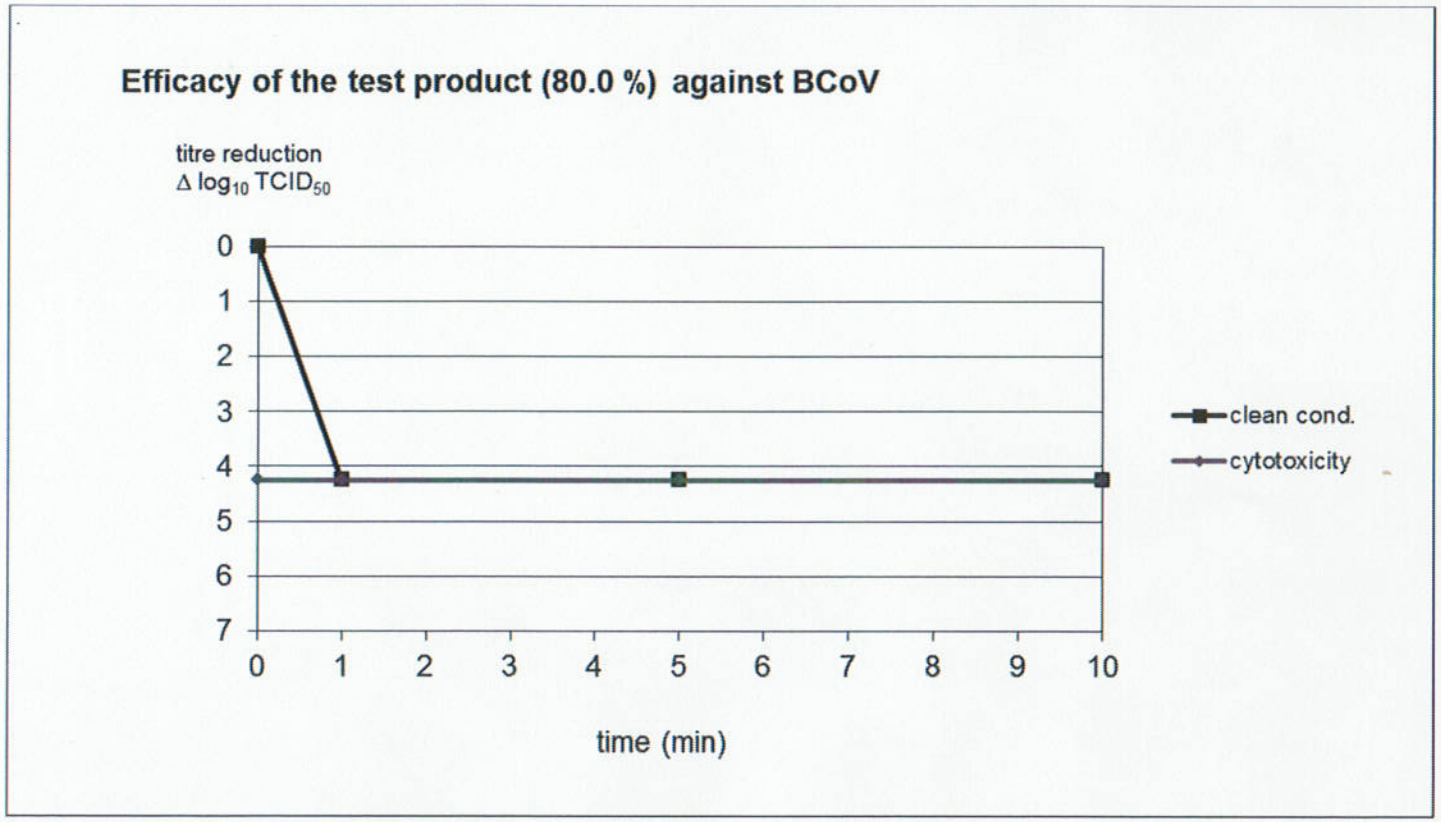

Figure 2: Virus-inactivating properties of formaldehyde (0.7 %)