DR. BRILL + DR. STEINMANN
INSTITUTE FOR HYGIENE AND MICROBIOLOGY

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14/12/2018

Test report L18/0869R.1

Evaluation of the effectiveness of

Chemisept med

Test virus:	human rotavirus strain Wa
Method:	based on EN 14476:2013+A1:2015 (dirty conditions) quantitative suspension test for the evaluation of virucidal activity of chemical disinfectants and antiseptics used in human medicine

Sponsor:
Chemi-Pharm AS
Pollu 132
EST – TALLINN 10917

TEST REPORT

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Location: Moldova

1. Identification of test laboratory

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

2. Identification of sample

Manufacturer	Chemi-Pharm AS
Name of product	Chemisept med
Confirmation no.	207507
Product diluent recommended by the manufacturer	-
Batch number	196291118
Application	hand disinfection
Production date	29/11/2018
Expiry date	29/11/2021
Active compound (s) (100 g)	72.5 g ethanol 7.5 g propan-2-ol
Appearance, odour	clear, colorless liquid product specific
pH-values	undiluted: 4.74 (20 °C)
Storage conditions	room temperature in the dark (area with restricted access)
Date of arrival in the laboratory	30/11/2018

3. Materials

3.1 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)
- 1.4 % formaldehyde solution (dilution of Roti®-Histofix 4 %, Carl Roth GmbH)
- Aqua bidest. (SG ultrapure water system, type Ultra Clear; serial no. 86996-1)
- PBS (Invitrogen, article no. 18912-014)
- BSA (Sigma-Aldrich-Chemie GmbH, article no. CA-2153)
- sheep erythrocytes (Fiebig Nährstofftechnik)

- Trypsin (SERVA Electrophoresis GmbH, article no. 37290).

3.2 Virus and cells

The human rotavirus strain Wa (serotype 1, subgroup II) was obtained by Prof. Dr. Holger Rabenau, Institute of Medical Virology of the Johann Wolfgang Goethe University of Frankfurt, DE - 60596 Frankfurt. Before the described tests, the virus had been passaged in *MA-104 cells* (embryonic rhesus monkey kidney cell line).

The cells (passage 48) 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)
- Cell culture flask (Nunc GmbH & Co. KG, Wiesbaden)
- Sealed test tubes (Sarstedt AG & Co., Nümbrecht).

4. Experimental conditions

Test temperature	20 °C ± 1.0 °C
Concentration of test product	undiluted (80.0 %) and as 50.0 % and 10.0 % (demonstration of non-active range) solutions
Appearance of product dilutions	no precipitation
Contact times	15 seconds and 30 minutes
Interfering substance	3.0 g/l bovine serum albumin + 3.0 ml/l erythrocytes (dirty conditions, EN 14476)
Procedure to stop action of disinfectant	immediate dilution
Diluent	Aqua bidest.
Stability of product in the mix with virus and interfering substance (80.0 % solution)	minor clouding, medium precipitation
Virus strain	human rotavirus strain Wa
Date of testing	30/11/2018 – 14/12/2018
End of testing	14/12/2018

5. Methods

5.1 Preparation of test virus suspension

After washing with serum-free Eagle`s Minimum Essential Medium twice, cells were incubated with EMEM without fetal calf serum for three hours to eliminate all FCS. This was followed by the addition of virus (stock virus suspension) to *MA-104 cells* in the presence of trypsin for two hours ± 10 minutes at 37 °C. After this time, medium with trypsin was added. If 90 % of the cells showed a cytopathic effect, cells were subjected to a rapid two-fold freeze-thawing procedure followed by a centrifugation at 1.620 g for 30 minutes at 4 °C in order to sediment cell debris. After aliquotation the supernatant was stored as test virus suspension at –80 °C.

5.2 Preparation of disinfectant (dilutions)

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

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

5.3 Infectivity assay

Infectivity was determined by means of end point dilution titration in a micro-procedure. For this, samples were serially diluted with ice-cold EMEM with trypsin and 100 µl of each dilution were placed after aspiration of the medium in eight wells of a sterile polystyrene flat bottom 96-well microtitre plate with a preformed *MA-104* monolayer. After one hour at 37 °C, 100 µl EMEM with trypsin were added. Incubation took place 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 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 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, 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 undiluted (80.0 %) and as 50.0 % and 10.0 % (demonstration of non-active range) solutions in Aqua bidest. at 20 °C based on EN 14476. 15 seconds 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 at the beginning of the test and after the longest exposure time (EN 5.5.7). One part by volume of test virus suspension was mixed with one part interfering substance and eight parts by volume of WSH or Aqua bidest. (RTU products).

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 hard 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 the wells of the microtitre plates with a preformed monolayer of *MA-104-cells*.

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 based on 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 $\geq 4.75 \pm 0.31$).

b) The test product showed cytotoxicity in the 1:10 dilutions (80.0 %) thus allowing the detection of a 4 $\log_{10}$ reduction of virus titre.

c) The comparative titration on pre-treated (disinfectant) and non-pre-treated (PBS) *MA-104 cells* showed no significant difference (< 1 $\log_{10}$; EN 5.7) of virus titre: 7.38 ± 0.25 (PBS) versus 7.13 ± 0.45 (1:100 dilution of disinfectant as 80.0 % solution) $\log_{10}$ $TCID_{50}$/ml.

d) The control of efficacy for suppression of disinfectant's activity (80.0 % solution) showed no decrease (≤ 0.5 $\log_{10}$; EN 5.5.5.1) in virus titre (7.63 ± 0.25 versus 7.25 ± 0.44 $\log_{10}$ $TCID_{50}$/ml).

e) One concentration demonstrated a 4 $\log_{10}$ reduction and (at least) one concentration demonstrated a $\log_{10}$ reduction of less than 4.

Since all criteria according EN 5.7 were fulfilled, examination with human rotavirus based on EN 14476 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 undiluted test product (80.0 %) was able to inactivate human rotavirus after 15 seconds of exposure time in this quantitative suspension test (table 1). The reduction factor was $\geq 4.75 \pm 0.31$ at this time point. This corresponded to an inactivation of ≥ 99.99 %.

The test product as 50.0 % solution was not able to inactivate human rotavirus within 15 seconds of exposure time in this quantitative suspension test (table 2).

The test product as 10.0 % solution was also not able to inactivate human rotavirus within 30 minutes of exposure time in this quantitative suspension test (table 3).

8. Conclusion

The hand disinfectant Chemisept med tested undiluted demonstrated effectiveness against human rotavirus after an exposure time of 15 seconds under dirty conditions.

Therefore, the hand disinfectant Chemisept med can be declared as active against human rotavirus as follows:

undiluted **15 seconds** **dirty conditions**

Bremen, 14/12/2018

- Dr. Britta Becker -
Head of Laboratory

- Dr. Dajana Paulmann -
Scientific Project Manager

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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 Dr. Brill + Partner GmbH and the sponsor will be stored in the archives at Dr. Brill + Partner GmbH.

11. Literature

1. EN 14476:2013+A1:2015: 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 Chemisept med (80.0 %) tested against human rotavirus
Table 2:	Raw data for Chemisept med (50.0 %) tested against human rotavirus
Table 3:	Raw data for Chemisept med (10.0 %) tested against human rotavirus
Table 4:	Raw data for formaldehyde solution (0.7 %) tested against human rotavirus
Table 5:	Raw data for control of efficacy for suppression of disinfectant activity (80.0 %)
Table 6:	Raw data (human rotavirus) for cell sensitivity (80.0 %)
Table 7 (a+b):	Summary of results with Chemisept med and human rotavirus

Legend to the Figures

Figure 1:	Virus-inactivating properties of Chemisept med (80.0 %)
Figure 2:	Virus-inactivating properties of formaldehyde (0.7 %)

Table 1: Raw data for Chemisept med (80.0 %) tested against human rotavirus at 20 °C (quantal test; 8 wells) (#5817)

Product	Concentration	Interfering substance	Contact time (min)	Dilutions (log_{10})								
				1	2	3	4	5	6	7	8	9
test product	80.0 %	dirty conditions	0.25	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.
			0.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			30	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
test product cytotoxicity	80.0 %	dirty conditions	n.a.	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.
virus control	n.a.	dirty conditions	0	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4444 4442	0000 0203	0000 0000	0000 0000
			60	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	3440 0404	0003 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 2: Raw data for Chemisept med (50.0 %) tested against human rotavirus at 20 °C (quantal test; 8 wells) (#5817)

Product	Concentration	Interfering substance	Contact time (min)	Dilutions (log_{10})								
				1	2	3	4	5	6	7	8	9
test product	50.0 %	dirty conditions	0.25	n.d.	4444 4444	4444 4444	4344 0344	0000 4000	0000 4000	0000 0000	n.d.	n.d.
			0.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			30	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
test product cytotoxicity	50.0 %	dirty conditions	n.a.	tttt tttt	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.
virus control	n.a.	dirty conditions	0	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4444 4442	0000 0203	0000 0000	0000 0000
			60	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	3440 0404	0003 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 3: Raw data for Chemisept med (10.0 %) tested against human rotavirus at 20 °C (quantal test; 8 wells) (#5817)

Product	Concentration	Interfering substance	Contact time (min)	Dilutions (log_{10})								
				1	2	3	4	5	6	7	8	9
test product	10.0 %	dirty conditions	0.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			0.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			30	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4040 4444	0040 0000	0000 0000	n.d.
test product cytotoxicity	10.0 %	dirty conditions	n.a.	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.
virus control	n.a.	dirty conditions	0	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4444 4442	0000 0203	0000 0000	0000 0000
			60	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	3440 0404	0003 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 4: Raw data for formaldehyde solution (0.7 %) tested against human rotavirus at 20 °C (quantal test; 8 wells) (#5817)

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	4444 4444	4444 4444	4444 4444	4403 3344	0020 0004	0000 0000	n.d.
			15	tttt tttt	tttt tttt	4444 4444	4444 4444	4444 4444	4030 2314	0000 0000	0000 0000	n.d.
			30	tttt tttt	tttt tttt	4444 4444	3334 4444	0030 4033	0000 0002	0000 0000	0000 0000	n.d.
			60	tttt tttt	tttt tttt	4444 4444	4400 0000	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.
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	4444 4444	4440 4240	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 (80.0 %) (#5817)

Product	Interfering substance	dilutions (log_{10})								
		1	2	3	4	5	6	7	8	9
test product	dirty conditions	tttt tttt	4444 4444	4444 4444	4444 4444	4444 4444	4344 4442	3000 0000	0000 0000	n.d.
corresponding virus control	dirty conditions	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	3440 0404	0003 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 6: Raw data (human rotavirus) for cell sensitivity (80.0 %) (#5817)

Product	Dilution	Dilutions (log_{10})								
		1	2	3	4	5	6	7	8	9
PBS	-	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4433 3404	0000 0000	0000 0000	n.d.
test product	1:100	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4400 4004	0000 0003	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 Chemisept med and human rotavirus

Product	Con-centration	Interfering substance	Level of cytotoxicity	log_{10} $TCID_{50}$/ml after ....min					> 4 log_{10} reduction after … min
				0.25	0.5	2	30	60	
test product	80.0 %	dirty conditions	2.50	≤2.50±0.00	n.d.	n.d.	n.d.	n.d.	0.25 (RF ≥ 4.75±0.31)
test product	50.0 %	dirty conditions	2.50	5.63±0.43	n.d.	n.d.	n.d.	n.d.	> 0.25 (RF = 1.63±0.62)
test product	10.0 %	dirty conditions	1.50	n.d.	n.d.	n.d.	7.38±0.41	n.d.	> 30 (RF = 0.00±0.61)

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

Table 7b: Summary of results with Chemisept med and human rotavirus

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	3.50	n.d.	7.63±0.41	7.25±0.33	6.13±0.45	4.75±0.33	> 60 (RF = 2.50±0.46)
virus control	n.a.	PBS	n.a.	n.d.	n.d.	n.d.	n.d.	7.25±0.33	n.a.
virus control	n.a.	dirty conditions	n.a.	7.75±0.33	n.d.	n.d.	n.d.	7.25±0.44	n.a.
suppression control	80.0 %	dirty conditions	2.50	n.d.	n.d.	n.d.	7.63±0.25	n.d.	n.a.
sens.control PBS	n.a.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	7.38±0.25	n.a.
sens. control test product	80.0 % → 1:100	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	7.13±0.45	n.a.

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

Figure 1: Virus-inactivating properties of Chemisept med (80.0 %)

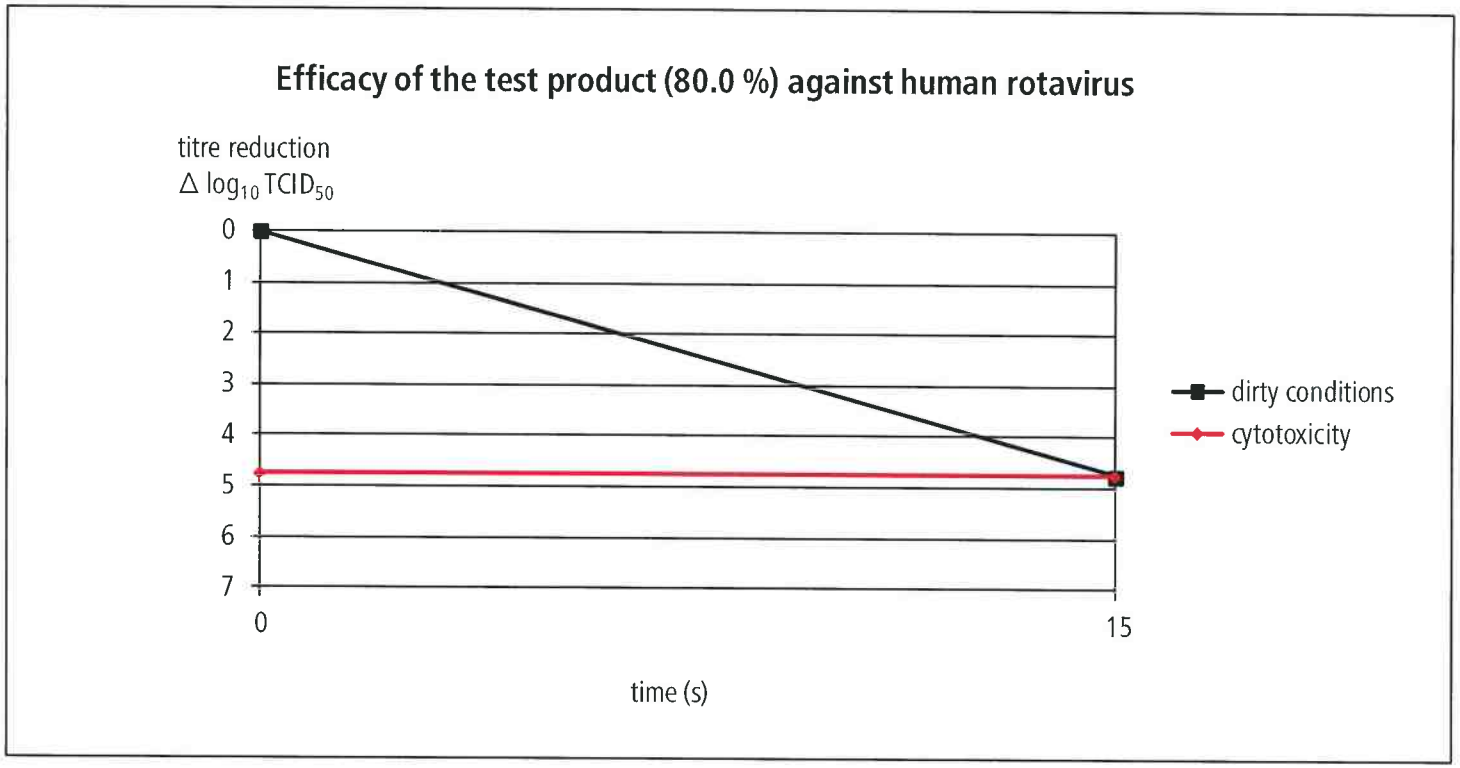

Figure 2: Virus-inactivating properties of formaldehyde (0.7 %)