Final report submitted to

Goldschmidt GmbH
Goldschmidtstrasse 100
D-45127 Essen
(http://www.goldschmidt.com)

Evaluation of the effectiveness of

TEGO 2000

against avian influenza virus A

Project number: G06ML271

Dr. Jochen Steinmann
MikroLab GmbH
Norderoog 2
D-28259 Bremen

phone: +49 (0) 421-27819102
fax: +49 (0) 421-2760283
e-mail: Mikrolab.GmbH@t-online.de
http://www.mikrolab-gmbh.de

2006-02-08

1. Identification of test laboratory

MikroLab GmbH, Norderoog 2, D-28259 Bremen

2. Identification of sample

Name of the product	TEGO 2000
Manufacturer	Goldschmidt GmbH
Lot no.	ES65833107
Appearance and smell of the product	clear, pale yellow liquid, product specific
Expiry time	-
pH-values	undiluted: 8.15 (20°C) 0.75%: 7.56 (20°C)
Date of receipt at laboratory	2006-01-25
Conditions of storage	room temperature in the dark (area with restricted access)
Active substance(s) and concentration(s)	20% amphoterics

3. Experimental conditions

Date of examinations	2006-01-25 – 2006-02-08
Test temperature	20°C ± 1°C
Dilution of product	0.75%
Contact times	5, 10, 15 and 30 minutes
interfering substance	0.03% serum albumin (clean conditions; EN 14476:2005) 0.3% serum albumin and 0.3% erythrocytes (dirty conditions; EN 14476:2005)
Diluent	Aqua bidest.
Procedure to stop action of disinfectant	gel filtration
Test virus	influenza virus A /duck/Ukraine/1/63 (H3N8)

4. Material and methods

4.1. Preparation of test virus suspension

The influenza virus A/duck/Ukraine/1/63 (H3N8) virus was obtained from PD Dr. Timm Harder, reference laboratory of avian influenza and Newcastle Disease, Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, D-17493 Greifswald - Isle of Riems. This virus strain was incorporated as test virus as surrogate of avian influenza virus A (H5N1) due to bio safety reasons. The MDCK cells were obtained from Dr. R. Riebe, cell bank for cell lines in veterinary medicine at the Federal Research Institute for Animal Health, D-17493 Greifswald - Isle of Riems.

To prepare the viral suspension, MDCK cells that had been cultured with Eagle's minimum essential medium (EMEM) and 10% or 2% fetal calf serum (FCS, Biochrom AG, Berlin, Germany) were inoculated with influenza virus A in 175 cm^2 cell culture flasks (Nunc GmbH & Co. KG, Wiesbaden, Germany). Once a cytopathic effect had been induced (approx. 24 hours), freezing and thawing was carried out once. The cell debris was removed by centrifugation at 3.000 rpm for ten minutes (4°C) and the supernatant was recovered as viral suspension (virus stock solution) and stored in aliquots at -80°C.

4.2. Inactivation tests

Tests were carried out in accordance to BGA and DVV guideline (1,2) with interfering substances as mentioned in EN 14476:2005. 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 substances, for clean conditions, 0.03% bovine serum albumin (BSA) fraction V was added, whereas testing dirty conditions 0.3% BSA and 0.3% washed sheep erythrocytes were included (EN 14476:2005; 5.2.3.3).
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.

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 removed after appropriate exposure times, and residual infectivity was determined. To reduce cytotoxicity, immediately at

the end of the exposure time the mixture was added to a MicroSpin™ S-400 HR column (see 4.5 reduction of cytotoxicity) and centrifuged.
In addition, in accordance with the guideline, virus controls were carried out.

4.3. Determination of infectivity

Infectivity was determined by means of end point dilution method using the microtitre process. For this, 100 µl aliquots of the samples, which had been serially diluted with ice-cold EMEM, were transferred to eight cups of a sterile polystyrol 96-well microtitre plate with a flat bottom (Nunc GmbH & Co. KG, Wiesbaden, Germany). Already on the previous day 100 µl aliquots of a freshly trypsinized MDCK cells (approx. 2.7×10^4 cells) had been placed in each well (preformed monolayer). Incubation took place at 37°C in a CO_2 incubator (5% CO_2 content) for five days. Finally, cultures were observed for cytopathic effects with a reversed microscope and the infective dose $TCID_{50}$/mL was calculated with the method of Kärber (3) and Spearman (4) with the following formula:

$$\log_{10}TCID_{50} = - (X_0 - 0.5 + \Sigma\ r/n)$$

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.

4.4. Determination of cytotoxicity

For the determination of the cytotoxicity, two volume parts PBS were mixed with eight volume parts of the disinfectant and, following serial dilution as outlined in 4.3, transferred to the 96-well microtitre plate with the preformed monolayer. The cytotoxic dose was calculated as $\log_{10}CD_{50}$ /mL (cytotoxic dose; analogous to $TCID_{50}$ value).

4.5 Reduction 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.

4.6. Calculation of virucidal efficacy

Virucidal efficacy of the test disinfectant was determined by calculating the titre reduction compared with the respective control titrations containing no disinfectant. Data are given as reduction factor (RF).

5. Results

In parallel with inactivation tests, cytotoxicity of the surface disinfectant TEGO 2000 (0.75%) and 0.7% formaldehyde were measured.
The formaldehyde solution was toxic for the MDCK cells in the 1:1000 dilutions. This corresponds to a $\log_{10}CD_{50}$/mL of 4.50 (Table 1).
Examinations showed that the tested surface disinfectant TEGO 2000 (0.75%) produced a cytotoxic effect at the dilution of 1:100. This means a $\log_{10}CD_{50}$/mL value (analogous to the $TCID_{50}$ value) of 3.50 (Table 1).
After treatment with the sephacryl columns TEGO 2000 (0.75%) showed no cytotoxicity in the 1:10 dilution ($\log_{10}CD_{50}$/mL value = ≤ 1.50).

These tests to measure the cytotoxicity are imperative, because in this way the lower detection threshold for non-inactivated influenza virus is determined.

Virus titres without treatment with MicroSpin™ S-400 HR columns were 5.75 (assay under clean conditions) and 5.88 (assay under dirty conditions) $\log_{10}TCID_{50}$/mL (data not shown in table).

Results of inactivation tests are found in table 2. Formaldehyde (0.70%) reduced the influenza virus titre after 5 minutes by ≥ 2.75 $\log_{10}$ steps. After 15, 30 and 60 minutes reduction factors were identical (≥ 2.75).
TEGO 2000 was tested as 0.75% solution. The exposure times were 5, 10, 15 and 30 minutes. Testing TEGO 2000 as 0.75% solution under clean conditions, after an exposure time of five minutes a reduction of the virus titre was measured (table 2). The reduction factor was 4.25. After 10 minutes no virus could be detected any longer (RF = ≥ 5.25)

Under dirty conditions, the contact time had to be extended to 15 minutes. After that time, the RF was 4.00. This corresponds to an inactivation of ≥ 99.99% meaning virus-inactivating properties.

According to the guideline of BGA/DVV and EN 14476:2005 (5), a disinfectant or a disinfectant solution at a particular concentration is having virus-inactivating properties if within the recommended exposure period the titre is reduced at least by four $\log_{10}$.
In summary, the following concentration and exposure time are recommended for avian influenza virus inactivation under clean and dirty conditions:

0.75% 15 min

Bremen, 2006-02-08

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

2. Kommentar zur 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

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

5. EN 14476:2005: Chemical disinfectants and antiseptics- virucidal quantitative suspension test – Test method and requirements (phase 2, step1)

Table 1: Cytotoxicity of TEGO 2000 (0.75%) 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	0.75%	clean conditions	+	-	-	-	-
product	0.75%	dirty conditions	+	-	-	-	-
formaldehyde	0.7%	without	+	+	+	-	-
after treatment	**conc.**	**soil load**	**dilutions**				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
product	0.75%	clean conditions	-	-	-	-	-
product	0.75%	dirty conditions	-	-	-	-	-
formaldehyde	0.7%	without	n.d.	n.d.	n.d.	n.d.	n.d.

n.d = not done

Table 2: Inactivation of avian influenza virus by TEGO 2000 (0.75%) 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
			5 min	10 min	15 min	30 min	
test product	0.75%	clean conditions	2.50	≤ 1.50	≤ 1.50	n.d.	5 min
test product	0.75%	dirty conditions	4.38	3.25	2.63	≤ 1.50	15 min
			5 min	**15 min**	**30 min**	**60 min**	
formaldehyde	0.70%	without	≤ 4.50	≤ 4.50	≤ 4.50	≤ 4.50	≥ 5 min
virus control	n.a.	clean conditions	n.d.	n.d.	n.d.	6.75	n.a.
virus control	n.a.	dirty conditions	n.d.	n.d.	n.d.	6.63	n.a.

n.d. = not done

n.a. = not applicable

Appendix table 1: raw data (avian influenza virus) of TEGO 2000 (clean/dirty) after treatment with MicroSpin S-400 HR columns

TEGO 2000	0.75%	clean conditions	5.0	4444 4444	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.
			10.0	0000 0030	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.
			15.0	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.
			30.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		dirty conditions	5.0	4444 4444	4444 4444	3443 4440	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.
			10.0	4444 4444	0444 0440	0000 0004	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.
			15.0	4444 4444	0000 0030	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.
			30.0	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.
TEGO 2000 cytotoxicity	0.75%	clean conditions	n.a.	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.
TEGO 2000 cytotoxicity	0.75%	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.	clean conditions	n.a.	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4000 0004	0000 0000	0000 0000	n.d.
		dirty conditions	n.a.	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4000 0000	0000 0000	0000 0000	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)

Appendix table 2: raw data (avian influenza virus) of formaldehyde control (20°C)

formaldehyde	0.7% (m/V)	PBS	5	tttt tttt	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.
			15	tttt tttt	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.
			30	tttt tttt	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	n.d.	n.d.	n.d.
			60	tttt tttt	tttt tttt	tttt tttt	0000 0000	0000 0000	0000 0000	n.d.	n.d.	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)