**Prof. Dr. med. H. W. Doerr**
Medical Microbiologist and Laboratory Physician
Director of the Institute for Medical Virology of the
J. W. Goethe-University Hospital Frankfurt/M.

---

Prof. Dr. med. H. W. Doerr, Paul Ehrlich Str. 40, D-60596 Ffm

BODE Chemie GmbH & Co.
Scientific Affairs
Melanchthonstraße 27

**22525 Hamburg**

Prof. Dr. Holger F. Rabenau
Paul Ehrlich Str. 40
D-60596 Frankfurt a. M.

Telephone:
(069) 6301-5312
(069) 6301-5219 (Office)
Telefax:
(069) 6301-83061

# EXPERT REPORT ON THE STUDY OF THE VIRUCIDAL EFFICACY OF MIKROBAC® FORTE AGAINST THE SARS-ASSOCIATED CORONA VIRUS (SARS-CoV)

| | |
|---|---|
| Sample amount submitted: | Minimum 500 mL |
| Sample name: | VP 355/1P |
| Lot: | 1225 227473 |
| Composition: | 100 g solution contain:<br>19.9 g Benzalkoniumchlorid<br>5.0 g Dodecylbispropylentriamin |
| Start of study: | 15.01.2004 |
| Study sponsor: | PD Dr. Günter Kampf<br>BODE Chemie GmbH & C., Scientific Affairs<br>Melanchthonstraße 27, 22525 Hamburg |
| Contact at<br>BODE Chemie GmbH & Co.: | PD Dr. Günter Kampf |
| Internal Test Code: | Mikrobac forte 28.7.04.doc |
| Person doing the experiments: | Ms. G. Bauer (MTA) (Medical Technician) |
| Investigators: | Prof. Dr. med. H. W. Doerr<br>Prof. Dr. rer. med. H. F. Rabenau<br>Institute for Medical Virology<br>in the Center for Hygiene of the<br>University Hospitals Frankfurt/Main<br>Paul Ehrlich Str. 40<br>60596 Frankfurt am Main |

## Introduction

Coronaviruses are widespread in humans and animals and were thought to be the cause of harmless infections of the respiratory and gastrointestinal tract in humans. In winter 2002/2003, an epidemic of “severe acute respiratory syndrome” (SARS) broke out in South China caused by a new, previously unknown corona virus probably transmitted from certain small animals (viverrids) to humans (Rota et al., Science 300 (5624): 1394-1399, 2003). The new infectious disease soon spread to other parts of the world before it could finally be stopped by strict hygiene measures. As with other respiratory infections, however, there is a risk of re-infection especially in winter. In fact, such cases have already been reported. The main human-to-human route of transmission appears to be via droplet infection, but other transmission routes are possible, although still not clearly defined. For example, it was reported from China that only 50 % of SARS patients had direct contact to other SARS cases (Liang et al. Emerg Infect Dis, 2004). The SARS coronavirus (SARS-CoV) also infects the intestinal tract and is excreted in large amounts for several weeks with the stools. Consequently, serious problems with hospital cross-infections occurred during the SARS epidemic. Testing and development of suitable disinfectants are therefore in the public interest, especially as another new, more pathogenic coronavirus has been discovered in humans (Berger et al., J Lab Med, 28(1), 42-55, 2004; Drosten et al., Trends in Molecular Medicine (9)8, 325-330, 2003; Rabenau et al., J Med Microbiol & Immunol, 2004; Rota et al., Science 300 (5624): 1394-1399, 2003; Seto et al., Lancet 361 (9368): 1519-1520, 2003; van der Hoek et al., Nature Medicine (10) 4, 2004).

BODE Chemie GmbH & Co. offers commercial antiviral disinfectants (including for hand, surface and instrument disinfection) and commissioned us to study the surface disinfectant Mikrobac® forte in a suspension test for its virucidal activity against SARS-CoV. The tests were performed in a cell culture system that had been established in our institute for identification and investigation of the SARS pathogen (Drosten et al., N Engl J Med. 348(20): 1967-1976, 2003; Cinatl et al., Lancet 362 (9390), 1158-1159, 2003, Cinatl et al., Lancet 361 (9374), 2045-2046, 2003). Every test was to be performed both under “clean conditions” (added protein increased by a factor of 10 from 0.03 % to 0.3 %), with addition of 10 % foetal calf serum (FCS), as well as under “dirty conditions” (according to EN).

Table 1: General parameters for the study of the virucidal efficacy of Mikrobac® forte against SARS-associated Coronavirus (SARS-CoV)

| Test substance or control | Concentration | Contact time |
|---|---|---|
| VP 355/1P | 0.5 % | 30 or 60 min. |
| Formalin control | 0.7 % | 10 min. |
| Control titration | | |
| Termination control | | |
| Cytotoxicity control | | |

**Materials and methods**

The general conditions for testing Mikrobac® forte were defined by us in agreement with the sponsoring company, as given in Table 1.

*Test material:*

The following coded test substance was sent to us to test for its anti-SARS-CoV activity:

Surface disinfectant

- VP 355/1P – Lot: 1225 227473

*Infection tests with cell cultures*

The Vero cell line used as the indicator cells was controlled regularly for contamination with mycoplasms and used only if the results were negative.

*Method:* The test was performed in 96-well microtiter plates in triplicate. The test substance was mixed (vortexer) with the virus suspension for a specified time (see below). Immediately after the contact time the test assay was diluted 1:10 (0.5 mL + 4.5 mL) in ice cold MEM and stored on ice (0 – 4 °C) until titration. Then this virus/test substance mixture was diluted serially (1:10 increments) and 50 µL of each dilution step filled into 8 indentations of a column ("wells"). Prior to this, 50 µL of the indicator cell suspension (in growth medium [MEM + 10 % FCS + penicillin/streptomycin (100 U/mL / 100 µg/mL)]) had already been placed in these wells. The whole assay was performed at room temperature (20 – 26 °C). The assay was incubated in a $CO_2$ incubator at 37 °C for 3-4 days before a final microscopic evaluation was performed.

The test setup named above was therefore as follows:

- 1 part virus + 1 part 3 % BSA sol. + 8 parts disinfectant (corresponds to “clean conditions” – added protein increased by a factor of 10 from 0.03 % to 0.3 %)
- 1 part virus + 1 part FCS + 8 parts disinfectant (corresponds to 10 % serum load)
- 1 part virus + 1 part 3 % erythrocyte solution** in 3 % BSA* + 8 parts disinfectant (corresponds to “dirty conditions”)

In detail:

- 300 µL virus + 300 µL 3 % BSA sol. + 2400 µL disinfectant (corresponds to “clean conditions” – added protein increased by a factor of 10 from 0.03 % to 0.3 %)
- 300 µL virus + 300 µL FCS + 2400 µL disinfectant (corresponds to 10 % serum load)
- 300 µL virus + 300 µL 3 % erythrocyte solution in 3 % BSA (corresponds to “dirty conditions”) + 2400 µL disinfectant
- 300 µL MEM + 300 µL FCS + 2400 µL disinfectant (toxicity test, with addition of 10 % serum load)

* 3 % BSA solution: 6 g bovine serum albumen (BSA) in 200 mL PBS, stir and sterilize by filtration.
** 3 % sheep erythrocyte solution: 3 mL erythrocyte sediment in 97 mL 3 % BSA solution (stable for 7 days at 4 °C)

To protect employees and in accordance with the WHO recommendations, special safety precautions (biosafety level (BSL) 3) were taken when doing all work with SARS-CoV.

*Controls run in parallel*

Two positive controls as well as one non-infected cell culture, a “termination control” and an assay with 0.7 % formalin solution were run with every test assay. The cytotoxicity of the test substances was determined in a separate test.

The positive controls were a titration of the “stock virus” used (initial virus) (called “stock virus” in tables 3 and 4) and a titration of the “stock virus” diluted analogously to the test assay 1:10 – without addition of protein (called “control titration” in tables 3 and 4).

The “termination control” was used to determine whether the 1:10 dilution of the test assay prepared immediately after completion of the contact time and stored intermediately on ice until titration had a “virus inactivating effect”. In other words this run served to control whether there was an “after-effect” of the disinfectant on the virus despite the 1:10 dilution.

The “intermediate storage phase” on ice lasted on average 5 and not more than 8 minutes. The virus used for the termination control – analogous to the actual test assay – was likewise pre-diluted 1:10 in the medium and incubated for 5-8 min on ice with the corresponding 1:10 dilution of the test substance. The test was performed without addition of protein (see table 4). A reference assay with formaldehyde (0.7 % formalin) as the disinfectant was performed with every test assay to verify the functionality of the test assay (“formalin control”). The test was performed, like that with the test substances, in triplicate. 200 µL of virus suspension were mixed with 800 µL of PBS and 1 mL of 1.4 % formaldehyde pH 7.0. Used as the starting preparation was approx. 37 % formalin (Sigma Chemie, Deisenhofen). The hydroxylammonium chloride method was used for the quantitative analysis of the formaldehyde concentration in the starting solution used (according to EU Standard: TC 216 WI 00216022).

The cytotoxicity control was used to determine the extent to which the test substances had a cytotoxic effect and therefore might interfere with the cell (indicator) system. These controls were performed analogously to the tests with the test substances – as a single assay with 10 % FCS load – (see above), but without addition of virus. The toxicity test was evaluated using MTT staining (Cinatl et al, Lancet 361: 2045-2046, 2003 and Lancet 362: 293-294, 2003).

*Virus:*

SARS-CoV was isolated from the saliva of a 32-year-old patient at Frankfurt University Hospital, cultured on Vero cells (monkey kidney cells; source, ATCC No. CCL-81) and characterized by electron microscopy, molecular biology and sequence analyses (Drosten et al., N Engl J Med. 348(20): 1967-1976, 2003). The virus (passage 6) was then concentrated by ultracentrifugation (2.5 h, 53,900 g; 4 °C, rotor model: Beckman FW 19) and used for testing the surface disinfectant in a titer of approx. $1 \times 10^9$ “50 % tissue cultured infectious dose” per mL ($TCID_{50}$/mL).

*Biometric test analysis and evaluation of the virucidal effect:*

The virus titer (highest infectious dilution of the virus suspension as the so-called $TCID_{50}$/mL) was calculated according to Kärber (see below) using a computer program. The program calculated the log-titer and its standard deviation before and after contact with the disinfectant as well as the difference between the two titer values (log reduction factor RF) and the variance of the RF. These parameters were used to evaluate the efficacy of the disinfectant mixture. The mixture is said to have a virucidal effect if the titer reduction is at least 4 $log_{10}$ units. We would like to thank Dr. Ackerman, ZInf, University hospital, Frankfurt for advising us on the statistical analyses.

*Calculation of the virus titer:*

The logarithmic infection titer was calculated for the $TCID_{50}$ according to Spearman and Kärber using the computer program Microsoft Excel or LB (Spearman, Brit J Psychol, 2, 227-230, 1908; Kärber, Naunyn-Schmideberg's Arch exp Path Pharm, 162, 380-384, 1931).

$$[\log_{10}]\ TCID_{50}\ /\ Inoculum = x_0 - \frac{d}{2} + \frac{d}{n} \times \sum x_i$$

where:

$x_0$ the positive exponent of the highest dilution at which all test objects show a positive reaction

d the logarithmic dose difference

n the number of test objects used per dilution

$x_i$ the sum of all test objects showing a positive reaction to the infection, from and including $x_0$

*Calculation of the virus reduction factor:*

The reduction factor (RF) is the ratio of the infection titer before ("control titration") to that after incubation of the virus with the disinfectant ("residual virus" balance) taking into account the volumes tested.

The reduction factor (RF) is calculated (for the same volumes) from:

$$RF = 10^{a'} / 10^{a''}$$
$$\Rightarrow \log RF = a' - a''$$

*"Control titration"*: Volume = v'; virus titer = $10^{a'}$ in $TCID_{50}$/mL

*"Residual virus" (balance):* Volume = v"; virus titer = $10^{a''}$ in $TCID_{50}$/mL

Natural variations occur in virus titrations. This fact is taken into account by determining the variance of the (log) reduction factor ($S_{RF}$):

$$S_{RF} = \sqrt{S^2_{T1} + S^2_{T2}}$$

(Thraenhart, Hyg Med 22, 223-224, 1997)

"$S^2_{T1}$" = Variance of the virus control (i.e. log virus titer of the control titration);

$S^2_{T2}$" = Variance of the virus titer of the "residual virus"( i.e. log virus titer of the "residual virus").

If there is no longer any virus detectable in the test assay with disinfectant (balance), the variance is calculated as follows:

$$S_{RF} = \sqrt{2 \times S^2_{T1}}$$

**Result**

Table 2 presents the cytotoxicity data for Mikrobac® forte. It shows that the product can be used in the test from a dilution of 1:100 without damaging the cell culture. This defines the lower limit for the sensitivity of virus detection as a log titer ≤ 3.80 $TCID_{50}$/mL, which was taken into account in each calculation of the reduction factor.

Compiled in tables 3 and 4 are the results of the studies on the SARS-CoV virucidal efficacy of Mikrobac® forte in the suspension test as well as those for the parallel controls. Within the defined contact time there was no longer any SARS-CoV detectable with the active concentrations tested. This applied to all three general test conditions (clean conditions [added protein increased by a factor of 10], with 10 % (FC) serum load, and under dirty conditions) (Table 3).

Since the results of the tests performed in triplicate showed no deviations of any kind and no "residual virus" was detectable, the variance of the log titer reduction was calculated according to the equation: $SRF = \sqrt{2 \times S^2T1}$.

Taking into account the cytotoxicity and the statistical analysis named above, a ($log_{10}$) reduction factor ≥ 3.75 ± 0.71 was calculated for Mikrobac® forte. This reduction factors were achieved regardless of the conditions, active concentration, contact time or type and concentration of added protein chosen.

The ratio of the virus titer of the "control titration" to the "residual virus" (after incubation of the virus with the disinfectant) was always used for calculation of the reduction factors.

The parallel controls (table 3 and 4) showed that under the experimental conditions chosen the termination controls had approximately the same titer as the "control titration" (difference ≤ 0.5 $log_{10}$ units) and the latter a titer that was approx. 1 $log_{10}$ unit lower than the stock virus. The formalin control (0.7 %) confirmed that "residual virus" was no longer detectable after 10 minutes' incubation time. Due to the high cytotoxicity, however, only a detectable ($log_{10}$) reduction factor of ≥ 3.0 ± 0 could be determined.

Table 2: Studies on the cytotoxicity of Mikrobac® forte in the suspension test (with 10 % serum load). Shown is the dilution level down to which a cytotoxic reaction can be detected (single tests performed).

| Test substance | Conc. | Contact time | Cytotoxic to a dilution of |
|---|---|---|---|
| VP 355/1P | 0.5 % | 30 or 60 min. | 1:100 |
| **Formalin control (0.7 %)** | | | |
| | 0.7 % | 10 min. | 1:10,000**** |

**** At 1:10,000 cell changes still detectable, but cell plaque evaluable

Table 3: Studies on the SARS-CoV virucidal efficacy of Mikrobac® forte in the suspension test; test virus: SARS-CoV (strain: FFM 1). Listed are the results of three separate test assays.

| Test substance or controls | Conc. | Contact time | Control titration* | Virus titer ($log_{10}$ $TCID_{50}$/mL ± 2s) (balance run) under | | | ($log_{10}$) Reduction factor (incl. variance) for | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | $Log_{10}$ $TCID_{50}$/mL±2s | Clean condition | 10 % serum load | Dirty condition | Clean condition | 10 % serum load | Dirty condition |
| VP 355/1P | 0.5 % | 30 min. | 7.55 ± 0.50 | ≤ 3.80 ± 0 (all 3 runs) | ≤ 3.80 ± 0 (all 3 runs) | ≤ 3.80 ± 0 (all 3 runs) | ≥ 3.75 ± 0.71 | ≥ 3.75 ± 0.71 | ≥ 3.75 ± 0.71 |
| | | 60 min. | | ≤ 3.80 ± 0 (all 3 runs) | ≤ 3.80 ± 0 (all 3 runs) | ≤ 3.80 ± 0 (all 3 runs) | ≥ 3.75 ± 0.71 | ≥ 3.75 ± 0.71 | ≥ 3.75 ± 0.71 |
| **Controls** | | | | | | | | | |
| Formalin control | 0.7 % | 10 min. | 7.80 ± 0** | ≤ 4.8 ± 0 (all 3 runs) | ≤ 4.8 ± 0 (all 3 runs) | ≤ 4.8 ± 0 (all 3 runs) | ≥ 3.0 ± 0 | ≥ 3.0 ± 0 | ≥ 3.0 ± 0 |

* The “stock virus” was diluted analogously to the test assay 1:10. Titer of the “stock virus” (± 2s): 9.0 ± 0.31 $log_{10}$ $TCID_{50}$/mL (mean of single measurements and mean of single standard deviations (mean standard deviation) from 5 runs [9.05± 0.33; 8.93 ± 0.25, 8.93 ± 0.25, 9.05 ± 0.35, 9.05 ± 0.35 $log_{10}$ $TCID_{50}$/mL])

** Titer of the “stock virus” used: 8.93 ± 0.25 $log_{10}$ $TCID_{50}$/mL

s = Standard deviation

Table 4: Results of termination controls of Mikrobac® forte in the suspension test (without addition of protein). Tests performed with single assays (titer of corresponding control titration: 7.68 ± 0.25 $\log_{10}$ $TCID_{50}$/mL – Titer of the "stock virus": 9.0 ± 0.31 $\log_{10}$ $TCID_{50}$/mL)

| Test substance | Conc. | Virus titer ($\log_{10}$ $TCID_{50}$/mL ± 2s) (balance run) | ($\log_{10}$) Reduction factor (incl. variance) |
|---|---|---|---|
| VP 355/1P | 0.5 % | 7.30 ± 0.38 | 0.38 ± 0.45 |

s = Standard deviation

**Overall evaluation**

The studies performed showed that under the specified test conditions SARS-CoV was inactivated quickly and efficiently by Mikrobac® forte, i.e. that the infectious titer decreased below the limit of detection determined by the cytotoxicity of the disinfectant. Accordingly, a ($\log_{10}$) reduction factor ≥ 3.75 ± 0.71 was determined for Mikrobac® forte with 0.5 % in 30 minutes regardless of the chosen test conditions (active concentration, contact time, clean conditions [added protein increase by a factor], with 10 % (FC) serum load, and under dirty conditions).

The parallel controls confirmed the validity of the test assays. For instance the control titrations showed that the titer of the 1:10 dilution was lower than that of the "stock virus" by approx. 1 $\log_{10}$ unit, as would be expected, that the termination controls had about the same values as the control titrations ($\log_{10}$ difference < 0.5), meaning there was no significant "after-effect" of the disinfectant, and that the standard deviations of all titrations were always ≤ 0.5 $\log_{10}$. Due to the high cytotoxicity (up to 1:10,000) only a ($\log_{10}$) reduction factor of ≥ 3.0 ± 0 could be determined for the formalin control.

A virucidal effect against other viruses cannot be derived from this study.

Frankfurt/Main, 28.07.2004

Prof. Dr. rer. med. H. F. Rabenau

Prof. Dr. med. H. W. Doerr