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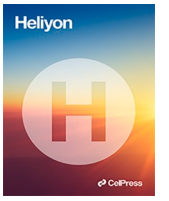

To reduce cytotoxicity when testing the virucidal activity of chemical disinfectants and biocides: The "T-25 method" as an alternative to "large-volume-plating"

F.V. Rheinbaben, J. Köhnlein, N. Schmidt, C. Hildebrandt, S. Werner *

HygCen Germany GmbH, Schwerin, Germany

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ABSTRACT

When testing the virucidal activity of biocides, the non-inactivated residual virus is titrated on cell cultures by the end point dilution method on 96-well tissue culture plates. However, residues of the biocide to be tested also come into contact with the cell cultures in varying concentrations and thus can lead to cytotoxic effects even at high levels of dilution. In the European standards for testing biocides, in particular disinfectants, methods such as *Large-Volume-Plating* (LVP) method and, in some guidelines, gel filtration procedures are described for reducing cytotoxic effects in the case of highly cytotoxic products, if the classical dilution method proves to be impractical.

In order to enable the testing of highly cytotoxic biocides for their activity against viruses, an alternative method for reducing cytotoxicity is introduced, which is based on a procedure of isolating infectious viruses from cytotoxic patients' materials such as stool and can be applied when the other methods fail.

1. Introduction

The determination of the virucidal activity of biocides has been standardized in recent years by a large number of basic test guidelines and standards. Nowadays, it is determined by a test procedure in which a suspension test as a so-called phase 2, step 1 test is to ensure the comparability of the different biocides. Subsequently, microbiological carrier tests of the so-called phase 2, step 2 test serve to determine the application parameters for their intended use, for example as surface or instrument disinfectants.

In the medical area, the activity levels to be determined are classified as "*virucidal activity against enveloped viruses*" and "*limited spectrum virucidal activity* (effective against enveloped viruses and additionally against adenoviruses, rotaviruses and noroviruses) or as "*virucidal activity*" (effective against enveloped and non-enveloped viruses) and are based on the activity against the test viruses specified by the respective European standards (EN 14476, EN 16777, EN 17111 [1–3]).

To perform a suspension test, 8 parts by volume of a solution of the biocide are added to one part by volume of the test virus suspension plus one part interfering substance (BSA and/or BSA + sheep erythrocytes as an additional interfering substance; Fig. 1). The biocide concentration is adjusted in such a way that the final concentration to be tested is reached only when all three components are combined. After the desired contact time has elapsed, but after 1 h at the latest (the longest accepted contact time of a biocide in European standards for the medical area), aliquots are taken from this preparation in order to be able to measure the virus titre, and thus the virucidal activity of the biocide with the aid of a dilution series. In parallel to the test procedure with the biocide, a test

* Corresponding author.
E-mail address: s.werner@hygcen.de (S. Werner).

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procedure with water is carried out as a control under the same conditions.

The test viruses are detected using permissive cell cultures by inoculating aliquot amounts from the dilution series onto the cell cultures. After the incubation period, which is specific for the respective virus species, the final evaluation is based on the typical cytopathic effect (CPE).

On the other hand, if tests are carried out with test surfaces, the respective test specimen, e.g. stainless steel plates, are contaminated with a virus preparation containing one part interfering substances and 9 parts virus suspension. After drying, the test specimens are covered with the biocide or immersed in the biocide and, after the contact time to be tested has elapsed, the test virus is taken up by eluting the test specimens in a recovery medium. Titration of the non-inactivated virus is then performed in the same manner as for the suspension test.

Both methods have in common that the titration fluids after the experiment contain residues of the biocide in addition to any virus that may still be present, which are also diluted in the dilution series of the titration and applied to the cell cultures.

The titre of the virus control should have a minimum concentration of 10^8 $TCID_{50}$/ml according to the standards mentioned, so that even in the case of high cytotoxicity, a sufficiently large measurement interval remains to be able to detect the required titre reduction of $\geq$4 l g levels.

Depending on their cytotoxicity, disinfectants usually destroy the culture cells in the first dilution steps, and thus minimize the measurement interval of the tests. This often happens in such a way that the required reduction rates of the virus titre compared to the controls of at least 4 l g levels cannot be detected at all or cannot be demonstrated reliably. For example, the detection limit of a product, which is cytotoxic up to the 1:1000 dilution, is $\leq$ 4.50 $TCID_{50}$.

This challenge in virucidal test method has been described in the literature. Lee et al. 2022 have tested the cytotoxicity of 72 commercially available surface disinfectants [4]. Among 19 Benzalkonium chlorides (BAC)-based disinfectants, 12 exhibited low (<10%) cytotoxicity and 4 exhibited <20% cytotoxicity. Conversely, 2 disinfectants exhibited high (100%) cytotoxicity and 1 disinfectant exhibited >80% cytotoxicity. Among the formulated Quaternary Ammonium Compounds (QAC)-based disinfectants, 5 exhibited <20% cytotoxicity and 27 exhibited >50% cytotoxicity. Interestingly, the three 2:3 formulated QAC-based disinfectants exhibited no cytotoxicity. The BAC/Didecyldimethylamonium chloride (DDAC)-based disinfectant was highly cytotoxic, whereas the BAC/DDAC/Polyhexamethylene biguanide hydrochloride (PHMB)-based disinfectant was not cytotoxic. Regarding the 2 Benzethonium chloride concentrate/2-propanol-based disinfectants, 1 Disinfectant was highly cytotoxic but another was not cytotoxic. Similarly, of the DDAC/PHMB-based disinfectants, 1 disinfectant was highly cytotoxic but another disinfectant was not cytotoxic. The disinfectants based on Sodium dichloroisocyanurate, Ethanol or Propanol mixed with Ethanol, Peracetic acid, Potassium peroxymonosulfate and Citric acid were not cytotoxic. However, the Hydrogen-peroxide-based disinfectant exhibited high cytotoxicity [4].

Moskowitz and Mendenhall 2020 evaluated the virucidal activity and cytotoxicity of mouth washes containing 1.5% Hydrogen peroxide, 0.2% Povidone, 0.12% Chlorhexidine and 100 ppm molecular Iodine for their ability to inactivate severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). They observed a cytotoxicity of 1.5% Hydrogen peroxide at a 1:1000 dilution and a cytotoxicity of 0.12% Chlorhexidine gluconate at a 1:100 dilution. The requested 4 l g reduction could not be demonstrated with these

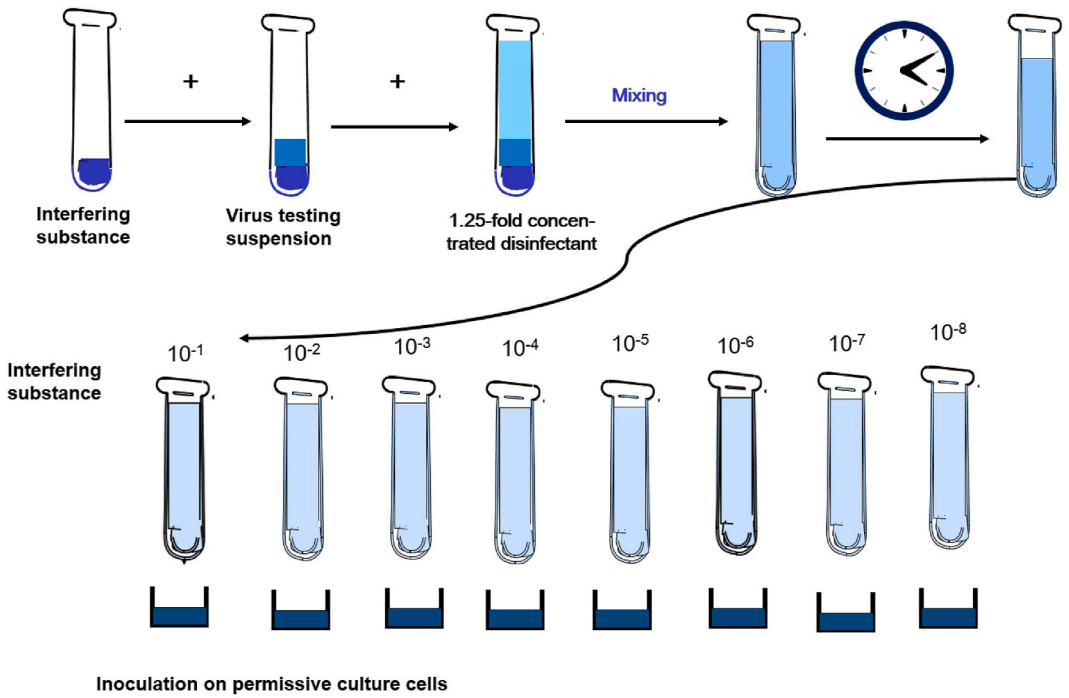

Fig. 1. Conducting a virus suspension test using the titration method (own illustration).

products [5].

Furthermore, Steinhauer et al. 2021 have tested 3 different formulations of disinfectants for hand or surface disinfection. With 2 of the formulations containing Alkyl (C12-16)dimethylbenzyl ammonium chloride, DDAC, Alkyl (C12e14)ethylbenzyl ammonium chloride resp. Propan-2-ol, the 4 l g reduction could likewise not be demonstrated with the titration method [6].

A special neutralization of the biocide effect cannot be performed, mainly because the biocide neutralisers in question and/or the reaction products of the biocides with the neutralisers used are usually cytotoxic themselves. For the elimination of cytotoxicity, therefore, only three procedures are described in the test standards so far: the dilution method, the *Large-Volume-Plating* (LVP) method and gel filtration with Sephadex columns [1–3]. However, the latter is only accepted in the national guideline of RKI/DVV if extensive controls are carried out (Guideline RKI/DVV, Annex 7 [7,8]). But the use of the Sephadex columns as proposed in the EN 14476 for detoxification requires an appropriate run in parallel without columns. By doing so, the lab can clearly notice whether parts of the test virus suspension from the test mixture are restrained in the columns which may result in false-positive results for the products.

In the virus control without disinfectant this phenomenon is often not seen and then wrong conclusions are drawn. The use of a Sephadex column may also be an explanation for favorable results in tests for virucidal activity. Formulations with a high own cytotoxicity may be tested with a Sephadex column which aims to reduce the cytotoxicity of the formulation. However, the Sephadex column will also prolong the contact time between the formulation and the test virus for some minutes with all possible implications for the test result [9].

This method described in the EN standards, in which the samples are freed from biocide residues and thus from cytotoxic side effects by means of gel filtration prior to titration, has generally proven to be less suitable, in particular because although it mitigates cytotoxic effects, it can also adsorb part of the virus particles and thus lead to titre losses in the filtrate and consequently to false-negative results. Therefore, a parallel approach without gel filtration must always be carried out. The *European Chemicals Agency* (ECHA, authorisation authority for biocides) has therefore rejected biocide tests with gel filtration several times due to the above mentioned reasons [9].

When using the LVP method, a dilution is prepared directly in a large volume preparation that lies outside the cytotoxic range (e.g. 62.5 µl of the test solution in 62.5 ml cell culture medium; Fig. 2). The number of microtitre plates used and therefore the sample volume used for the test determines the detection limit. For the experiments presented, the complete volume of the sample dilution was always tested. It is also possible to test only parts of the volume. It is possible to lower the detection up to 2 l g steps with the LVP method compared to the titration method.

In the meantime, however, biocides and especially biocidal products (disinfectants) are increasingly being tested whose toxicity for cell cultures is so high and the non-cytotoxic dilution in the LVP method so great that the lowering of the detection limit is not sufficient to detect the ≥4 l g reduction. In these cases, the LVP methodology does not help either. This is especially true for surfactants, which are added to the preparations as active ingredients as well as excipients, and which destroy the membranes of the culture cells even in very high dilutions due to their lipophilic effect [10].

Therefore, an urgent need for further recognised methods to reduce cytotoxicity in biocide testing exists. Otherwise, the virucidal activity of such products would not be detectable according to the currently valid standards.

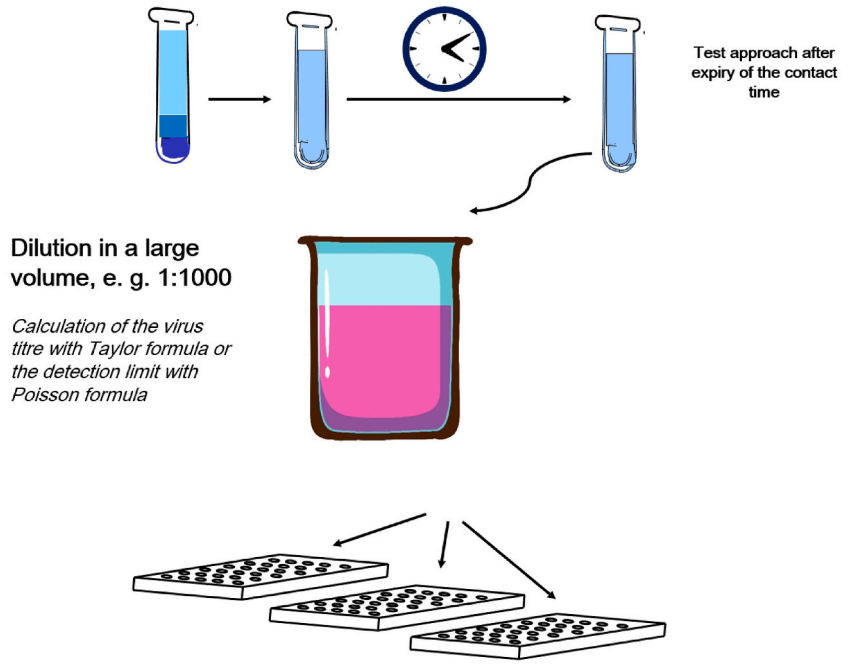

Fig. 2. Procedure of a virus suspension test using the LVP method (EN 14476:2019–10, Annex B3 [1]; own illustration).

In the following study, we therefore tested the suitability of a fourth method, which we named the *T-25 method for reducing cytotoxicity*. The aim was to extend the measurement interval when testing virucidal activity, especially that of chemical disinfectants for use in the medical sector (and possibly also for the institutional and veterinary sectors).

2. Materials and methods

2.1. Test viruses and cell cultures

- Murine norovirus (MNV) grown and titrated on RAW cells (RAW 264.7)
- Modified vaccinia virus strain Ankara (MVA) grown and titrated on BHK-21 cells (CCLV-RIE 179)
- Adenovirus type 5, strain Adenoid 75, ATCC VR5, isolated and titrated on HeLa cells
- Poliovirus type 1, strain LSc-2ab, isolated and titrated on HeLa cells
- Bovine virus diarrhoea virus (BVDV) ST UG59 isolated and titrated MDBK cells (CCLV-RIE 261)

T-25 cell culture flasks from Sarstedt with 25 cm^2 culture growth surface and ventilation caps, order number 83.3910.002.

2.2. Test methods

The detection of residual virus from cell culture supernatants from T-25 cell flasks as well as the control titrations were carried out according to the following standards in each case.

- EN 14476 (Suspension test phase 2, step 1 for testing chemical disinfectants for human medical use (EN 14476 [1]),

as well as according to the.

- suspension test according to the RKI/DVV test method [7].

2.2.1. End point titration (quantal experiment)

To prove the virucidal activity in the suspension test according to EN 14476, suspensions of 8 parts by volume of the disinfectant, product test solution were incubated with one part interfering substance (0.3% BSA + 0.3% sheep erythrocytes for the test of "dirty conditions" or 0.03% BSA for the test of "clean conditions") and one part by volume of virus suspension with a titre of at least 10^7 $TCID_{50}$/ml. After the exposure time, the effect of the disinfectant was stopped by dilution. For this purpose, the residual virus content was diluted 1:10/1:100/1:1000 etc. in a dilution series. The dilution steps were applied in a microtitre system on 96-well plates with 100 µl/well and 8 wells per dilution step (Fig. 1). For the verification of the test results, the validation controls as defined in EN 14476 were performed [1] and found to be effective in all experiments indicating the validity of presented data.

The calculation of the results was done according to *Spearmann* 1908 and *Kärber* 1931 [11,12] and was calculated as the difference between the titre of the virus control and the titre of the biocide test solution, including a 95% confidence interval.

$$m = x_k + d/2 - d\sum p_i$$

m = *negative decimal logarithm of the titre based on the test volume*
x_k = *logarithm of lowest dose (dilution level) at which all test objects exhibit a positive reaction*
d = *logarithm of dilution factor*
p_i = *observed reaction rate*.

2.2.2. Large-volume-plating (LVP) method

The LVP method can be used when it is not possible to show the minimum measurement interval of ≥4 l g reduction due to cytotoxicity. For this purpose, the biocidal virus suspension was diluted with cell culture medium immediately after the contact time in a mixing ratio of 1:1000 or 1:10,000, the complete volume (e.g. 62.5 ml) of the dilution step was divided into e.g. 125 µl/well portions and completely inoculated onto 4 to 5 micro-titre plates with permissive cells (Fig. 2). If, after the incubation period, a CPE can still be detected by microscopic examination due to active virus particles, the virus titre is calculated according to the Taylor formula:

$$c = \frac{D}{V_w} \times \left(- ln \frac{n - n_p}{n} \right)$$

c = Concentration of infectious virus particles.
D = Dilution factor of the pre-diluted sample.
V_w = Plated volume per well
n = Number of inoculated wells
n_p = Number of infected wells.
If no more active virus can be detected, the detection limit is determined using the Poisson formula. At a 1:1000 dilution, the

detection limit was ≤1.84 l g. If the test solution was diluted 1:10,000 this resulted in a detection limit of ≤2.84 l g.

$$C = \frac{\ln p}{-v}$$

c = Concentration of virus particles in the test mixture
p = 95% probability of detecting viruses (p = 0.05, ln p = −2.99).
V = Total test volume in ml.

2.2.3. T-25 method

The T-25 method follows the principle of a standard method previously used in routine diagnostics for the detection of active virus particles from patient materials, especially from stool, because stool suspensions are often still highly toxic for culture cells even in high dilution:

After incubation of the virus-biocide mixture following the respective test specifications of the biocide as well as the specifications of EN 14476 (8 parts of the pre-concentrated biocide + 1 part of interfering substances + 1 part of virus suspension), an aliquot (0.5 ml) was taken from the test mixture at the end of the respective contact time and diluted in a titration series in 1:10 dilution steps, whereby 4.5 ml of cell culture medium was specified in each case.

To reduce the cytotoxicity of the samples, 100 µl aliquots of each dilution step of the titration series were then pipetted into T-25 cell culture flasks containing 10 ml of cell culture medium and 25 cm^2 of growth area instead of micro-titre plates (Fig. 3). At least, one T-25 cell culture flask was inoculated per dilution level and up to 3 parallel studies were set up per dilution level.

Cell seeding was ~ 1×10^6 cells per flask. After their pre-incubation over a period of 18 h–24 h at 37 °C in a CO_2 incubator (5% CO_2), aliquots were dispensed directly into the flask supernatant. The selection of the permissive cells depends on the virus to be titrated. This also applies to the serum content in the cell culture medium, which is, however, usually 2% fetal bovine serum (FBS). The cells are not washed before seeding.

Four to six hours after pipetting the aliquot into the T-25 flasks, all the culture medium was replaced with 10 ml of fresh medium.

The observation period of the respective cell cultures extended over a total period of 3 d–8 d and was predetermined by the respective control titrations of the respective virus suspensions on 96-well plates. The evaluation was done by microscopic control for viral cytopathogenic effects (CPE).

If the titration on T-25 flasks did not lead to a clear result, cell culture supernatant of the test solutions as well as the controls in T-25 flasks and on 96-well plates were over-inoculated with new cell cultures.

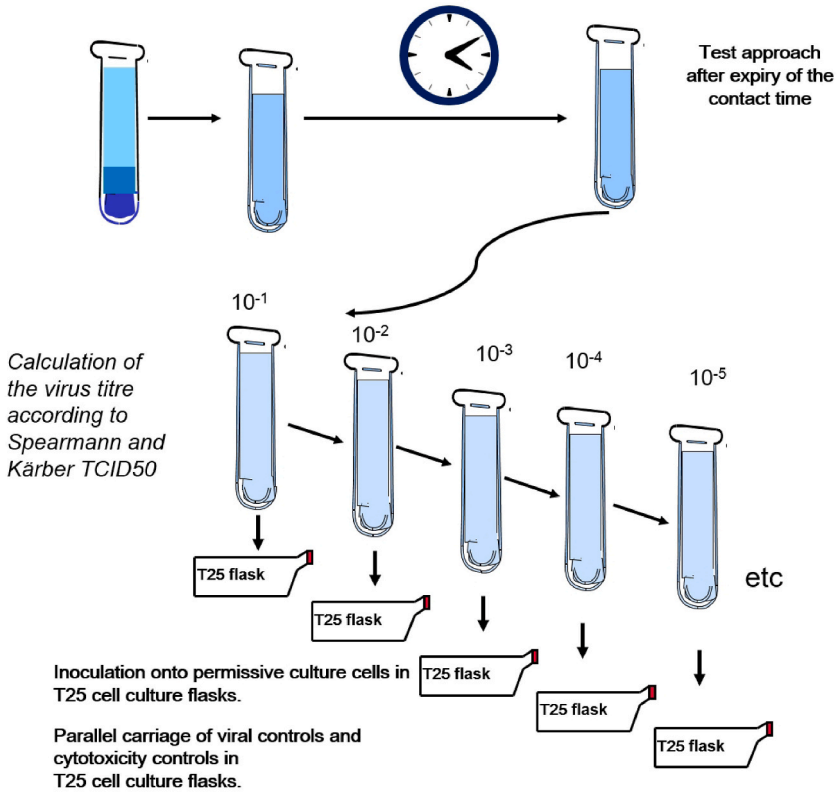

Fig. 3. Procedure of a virus suspension test using the T-25 method (own illustration).

Table 1
Elimination of cytotoxicity and extension of the measurement interval by using the T-25 method when testing highly cytotoxic biocides compared to the LVP method and the conventional dilution method.

Product No.	Recipe number/active ingredient base (in 100 g)	Test standard/ test conditions	Test virus	Reduction factor (lg RF)			Cytotoxicity ($lg\text{-}CD_{50}$)/ detection limit (lg RF)	Results
				Dilution method*	LVP method**	T-25 method***		
1	Chlorhexidine digluconate, Phenoxyethanol	EN 14476 50% (v/v), 0.5 min Dirty conditions	*Modified vaccinia virus Ankara (MVA)*	≥1.13	n.d.	**≥ 4.00**	≤4.50* ≤1.50***	Detection of ≥4 l g possible with T-25 method
2	Alkyl (C12-16) dimethylbenzyl ammonium chloride, Didecyldimethyl-ammonium chloride	EN 14476 80% (v/v), 15 min Clean conditions	*Murine Norovirus (MNV)*	≥1.75	2.80	2.00	≤4.50* ≤2.84** ≤2.50***	Inactivity of the product proven with LVP method and T-25 method
3	Chlorhexidine digluconate	EN 14476 80% (v/v), 1 min	*Poliovirus*	≥2.00	n.d.	3.00	≤5.50* ≤2.50***	Inactivity of the product proven with T-25 method
		Clean conditions	*Adenovirus*	≥2.50	n.d.	3.00	≤5.50* ≤3.50***	Inactivity of the product proven with T-25 method
4	Alkyl (C12-16) dimethylbenzyl ammonium chloride, Didecyldimethyl-ammonium chloride	EN 14476 3%, 5 min Dirty conditions	*MVA*	≥0.75	n.d.	≥3.00	≤4.50* ≤2.50***	Not detectable even with the T-25 method due to extremely high cytotoxicity ≥4 l g
		EN 14476 0.5%, 5 min Dirty conditions	*MVA*	≥1.75	n.d.	**≥ 5.00**	≤3.50* ≤1.50***	Detectable with T-25 method ≥4 l g
5	Octenidine hydrochloride	RKI/DVV 2015 90%, 0.5 min	*BVDV*	≥2.13	**≥ 4.79**	**≥ 4.00**	≤4.50* ≤2.84** ≤2.50***	Activity of the product proven with LVP method and T-25 method
		80% 0.5 min, FKS	*BVDV*	≥1.88	**≥ 4.54**	**≥ 4.00**	≤4.50* ≤2.84** ≤2.50***	Activity of the product proven with LVP method and T-25 method
		90%, 15 s	*MVA*	≥2.00	≥3.66	**≥ 4.00**	≤4.50* ≤2.84** ≤2.50***	Activity of the product proven with T-25 method
		RKI/DVV 2015 80%, 15 s FKS	*MVA*	≥3.00	≥3.16	**≥ 5.00**	≤4.50* ≤2.84** ≤2.50***	Activity of the product proven with T-25 method
6	Cocospropylenediamine-guanidinium diacetate, Phenoxy propanol, Benzalkonium chloride	EN 14476 1%, 5 min Dirty conditions	*MVA*	≥2.13	n.d.	**≥ 4.00**	≤4.50* ≤2.50***	Activity of the product proven with T-25 method
7	Dimethyl ammonium chloride, Phenoxy propanol, Alkyl guanidine acetate, Laurylpropylenediamine	EN 14476 1% (v/v), 5 min Dirty conditions	*MVA*	≥2.00	≥3.66 .	**≥ 4.00**	≤4.50* ≤2.84** ≤1.50***	Activity of the product proven with T-25 method
8	Didecyldimethyl-ammonium chloride, N-(3aminopropyl)-N-dodecyl-propane-1.3-diamine	EN 14476 1.5% (v/v), 15 min Dirty conditions	*MVA*	≥1.88	≥3.54 .	**≥ 4.00**	≤4.50* ≤2.84** ≤2.50***	Activity of the product proven with T-25 method
9	Alkyl dimethylammonium chloride	EN 14476 1.5% (v/v), 5 min Dirty conditions	*MVA*	≥1.38	≥3.54 .	**≥ 4.00**	≤4.50* ≤2.84** ≤2.50***	Activity of the product proven with T-25 method
10	Didecyldimethyl-ammonium chloride, N-(3-aminopropyl)-N-dodecylpropane-1.3-diamine	EN 14476 1% (v/v), 15 min Clean conditions	*MVA*	≥2.00	≥3.54	**≥ 5.00**	≤4.50* ≤2.84** ≤2.50***	Activity of the product proven with T-25 method

(*continued on next page*)

Table 1 (*continued*)

Product No.	Recipe number/active ingredient base (in 100 g)	Test standard/ test conditions	Test virus	Reduction factor (lg RF)			Cytotoxicity ($lg\text{-}CD_{50}$)/ detection limit (lg RF)	Results
				Dilution method*	LVP method**	T-25 method***		
12	Chlorhexidine digluconate, Ethanol	EN 14476 80% (v/v), 1 min Clean conditions	*Adenovirus*	1.00	n.d.	3.00 .	≤4.50* ≤1.50***	Inactivity of the product shown with endpoint dilution and T-25 method
			MNV	≥1.13	n.d.	**≥ 4.00**	≤4.50* ≤1.50***	Activity of the product proven with T-25 method
			Poliovirus	≥3.00	n.d.	3.00	≤4.50* ≤1.50***	Inactivity of the product proven with T-25 method
14	N,N-didecyl-N,N-Dimethylammonium chloride, N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine	EN 14476 97% (v/v), 1 min Clean conditions	*MNV*	≥1.13	n.d.	≥3.00	≤5.50* ≤3.50***	Not detectable with T-25 method ≥4 l g
		EN 14476 97% (v/v), 5 min Clean conditions	*MNV*	≥1.13	n.d.	≥3.00	≤5.50* ≤3.50***	Not detectable with T-25 method ≥4 l g
		EN 14476 80% (v/v), 1 min Clean conditions	*MNV*	≥0.50	n.d.	1.00	≤5.50* ≤2.50***	Inactivity of the product proven with T-25 method
14	N,N-didecyl-N,N-Dimethylammonium chloride, N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine	EN 14476 80% (v/v), 5 min Clean conditions	*MNV*	≥0.50	n.d.	2.00	≤5.50* ≤2.50***	Inactivity of the product proven with T-25 method

BVDV = Bovine virus diarrhoea virus, MVA = Modified vaccinia virus strain Ankara, MVN = Murine norovirus, n.d. = not done.
* = when using the dilution method, ** when using the LVP method, *** when using the T-25 method.

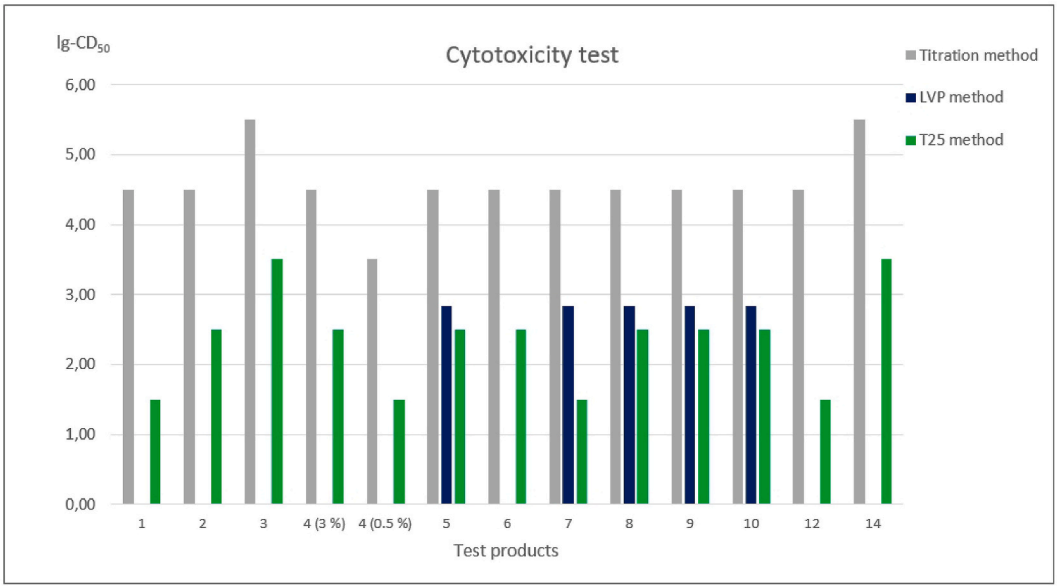

Fig. 4. Cytotoxicity of the test products using the different methodologies (own illustration).

In addition to the titration of the test solutions and their total dilutions, both cytotoxicity controls in T-25 flasks and virus controls on T-25 flasks as well as on 96-well plates were prepared. However, the calculation of the virus titre reduction was always based on the result of the virus control in T-25 flasks.

2.3. Test substances/test parameters

12 different biocides were tested in comparison. These were commercially available disinfectants. Their ingredients as well as the tested conditions of use are listed in Table 1.

3. Results

All test samples were found to be highly cytotoxic in the endpoint titration and destroyed the permissive cells up to the 1:1000 (lg-CD_{50} 4.50) and 1:10,000 (lg-CD_{50} 4.50) dilution, respectively (Fig. 4). With the conventional dilution methods, the demonstration of a titre reduction of the test viruses by 4 l g therefore proved to be impossible. The active substances were Quaternary Ammonium Compounds, Chlorhexidine and different tensids. Lee et al. 2023 and Moskowitz et al. 2020 have described the high cytotoxicity of these substances [4,4,5,5].

In the case of 12 different products, the titration method and the T-25 method was carried out 21 times each. The LVP method was performed in 9 cases.

By using the LVP method, the cytotoxicity could be significantly reduced and the measurement interval extended, but the LVP method also proved to be insufficiently efficient for products containing QAC as active substances (see Table 1, Fig. 5). In contrast, the required ≥4 l g reduction was detectable in 9 different products using the T-25 method (product No. 1, No. 4 [0.5%], Nos. 5–10 and No. 12 [MNV]).

For the tests with BVDV with 10% FBS inference substance and without interference substances ≥4 l g reduction could be shown for sample No. 5 using both the LVP method and the T-25 method (Fig. 6).

Conversely, testing of product No. 12 and 14 showed that the T-25 method, in contrast to endpoint titration, also clearly demonstrated their inactivity against Poliovirus (product 12) respectively MNV (product 14 with a concentration of 80%).

In the case of samples No. 4 (MVA, 3%) and No. 14 (MNV, 97%), the cytotoxicity could only be reduced to ≤ 2.50 lg resp. ≤ 3.50 lg even with the T-25 method, and therefore the required titre reduction could not be demonstrated. The active substance of product No. 4 and 14 is DDAC, a QAC and therefore a well-known biocide with high cytotoxicity. As shown before, Lee et al. 2023 have investigated the cytotoxicity of this substance group [4].

When using the T-25 method, it was possible in many cases to achieve a significant reduction in cytotoxicity and thus the necessary extension of the measurement interval.

All results of the T-25 method are summarised in comparison with the required endpoint titration and/or the LVP method in Table 1. They show that the sensitivity of titration for residual virus in test samples with high cytotoxicity can be increased with this method, especially for disinfectants with high surfactant contents.

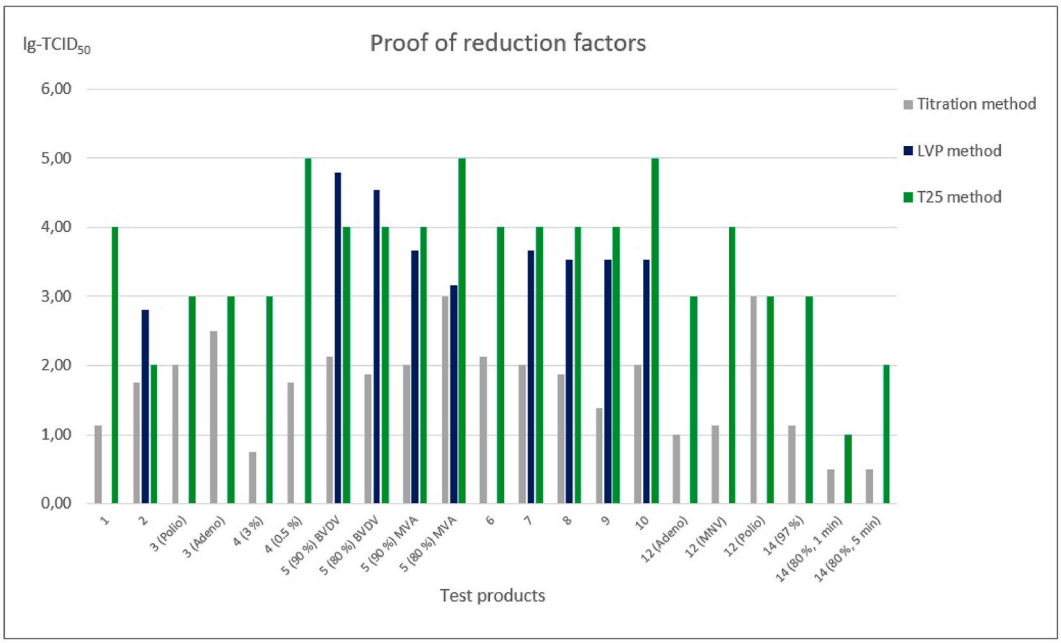

Fig. 5. Proof of reduction factors using the three different methods (own illustration).

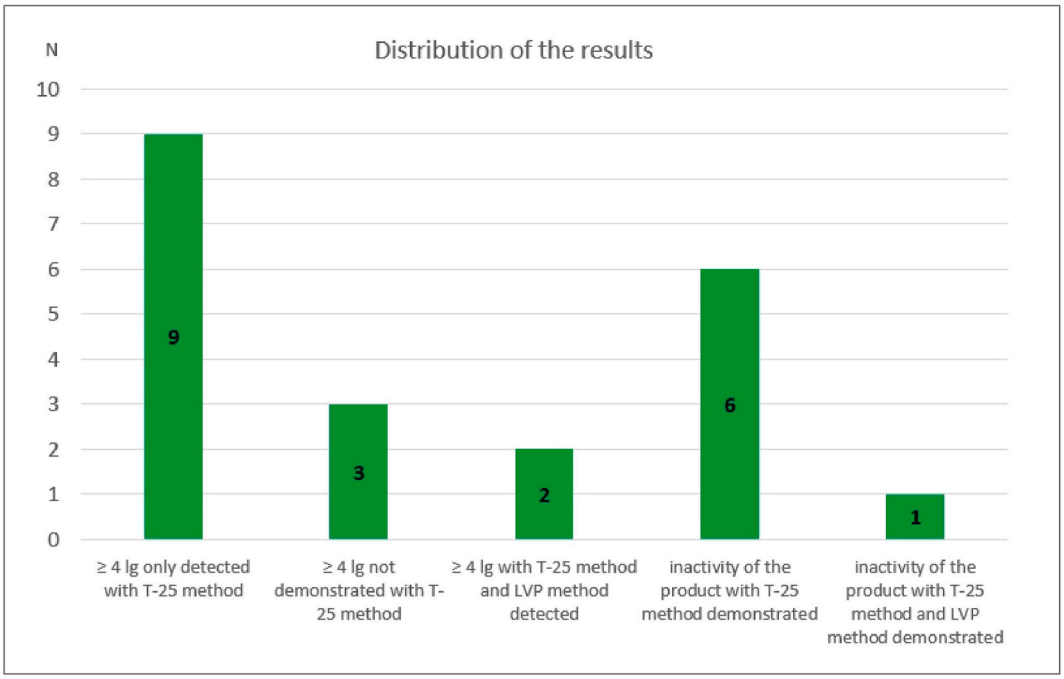

Fig. 6. Distribution of results with regard to the test methodology (own illustration).

4. Discussion

Chemical disinfectants for use on humans or for surfaces, instruments and laundry disinfection in the human medical area in Europe must meet the test requirements of the European Standards EN 14476 (suspension test phase 2, step 1). In addition, to demonstrate virucidal activity, they must comply with EN 16777 (germ carrier test for surface disinfectants, phase 2, step 2 test [2]; or EN 17111 (germ carrier test for instrument disinfectants, phase 2, step 2 test [3]), depending on their intended use.

An aliquot taken from a disinfectant-virus mixture contains not only active residual virus particles, if any, but also always the biocide to be tested and a proportion aliquot of the required additional interfering substances. In the dilution series subsequently prepared for titration, these components are diluted along with any residual virus that may still be present. To inhibit the biocide activity, a dilution of 1:10 to 1:100 is performed. However, the first dilution levels still have a pronounced cytotoxicity for the permissive cells needed for virus detection, so that they cannot be used to assess a cytopathic effect. The virucidal activity of disinfectants or biocides with a cytotoxicity of more than 1:10,000, on the other hand, can no longer be demonstrated using conventional methodology. Other studies have shown these cytotoxic effects and the limitations of testing the virucidal activity of disinfectants [4, 5].

Although there have been repeated attempts in the past to apply special neutralisers, as has been standard for many years in studies on the bactericidal and fungicidal activity of disinfectants [13], they all failed to produce satisfying results in viral titrations.

For example, experiments have been conducted with Tryptose phosphate, Sodium thiosulphate, Sodium thioglycolate, Sodium hydrogen sulphate, Lecithin, Sodium bisulphite, Histidine, L-cysteine and even skimmed milk. Semicarbazide (10% solution in a 1:1 ratio to neutralise 0.7% Formaldehyde) was used to inactivate Formaldehyde and additions of Glutathione were used to neutralise Peracetic acid. For the inactivation of 1% Glutaraldehyde, 4% Na-bisulphite was tested [10]. However, all these experiments were not suitable for replacing the dilution method for viruses, because all the chemicals used for inactivation were also more or less cytotoxic.

It turned out that neutralising agents should have been chosen in such a way that they chemically reacted with the biocide to form a non-toxic cell culture-compatible compound without toxic residues of both initial reactants remaining after the reaction. This is difficult to realise in practical biocide testing, especially since biocidal products often consist of more than half a dozen different individual components and, in the case of disinfectants, they may also contain cytotoxic auxiliary substances.

The detection of the virus titre is therefore always carried out in accordance with the applicable test methods via the preparation of a dilution series, in which the concentration of the biocide also decreases with each dilution step and, in parallel, its cytotoxicity. However, if a cytotoxic effect is still detectable up to the dilution of 1:1000 or 1:10,000, the LVP method must be used in accordance with the EN standards.

In the case of highly cytotoxic biocides, changing the culture supernatant approx. 2–3 h after inoculation of the dilution series is then the only possible procedure to reduce cytotoxicity after the virus has entered the indicator cells. However, such a procedure is not yet specified in the European testing standards.

Based on early experience from routine diagnostics of human viral infections, where it was a matter of isolating active virus from

cytotoxic patient materials such as stool and growing it on cell cultures in the presence of residues of these patient materials, we chose cell cultures in T-25 cell flasks in our approach, and thus were able to determine the virucidal activity even of biocides that would otherwise not have been testable with reasonable effort. Instead of T-25 cell culture flasks, 6-well cell culture plates can also be used in our experience. Likewise, larger flasks, especially with a culture growth area of 75 cm^2 can be used. However, due to the better manageability and economical use of materials, T-25 bottles have proven to be particularly suitable in our experience. The method could thus be considered, similar to the LVP method, as an additional alternative in case the usual dilution method should prove to be inapplicable in preliminary tests.

4.1. Study limitations

It was not possible to reduce the cytotoxicity of every tested product in such a way that the required 4lg reduction could be demonstrated. A cytotoxic reference substance would have been helpful to show the reproduceability of the T25-method.

5. Conclusion

Based on our findings, we propose the T-25 method as a viable method in addition to the endpoint titration method and the LVP-method for testing the virucidal activity of biocides. However, prior to inclusion in the biocide testing standards, method comparisons with highly cytotoxic and less cytotoxic biocides should be performed in the ring test to evaluate the procedure.

CRediT authorship contribution statement

F. V. Rheinbaben: Conceptualization, Formal analysis, Supervision. **J. Köhnlein:** Data curation, Investigation, Methodology, Project administration, Visualization, Writing – original draft, Writing – review & editing. **N. Schmidt:** Investigation, Methodology. **C. Hildebrandt:** Conceptualization, Formal analysis, Project administration. **S. Werner:** Supervision.

Declaration of competing interest

I wish to submit an revised original research article entitled *"To reduce cytotoxicity when testing the virucidal activity of chemical disinfectants and biocides: The "T-25 method" as an alternative to "Large-Volume Plating""* for consideration by the Journal of Virological Methods.

I confirm that this work is original and has not been published elsewhere, nor is it currently under consideration for publication elsewhere.

All comments of the first review have been revised in this version.

We have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20728.

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