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Establishment of a standardized phase 2/step 2 method for virucidal surface disinfection: results of an interlaboratory ring trial (4-field test)

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SUMMARY

Introduction: Evaluating the virucidal efficacy of surface disinfectants under practical conditions with mechanical action remains a major challenge. Current standards primarily rely on suspension tests and carrier tests without mechanical action, whereas mechanical, surface-based methods are not yet sufficiently validated for virucidal testing. This methodological validation study reports the results of an interlaboratory ring trial on the 4-field test for viruses with hard water. The objective of the present study was to validate a practical test method aligned with European standards and to support the development of a new Work Item within the Working Group 1 of CEN Technical Committee 216 activities.
Methods: Thirteen European and international laboratories tested three human pathogenic model viruses – Adenovirus type 5, Modified Vaccinia Virus Ankara, and Murine

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4-field test
Ready-to-use

Norovirus — on non-porous surfaces in the 4-field test using hard water. Virus recovery was assessed across multiple contact times, and repeatability and reproducibility were calculated according to ISO 13528.

Results: All viruses were reliably recovered on test field 1. The method was largely robust across laboratories, although variations in physical parameters affected interlaboratory consistency, underscoring the relevance of mechanically driven application factors.

Conclusion: The virucidal 4-field test demonstrates promising applicability as a phase 2/step 2 method and represents a valuable candidate for incorporation into future European standards. The method is particularly relevant for assessing ready-to-use wipes as virucidal efficacy depends on the combined effects of wipe material, released liquid, and mechanical action, which may substantially differ from results obtained in the absence of mechanical application. Representative-of-use validation methods are therefore essential for assessing disinfectant efficacy in hospitals, long-term care facilities, and public spaces.

Introduction

Viruses on environmental surfaces represent a significant challenge in infection prevention, particularly in healthcare facilities, nursing homes, public institutions, and other communal facilities, where surface disinfection is predominantly performed using ready-to-use (RTU) wipes [1]. Numerous studies have shown that clinically relevant viruses such as noroviruses, adenoviruses, and poxviruses can remain viable on inanimate surfaces for extended periods — ranging from several hours to multiple days — depending on the surface type, ambient humidity, and temperature [2]. These findings underscore the importance of targeted virucidal surface disinfection, especially in settings involving immunocompromised individuals or potential viral gastroenteritis outbreaks.

Despite the availability of effective disinfectants, the evaluation of virucidal efficacy has historically relied on suspension-based test methods, primarily EN 14476 [3], which are essential for defining the fundamental activity spectrum of chemical agents under controlled conditions. However, suspension tests do not reflect the practical use of disinfectants on surface, as they neither include mechanical action nor adequately account for evaporation, drying dynamics, or the potential spread of contamination. Since 2019, the only surface-based virucidal tests currently available in Human medicine (Working Group 1 of European Norm series) are EN 16777 for surface tests without mechanical action and EN 17111 [4,5] for instrument reprocessing by immersion, which, while useful, do not include mechanical wiping and therefore cannot fully simulate routine disinfection procedures of all products used by mechanical action (e.g. RTU wipes) [6]. In practice, the virucidal efficacy of a surface product applied by wiping or mopping is determined by the complex interplay between the wipe material (cleaning effect) and the impregnated disinfectant solution, which must be carefully aligned. This interaction can result in a substantially broader and more realistic virucidal effect than that observed in suspension or immersion tests [7]. Accordingly, recent publications have increasingly focused on carrier-based surface tests using stainless steel carriers to better reflect practical wiping conditions, including studies addressing the performance of disinfectants applied via wipes under realistic use scenarios. In particular, the work by E. Steinmann *et al.* has demonstrated the relevance of carrier tests for assessing virucidal efficacy on stainless steel surfaces, thereby underlining the need for practice-oriented test methods suitable for wiping applications [8—10]. European tiered approach grades virucidal efficacy into three levels, each defined by corresponding model test viruses: virucidal activity against enveloped viruses (e.g. influenza, herpes viruses, Corona viruses), limited spectrum of virucidal activity against enveloped viruses plus noroviruses, rotaviruses, and adenoviruses, and virucidal activity against enveloped and non-enveloped viruses [11].

Within the European standardization framework established by CEN TC 216 Chemical disinfectants and antiseptics, phase 2/step 2 methods are considered critical for evaluating disinfectants under simulated practical conditions. In bactericidal and yeasticidal testing, the EN 16615 '4-field test' has become a well-established surface method, offering high reproducibility and close simulation of wiping disinfection [12]. In addition, in 2023, the 4-field test concept was further extended through the publication of EN 17846 for sporicidal testing [13]. This development demonstrated that the methodology is not limited to bacteria and yeasts, but can also be successfully applied to bacterial spores under standardized wiping conditions, thereby confirming its broader applicability to additional groups of micro-organisms. However, for virucidal testing, no validated or harmonized surface test incorporating mechanical action has yet been formally adopted at this level, particularly none address wiping/mopping as an application principle. In 2015, a virucidal 4-field test procedure based on EN 16615 was described within CEN TC 216 as Work Item 00216151 [14]. However, this approach was not pursued further for several years. In 2018, Maren Eggers demonstrated the practical feasibility of a virucidal 4-field test for vaccinia virus within preliminary internal tests, thereby providing the first evidence that such a method could be applied to viruses under realistic wiping conditions. Building on these findings, the development of a standardized, practical test method to evaluate the virucidal efficacy of disinfectants on surfaces using realistic wiping/mopping scenarios, with a particular focus on RTU wipes, was taken up again. To address robustness of the experimental method, virus recovery, desiccation, and transfer dynamics, the Working Group 1 of CEN Technical Committee 216 (CEN TC216 WG1), in collaboration with international expert laboratories, initiated a process to adapt the bactericidal 4-field test for virucidal purposes. A dedicated Task Force, supported by the German Association for Applied

Hygiene (VAH), was established to develop a suitable test protocol for a methodological validation study with hard water and organize an interlaboratory ring trial as a prerequisite for standardization.

This ring trial was designed to assess the methodological reproducibility, reliability, and discriminatory capacity of a modified 4-field test applied to viruses. Three test viruses were selected to represent different structural and resistance characteristics and, at the same time, correspond to standard test viruses used in other European virucidal norms: Adenovirus type 5 (AV5) as a non-enveloped, intermediate-resistant virus; Murine Norovirus (MNV) as a surrogate for human noroviruses, known for their high environmental stability and disinfection resistance [8]; and Modified Vaccinia virus Ankara (MVA) as a model enveloped virus and surrogate for vaccinia virus and other enveloped viruses, such as influenza or Corona virus [15]. Like other carrier tests such as EN 16777, the use of poliovirus does not allow testing on dried surfaces due to a high loss of viral titre during the drying stage and was therefore not considered in the study design. Importantly, the trial was conducted using hard water alone, without any active chemical agents, in order to isolate and evaluate the test method itself, particularly the mechanical and procedural aspects, prior to applying it in product-specific efficacy studies.

The present article reports on the design, execution, and results of this interlaboratory ring trial and evaluates the performance of the modified 4-field test. The findings are discussed within the framework of existing European test standards and aim to inform the development of a new European standard for the virucidal testing of surface disinfectants (Work Item 00216151) [16]. This work represents a key step towards establishing a harmonized, practice-relevant method for assessing surface virucidal efficacy, especially for widely used wiping disinfectant products, thereby supporting improved infection prevention through evidence-based product evaluation.

Material and methods

In 2023, an interlaboratory ring trial was conducted with the support of the CEN TC216 WG1, in collaboration with the Task Force "4-Field Test for Viruses" and the VAH. The primary objective was to validate a standardized phase 2/Step 2 method for assessing the virucidal efficacy of disinfectants on non-porous surfaces under simulated practical conditions.

A total of 13 laboratories from Germany, other European countries, and Asia participated in the trial (see Table I). One laboratory was excluded due to substantial deviations from the protocol. In this case, all test fields 1–4 were contaminated with the inoculum, rather than just the first one, therefore the results could not be included in the analysis. Laboratory identifiers were anonymized and randomly assigned. All laboratories followed a uniform, harmonized protocol developed specifically for this ring trial. It should be noted that not every laboratory tested all test viruses or all conditions, so that not all 13 participating laboratories always appear in the final evaluation.

The study was conducted following a modified version of the EN 16615:2015 '4-field test,' originally developed for evaluating the bactericidal and yeasticidal efficacy of surface disinfectants administered with wipes that include RTU wipes. The method is designed to simultaneously assess the removal of viral contamination from an initially contaminated surface and the potential transfer of infectious virus to previously uncontaminated areas during a wiping procedure. In this study, the method was adapted for the recovery and quantification of viral particles under clean (0.3 g/L BSA) and dirty conditions (3 g/L bovine serum albumin [BSA] +, 3 ml/L sheep erythrocytes).

The test viruses AV5, MNV, and MVA were obtained by each laboratory from different suppliers. As is generally accepted, different cell lines were used depending on virus type and laboratory (see Table II). For tests with AV5, most laboratories used epithelial cells of a cervical carcinoma or

Table I
Participants of the ring trial 2023 Quantitative test method on non-porous surfaces with mechanical action (virucidal 4-field test)

Laboratory	Location
BluTest Laboratories Ltd	Glasgow (United Kingdom)
Dr. Brill + Partner GmbH	Bremen (Germany)
Ekolabos	Wrocław (Poland)
Eurovir Hygiene-Labor GmbH	Luckenwalde (Germany)
HygCen Austria GmbH	Bischofshofen (Austria)
HygCen Germany GmbH	Schwerin (Germany)
Institut für Hygiene and Public Health	Bonn (Germany)
IKI - Institut für Krankenhaushygiene und Infektionskontrolle GmbH	Gießen (Germany)
Laboratoires Anios (Ecolab)	Lezennes (France)
Labor Prof. Dr. G. Enders MVZ GbR	Stuttgart (Germany)
MICROMUN GmbH	Greifswald (Germany)
Robert Koch Institut	Berlin (Germany)
TECOLAB Sdn. Bhd.	Kuala Lumpur (Malaysia)

Table II
List of test viruses with their used cell cultures sorted by laboratory

Laboratory	Virus AV 5/ Cell culture	Virus MNV/ Cell culture	Virus MVA/ Cell culture
LC001	A549	RAW 264.7	BHK-21
LC002	HEK293	RAW 264.7	BHK-21
LC003	Hep-2	RAW 264.7	BHK-21
LC004	HeLa	RAW 264.7	Vero
LC005	Vero	RAW 264.7	BHK-21
LC006	A549	RAW 264.7	BHK-21
LC007	A549	RAW 264.7	n.d.
LC008	A549	RAW 264.7	BHK-21
LC009	HEK293	RAW 264.7	BHK-21
LC010	HeLa	RAW 264.7	BHK-21
LC011	n.d.	n.d.	BHK-21
LC012	HeLa	RAW 264.7	Vero
LC014	HEK293	RAW 264.7	DF1

'n.d.', not done.
A549, adenocarcinomic human alveolar basal epithelial cells; RAW 264.7, immortalized mouse (murine) macrophage cell line; BHK-21, Baby hamster kidney cells; HEK293, Human embryonic kidney cells 293; Hep-2, immortalized human tumour cells; HeLa, epithelial cells of a cervical carcinoma; Vero, monkey kidney epithelial line; n.d., not determined.

adenocarcinomic human alveolar basal epithelial cells (others were monkey kidney epithelial line cells, human embryonic kidney cells, and immortalized human tumour cells , see Table II). Only one cell culture was used for tests with MNV (immortalized mouse (murine) macrophage cell line from Friedrich-Loeffler-Institut (FLI) (Federal Research Institute for Animal Health), see Table II). For tests with MVA, most laboratories, except three laboratories, used baby hamster kidney cells as cell culture (LC004 and LC012 used Vero cells and LC014 used DF1 cells (chicken embryo fibroblasts, ATCC UMNSAH/DF-1; Embryonic Fibroblast; Chicken (Gallus gallus), Supplier: ATCC CRL3586), see Table II). The viruses were tested as specified in the protocol.

A polyvinylchloride plate free foam FOREX classic (dimensions: 20 cm × 50 cm) was marked with four fields (each 5×5 cm), arranged in a linear row, and spaced 5 cm apart (see Figure 1). At the beginning of each experiment, only test field 1 was contaminated, while test fields 2–4 remained virus-free, thereby enabling the evaluation of virus transfer during wiping. A defined virus inoculum (100 µL) mixed with interfering substance was applied to test field 1 on the surface, evenly spread using a sterile glass spatula, and allowed to dry under controlled conditions. After drying, the wiping procedure was performed using a standardized wipe (Tork Standard wipe, art. no. 90491; 55% pulp, 45% polyethylene terephthalate), premoistened with 16 ml of hard water [12]. To ensure reproducibility, the wipe was folded and fixed beneath a standardized weight (~2.5 kg with 12.1 cm length, 8.6 cm width, and 8.6 cm height). This weight was placed in front of test field 1 and moved across all four test fields in a continuous motion within 1 s towards test field 4, followed by an immediate return movement after test field 4 to test field 1 within an additional second (see Figure 1). This procedure mimics a standardized mechanical wiping action and enables the redistribution of the initial contamination across the surface. Following the defined contact times (1 min, 5 min, 30 min, and 60 min), viral recovery was performed from each of the four test fields using one premoistened and one dry nylon swab (FlOQSwab, Copan Diagnostics, Italy). Recovery from test field 1 was used to quantify the removal and/or inactivation of virus at the site of initial contamination. In contrast, recovery from test fields 2–4 allowed assessment of potential virus transfer during the wiping process, thereby reflecting the risk of cross-contamination. Swabs were transferred into 5 ml of minimum essential medium (MEM) and vortexed for 30 s to release viral particles. As chemical neutralization was not feasible due to potential interference with the cell culture system, immediate serial 10-fold dilutions of the test mixtures in ice-cold maintenance medium were performed. All experiments were carried out in three independent runs.

To ensure data reliability and interpretability, the following controls were integrated into each test run: Initial virus control (VC): A 100-µL aliquot of the virus inoculum with interfering substance was mixed with 5 ml MEM to determine the initial virus titre prior to drying. This control served as the baseline for virus titre determination before surface application and drying. Drying controls without wiping (D_{C0} and D_{Ct}): These controls assessed viral stability on surfaces during the drying process. D_{C0} samples were recovered immediately after drying, quantifying virus loss during desiccation. D_{Ct} was recovered after drying and the respective contact time, serving as the reference value for calculating +log reduction. Cytotoxicity control: To define the limit of detection for each test condition, test field 1 was inoculated with MEM without virus mixed with interfering substance, followed by the standard wiping procedure. The eluate from this control was applied to the cell culture system to identify potential cytotoxic effects of the wipes, swabs, or residual substances, ensuring that measured cytopathic effects in test samples were virus-specific.

Virus titres from eluates were immediately determined using endpoint titration dilution on appropriate cell cultures in accordance with EN 14476:2019 [3]. Results were expressed as lg $TCID_{50}$ (Tissue Culture Infectious Dose 50%)/mL using the Spearman–Kärber–method with 95% confidence intervals, and reductions were calculated by comparing viral titres of test field 1 to virus control titres (after drying and at chosen contact time) [17,18]. Interlaboratory and intralaboratory variability was assessed according to DIN EN ISO 13528 [19]. z-Scores were calculated using PROLab software (QuoData, Version 2023.8.2.0) to assess the laboratories' performances.

Results

Virus controls during desiccation

Twelve out of thirteen participating laboratories provided valid data sets in accordance with the predefined protocol.

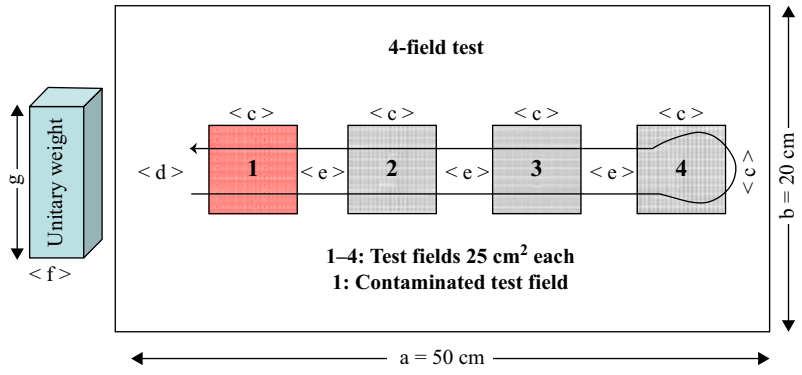

Figure 1. Diagram of the 4-field test showing test surface (20x50 cm) with four test fields (5x5 cm) and stipulated wiping route of the wiping cloth. a=50 cm, b=20 cm, c=5 cm, d=10 cm, and e=5 cm; the f and g dimensions of the unitary weight were at least 8.6 cmx12.1 cm, respectively, and the block weighs 2.5 kg. The wiped area includes test fields 1–4, with the turnaround at test field 4. Test field 1 is contaminated.

Table III
Results and statistical parameters of the virus control before drying (VC), after drying (D_{C0}), and after the specified contact times of 1 min, 5 min, 30 min, and 60 min (D_{c1}, D_{C5}, D_{C30}, D_{C60}) of **AV5** under clean and dirty conditions according to the protocol

		VC	D_{C0}	D_{C1}	D_{C5}	D_{C30}	D_{C60}
Clean conditions	Number of participants	10	10	10	10	10	10
	Mean lg $TCID_{50}$/mL ± 95% CI*	7.06 ± 0.61	6.02 ± 0.42	5.81 ± 0.43	5.79 ± 0.39	5.50 ± 0.32	5.48 ± 0.44
	Repeatability s.d. S_r	0.29	0.36	0.35	0.34	0.34	0.32
	Reproducibility s.d. S_R	1.00	0.73	0.74	0.68	0.58	0.74
Dirty conditions	Number of participants	12	12	12	12	12	12
	Mean lg $TCID_{50}$/mL ± 95% CI*	7.01 ± 0.38	6.05 ± 0.41	5.92 ± 0.45	5.78 ± 0.43	5.59 ± 0.40	5.57 ± 0.45
	Repeatability s.d. S_r	0.35	0.48	0.30	0.37	0.36	0.37
	Reproducibility s.d. S_R	0.72	0.81	0.82	0.80	0.76	0.83

The controls are expressed in lg TCID50/mL with the confidence interval.
The number of participants for each value, the repeatability s.d Sr, and the reproducibility s.d. SR are included.
*CI, Confidence Interval; s.d., standard deviation.

Nevertheless, not all conditions were always tested by all participants. The treatment with hard water did not affect cell morphology and growth or susceptibility for the test organism in the dilutions of the test mixtures, which are necessary to demonstrate a lg of 4 reduction of the virus. Small variations in cytotoxicity with a treatment of hard water on test field 1 can be observed. It is assumed that the swabs, used in the recovery process, can cause a higher cytotoxicity.

The dispensing quantity by the cloth (g) during the wiping process provided by laboratories had a mean value of 0.94 g ± 0.14 g, without generating abnormal z-scores for this parameter by any laboratory. The weight (kg) for wiping used by the laboratories was within the acceptable range of 2.3–2.5 kg and had a mean value of 2.47 kg ± 0.03 kg [12].

Tables III–V present the mean viral titres (10-logarithmic value of the TCID50 titre per millilitre) of the virus controls before drying (VC), after drying (D_{C0}), and after the specific contact times (D_{Ct}) based on the repetitions of the three measurements and the number of laboratories and associated repeatability and reproducibility values for the three test viruses –AV5, MNV, and MVA – under clean and dirty conditions. On average, one lg level is lost during the drying process as can be seen when comparing VC and D_{C0}. The VC represents the baseline maximum recoverable virus without drying, while the drying controls (D_{C0} and D_{Ct}) are used to quantify virus loss during desiccation and to serve as reference points for lg-reduction calculations. For all viruses, a gradual decline in viral titres was observed over time following the drying procedure immediately after being visibly dry (D_{C0}) and after the defined contact time (D_{Ct}), indicating progressive viral inactivation during the drying process.

Under clean conditions, the mean AV5 titre decreased from 7.06 ± 0.61 lg $TCID_{50}$/mL before drying (VC) to 5.48 ± 0.44 lg $TCID_{50}$/mL after 60-min additional waiting time, corresponding to a reduction of approximately 1.6 lg (see Table III). Under dirty conditions, a similar decrease from 7.01 ± 0.38 lg $TCID_{50}$/mL to 5.57 ± 0.45 lg $TCID_{50}$/mL was observed. Repeatability ranged from 0.29 to 0.36, and reproducibility improved from 1.00 at VC to 0.74 at D_{C60} under clean conditions, indicating consistent performance across laboratories at later time points. MNV demonstrated a titre decline from 6.62 ± 0.77 lg $TCID_{50}$/mL to 5.19 ± 0.87 lg $TCID_{50}$/mL under clean conditions and from 6.57 ± 0.57 lg $TCID_{50}$/mL to 4.93 ± 0.70 lg $TCID_{50}$ under dirty conditions, corresponding to a reduction of 1.4–1.6 lg (see Table V). Repeatability was low (0.17 to 0.37), reflecting good within-laboratory consistency, while reproducibility

Table IV
Results and statistical parameters of the virus control before drying (VC), after drying (D_{C0}), and after the specified contact times of 1 min, 5 min, 30 min, and 60 min (D_{c1}, D_{C5}, D_{C30}, D_{C60}) of **MNV** under clean and dirty conditions according to the protocol

		VC	D_{C0}	D_{C1}	D_{C5}	D_{C30}	D_{C60}
Clean conditions	Number of participants	9	9	9	8	7	7
	Mean lg $TCID_{50}$/mL ± 95% CI*	6.62 ± 0.77	5.77 ± 0.60	5.72 ± 0.71	5.58 ± 0.83	5.37 ± 0.90	5.19 ± 0.87
	Repeatability s.d. S_r	0.29	0.17	0.18	0.31	0.22	0.30
	Reproducibility s.d. S_R	1.17	0.91	1.07	1.20	1.20	1.18
Dirty conditions	Number of participants	10	10	10	10	10	10
	Mean lg $TCID_{50}$/mL ± 95% CI*	6.57 ± 0.57	5.80 ± 0.60	5.71 ± 0.60	5.62 ± 0.52	5.27 ± 0.71	4.93 ± 0.70
	Repeatability s.d. S_r	0.21	0.22	0.37	0.36	0.35	0.33
	Reproducibility s.d. S_R	0.91	0.97	0.99	0.87	1.16	1.13

MNV, murine norovirus.
The controls are expressed in [lg TCID50/mL] with the confidence interval.
The number of participants for each value, the repeatability s.d S_r, and the reproducibility s.d. S_R are included.
*CI, confidence interval; s.d.: standard deviation.

Table V
Results and statistical parameters of the virus control before drying (VC), after drying (D_{C0}), and after the specified contact times of 1 min, 5 min, 30 min, and 60 min (D_{c1}, D_{Ct5}, D_{C30}, D_{C60}) of **MVA** under clean and dirty conditions according to the protocol

		VC	D_{C0}	D_{C1}	D_{C5}	D_{C30}	D_{C60}
Clean conditions	Number of participants	9	9	9	9	9	9
	Mean lg $TCID_{50}$/mL ± 95% CI*	6.03 ± 0.46	5.56 ± 0.37	5.50 ± 0.52	5.37 ± 0.58	5.20 ± 0.57	5.01 ± 0.66
	Repeatability s.d. S_r	0.31	0.51	0.48	0.52	0.50	0.50
	Reproducibility s.d. S_R	0.73	0.69	0.87	0.97	0.94	1.07
Dirty conditions	Number of participants	10	10	10	10	10	10
	Mean lg $TCID_{50}$/mL ± 95% CI*	6.09 ± 0.46	5.59 ± 0.41	5.53 ± 0.43	5.56 ± 0.47	5.36 ± 0.49	5.28 ± 0.60
	Repeatability s.d. S_r	0.28	0.32	0.28	0.24	0.43	0.31
	Reproducibility s.d. S_R	0.77	0.70	0.72	0.77	0.86	0.98

MVA, modified vaccinia virus Ankara.
The controls are expressed in [lg TCID50/mL] with the confidence interval.
The number of participants for each value, the repeatability s.d S_r, and the reproducibility s.d. S_R are included.
*CI, confidence interval; s.d., standard deviation.

values (~1.0 to 1.2) indicated greater interlaboratory variability than the other test viruses. For MVA, drying resulted in a smaller titre reduction of approximately 1.0 lg (clean conditions: 6.03 ± 0.46 lg $TCID_{50}$/mL to 5.01 ± 0.66 lg $TCID_{50}$/mL; dirty conditions: 6.09 ± 0.46 lg $TCID_{50}$/mL to 5.28 ± 0.60 lg $TCID_{50}$/mL, see Table IV). Repeatability values remained within 0.24–0.52, and reproducibility was between 0.69 and 1.07. The data indicate relatively high stability of MVA during drying, with comparable results under clean and dirty conditions.

Overall, drying resulted in a modest yet consistent reduction in viral infectivity, with the extent of the effect depending on the virus type. At prolonged contact times, D_{Ct} values may decrease to levels at which demonstrating a lg of 4 reduction becomes challenging (see Tables III–V).

Virus recovery from test field 1

The statistical evaluation of the lg $TCID_{50}$/mL values for the three test viruses – AV5, MVA, and MNV – under clean and dirty conditions using hard water provides insights into the reproducibility and efficacy of the test protocol across varying contact times (1–60 min). Tables VI–VIII summarize the statistical variation in viral inactivation (lg $TCID_{50}$/mL). The observed mean lg $TCID_{50}$/mL detected on test field 1 across all valid laboratories showed a clear trend of reducing lg $TCID_{50}$/mL with prolonged contact time for AV5, MVA, and MNV (see Tables VI–VIII) Supplementary Table S1.

For AV5, mean lg $TCID_{50}$/mL values under clean conditions decreased from 3.73 ± 0.22 lg $TCID_{50}$/mL after 1 min to 2.31 ± 0.43 lg $TCID_{50}$/mL after 60 min. Under dirty conditions, the mean lg $TCID_{50}$/mL values decreased from 3.51 ± 0.54 lg $TCID_{50}$/mL to 2.04 ± 0.38 lg $TCID_{50}$/mL within the same contact period. Repeatability (s.d. S_r = 0.33–0.69) and reproducibility (s.d. S_R = 0.48–0.98) values remained mainly within the acceptable range for both conditions. For MNV, the mean lg $TCID_{50}$/mL values under clean conditions decreased from 3.22 ± 0.86 lg $TCID_{50}$/mL to 2.25 ± 0.87 lg $TCID_{50}$/mL after 60 min and from 3.03 ± 0.58 lg $TCID_{50}$/mL to 1.75 ± 0.46 lg $TCID_{50}$/mL under dirty conditions. The corresponding repeatability values ranged from 0.19 to 0.40, while reproducibility values were between 0.76 and 1.32. For MVA, under clean conditions, mean values declined from 2.88 ± 0.59 lg $TCID_{50}$/mL at 1 min to 2.05 ± 0.70 lg $TCID_{50}$/mL at 60 min. In dirty conditions, a comparable decrease from 2.93 ± 0.39 lg $TCID_{50}$/mL to 2.04 ± 0.52 lg $TCID_{50}$/mL was observed. The repeatability standard deviation ranged between 0.26 and 0.67, while reproducibility standard deviation ranged between 0.73 and 1.30, with higher variability noted under clean conditions.

Table VI
Results and statistical parameters for the virus recovery on test field 1 [lg $TCID_{50}$/mL] of test virus **AV5** after treatment with hard water for 1 min, 5 min, 30 min, and 60 min according to the protocol under clean and dirty conditions

	Conc./time relation	Hard water 1 min	Hard water 5 min	Hard water 30 min	Hard water 60 min
Clean conditions	Number of participants	10	10	10	10
	Mean lg $TCID_{50}$/mL on TF 1 ± 95% CI*	3.73 ± 0.22	3.08 ± 0.50	2.41 ± 0.32	2.31 ± 0.43
	Repeatability s.d. S_r	0.41	0.69	0.68	0.37
	Reproducibility s.d. S_R	0.48	0.97	0.75	0.75
Dirty conditions	Number of participants	12	12	12	12
	Mean lg $TCID_{50}$/mL ± 95% CI*	3.51 ± 0.54	2.78 ± 0.43	2.19 ± 0.39	2.04 ± 0.38
	Repeatability s.d. S_r	0.36	0.36	0.35	0.33
	Reproducibility s.d. S_R	0.98	0.81	0.73	0.71

AV5, adenovirus type; TF 1, test field 1.
The number of participants for each value, the repeatability s.d S_r, and the reproducibility s.d. S_R are included.
*CI, Confidence interval; s.d., standard deviation.

Table VII
Results and statistical parameters for the virus recovery on test field 1 [lg $TCID_{50}$/mL] of test virus **MNV** after treatment with hard water for 1 min, 5 min, 30 min, and 60 min according to the protocol under clean and dirty conditions

	Conc./time relation	Hard water 1 min	Hard water 5 min	Hard water 30 min	Hard water 60 min
Clean conditions	Number of participants	9	8	7	7
	Mean lg $TCID_{50}$/mL on TF 1 ± 95% CI*	3.22 ± 0.86	2.77 ± 0.82	2.51 ± 0.60	2.25 ± 0.87
	Repeatability s.d. S_r	0.30	0.39	0.33	0.19
	Reproducibility s.d. S_R	1.32	1.21	0.84	1.16
Dirty conditions	Number of participants	10	10	10	10
	Mean lg $TCID_{50}$/mL ± 95% CI*	3.03 ± 0.58	2.58 ± 0.45	2.11 ± 0.54	1.75 ± 0.46
	Repeatability s.d. Sr	0.21	0.40	0.36	0.26
	Reproducibility s.d. S_R	0.94	0.78	0.90	0.76

MNV, murine norovirus; TF 1, test field 1.
The number of participants for each value, the repeatability s.d S_r, and the reproducibility s.d. S_R are included.
*CI, Confidence interval; s.d., standard deviation.

Virus transfer from test field 2 to 4

In addition to the viral recovery on test field 1, also the transfer of virus to test fields 2–4 (accumulation factor of test fields: 2–4) was analysed to assess potential cross-contamination during the wiping process. For this purpose, the individual titres on test fields 2–4 were evaluated. This parameter reflects the extent to which infectious virus is redistributed from the initially contaminated area (test field 1) to previously uncontaminated surfaces (test fields 2–4).

Overall, virus transfer to test fields 2 to 4 was detectable at shorter contact times, indicating that mechanical wiping can lead to redistribution of viral particles across the surface. However, with increasing contact time, viral recovery from test field 2 to 4 decreased markedly, and in many cases, no infectious virus could be recovered, particularly under conditions where virus stability was already reduced [see Table VIII]. Especially for long contact times (30–60 min), it is hard to recover any virus from test fields 2–4. Given this strong dependence on virus stability and recovery limits, the expert group decided that defining a fixed quantitative acceptance criterion for transfer, analogous to bactericidal testing, would not be appropriate for virucidal testing. Instead, a comparative approach was adopted, using the water control as a worst-case reference for maximum expected transfer under purely mechanical conditions. This approach with water was intended to maintain a stable baseline below a lg of 4 reduction while still allowing recovery of detectable virus particles. Accordingly, the accumulation of virus on test fields 2–4 in the test product approach should be lower than that observed with the water control. In cases where no residual virus can be found on test fields 2–4 in the water control, the corresponding test product should likewise show no detectable virus on test fields 2–4. This approach allows for a context-dependent interpretation of transfer dynamics while accounting for limitations in virus recovery and environmental stability.

Discussion

In an era marked by emerging respiratory viruses, antimicrobial resistance, and increased public health expectations, effective environmental disinfection remains a cornerstone of infection prevention. Beyond direct person-to-person transmission, indirect transmission via contaminated surfaces and fomites plays a critical role in the spread of many clinically relevant viruses. Numerous outbreak investigations and experimental studies have demonstrated that viruses deposited on frequently touched surfaces can remain infectious for extended periods and contribute to onward transmission, particularly in healthcare and long-term care settings where vulnerable populations are exposed [10,20]. Studies have shown that both enveloped and non-enveloped viruses can remain viable on surfaces for hours to days, thereby contributing to indirect transmission via contaminated fomites [2].

Table VIII
Results and statistical parameters for the virus recovery on test field 1 [lg $TCID_{50}$/mL] of test virus **MVA** after treatment with hard water for 1 min, 5 min, 30 min, and 60 min according to the protocol under clean and dirty conditions

	Conc./time relation	Hard water 1 min	Hard water 5 min	Hard water 30 min	Hard water 60 min
Clean conditions	Number of participants	9	9	9	9
	Mean lg $TCID_{50}$/mL on TF 1 ± 95% CI*	2.88 ± 0.59	2.71 ± 0.86	2.53 ± 0.75	2.05 ± 0.70
	Repeatability s.d. S_r	0.30	0.26	0.67	0.38
	Reproducibility s.d. S_R	0.91	1.30	1.25	1.10
Dirty conditions	Number of participants	10	10	10	10
	Mean lg $TCID_{50}$/mL ± 95% CI*	2.93 ± 0.39	2.63 ± 0.42	2.20 ± 0.40	2.04 ± 0.52
	Repeatability s.d. S_r	0.51	0.46	0.47	0.38
	Reproducibility s.d. S_R	0.74	0.76	0.73	0.87

MVA, modified vaccinia virus Ankara. TF 1, test field 1.
The number of participants for each value, the repeatability s.d S_r, and the reproducibility s.d. S_R are included.
*CI, confidence interval; s.d., standard deviation.

Consequently, there is an urgent need for phase 2/step 2 methodologies that realistically assess the efficacy of surface disinfectant practices, particularly those based on wiping, where mechanical action, liquid release, and subsequent drying play a decisive role.

The virucidal 4-field test was developed to address this gap by transferring a well-established surface test concept from bactericidal and yeasticidal efficacy testing (EN 16615), as well as from sporicidal surface testing (EN 17846), to virucidal efficacy testing. A key rationale for this approach is the recognition that mechanical effects, such as wiping pressure, friction, liquid distribution, and re-drying, can substantially influence virus removal, inactivation, and transfer. These physical effects may either enhance or counteract the chemical activity of disinfectants and are therefore highly relevant for evaluating wiping-based applications, where efficacy cannot be attributed solely to the intrinsic virucidal activity of the formulation. This approach has been discussed within CEN/TC 216 as a potential European Standard (Work Item WI 00216151) [16] and aims to provide a practice-oriented framework for evaluating virucidal surface disinfection under realistic conditions. In addition to assessing reduction in viral load, the test allows the investigation of cross-contamination, mechanical transfer, and application-related variables, aspects that are not captured by suspension tests or surface tests without mechanical action.

The present interlaboratory ring trial was designed to address a central and critical question: whether a standardized wiping-based surface test can be applied to viruses in a reproducible and robust manner and whether the viral load recovered from test field 1 after drying and sampling is sufficiently high to allow the demonstration of a lg of 4 reduction in viral load in product efficacy tests, as required for virucidal claims. In contrast to bactericidal testing, virucidal efficacy testing is frequently hampered by lower initial virus titres, losses during drying, cytotoxic effects in cell culture systems, and the lack of neutralizing agents. To isolate and evaluate the methodological performance of the test itself, the ring trial was deliberately designed to focus exclusively on the mechanical component of wiping by using hard water without active chemical ingredients. This design allowed assessment of virus recovery, transfer, drying effects, and reproducibility independent of chemical virucidal activity.

While current virucidal standards — such as the EN 14476:2025 [21] suspension test, recently published without changes to its technical requirements — are essential for characterizing the intrinsic activity of surface disinfectant formulations, they do not encompass practical application conditions. Moreover, existing surface tests designed for products applied without mechanical action, including EN 16777 [4], do not sufficiently capture the mechanical, physical, and environmental processes occurring during routine wiping-based surface disinfection. This limitation is particularly relevant for surface product administered with wipes (including RTU wipes, surface sprays, and concentrates) [1]. For disinfectants applied on surface by wipes, the interaction between the wipe material and size, the released liquid, mechanical action, and drying dynamics is critical and can lead to substantial differences in efficacy than tests performed without mechanical action. The virucidal 4-field test was therefore conceived to bridge this methodological gap and to provide a more realistic basis for evaluating wiping-based disinfection performance.

Across all test conditions, the data demonstrate a gradual and reproducible reduction in viral load over time for all three test viruses, irrespective of the interfering substance applied (see Tables III—V). The observed decrease in lg $TCID_{50}$/mL for test field 1 confirms the time-dependent nature of virus recovery on surfaces. Each wiping step introduces moisture onto the surface, followed by renewed drying, resulting in repeated cycles of partial virus inactivation. Drying-related losses were observed for all viruses and varied according to viral structure, with more pronounced reductions for the non-enveloped viruses MNV and AV5 than the enveloped MVA (see Tables III—V). MVA exhibited the highest resistance to drying, consistent with its well-documented environmental robustness and previous findings describing its stability across a wide range of physical conditions, including desiccation and temperature fluctuations [22]. In contrast, AV5 and MNV showed more pronounced titre reductions during the drying phase. This observation suggests that, while non-enveloped viruses are generally more resistant to chemical disinfectants, they may be comparatively more susceptible to dehydration-associated stress, as reported for adenoviruses on stainless steel and other hard surfaces [23—25]. These findings are consistent with virus-specific differences in environmental stability and highlight the relevance of drying as a key determinant in surface-associated virus persistence.

The presence of organic material exerted a protective effect on viruses, most likely by partially shielding viral particles from desiccation-induced structural damage. This observation is in line with previous reports describing the shielding effect of organic load against desiccation-induced and stress-induced viral damage for both enveloped and non-enveloped viruses and emphasizing the importance of considering soiling in disinfection efficacy testing [26—28].

Importantly, despite drying-associated losses and virus-specific variability, the overall performance of the test procedure was sufficient to provide an initial viral load high enough to allow a demonstration of lg of 4 reduction under controlled conditions. Demonstrating the feasibility of reliably achieving a lg of 4 reduction was a central objective of the interlaboratory ring trial. While the majority of participating laboratories achieved consistent recovery of the virus, a small number of them experienced greater variability. This variability emphasizes the sensitivity of virucidal surface testing to experimental conditions and underscores the importance of high initial virus titres, extensive laboratory experience with cell culture-based virus quantification, and an awareness of virus-specific behaviour and environmental influences.

Methodological evaluation showed low repeatability values (Sr approximately ~0.2—0.7, see Tables VI—VIII), suggesting consistent intralaboratory performance, while reproducibility values (SR approximately ~0.7—1.3, see Tables VI—VIII) showed moderate interlaboratory variability. Such variability is typical in interlaboratory ring trials involving biological systems and has been reported previously for virucidal and bactericidal efficacy testing. Importantly, the observed reproducibility remained within the expected and acceptable range for interlaboratory ring trials, supporting the overall robustness and reliability of the applied standardized protocol [29,30].

In comparison to bactericidal 4-field testing and spore-based applications such as those described for *Clostridioides difficile* [31], a higher degree of variability was observed in the present study. This is likely attributable to the inherent

complexity of virucidal testing, which relies on two coupled biological systems (virus and cell culture) and is generally less standardized than bacteriological methods (e.g. cell culture of AV5, see Table II). Factors such as differences in cell lines, culture media, and supplements have been shown to significantly influence virus stability and inactivation outcomes [32]. In addition, the test suspension itself is less strictly defined than in comparable bactericidal standards. While bacterial tests specify a narrow concentration range, virucidal testing generally only requires a sufficiently high initial viral titre to allow demonstration of a $\log_4$reduction. As a result, laboratories may enter the test with different starting titres, which may contribute to interlaboratory variability, particularly in the water controls. Furthermore, surface-based practical tests inherently introduce greater variability than suspension tests due to additional factors such as drying dynamics, recovery efficiency, cytotoxicity, and mechanical handling. All in all, it can be said that a great deal of experience is necessary. These findings suggest that variability in virucidal efficacy testing may in part be influenced by parameters that are not fully standardized, such as cell culture conditions, test suspensions, and recovery procedures. Further experience with the method and additional repeatability data may help to better assess the robustness of the approach and improve interlaboratory comparability.

The recently published EN 17846 (2023), which applies the 4-field test principle to *Clostridioides difficile* spores, provides a relevant methodological reference for the present study [13]. Despite fundamental biological differences, comparable effects were observed in the water controls, where reductions of approximately 2–3 lg or even higher were achieved, reflecting the contribution of drying and mechanical removal alone. Transfer to test fields 2–4 was observed in both approaches, confirming that mechanical wiping can redistribute contamination to previously clean areas [33]. In our study, this effect decreased with increasing contact time and was in some cases no longer detectable, consistent with reduced virus stability and recovery. A summary of mean viral recovery on test fields 2–4 is provided in Table VIII. This demonstrates that wiping may not only remove but also redistribute viral contamination if not properly controlled, which supports a comparative evaluation against the water control rather than a fixed acceptance criterion. Overall, EN 17846 supports the robustness of the 4-field test framework while underlining the importance of tailoring the approach to virus-specific characteristics.

Overall, the ring trial demonstrated that the modified 4-field test for surface virucidal efficacy produced consistent and reproducible results across different laboratories, virus types, and time points. From a broader perspective, these findings have important implications for standardization and regulatory evaluation. The ability to reproducibly assess surface virucidal efficacy under realistic wiping conditions supports the inclusion of mechanical action as a mandatory component of future surface disinfection standards. For manufacturers, such a standardized approach provides a more meaningful framework for product development and performance claims, while for regulators and end users, it offers greater confidence that test results reflect real-world application scenarios. Robust virus recovery from test field 1 for AV5, MVA, and MNV confirmed consistent application, wiping and drying conditions across laboratories. The ability to generate meaningful results in the absence of active chemical agents further validates the mechanical and procedural integrity of the method itself. Occasional deviations, reflected by isolated z-score outliers, could be attributed to differences in parameter such as wiping pressure, pH values, cloth saturation, or local handling procedures, underlining the necessity of strict procedural standardization in future applications.

The present ring trial was intentionally designed to focus on viral recovery and mechanical reproducibility, using hard water. Future studies should address cell line variability, test suspension, sources, media, handling the weight under the bench, and cytotoxicity control, as even minor differences in these parameters can substantially affect virus detection limits and result interpretation. This represents a limitation of the current study design and should be addressed in future investigations.

In conclusion, the modified virucidal 4-field test represents a robust and reproducible tool for assessing surface virucidal efficacy under standardized clean and dirty conditions. It's particularly relevance lies in the evaluation of wiping-based disinfection, especially for RTU wipes, where mechanical action, liquid release, and drying behaviour jointly determine performance. The establishment of a harmonized virucidal wiping test therefore constitutes a critical prerequisite for realistic product evaluation and provides strong scientific support for the timely development of a corresponding European standard (CEN WI 00216151) [16]. While a higher degree of variability than bactericidal or sporicidal applications was observed, this reflects the inherent complexity of virucidal testing due to two biological systems and highlights the need for further standardization. Further interlaboratory ring trials should be conducted with the objective of evaluating disinfectants taking into account the different ways of application of surface products with a wipe (soaking/spraying etc). The feasibility with disinfectants must be tested, as well as the extent to which variations occur between laboratories.

CRediT authorship contribution statement

K-M. Roesch: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **M. Eggers:** Writing – review & editing, Validation, Resources, Methodology, Investigation, Conceptualization. **N. Bonner:** Resources, Methodology, Investigation. **F.H.H. Brill:** Writing – review & editing, Resources, Methodology, Investigation, Conceptualization. **M. Cavalleri:** Resources, Methodology, Investigation, Conceptualization. **J. Gebel:** Writing – review & editing, Supervision, Software, Methodology, Investigation, Funding acquisition, Conceptualization. **B. Großjohann:** Resources, Methodology, Investigation. **K. Konrat:** Resources, Methodology, Investigation. **C.S. Lee:** Resources, Methodology, Investigation. **S. Loeffert:** Writing – review & editing, Validation, Resources, Methodology, Investigation, Conceptualization. **N.T. Mutters:** Supervision, Funding acquisition. **D. Paulmann:** Writing – review & editing, Validation, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **F-A. Pitten:** Resources, Methodology, Investigation. **C. Rubrecht:** Resources, Methodology, Investigation, Conceptualization. **I. Schwebke:** Writing – review & editing, Methodology, Investigation, Conceptualization. **K. Steinhauer:** Resources,

Methodology, Investigation, Conceptualization. **C. Hildebrandt:** Writing — review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

Ethical approval

Not required.

Data availability

The datasets used and/or analysed during the current study are available from the first author on reasonable request.

Declaration of Generative AI and AI-assisted technologies in the writing process

The author(s) declare that generative AI (ChatGPT) was used exclusively to assist with language editing, grammatical refinement, and improvement of clarity of expression. The author(s) reviewed and edited the manuscript and take full responsibility for its content.

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Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2026.05.040.

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