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Review

Methods to assess environmental surface disinfectants against viruses: the quest and recommendations for a globally harmonized approach to microbicide testing

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SUMMARY

Viruses pose a wide-ranging and significant risk to human health through acute and persistent infections that may confer risks for sequelae including musculoskeletal, immunological, and oncological disease. Infection prevention and control (IPAC) remains a highly effective, generic, global, and cost-effective means to mitigate virus spread. IPAC recommends proper disinfection of high-touch environmental surfaces (HITES) to reduce the risk of direct and indirect virus spread. The United States, Canada and many other countries mandate pre-market assessments of HITES disinfectants against viruses and other types of microbial pathogens. However, there are basic disparities in the regulation of disinfectants. Such incongruity in test protocols interferes with the determination of the true breadth of the microbicidal potential of a given product in the field where target pathogens are often unknown or may be encountered as mixtures. This review examines the various methodological disparities and recommends a more cohesive and harmonized approach. While there is particular emphasis on viruses here, an overall harmonization in microbicide testing of HITES disinfectants will greatly assist the numerous stakeholders involved in IPAC.

Background and introduction

Since their discovery in 1901 [1], human pathogenic viruses have been incriminated in the aetiology of many serious and wide-spread ailments. A systematic analysis reveals over 200 virus types that are either causative or associated with acute disease. This number is continually rising with on average, three new types identified each year [1–3]. Table I is a listing of human pathogenic viruses that have been discovered over the last 25 years.

The threat of pathogenic viruses is not only limited to acute infection. Secondary bacterial and fungal infections are common in the infected, particularly in the context of those causing immunodeficiency such as HIV and HTLV-1 [4]. In addition, there is an increased risk of certain non-communicable diseases associated with persistent viral

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Table I
Human pathogenic viruses discovered in the past 25 years (modified from Sudhan & Sharma, 2020 [65])

Name	Year of discovery	Type	Host	Mode of spread	Status
Puumala virus	1980	Enveloped	Rodent, Human	Aerosol	Rare
Hepatitis E virus	1983	Non-Enveloped	Human, Pigs	Food, Water	Endemic in various countries
Torovirus	1984	Enveloped	Humans, Pigs	Food, Water	Endemic
Rotavirus C	1986	Non-Enveloped	Humans, Pets	Water	Endemic
Sin Nombre	1993	Enveloped	Humans, Rodents	Rodents and Aerosols	Endemic
Hendra virus	1994	Enveloped	Human, Pigs, Horses, Bats	Bodily fluids	Endemic
Menangle virus	1998	Enveloped	Humans, pigs, bats	Bodily fluids	Endemic
Nipah virus	1999	Enveloped	Humans, bats, pigs	Bodily fluids	Endemic
Metapneumovirus	2001	Enveloped	Humans, Avian	Respiratory secretions	Endemic
SARS coronavirus	2003	Enveloped	Humans, mammals	Respiratory secretions	Eliminated
Bocavirus	2005	Enveloped	Humans	Respiratory secretions	Endemic
Parvovirus 4 (genotype 2)	2005	Enveloped	Humans	Respiratory secretions	Endemic
Klassevirus	2009	Non-Enveloped	Humans	Food,	Endemic
H1N1pdm	2009	Enveloped	Humans, avians, pigs	Respiratory secretions, bodily fluids	Eliminated
MERS (Middle East Respiratory Syndrome	2013	Enveloped	Humans, Dromedaries	Respiratory secretions	Rare
mPox (West Africa clade)	2017	Enveloped	Humans	Bodily fluids	Epidemics
SARS-CoV-2	2019	Enveloped	Humans, other mammals	Respiratory secretions	Pandemic
Langya	2022	Enveloped	Humans, shrews, rodents, dogs	Respiratory secretions	Rare

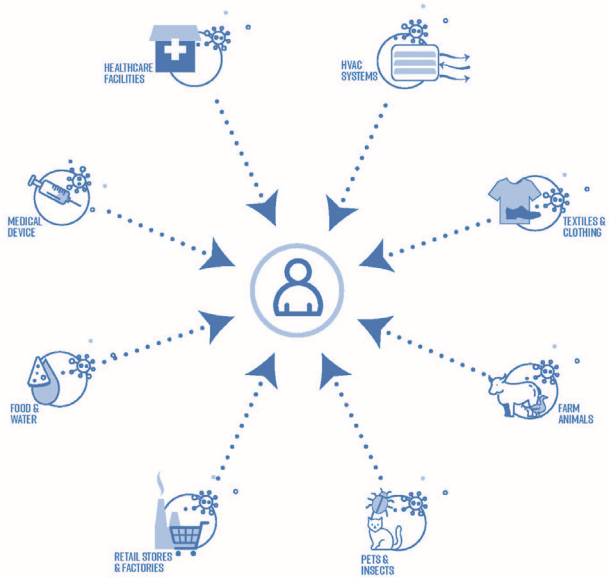


Figure 1. Vehicles known to be associated with environmental spread of human pathogens including viruses.

infections. For example, several viruses have been incriminated in the aetiology of benign or malignant tumours [5]. Other potential diseases associated with viral infection include autoimmunity [6], cardiovascular and kidney diseases [7], diabetes [8], mental health disorders [9], multiple sclerosis [10] and putative links with Alzheimer's Disease [11].

Drug therapy and vaccination have been the public health hallmarks of reducing the impact of viral infections. A different approach to reducing the risk of infections involves inactivation and/or removal of pathogens from a given vehicle prior to contact with a susceptible host. This process, also known as Infection Prevention and Control (IPAC), has become a significant factor in reducing the burden of infections in general. From an environmental perspective, a major risk of pathogen spread lies with high-touch environmental surfaces (HITES) [12—14] and as such, an appropriate IPAC strategy must be incorporated to reduce the potential for pathogen spread via HITES. A successful strategy must include the following criteria:

(1) ***Generic in nature:*** The deployment of an effective IPAC measure against major types of HITES-borne pathogens can interrupt their spread.
(2) ***Preventative approach:*** By its very nature, IPAC constitutes a preventative approach. In contrast, it is generally not feasible or economical to administer anti-viral drugs as prophylactics on a community-wide basis. Nor are such drugs currently available with a broad-spectrum of anti-viral activity. The same limitations apply to vaccines, particularly the injectable kind.
(3) ***Universal applicability:*** HITES disinfection can be applied at a variety of sites using the same products and procedures with a documented safety profile and, often without the need for specialized/expensive devices.
(4) ***Lower cost of product development and registration for use:*** The introduction, testing and registration of disinfectants for use on HITES can be much faster and less expensive compared to drug and vaccine development.

While these principles are easy to appreciate for direct contact, the application of their approach is more complex due to the reality of pathogen spread through indirect contact. This phenomenon, generally referred to as 'environmental spread' [15], is mediated via a variety of vehicles, many of which are also HITES, that may not be naturally instinctively associated with a risk for infection spread (Figure 1).

For the most part, interruption of both direct and indirect spread can be accomplished using chemicals with known microbicidal activity against viruses [16]. Research has shown that an increased emphasis on the use of certain types of antimicrobials — disinfectants — can considerably reduce the potential of HITES as vehicles for viruses [17—19].

While there is little doubt on the benefits of using disinfectants in a HITES-targeted IPAC strategy, the effectiveness of individual disinfectants must continually be questioned by those responsible for implementing these measures. Since the dawn of disinfectants in the 1800s [20], test methods have been developed to ensure that disinfectants are able to inactivate and/or kill their microbial targets. Currently, numerous national regulatory agencies determine whether an active ingredient or a formulated product containing it meets the criteria for sale in each jurisdiction. However, the focus has been on pathogenic bacteria or their surrogates [21]. Testing

Table II
Test methods for virucidal activity

Medium	European Union	United States	Canada	Australia
Hard nonporous surfaces	EN 14476 EN16777	ASTM E1053 (810.2200)	ASTM E1053	ASTM 1052 ASTM 1053 EN 14476
Air disinfection	EN 14476 EN 17272	EPA accepted protocol based on ASTM E3273	ASTM E3273-21	EN17272
Textiles	EN 14476 ASTM E2274 ASTM 2406	Modified E1053; New EPA Method/Guide	Modified E1053	NA
Laundry	EN 14476 ASTM E2274 ASTM 2406	ASTM E2274 and ASTM E2406	ASTM E2274 and ASTM E2406	NA
Wipe	EN 14476 EN 16777	ASTM E2362	ASTM E1053	NA

for virucidal activity became available although its adoption has been slow at best. For example, AOAC International still does not include any methods for testing virucidal activity [22].

Table II lists the main regulatory agencies around the world that review and register HITES disinfectants for sale in their respective jurisdictions. The regulatory agency such as U.S. Environmental Protection Agency (EPA) or standards-setting organizations may validate the performance of such test protocols.

While many countries do not have a formalized system for regulating microbicidal agents, some rely on one of the listed entities as their guide, leaving a large part of the world with no indigenous regulations. This fact came to the forefront in face of the COVID-19 pandemic, with an upsurge of need for conventional as well as innovative technologies for HITES decontamination [23].

Regulatory agencies, even in relatively high-income regions of the world, simply do not have the resources to keep pace with such a demand. Perhaps more troubling is the rise of HITES disinfectant technologies for which standard testing models are not reflective of their applicability. A good example is the upsurge of interest in products with long-lasting microbicidal activity [24]. It is incumbent upon regulatory agencies to weigh the microbicidal activity of a technology against its safety for humans and the environment [25] while addressing the need of stakeholders such as disinfectant manufacturers, end-users, and testing laboratories.

Scope of the review

While the concerns are universal for all microbicidal products, there is a greater priority on regulation of virucidal products due to the rise of viral threats worldwide. This review aims to find a path to a harmonized protocol for virucidal testing through a dissection of the disparities that exist between current protocols and subsequent synthesis of recommendations that can act as a foundation for protocol development. This review will also provide perspective on the development of protocols through the lens of various stakeholders such that protocols will gain universal confidence in the effectiveness of disinfectants. In the process, we will express our views on the relative strengths and shortcomings of existing standard methods as mandated by a critical review.

Harmonization of test methods

It should be noted that the idea of harmonization had not been a significant priority in the IPAC community for quite some time. That changed with the COVID-19 pandemic. As the causative agent was a virus, SARS-CoV-2, the hope for universal virucidal testing turned into a need and agencies were required to face their regulatory shortfalls. Yet, instead of focusing on developing harmonized protocols, The EPA introduced the 'Emerging Pathogens Policy' [26] on acceptable virus surrogates for COVID-19. While this approach appeased the varied requests of stakeholders (Table III) for some kind of protocol, it was also one that was afforded by chance. The change in policy was due to the fact the causative virus was a coronavirus, for which there are already test methods in existence for HITES disinfection [27]. However, should this have been a completely new virus for which there are no known test methods, this approach may not have been possible.

Table III
Stakeholder benefits from a harmonized test protocol

Disinfectant manufacturers	• Reduced cost and speed of product development • Improved stringency in testing • Higher relevance to actual product use • Simpler label claims
Regulators	• More unified and cohesive product registration regulations
End-users	• Better reflection of microbial contamination under field conditions • Higher confidence in the overall quality of the label claims • Label claims easier to read and follow • Testing based on reduced and more field-relevant contact times

At the bare minimum, a test method needs to ascertain several parameters associated with microbicidal activity. The method needs to be universal such that the most critical components of the test, such as reagents, can be sourced from numerous different suppliers. The test itself needs to be reproducible both within an institution as well as across external laboratories with minimal statistical error. All tests should also be relatively user friendly to reduce the necessity for significant and often expensive training of laboratorians. Most importantly, harmonization requires that all testing, irrespective of the type of target microbe under test, be conducted in a similar fashion such that there is no difference regardless of the environment in which the test is being conducted. Such an approach is essential to determine the true width of microbicidal activity of a given formulation.

While there appears to be global agreement on many of these tenets, disparities do exist in test methods between various regulatory jurisdictions as well as standards-setting organizations. Despite decades of discussions between various groups, there was no consensus on how best to harmonize methods so that all jurisdictions could act in a similar manner. By the turn of the millennium, it was clear that an organization needed to spearhead the efforts to at least determine the challenges such that a strategy for harmonization could at least be discussed if not implemented.

The Organization for Economic Co-operation and Development (OECD, Paris, France), with its 38 (as of May 2023) industrialized member countries, took on the task in 2002 when it hosted the first workshop with 100 invited participants (Washington, D.C.) on methods to test HITES disinfectants [28]. *Inter alia*, the focus of the workshop was on a comparison of available quantitative carrier test protocols to zero in on one with the greatest potential for harmonized testing against the five major classes of human pathogens (bacterial spore-formers, vegetative bacteria, fungi, mycobacteria and viruses). This was with the expectation that such a harmonized and unified method would be adopted by all the OECD member countries, thus contributing to the OECD's mandate for reducing trade barriers across the globe by creating a level playing field.

Issues requiring harmonization

The OECD has held several more workshops in Europe and North America and organized round-robin testing with generally encouraging results using the candidate test protocol. Concurrently, the EPA is also pursuing effort with its own resources (with participation from the U.S. industry and Canadian disinfectant testing laboratories through ASTM International) to include the testing against viruses as a part of this exercise in developing a harmonized method for HITES disinfectants. However, there continues to be a lack of congruence among various jurisdictions. Below are the main components of the test that still require harmonization.

Laboratory

Obviously, for any such work to be performed, a laboratory must be properly credentialed as a minimum Containment Level 2 working environment and be compliant with Good Laboratory Practice (GLP) procedures. In addition, staff require proper safety training to ensure that they are kept safe from viruses and other types of pathogens, which can locally spread not only from surfaces but also as droplets and aerosols.

As viruses (except bacteriophages) need *in vitro* cultures of eukaryotic cells, laboratories require staff training and additional resources for equipment (e.g., liquid nitrogen dewars and ultra-low freezers) and protocols for storing and handling such cells. Other required equipment for virucidal testing include gas-infused incubators, and autoclaves. Not surprisingly, these additional requirements for a microbiology laboratory limit the number of facilities capable of conducting virucidal testing. In this context, the laboratory is not considered an issue for harmonization.

Methodology

Currently, the U.S. ASTM's method E1053 is widely accepted for testing the virucidal activity of HITES disinfectants. While E1053 is relied upon and can be readily adapted to work with bacteria and fungi for harmonization, there are several concerns with its basic design: (a) the microbial inoculum volume (0.2 mL) as well as the volume of the test formulation (2.0 mL) to be overlaid on it are too large to be reflective of HITES decontamination; (b) the inoculum volume per carrier would also deplete more quickly than the generally limited and high-titered infectious virus pools needed for testing; (c) the need for adding at least 2.0 mL of a neutralizer to the carrier further dilutes the amount of any viable virus; and (d) the use of gel columns to remove the active ingredient(s) unavoidably adds 10—15 seconds to the contact time.

It should also be noted here as to how critical proper neutralization of the microbicidal activity of the test formulation is in E1053 before scraping the carrier surface to collect the test inoculum; a lack of proper timing here can readily turn the carrier test into a suspension test protocol. Additionally, while EPA has allowed adaptation of the method to support the registration of viral claims for towelette products for decades, the current version of E1053 does not incorporate specific instructions for use of towelettes. An ASTM work group plans to add this language in the upcoming revision based on the testing conducted since 1985. Additionally, in 2015, the development of the wipe test has offered hope for a more harmonized future regarding the now oft used towelettes.

Yet, even this supposedly simple approach has become complicated as two versions of the wipe test method were unveiled in 2015. They were the ASTM approved wipe test method, E2967 [29] and the EU ring trial tested EN 16615 [30]. Both were designed to re-create the real-world experience while maintaining statistically reproducible results. However, only one addressed the above concerns, namely E2967, while the other merely modified the currently approved protocol, EN14476 and added a wiping step [31]. The differences between the two methods highlight the impact of tradition and preference.

The EN 16615 test, also called the 4-field test, utilizes tiles (5 X 5 cm or 2-inch squares) of polyvinyl chloride with a polyurethane coating, to assess the bactericidal and yeasticidal activities of HITES disinfectants with manual wiping. The test microbes on each tile are collected separately via a swab and the swabs individually dipped into tubes with the recovery medium. From a purely IPAC perspective, there are several issues with this approach. The size of the tiles would not permit their complete immersion and elution in a relatively small volume (say, 1—5 mL depending on the size of each carrier) of eluent. Neither the swabbing of the tiles nor the dipping of the swabs into a recovery medium lend themselves well to virus recovery with potential for variability in the results. The relatively smooth surfaces of the tiles may compromise the stringency of the testing [28]. And finally, the wiping is manual allowing for significant inter- [21] and intra-user variability and statistical error [32].

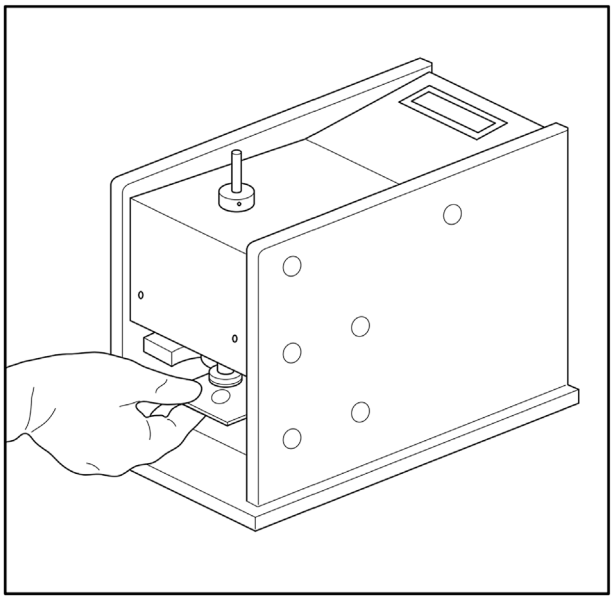


Figure 2. Schematic of the Wiperator (Based on ASTM standard E2967). This device called 'the Wiperator', is designed to wipe hard, non-porous surfaces with a disinfectant-soaked wipe under defined conditions of pressure during the contact, duration of wiping as well as the number of wiping strokes during the set wiping time. It accepts carriers (1 cm diam.) of metal or other common hard, non-porous environmental surfaces with a dried inoculum on them. The device is suitable for work all major classes of pathogens.

In contrast, the E2967 has a base in the ASTM E2197 Quantitative Disk Carrier Test, published in 2002. But the protocol changes such that it includes a mechanical wiping device known as the Wiperator (Figure 2). The Wiperator is designed to provide a consistent wiping motion that reduces the error associated with manual wiping [14]. While this may appear to be a similar approach to EN 16615, the difference lies in the removal of variability using automation. The results are significantly more reproducible and can be utilized in any environment without worry of inter- or intra-laboratory variation.

The emergence of the EN16615 and the E2967 and their different approaches to testing bring up a long-standing question with respect to harmonization. Is relevance to use conditions more important than reproducibility? Tests should be closer to the practical application conditions under worst-case scenarios. However, there is also the need for reproducibility and repeatability. As wiping is a rather generic term that could mean a variety of actions depending on the individual, a significant amount of training and practice would be needed to ensure the reproducibility required to meet the requirements for approval. On the other hand, a mechanical system can meet the required stringency.

Contact time

For government registration, all HITES disinfectants must state a contact (dwell) time on the product label. Until recently in North America, the predominant contact time on product labels for HITES disinfectants was 10 minutes, but there is a trend towards contact times as short as a few seconds, which is more reflective of a given product's field use [33].

Test carriers

This is certainly among the most crucial considerations in designing a harmonized test for virucidal activity. As compared to working with bacteria and fungi, producing pools of high-titered viruses can be expensive and technically demanding, thus requiring economy in their use. A proper carrier for testing should allow for the accurate placement of a relatively small (e.g., 10—20 μL) test inoculum, making flat carriers more suitable for this. Also, the size of the carrier should allow for its complete immersion in a relatively small (e.g., 1.0—5.0 mL) volume of an eluent/neutralizer to permit the processing of most, if not all, of the eluent.

The surface of the carrier should be reasonably uneven (e.g., brushed) to represent HITES surfaces with uneven topography under field conditions. Surfaces with an uneven topography also add to the stringency of the testing by assessing the ability of the test product to better penetrate areas where pathogens may be sequestered. It is assumed here that a product that works on an uneven surface will also work on a smooth one. Disks of stainless steel AISI 304 would be one type of carrier recommended for use in a harmonized test protocol. Swabbing and scraping of the carrier surface to recover the microbial inoculum is not considered desirable because of their inefficient and variable recovery rates.

If desired, the carrier disks of stainless steel or other types of hard, nonporous materials as HITES can be readily rendered magnetic by painting one side of the carrier with a magnetic paint (e.g., Rustoleum brand); such commercially available paints are non-toxic and can also readily withstand autoclaving. In our experience, magnetized disks are easier to hold in place during their washing and rinsing by placing a block magnet outside the bottom of Nalgene vials.

Soil load

In nature, pathogens are released from the host in one form of body fluid causing environmental contamination. Pathogens may gather additional contaminants from the surroundings once released. Such contaminants may protect the viability of the discharged pathogen while also potentially interfering with the activity of the disinfectant applied. Thus, in disinfectant testing, a 'soil load' (as a mixture of organic and inorganic substances) is added to the test microbial suspension to simulate the presence of such contaminants to better field conditions.

Often, 5% (final concentration) fetal bovine serum (FBS) is used as the soil load [34]. We regard this as unsuitable due to its: (a) high cost, (b) supply chain issues, (c) presence of specific and non-specific inhibitors against test microbes, (d) potential for batch-to-batch variations and (e) possible contamination with viruses. To address these limitations, a tripartite soil load consisting of bovine serum albumin (large molecular weight protein), bovine mucin (mucilaginous substance) and yeast extract (short peptides with amino acid terminals) could be considered. A blend of these chemicals [35] is added to the test microbial suspension to yield a total level of protein of 5% which is equal to that in 5% FBS. All three components of the soil load mentioned above are available from reputable biological supply houses and are relatively inexpensive. The mixture is compatible with test viruses and their host cells as well as with bacteria and fungi to be used in the testing.

In Australia, most virucidal claims may use the same soil; however, claims made against bloodborne pathogens such as HIV must demonstrate activity in the presence of 50% whole blood as soil [36]. The mandated use of whole blood needs better justification while also requiring additional controls and safety issues. It is also incongruous considering that HITES are often manually cleaned to remove gross contamination before disinfection. The need for blood would be invalidated.

In contrast to this generally harmonized view on the soil load, the European Union has a multi-structured approach that focuses on the purpose rather than the active. For example, disinfectants destined for use in healthcare must demonstrate activity in the presence of 0.3g/L BSA, which is denoted as 'clean', and a combination of 3g/L BSA and 3mL/L sheep erythrocytes, which is called 'dirty' [37]. For food contact surfaces, clean is the same as healthcare while dirty removes the requirement for erythrocytes. [37].

While the two-tiered approach of 'clean' and 'dirty' may appear to reflect real-world settings, there is little likelihood that the clean situation would be of any relevance. Indeed, even the EU biocidal products regulation (BPR) states that all testing should reflect the worst-case scenario, which in this case would be the 'dirty'. While there continues to be little agreement on the harmonization of soil load, there is significantly more evidence to suggest a universal approach may be the most appropriate.

Neutralization/removal of virucidal activity

As mentioned earlier, to ensure the accuracy of the label-claim, the microbicidal activity in the test formulation must be safely and effectively removed/neutralized right at the end of its contact time. This presents varying degrees of challenge depending on the nature of the active ingredient and its concentration while also making sure that the neutralization process is harmless to the viability of the test microbe(s) and innocuous for any host cells involved when dealing with viruses.

When possible, a simple dilution of the test formulation/microbe mixture at the end of the contact time may render it non-microbicidal. However, this dilution-based approach is only feasible when the viability titer of the test microbe(s) is high enough to enable the demonstration of the required level of $\log_{10}$ (or percent) reduction to meet the product's effectiveness criterion. In some instances, the formulation/microbe mixture was ultracentrifuged to sediment out the test virus [38], which was then resuspended for the testing. Obviously, this is not a desirable approach for its dependence on an ultracentrifuge and the inevitable and substantial extension in the contact time.

Alternatively, one or more chemicals (e.g., sodium thiosulfate, or lecithin) may be added to the product/microbe mixture at the end of the contact time to chemically neutralize the microbicidal activity. To achieve a physical separation of the active ingredient from the mixture, it can be passed through a molecular sieve (e.g., Sephadex), which can retain relatively small molecules and lets the test microbe (in the form of large molecules), pass through. In contrast with chemical neutralization, the use of such gel columns inevitably adds to the contact time.

There is little doubt innovation is needed to neutralize the virucidal activity of microbicides safely and effectively at the end of the contact time. This is particularly important in view of the rapid reductions (one minute or less) in contact times being claimed on product labels [39].

Test results

There continues to be a significant discrepancy in the way the results for virucidal activity are determined and reported. Most often it is done through assessments of the 50% tissue (cell) culture infectious dose or $TCID_{50}$ $CCID_{50}$. This approach is popular because it is relatively simple, less expensive and less time-consuming. However, it is semi-quantitative at best while relying entirely on the skill and experience of the operator to read a given virus's cytopathology.

An alternate approach is the use of plaque assays to determine the viability titer of the test virus. Doubtless, this approach can be more expensive and takes more time to perform. However, its results are more accurate and less subjective, while also recognizing that not all test viruses may readily form plaques. On the other hand, it is quantitative, which requires an assessment based on the actual counting of plaques the direct equivalent to counting colonies of bacteria and fungi.

The choice of one over the other comes down to the overall requirement of the test method. If the goal is to simply determine if there is virucidal activity, the $TCID_{50}$ may be best as it can support high throughput and determine whether an active may be suited for the intended claim. However, if the goal is to have an accurate assessment of the reduction in the number of viable virus particles, which is essentially the need for any test method, then the plaque assay is the best option.

While these two tests are the most common, there are others that may be considered moving forward. The focus forming assay is a modification of the plaque assay in which antibodies are used to identify the virus as it is causing infection. However, the test relies on a monoclonal or polyclonal antibody. While it may be faster than the plaque assay, it may not be as easy or cost-effective.

In addition to infectivity tests, PCR or RT-PCR tests may also be used to quantitate the level of virus within a test environment. This is both economical and fast. However, there is the crucial issue of viability. While a plaque assay and $TCID_{50}$ will provide information on the viability of the virus, PCR or RT-PCR can only demonstrate the presence of viral genetic material. This does not equate to viability and may lead to inaccurate results.

Surrogates for human pathogenic viruses

It goes without saying that a 'surrogate' can never be the 'real thing'. Yet, it is neither technically possible nor safe to handle certain types of human pathogenic viruses in laboratories that conduct R&D and/or routine testing of microbicides. This is because certain types of viruses are not generally available for the testing even from repositories such as the American Type Culture Collection (ATCC) or national agencies such as the Centers for Disease Control and Prevention (CDC) or the Canadian Public Health Agency (PHAC). In view of this, a hierarchy was developed based on the susceptibility of different types of vertebrate viruses to chemical disinfectants [40]. This topic was also the subject of a workshop organized by the EPA in Feb. 2016 [41].

When a surrogate is needed, its choice is based on the following factors: (a) ready availability, (b) safety and suitability for work at biosafety/containment level 1 or 2, (c) ability of growth in cell cultures to high enough titers to assess the product performance criterion, (d) the ability to withstand the inoculum drying process on test carriers well enough to accommodate the assessment of the product performance criterion, (e) microbicide susceptibility expected to be at least equal to that of the target virus, and (f) ability to yield results in a reasonable period of time.

Both Canada and the U.S. require testing against each type of virus to be claimed on the product label. However, and as far as we know, attempts are underway at the EPA to change the situation in favor of accepting surrogates with research and development underway [42].

Globally, the issue of the use of viral surrogates became more urgent when the World Health Organization (WHO) placed an embargo on the use of the Sabin vaccine strains of polioviruses in anticipating the eradication of poliomyelitis [43]. To comply with the embargo, all laboratories were directed to purge their stocks of virulent as well as vaccine strains of polioviruses. However, countries such as the U.S [43,44] still allow the use of the vaccine strains of polioviruses in disinfectant testing with an updated vaccination status of the staff and needed biosafety precautions. Nevertheless, polioviruses no longer should be considered as an available option among virus surrogates. One of several non-polio enteroviruses may take their place, but thus far there is no consensus on which enterovirus.

For a specific claim, a virucidal agent must be tested against the actual type of virus described. In the case of a need for a surrogate virus, such as hepatitis B virus, hepatitis C virus, and human norovirus, the choice should be a closely related species such as duck hepatitis virus, bovine viral diarrhea virus, and mouse norovirus/feline calicivirus, respectively. Currently, where the use of surrogates is allowed, the list differs depending on the jurisdiction, but tends to include several of the following:

- human adenovirus type 5 (U.S., CAN, EU)
- parvovirus (bovine, canine, or murine) (U.S., CAN, EU, Australia Therapeutic Goods Administration (TGA))
- murine norovirus (EU)
- herpes simplex virus (TGA)
- modified vaccinia virus Ankara (MVA) (EU)
- poliovirus type 1 (U.S., EU, TGA)

As mentioned above, one of the most concerning issues with this list is the continued requirement for testing against the live vaccine strains of poliovirus. There is an ongoing effort to eradicate poliomyelitis and restrictions on the use of polioviruses will widen across all sectors including test laboratories. Although testing against MVA is not mandatory, that virus also need not be used considering the fact numerous other enveloped viruses are available for use including (e.g., bacteriophage Φ6), [45—48] for Ebola [48] and SARS-CoV-2 [49]). Considering the continued introduction or re-emergence of novel viral pathogens such as mPox and Ebola, for example, the EPA has initiated the development of a guideline on 'Emerging Viral Pathogens' with industry collaboration [26].

In terms of potentially harmonizable guidelines for virus species, there are three that should be considered. First, murine norovirus or feline calicivirus (FCV) to represent small, non-enveloped viruses and replace polioviruses. Next, adenoviruses, which will suffice as medium-sized non-enveloped viruses. Finally, Phi6 to represent enveloped viruses. The coliphage MS-2 is another potential surrogate for small, non-enveloped viruses; it is easier and safer to handle and has already been accepted by the U.S. EPA as a surrogate in testing against such viruses to study indoor air decontamination.

The feline calicivirus (FCV) is recommended as a surrogate for testing the virucidal activity of HITES disinfectants because of its attributes as listed. It is recognized that certain classes of microbicides (e.g., quaternary-based formulations) may require reformulation for activity against it, FCV would be considered a good predictor of the general virucidal activity of HITES disinfectants. However, some investigators have found FCV to be less stable under acidic and alkaline conditions [50].

End-user friendliness

Although end-users are not considered among the main foci of a test method, they are an important stakeholder. The ultimate success of a given formulation in IPAC depends on how well an end-user applies it in the field [51]. Therefore, the label claims and use directions must not only be readily understandable by end-users but also relevant to field use. Label directions requiring different dilutions of the same product for specific uses against different types of pathogens has the potential for misuse of the product. In addition, the request for label claims against emerging viruses such as Ebola, Marburg, and SARS-CoV-2 suggests having a broad-based virucidal claim may better streamline the current EPA Emerging Viral Pathogen Guidance [26].

Briefly, the guidance focuses on the ability of a formulated disinfectant product to kill one of the three different types of viruses, enveloped, large non-enveloped, and small non-enveloped. If the virucidal active was able to inactivate the above-mentioned recommendations of murine norovirus, adenovirus, and MS2, there would be no need to categorize actives. They would most likely be applicable for all emerging viruses as well and thus could receive approval for label claims faster.

Apart from the test organism, another component of user friendliness is the contact time. In essence, a lack of compliance at the user level is to be expected. Ideally, users will allow for wet contact between the disinfectant and the surface for that period of time before neutralizing, rinsing, or wiping. However, it has long been known that there is a significant discrepancy between the contact times stated on the label and the actual contact times during use. A lack of patience or available time on the part of the user renders the label contact timeaspirational. Moreover, in the case of some enveloped viruses, such as SARS-CoV-2, longer contact times may allow for detergents to be considered as disinfectants. For example, a contact time of 10 minutes in soapy water can reduce the concentration of the virus by the required 4 log in a suspension test. Of course, when a proper carrier test is performed, that number drops to less than 0.5 log [52].

Other factors to consider

As much as virus inactivation is an important aspect of approvals, there are numerous concerns that have grown in importance regarding the field use of disinfectants. As harmonization is discussed, it may be time to consider adding these criteria to any guidance for approvals.

Low toxicity

One Health has become a significant factor in the realm of public health. Humans must be aware of animals and the environment as decisions are made regarding their use of products *en masse*. Based on the Berlin Principles of One Health [53], a product should be active when used but either inactive or inert when disposed. While some products are rendered this way when placed into the drain, such as chlorine-based disinfectants, hydrogen peroxide, and citric acid, others have been known to persist in the environment and may potentially lead to toxic effects. Regulatory agencies such as the EPA and Health Canada currently review each disinfectant active for its potential to cause chronic issues. That then determines the labeling directions for the disposal of a given product.

A standardized *in vitro* or if possible *in vivo* test such as the zebrafish embryo acute toxicity test or the OECD Fish, Acute Toxicity Test [54] for compatibility with the environment may help to alleviate the concerns with respect to toxicity.

Does not contribute to antimicrobial resistance

As mentioned in the Guidance on the Biocidal Product Regulation [55], disinfectant chemicals should be screened for their potential to generate cross resistance to antibiotics. Further, for over 30 years, certain disinfectant actives have been suspected of contributing to the emergence of resistance

in various bacterial species. The most known example is triclosan, which was first suspected in 2000 [56] and eventually banned from soaps in 2016 [57]. Although the mechanism of contribution was only identified in 2020 [58], the association was enough to cause doubt in the active and eventually lead to its removal. Numerous laboratory studies have demonstrated the potential or actual development of resistance not only to antibiotics but to the disinfectants themselves [59]. While individual laboratories have developed tests to identify the contribution, there is a gap at the test method level to provide assurances that widespread use of the active will not lead to resistance either in the local or downstream environments. A concerted effort to test for the development of resistance with exposure should be considered. The triclosan example may be a harbinger of more to come particularly amid the significant increase in the use of disinfectants during the COVID-19 pandemic. While no link has yet been established, there are already concerns that we may see even more antibiotic resistant isolates in the years to come.

Material compatibility

Disinfectants can be harsh on materials and possibly lead to degradation, corrosion and weakening of the overall structure. This is especially important considering some of the novel methods for disinfection do not adhere to the more common liquid on a surface model. Many disinfectant companies employ methods to determine the compatibility of their products with various surfaces. However, there is no single harmonized method to test disinfectant compatibility. Although this may not be a priority in the context of harmonized test methods, a possible method may already exist in the airline industry [60]. However, a careful review of that method is essential to ascertain its suitability for HITES disinfectants in general.

The continuing search for broad-spectrum and fast-acting HITES disinfectants

Despite decades of experience with the formulation and use of HITES disinfectants, there remain major gaps in the technology. Up to a time, the pathogens for inactivation were bacteria only. This is illustrated by the fact AOAC International, a standards-setting organization, continues to lack any test protocols for virucidal activity despite an otherwise broad-based coverage of its standards. While in North America, testing using suspension protocols is allowed in limited situations, other jurisdictions continue to rely on such testing (e.g., in EU). By their very nature, suspension tests for HITES disinfectants are easier to pass and not predictive of the activity of a given formulation in carrier tests. Therefore, such suspension tests may be limited for use in the initial R&D and not for any regulatory purposes.

Testing with a mixture of microbial pathogens as another approach to protocol harmonization

In the field, we may encounter more than one type of pathogen on a given HITES. Thus, it is conceivable that in such situations a disinfectant with a limited microbicidal potential may leave behind on the treated surface one or more harder to kill pathogens. Could this issue be addressed by testing HITES disinfectants using a mixture of HITES-borne pathogens for a broad-spectrum outcome? The feasibility of such an approach has been demonstrated using a mixture of five types of human pathogens representing vegetative bacteria (*Staphylococcus aureus*), a mycobacterium (*M. bovis* BCG), a filamentous fungus (*Trichophyton interdigitale* conidia), a small non-enveloped virus (Sabin strain of poliovirus type 1) and a bacterial spore former (*Geobacillus stearothermophilus*). With this protocol, 11 different types of commercially available formulations were tested showing the breadth of their microbicidal activity in a carrier test with a soil load [61].

Example of a harmonized but static test protocol

ASTM International's test protocol E2197 exemplifies a harmonized but static method (meaning no surface wiping action involved) for testing HITES disinfectants [27]. This method was developed in the early 1980s with financial support from the EPA and was eventually adapted as an ASTM standard. Its mandate was indeed to design a test protocol which would use essentially the same basic materials and method across all major groups of human pathogens (spore-forming bacteria, vegetative bacteria, fungi, mycobacteria, and viruses) except the microbial culture and quantitation methodologies.

Disks (one cm in diam.) of brushed stainless steel are used in it as archetypical hard surface carriers. Each disk receives a relatively small microbial inoculum (10 μL) with an added soil load. The inoculum is dried and overlaid with 50 μL of the test substance for a defined contact time of as short at 10 seconds under ambient conditions. In testing for virucidal activity, each disk is eluted in only one mL of an eluent/neutralizer and the eluates assayed for viable viruses in cell cultures along with the needed controls. It, therefore, represents a closed system allowing for the capture of all viable input viruses in control and test samples. The use of brushed carriers increases the level of stringency of the testing by better simulating the uneven topography of environmental surfaces in the field. The application of a small volume of the test substance better represents the field application of HITES disinfectants while making it easier to neutralize the active ingredients and reducing the loading of the environment. This harmonized test protocol has already been successfully used to test HITES microbicides against vegetative bacteria, fungi [62], mycobacteria [63], bacterial spore-formers [64], and viruses [35].

Example of a harmonized and dynamic test protocol

While E2197 is a static protocol (no wiping or mechanical action), the afore-mentioned Wiperator method (E2967) expands the application of E2197 to the dynamic in-use method [29]. All the parameters of the test are the same as E2197 including carrier, soil load, inoculum, drying time, and neutralizer. The difference lies in the addition of a wiping and transfer step, which is accomplished with the Wiperator. The method not only tests the efficacy of disinfection of the test surface but also quantitatively examines the transfer of pathogen from one surface to another via the wipe itself. This addition to the test results closes an important gap in IPAC, namely, the potential for transfer of pathogens through the

wipe substrate. By ensuring that both the test surface and subsequently touched surfaces remain at acceptably low levels of microbes, the protocol reveals a possible future for test methods in which the overall disinfection action is tested as opposed to simply the disinfectant itself.

More recently, another protocol has been developed to quantitatively assess the disinfection of HITES by wiping in a field-relevant manner [14]. This protocol as described in Figures 3 and 4 utilizes a Teflon-based platform with nine brushed stainless-steel disks (1 cm in diam.) embedded in it. Each disk is contaminated with 10 μL of the test microbial suspension in a soil load and the inocula dried under ambient conditions. The disks are wiped with a disinfectant-prewetted wipe in a manner simulating field use. The disks are then separately and simultaneously retrieved in plastic vials with 1.0 mL of an eluent/neutralizer for viability assays. Alternatively, the contaminated disks on the platform can be sprayed with the test disinfectant and then wiped. The used wipe can be tested on a separate platform with sterile disks to assess any viable microbial transfer.

This platform is another possible approach for a harmonized test protocol as it has already been successfully tested with two types of vegetative bacteria [14] as well as aerobic and anaerobic bacterial spore-formers, *Candida albicans* and vertebrate viruses (hepatitis A, MNV, coronaviruses 229E and OC43), attesting to its potential as a candidate dynamic test for harmonization.

Limitations to harmonization

Although both protocols are well established and have been shown to be universally feasible, there are challenges to wide-scale implementation. One of the most contentious is the concept of soil load or interfering substances. The harmonized approach is highly stringent and will only allow certain products to achieve the required reduction. However, some regulations do not require this level of stringency for some products to be able to make claims. That reduced stringency, however, is not an issue that stems from the protocol but rather the intent of the jurisdiction. This may stand in the way of a harmonized protocol.

Perhaps even more contentious is the issue of contact time. Non-enveloped viruses in general require longer contact times to achieve the appropriate level of inactivation. Asking a user to wait 10 minutes or longer for proper a $\log_{10}$ reduction is neither appropriate nor in line with field usage of a given product. However, a shorter contact time may not achieve an acceptable level of activity in the harmonized test method. This again is not an issue with the protocol but the interest of stakeholders, particularly those who manufacture and use these products. In essence, no matter what a label claim might say, impatience may be hard to overcome.

This naturally leads to the greatest issue with harmonized test methods, the choice of test virus(es). At the outset, it must be stated that surrogates are not 'perfect' but ersatz in nature when it comes to selecting one for microbicidal tests. Moreover, despite the availability of attenuated strains of viruses and surrogates such as mouse norovirus, feline calicivirus, adenoviruses, and enveloped and non-enveloped bacteriophage, there continues to be a focus on antiquated test agents, particularly attenuated polioviruses and MVA. This limitation is again separate from the test. Instead, it is a matter of tradition. Perhaps ironically, this is the issue that could be resolved the quickest as both viruses are targets of eradication which has already been achieved for smallpox and soon anticipated for poliomyelitis. Quite simply, pox- and polioviruses should not be used no matter at what biosafety level. However, the use of these viruses continues to be recommended in test methods as does another traditional method for enumerating them, the Median Tissue Culture Infectious Dose

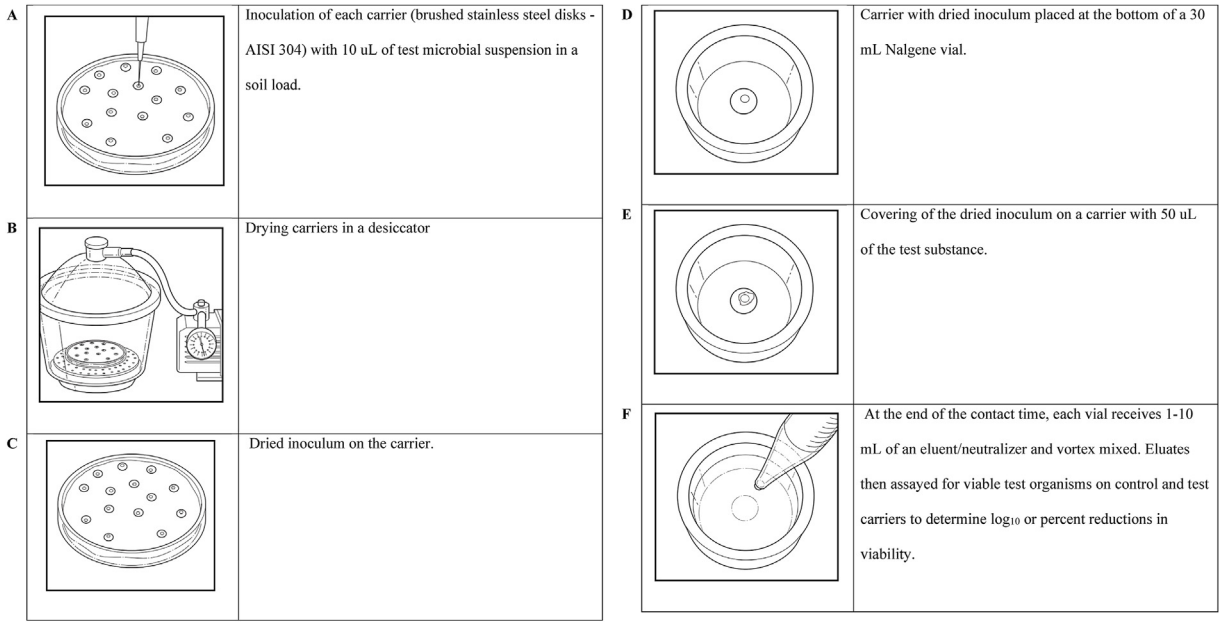


Figure 3. Materials and main steps in the static disk-based, quantitative carrier test (Based on ASTM method E2197).

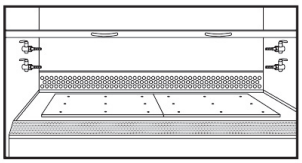
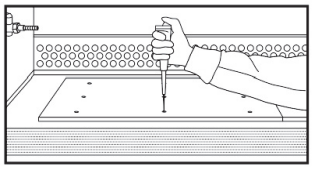
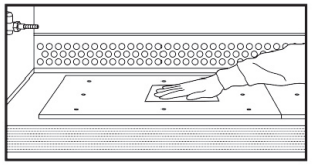
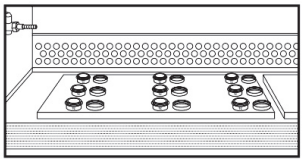
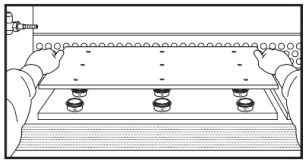
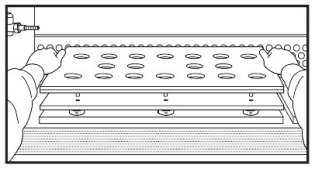
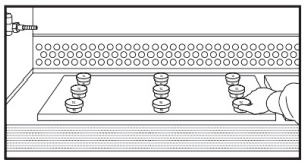
A		Two carrier-loaded platform sitting inside an operating biosafety cabinet
B		The carriers on the platform (contaminated platform) to the left receiving test microbial inoculum for subsequent drying.
C		The platform with the contaminated carriers is being wiped with a prewetted fabric in a standardized fashion. The used wipe is then applied over the platform with the clean carriers to study microbial transfer.
D		Each platform is placed over a tray containing nine plastic vials to separately capture the carriers directly into an eluent/neutralizer.
E		Carrier retrieval device with nine prongs to push the carriers into the collection vials.
F		The carrier retrieval device being placed on the platform for carrier collection.
G		The tray with the vials containing the retrieved carriers for viability assays.

Figure 4. The materials and main steps in the dynamic, disk-based test for HITES disinfectants (Based on ASTM E2197).

($TCID_{50}$). Despite the value and accuracy of a plaque assay, which can be done with all the viruses mentioned above, there continues to be a lack of movement towards it. The slight extra effort needed for plaque assays compensates for their accuracy.

Can we achieve a harmonious future?

Table IV provides a list of recommendations that address many of the concerns listed above and can be found in the examples of harmonized static and dynamic test methods. Most

Table IV
Basic tenets in the design of a harmonized carrier test protocol to assess the microbicidal activities of HITES disinfectants

Tenet	Purpose	Comments	Recommendations
Methodology	To develop a carrier test protocol which uses common and basic materials and procedures to assess HITES disinfectants against all major classes of human pathogens.	The protocol will entail the use of basically the same materials and procedures to test the microbe under investigation. The test microbe and the procedures for its culture and quantitation will necessarily be different.	Harmonized methods need to be designed to accommodate the special needs of viruses. They should focus on reproducibility such that the results can be provided with confidence to testing laboratories. While practical application is an obvious parameter, it should not come at the sacrifice of strong statistically significant data.
Contact time	To ensure that the end-user applies the product for the required duration to achieve the desired result.	To reduce the disparity between product label claims and actual field use of a given product. This is particularly important in a harmonized test protocol to make sure that the contact time can achieve the required level of inactivation of all the pathogens in the mixture.	Harmonized test methods must allow for the incorporation of much shorter and field-relevant contact times which can be determined and reproduced accurately during the testing. The length of the contact time must also take into consideration the realities of how long it would take for an operator to perform the testing.
Assessing the breadth of microbicidal activity	To cover testing against all major groups of human pathogens including vegetative bacteria, spore-forming bacteria, enveloped and non-enveloped viruses, mycobacteria and filamentous and non-filamentous fungi.	Such testing would enable the stakeholders to assess the breadth of the formulation's microbicidal activity under a harmonized set of test conditions.	Testing of microbicides should utilize the same harmonized test methods over a broad scope of test agents.
Microbes to be tested	Any pathogen or its surrogate relevant to the type of claim to be made.	The test microbe to be selected must allow ready *in vitro* culture to high enough viability titers to comfortably assess the formulation's effectiveness criterion.	Testing of microbicides against viruses should not be based on the 'fear factor' alone and in the absence of evidence for the potential of a given virus to spread via HITES. A good example here is that of the human immunodeficiency virus (HIV). Although HIV can cause a serious and potentially fatal infection, it can rarely spread by environmental means. Besides, its enveloped nature makes it relatively fragile outside the body of the host and susceptible to the action of even mild detergents (e.g., soap) and disinfectants [66,67].
Input level of microbial target	The formulation to be tested must meet a certain performance criterion with regards to its microbicidal activity to qualify for registration.	Such a performance criterion should be reasonable in terms of $\log_{10}$ or percentage reductions in viability after exposure to the test substance. However, the criterion must be set	In case a mixture of target microbes is used in the testing, testing must be performed to make sure that individual members of the mixture are not incompatible with each other.

		considering the technical limitations of generating pools of viable organisms as well as the test microbe's ability to withstand the drying of the inoculum on test carriers. Ideally though, a health risk-based approach would be highly desirable in this context. However, such information on all major classes of HITES-borne pathogens does not exist to enable this. We, therefore, recommend a minimum 3-$\log_{10}$ (99.9%) reduction in the viability of all test organisms to create a level playing field and an attainable target across the board.	
Soil load	To be added to the test microbial suspension to represent the presence of body fluids and other contaminants.	A soil load must be harmless to the test microbe as well as its host cells, if any; it should also be readily and widely available as well-characterized chemicals. It is recognized that this issue needs further discussion.	The tripartite soil load should be adopted for harmonization in view of its safety, low cost, ready availability, reproducibility, and compatibility with a wide variety of viruses, their host cells and other types of test organisms.
Carriers	Relatively small (e.g., 1.0 cm diam.), flat disks of stainless steel with a brushed surface are recommended as archetypical HITES.	Disks of other materials with similar dimensions may be used, if desired. The recommended size and shape of the carriers permits their accurate inoculation with relatively small (e.g., 10 μL) volumes of the microbial inoculum. In the case of a static test method it permits the exposure of the dried inocula to 20 −50 μL of the test substance.	Test carriers should be harmonized to a brushed steel surface of not more than 1 −2.5 cm in diameter (or one inch square) with the ability to be covered with no more than 50 μL of the test liquid. These can be availed readily due to the ubiquitous nature of stainless steel. Carriers with other types of HITES materials may be used in the testing in addition to the default metallic carriers for added label claims.
Neutralization	The eluent should contain a validated neutralizer for the immediate quenching of the test formulation's microbicidal activity	This crucial step requires the addition of a single chemical or a combination of chemicals with validated activity against the active(s) in the test formulation. The chemical(s) used for the purpose must be known to be compatible with the test microbe and its host cells, if any.	The use of chemical neutralizers and/or gel columns should continue as an effective means of arresting the virucidal activity. However, this aspect of virucidal testing requires further research and development.
Carrier elution procedure	By complete immersion of each carrier into a relatively small volume of the eluent/neutralizer.	When working with viruses, a 1.0−5.0 mL of the eluent/neutralizer is recommended to allow for the assay of most or all the eluate for accuracy of the results. Volumes as large as 10 mL/ carrier may be used when working with other classes of test microbes followed	

(*continued on next page*)

Table IV (*continued*)

Tenet	Purpose	Comments	Recommendations
		by membrane filtration of the eluates or their dilutions.	
Viability assay	For viruses, tissue culture infective dose 50% ($TCID_{50}$) or plaque assay. For the other microbes, spread-plating or membrane filtration.	Plaque assays for viruses and membrane filtration for bacteria and other types of microbes are recommended for their higher accuracy, allowing the processing of most or all the eluate sample and direct visual indication of the results. Membrane filtration for bacteria and fungi can not only lower the limit of detection but can also assist in the removal of disinfectant residues by washing of the filters.	Whenever possible, the use of plaque assays in testing virucidal activity should be considered. The use of PCR- and RT-PCR-based titrations are not considered suitable in this context as opposed to their use in testing clinical samples. The use of plaque assays also provides a better means to assess cytotoxicity and virus neutralization.
End user friendliness			Incorporate the user as a stakeholder in the development of harmonized test methods and the corresponding use directions. This includes considering a lower maximum contact time and field-relevant directions to ensure compliance.
Wiping of carriers for dynamic testing	While 'static' test protocols require no wiping of the carriers, that is an essential component in 'dynamic' methods.	While there is a wide variety of substrates available for on-site wiping of HITES, testing must be based on one or two most common types of wipe materials respecting cost and time limitations. In addition, the selection of biodegradable/compostable wipe substrates would be preferable to reduce the burden on the solid waste stream.	A static test protocol may be used for the initial assessment of a formulation's microbicidal activity. However, and whenever possible, that should be followed up with testing using a suitable dynamic method to better the field use of the formulation.,
Style of wiping	The number of wiping strokes applied during the wiping period as well as the style of wiping of contaminated carriers is crucial in any dynamic test protocol.	The disks can be wiped for disinfection in a back-and-forth or circular motion. Either one of these would be acceptable if the process is described well in the report. The EPA provides guidance in the matter [68].	Surfaces to be disinfected are wiped in different ways by different operators. For consistent results, the same style of wiping should be chosen and used in assessing a given formulation.
Weight applied during the wiping	The weight applied during the wiping of the carriers will impact the efficiency of the decontamination process.	This is a variable and difficult to control aspect of carrier decontamination during the manual wiping process. One approach would be to place a sand bag with 1 kg weight over the wiping hand during the contact with the carrier being wiped while applying no extra pressure [14]. The Wiperator method allows for the application of at least two levels of weight during the wiping [14]	The use of the Wiperator largely addresses this issue.

of these are either already implemented in some fashion within one or more test protocols. Those that are not can be adopted in any laboratory regardless of income status. Yet even with these recommendations, it is fair to ask whether a harmonious future with regards to the testing and use of HITES disinfectants against all major classes of pathogens is a realizable objective. Harmonization is not an easy task and will take time and significant compromise to be achieved. A harmonized and potentially more reproducible test protocol may also prove to be more stringent to pass against viruses for certain types of formulations. However, considering the most recent pandemic, and the threat of others that could be even more devastating than COVID-19, there is an unprecedented need to come back to the table and resume the process. The OECD experience with hard, non-porous surfaces was challenging but also enriching as it proved harmony can be achieved. There is hope that the same can happen for virucidal testing in time for the next major viral challenge to public and indeed global health. The only question is whether we are ready to make it happen or, to paraphrase Pogo, will we come to see the enemy is not the virus, but rather, ourselves.

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SAS developed the original concept of this article. JAT and SAS conducted the literature review and synthesized the findings. SAS, JAT and BZ contributed equally to writing the initial draft and reviewed the final draft.

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The authors declare no competing interests.

Ethics

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