CHAPTER 12

Chemical Disinfection Strategies Against Food-borne Viruses

Syed A. Sattar and Sabah Bidawid

1.0. INTRODUCTION

The impact of food-borne viral pathogens on human health can be substantial (Lüthi, 1997; Meade et al., 1999; Appleton, 2000; Sair et al., 2002) even though significantly fewer viruses than bacteria can spread via foods (Cliver, 1997). General difficulties in recovering and identifying viruses from foods and clinical specimens collected during food-borne outbreaks grossly underestimate the true role of viruses as food-borne pathogens (Collins, 1997; Bresee et al., 2002; Koopmans et al., 2002), reinforcing the need for proper inactivation of food-borne viruses. The main focus of this chapter is on the testing and application of chemicals (microbicides) that can be used to inactivate viruses on inanimate and animate food contact surfaces as well as for the decontamination of foods consumed raw or with minimal processing. Table 12.1 defines the common terms to be used in this chapter.

2.0. BASIC CONSIDERATIONS

Whereas the use of microbicides in reducing the risk from food-borne infections is widespread, there are many aspects of this practice that require reevaluation, especially those for interrupting the spread of food-borne viruses. A clear understanding of the following factors is necessary for the development of any successful strategy for the use of microbicides in preventing and controlling the spread of food-borne viral infections:

1. Hepatitis A virus (HAV), an important food-borne pathogen, can survive better than many enteric bacteria on inanimate and animate surfaces (Sattar et al., 2000); recent studies have shown this to be the case with caliciviruses as well (Bidawid et al., 2003).
2. A microbicide shown to be effective against vegetative bacteria may not be suitable in inactivating viruses, particularly the nonenveloped ones (Ansari et al., 1989).
3. Unlike many types of bacteria, viruses cannot replicate in contaminated foods; thus, holding of foods at an inappropriate temperature as such is not a risk factor in case of viral contamination. But viruses may remain viable in contaminated foods for several days, especially under refrigeration.

Table 12.1 Glossary of Common Terms Used in this Chapter

Term	*Explanation*
Antimicrobial agent	A physical or chemical agent that kills microorganisms or suppresses their growth.
Antiseptic	An agent that destroys pathogenic or potentially pathogenic microorganisms on living skin or mucous membranes.
Carrier	An inanimate surface or object inoculated with a test organism.
Cleaning (precleaning)	Removing, by physical and/or chemical means, visible soil, dirt, or organic debris from a surface or object.
Microbial contamination	The presence of viable microorganisms in or on a given material or object.
Decontamination	Freeing a person, object, or surface of harmful microorganisms, chemicals, or radioactive materials.
Disinfectant	A physical or chemical agent that destroys pathogenic or potentially pathogenic microorganisms in or on inanimate surfaces or objects.
EBSS	Earle's balanced salt solution.
Eluate	An eluent that contains microorganism(s) recovered from a carrier.
Eluent	Any solution that is harmless to the test organism(s) and that is added to a carrier to recover the organism(s) in or on it.
Chemical microbicide	A chemical that kills pathogenic or potentially pathogenic microorganisms in or on inanimate surfaces/objects and on living skin/mucous membranes.
High-level disinfectant	A chemical or a mixture of chemicals that is bactericidal, fungicidal, mycobactericidal, and virucidal; may also be sporicidal with an extended contact time.
Intermediate-level disinfectant	A chemical or a mixture of chemicals that is bactericidal, fungicidal, mycobactericidal, and virucidal, but not sporicidal.
Label	Written, printed, or graphic matter on, or attached to, the microbicide containers or wrappers.
Low-level disinfectant	A chemical or a mixture of chemicals that kills only vegetative bacteria and enveloped viruses.
Microbicide (microbiocide)	A physical or chemical agent that kills microorganisms.
Neutralization	Quenching the antimicrobial activity of a test formulation by dilution of the organism/test formulation mixture and/or addition of one or more chemical neutralizers to the mixture.
OTC	Over-the-counter topicals.
Pathogen	Any disease-producing microorganism.
Pesticide	Any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest.

Table 12.1 *Continued*

Term	*Explanation*
Potency	The degree of strength or power of a microbicide to render disease-causing microorganisms noninfectious.
QCT-1	Quantitative carrier test—tier 1.
QCT-2	Quantitative carrier test—tier 2.
Sanitization	A process that reduces the microbial load on a surface or object.
Soil load	A solution of one or more organic and/or inorganic substances added to the suspension of the test organism to simulate the presence of body secretions, excretions, or other extraneous substances.
Sterile	Free from living microorganisms.
Sterilization	A process that kills all forms of microbial life.
Stringency of test method	The level of rigor, strictness, or severity built into the method to reflect factors the test formulation may encounter under in-use conditions.
Test formulation	A formulation that incorporates antimicrobial ingredients.
$TCID_{50}$ (50% tissue culture infective dose)	The dilution at which 50% of all infected cell cultures show evidence of virus infection.
Test organism	An organism that has readily identifiable characteristics. It also may be referred to as a surrogate or a marker organism.
Use-dilution	The level to which a concentrated microbicide is diluted for use.
Virucide (viricide)	An antimicrobial agent that kills (inactivates) viruses.
Water hardness	The measure of the amount of metallic (e.g., calcium) salts in water.

4. Foods such as shellfish harvested from fecally polluted waters do not lend themselves readily to decontamination by chemicals.
5. Hands can readily acquire or donate infectious virus particles under conditions encountered during the handling and preparation of foods (Sattar and Springthorpe, 1996).
6. Suitable microbicides, when properly used, can interrupt the transfer of viruses from contaminated surfaces to foods (Bidawid et al., 2000).
7. Safety considerations exclude the use of certain types of microbicides (e.g., phenolics) on food contact surfaces (Gulati et al., 2001).
8. Microbicides often used on food contact surfaces are neither required to nor are tested against common types of food-borne viruses.
9. In the United States, there are no officially accepted methods for evaluating the virucidal activity of handwash and handrub agents; nor is there any regulatory framework to allow such products to make claims against viruses (Sattar et al., 2002).

10. Recognized flaws in current methods to assess microbicidal activity can compromise the label claims of disinfectants in general.

3.0. TEST METHODOLOGY TO DETERMINE VIRUCIDAL ACTIVITY

The virucidal potential of microbicides is normally assessed by "suspension" or "carrier" tests (Springthorpe et al., 1986; Springthorpe and Sattar, 1990; Quinn and Carter, 1999). In suspension tests, a known volume of the challenge virus, with or without a soil load, is mixed with a 5- to 10-fold larger volume of the test microbicide. For control, the virus is suspended in an equivalent volume of a liquid known to be harmless to the virus. The mixtures are held for a defined contact time at a specified temperature, neutralized to stop virucidal activity, titrated for infectious virus, and the degree of loss in virus viability calculated (ASTM, 2002a). While suspension tests are easier to perform, they are also easier to pass (Sattar et al., 1986; Abad et al., 1997) and are thus suitable for screening the activity of microbicides under development. Regulatory agencies in North America do not accept claims of virucidal activity based on suspension tests for product registration purposes.

Under most field conditions, the target virus is present on an animate or inanimate surface. In view of this, carrier tests, where the challenge virus is first dried on a representative surface and then exposed to the test formulation, are considered more suitable in assessing the potential of microbicides under in-use conditions (Springthorpe and Sattar, 1990).

4.0. FACTORS IN TESTING FOR VIRUCIDAL ACTIVITY

4.1. Test Viruses

With the exception of certain blood-borne viruses, the U.S. Environmental Protection Agency (EPA) so far does not accept surrogates in tests for virucidal activity of microbicides but requires that a given product be tested against each virus to be listed on the product label. On the other hand, Health Canada currently allows for a general virucidal claim when a given product shows the required level of activity against the Sabin strain of poliovirus type 1 (CGSB, 1997). The use of this nonenveloped virus, which is also safe to handle and is relatively resistant to microbicides, makes product development easier and label claims simpler and reliable. However, one or more substitutes for poliovirus may be needed soon in view of the anticipated eradication of poliomyelitis and the expected ban on the laboratory use of all types of polioviruses (Aylward et al., 2003).

What should one look for in selecting viruses to assess the activity of microbicides against food-borne viruses? Fortunately, the list of major food-borne viruses is short, and identification of potential surrogates for them is easy. The two most suitable viruses in this regard would be cell

culture–adapted strains of HAV (e.g., HM-175) and the F9 strain of feline calicivirus (FCV). Indeed, investigations in the past decade have already demonstrated the feasibility of using such strains in testing disinfectants and antiseptics to be used in settings where foods are processed and handled (Doultree et al., 1999; Gulati et al., 2001; Sattar et al., 2000). Rotaviruses can also cause food-borne outbreaks (Sattar et al., 2001), and it is feasible to use the cell culture–adapted Wa strain of human rotavirus (HRV) to evaluate microbicides against them (Sattar et al., 1994).

HAV shows the highest level of microbicide resistance of the food-borne viruses tested so far (Mbithi et al., 1992), and it would thus make a good surrogate if the selection were to be based on this factor alone. Working with this virus has become safer because an effective vaccine against it is now available. The possible drawbacks in the use of HAV are that the turnaround time for test results is at least 1 week and that many formulations in current use may fail against this virus. This points to the need for further discussions on the justification of using one or more surrogates in testing microbicides against food-borne viruses, and a consensus between major stakeholders is needed on which virus(es) may be the most suitable for this purpose.

4.2. Nature and Design of Carriers

The three categories of surfaces to be discussed here are inanimate nonporous environmental items that may contact foods during storage, preparation, and serving; fruits and vegetables that are consumed raw or with minimal processing; and hands of food handlers.

4.2.1. Environmental Surfaces

Food contact surfaces vary widely in their nature, usage, and level of cleanliness. The microtopography of a given surface may also change with the type and extent of use, which may provide either more or less protection to viruses deposited on it (Springthorpe and Sattar, 1990). Because it is impractical to test microbicides on all types of food contact surfaces prior to product registration, it would be logical to develop and use a “surrogate” surface. The selection of such a surrogate, inanimate, food contact surface should take the following into consideration: (a) how frequently it contacts foods and hands of food handlers; (b) how readily it releases infectious viruses it carries; (c) it must not inactivate the test virus or irreversibly bind or sequester it such that virus elution from it becomes difficult; (d) its surface should be uneven enough to represent those in the field; (e) if meant for reuse, it should readily withstand repeated decontamination and sterilization; and (f) it should be resistant to microbicides commonly used in decontamination of food contact surfaces.

Further, any carriers made out of such a surrogate material should allow the convenient deposition of the desired volume of the test virus as well as the test microbicide, and the entire carrier should be submersible in a reasonably small volume of the eluent without any wash-off. The need for keeping the eluent volume per carrier as small as possible is particularly rel-

evant when working with viruses, because unlike tests against bacteria, membrane filtration cannot be readily used to trap viruses from large volumes of eluates. The need for cell cultures for detection and quantitation of infectious virus in test samples also restricts the eluate volumes that can be easily and economically processed.

Disks (~1 cm diameter) of brushed stainless steel offer almost all the desired attributes of a surrogate surface in testing microbicides against food-borne viruses (Springthorpe and Sattar, 1990; Sattar and Springthorpe 2001a, 2001b). The microtopography of the disk surface is sufficiently uneven, and the carriers can be handled in a closed system so that wash-off of the test virus does not occur. If needed, disks similar in size to those described above can be readily prepared from other types of food contact surfaces (Lloyd-Evans et al., 1986). Porous materials can also be made into disks as carriers (Traoré et al., 2002), but they are generally more difficult to work with in testing microbicides because their absorbent nature reduces the efficiency of recovery of test organisms. Besides, such materials are rarely meant to be decontaminated using microbicides.

4.2.2. Food Items

In view of the potential of fresh produce to spread viruses (Seymour and Appleton, 2001), such items may be treated with microbicides before consumption (Beuchat, 2001). The use of microbicides for this purpose requires that they be evaluated for their virucidal efficacy on representative types of vegetables and fruits that are eaten raw or after minimal processing. A carrier test using small disks or pieces of items such as lettuce or strawberries represents a feasible approach (Bidawid et al., 2000).

4.2.3. Hands

Although virucides intended for use on human skin are often tested using hard inanimate surfaces, comparative testing has found skin to present a stronger challenge to microbicides (Woolwine and Gerberding, 1995). This reinforces the need for using carriers of a suitable animate surface for evaluating the virucidal activity of formulations for the decontamination of hands. Virucides can be tested using the entire surface of both hands of an adult subject (ASTM, 2002a), but the disadvantages of such an approach include high variability in results, inability to run controls and test samples simultaneously, lack of statistical power, and the need for larger volumes of high-titered virus pools (Sattar and Ansari, 2002). The fingerpad method (Ansari et al., 1989), which is a standard of ASTM International (2002a), avoids these drawbacks by using the thumb- and fingerpads of adult subjects as *in vivo* carriers. In this method, the test virus is placed on targeted areas on the hands, allowed to dry, and then exposed to a handwash or handrub formulation for a suitable contact time. It also allows for the determination of reduction in virus infectivity after exposure to the test formulation alone or after post-treatment water rinsing and with or without drying using cloth, paper, or warm air (Ansari et al., 1991). The fingerpad method has already been applied to assess the microbicidal activity of food-borne viruses such

as HAV (Mbithi et al., 1992), FCV (Bidawid et al., 2003), and HRV (Ansari et al., 1988).

4.3. Nature and Level of Soil Loading

The organic matrix or "*soil load*" surrounding viruses, whether they are in body fluids or sewage/sludge, enhances their survival in the environment. Normal precleaning of surfaces and items to be disinfected may reduce the amount of such soil, but enough of it remains and can interfere with the activity of the applied microbicide by either binding to it or by preventing its access to the target virus. Any good method for virucidal activity must, therefore, simulate the presence of such soil by incorporating in the test virus suspension a certain amount of organic and inorganic material, and this is now a requirement in several standard protocols (ASTM, 2002a, 2002b; CGSB, 1997).

Although many different types and levels of substances are used as the soil load in testing microbicides, extra precautions are needed in their selection and use when working with viruses. For example, animal sera may contain specific antibodies or nonspecific inhibitors against viruses such as rotaviruses. Fecal suspensions, which have been used in testing microbicides against HAV (Mbithi et al., 1990), are inherently variable and thus unsuitable as a soil load for standardized test protocols. To overcome these difficulties, a "universal" soil load has been developed for testing microbicides against viruses as well as other pathogens (Springthorpe and Sattar, 2003); it consists of a mixture of bovine mucin, tryptone, and bovine albumin in phosphate buffer. The concentrations and ratios of the three ingredients are designed to provide a challenge roughly equivalent to that in 5–10% bovine serum. This soil has been found to be compatible with all viruses as well as other types of pathogens tested thus far (Sattar and Springthorpe, 2003).

4.3.1. Diluent for Test Microbicide

Many microbicides are tested by manufacturers using distilled water as the product diluent, and because this is not clearly specified in label directions, most users use tap water instead. Formulations with marginal virucidal activity may work with distilled water but fail when tap water is used as the diluent (Sattar et al., 1983). This highlights the importance of choosing the right diluent during product development and to clearly specify it on the label.

Although tap water is commonly used in the field and may represent a stronger challenge to microbicides under test, it is unsuitable as a diluent in standardized tests for virucidal activity. This is because the quality of tap water as well as the nature and levels of disinfectants in tap water vary both temporally and geographically. In view of this, water with a standard hardness level of 200–400 parts per million $CaCO_3$ is considered a more desirable diluent in such tests (Sattar et al., 2001b).

4.3.2. Dried Virus Inoculum as the Challenge

As stated above, a carrier with the test inoculum dried on it presents a stronger challenge to the microbicide being evaluated. Although this may be

possible with some viruses, certain commonly used surrogates (e.g., polioviruses) lose high levels of infectivity on drying (Mbithi et al., 1991) especially at low levels of relative humidity (RH). A fine balance may therefore be required to achieve the right degree of drying of the virus inoculum on carriers or by selecting a surrogate that is more stable during the drying of the test inoculum. HAV, FCV, and HRV are all more resistant to drying than enteroviruses in general (Sattar and Ansari, 2002). Suitable controls must be included to determine the loss in the infectivity of the test virus during the drying process, and the level that survives becomes the baseline for measuring the extent of virus inactivation by the test formulation (Springthorpe and Sattar, 2003).

4.4. Time and Temperature for Virus-Microbicide Contact

Except for products that are meant for prolonged soaking of items to be decontaminated, the contact between the target virus(es) on an environmental surface and microbicide under in-use conditions is generally very brief. This should be properly reflected in the design of a carrier test for virucidal activity, and such contact times should not be longer than about 3 min to allow for relatively slow-acting but commonly used actives such as ethanol. This is in contrast with currently accepted microbicide test protocols that incorporate a minimum contact of 10 min, which is much too long to simulate the use of environmental surface disinfectants in the field (AOAC, 1998).

Formulations to be used on environmental surfaces are tested at an air temperature of 20°C; this is lower than the ambient temperature indoors in many work settings and requires the use of suitable climate control chambers to maintain the desired temperature. Air temperatures higher than 20°C may enhance the activity of microbicides while also accelerating the rate of their evaporation from the carrier surface. Products to be used outdoors during winter months or indoors in refrigerators must be shown to be effective against viruses at lower temperatures.

4.5. Elimination of Cytotoxicity

Cytotoxicity of the test formulation to host cells is an important consideration in virucidal tests (Quinn and Carter, 1999) because it can interfere with the reading and interpretation of test results. In addition, any material(s) and procedure used to remove and/or neutralize cytotoxicity must itself be safe for the test virus.

A 10- to 100-fold dilution of the virus-microbicide mixture at the end of the contact time is one simple and potentially viable approach to reducing cytotoxicity (Lloyd-Evans et al., 1986). This approach, however, requires relatively high titered pools of the test virus and may not work on its own for chemicals that are highly cytotoxic. Microbicides such as formaldehyde can effectively kill host cells without detaching them or producing any apparent damage to them. Such cytotoxicity can be misleading because host cell monolayers may appear to be undamaged but are unable to support virus replication. Moreover, one should note that even when toxicity appears to be

visibly removed, subtle effects on the cells and potentially on their ability to support virus replication may remain. This needs to be examined through a low-level virus challenge (Sattar et al., 2003).

Gel filtration (ASTM, 1998) or high-speed centrifugation (Doultree et al., 1999) of virus-microbicide mixtures may be effective in the removal of cytotoxicity, but such steps invariably extend the contact of the virus with the test microbicide by several minutes or more and bring into question the accuracy and relevance of claims of virucidal activity for many applications. Other considerations in the selection and use of procedures for the elimination of cytotoxicity have been described before (Sattar and Springthorpe, 2001a).

4.6. Neutralization of Virucidal Activity

For accurate and reproducible results, the microbicidal activity of the test formulation must be arrested immediately at the end of the contact time (Sutton, 1996). This can be achieved by either the addition of a neutralizer or dilution of the virus-microbicide mixture or a combination of both. Whichever approach is adopted, its effectiveness must be properly validated before the test results can be considered as meaningful.

The difficulties in choosing a suitable chemical neutralizer are somewhat similar to those enumerated above for cytotoxicity removal. Although a 100-fold dilution of the virus-microbicide mixture soon after the end of the contact time has proved effective in dealing with most types of microbicides (Lloyd-Evans et al., 1986), this procedure requires that the volume of the diluent be kept relatively small to allow for the titration of most of the eluate.

4.7. Quantitation of Virus Infectivity

The availability of a simple and reproducible method for assaying infectious virus in the test and control samples is absolutely essential for determining virucidal activity. Indirect measures of virus infectivity based on assays for antigens, enzymes, or nucleic acids are not recommended because of the lack of demonstrated correspondence between their concentrations and those of infectious virus in the samples being assayed.

It is noteworthy that the presence of microbicide residues, even in diluted eluates, may increase or decrease the susceptibility of the host cells to the test virus. In case of decreased susceptibility, the host system could overestimate the activity of the tested microbicides by not being able to detect the presence of low levels of infectious virus in the inoculum. An increase in the level of infectivity could possibly be due to any one or a combination of (a) unmasking of more viral receptors on the host cell surface, (b) inactivation of specific or nonspecific virus inhibitors, (c) altering the electrostatic charges on the virus and/or the cell surface, and (d) deaggregation of viral clumps. Controls must, therefore, be included in virucidal tests to rule out the presence of such interference and for the results to be considered valid. The best way to approach this is to first expose the cell monolayer to a non-cytotoxic level of the test microbicide and subsequently challenge the cells to the test virus diluted to yield countable infectious foci such as plaques. If the number

of infectious foci in such pre-exposed monolayers is not statistically different from those in the monolayers treated with a control fluid, the product can be assumed to be free from such interference.

4.8. Number of Test and Control Carriers

Enough test and control carriers must be included to make the results statistically meaningful. This requires some knowledge of the degree of reproducibility of the assay methods; because viruses require a host system, the results tend to be inherently more variable than those observed for bacteria and fungi. In general, methods that determine virus plaque- or focus-forming units are more accurate than the most probable number (MPN) techniques. Each measure of reduction in virus infectivity by a microbicide is obtained by comparison with controls not exposed to microbicide. Therefore, it is important that sufficient numbers of such controls are included to obtain an accurate mean value against which each test carrier can be assessed.

4.9. Product Performance Criteria

For government registration, microbicidal products must meet a performance criterion that is based on practical considerations rather than on sound public health science. A 3–4 $\log_{10}$ reduction in virus infectivity titer after exposure to the test formulation is regarded as satisfactory virucidal activity. The CGSB (1997) standard, for example, requires the tested product to show a >3 $\log_{10}$ reduction (beyond the level of cytotoxicity) in the level of infectious virus to meet its requirements. This criterion is lower than the minimum 5–6 $\log_{10}$ reductions required for other classes of pathogens because of the general difficulties in generating high-titered virus pools.

5.0. CURRENTLY AVAILABLE TESTS

Table 12.2 lists the methods currently accepted or under consideration as standard test protocols for testing the virucidal activity of microbicides.

5.1. Quantitative Suspension Tests

The ASTM suspension test for virucidal activity (E-1052) has recently been revised, and the current version incorporates several changes. This test is for special applications of virucides such as inactivation of viruses in contaminated wastes and as a first step in determining the virucidal potential of liquid chemical microbicides, liquid hand soaps, over-the-counter (OTC) topical products, or other skin care products.

Another quantitative suspension test for virucidal activity of chemical disinfectants and antiseptics is being drafted by CEN (Comité Européen de Normalisation) Technical Committee (TC) 216. An adenovirus and a vaccine strain of poliovirus are listed as test viruses. The contact time at 20 ± 1°C ranges from 30 to 60 min depending on the intended use of the product. The formula being evaluated is tested with and without an added protein load in

Table 12.2 Standard Test Methods for Evaluating the Virucidal Activity of Microbicides Designed to Be Used on Environmental Surfaces or Human Hands

Organization	*Title of Standard*	*Document No.*
ASTM International	Standard Test Method for Efficacy of Antimicrobial Agents Against Viruses in Suspension	E-1052
	Standard Test Method for Efficacy of Virucidal Agents Intended for Inanimate Environmental Surfaces	E-1053
	Standard Test Method for Neutralization of Virucidal Agents in Virucidal Efficacy Evaluations	E1482
	Standard Test Method for Determining the Virus-Eliminating Effectiveness of Liquid Hygienic Handwash and Handrub Agents Using the Fingerpads of Adult Volunteers	E-1838
	Standard Test Method for Evaluation of Handwashing Formulations for Virus-Eliminating Activity Using the Entire Hand	E-2011
	Standard Quantitative Disk Carrier Test Method for Determining the Bactericidal, Virucidal, Fungicidal, Mycobactericidal, and Sporicidal Activities of Liquid Chemical Germicides	E-2197
Canadian General Standards Board	Assessment of Efficacy of Antimicrobial Agents for Use on Environmental Surfaces and Medical Devices (Canadian national standard)	CAN/CGSB-2.161-M97

the form of either 0.3% bovine serum albumin or 5% defibrinated sheep blood. The product performance criterion is a minimum $4\log_{10}$ reduction in the infectivity titer of the test virus.

5.2. Quantitative Carrier Tests

There are four methods in this category in North America. The first is an ASTM (2002a) standard, which has also been revised recently (E-1053). It is meant for evaluating the activity of liquid or pressurized antimicrobials against viruses on inanimate, nonporous, environmental surfaces. This standard lists 10 different viruses with varying degrees of resistance to liquid chemical microbicides. It recommends, however, that the test formulation be evaluated at least against a poliovirus, a herpesvirus, and an adenovirus to qualify for a general virucidal claim. The test virus suspension is first dried on a glass Petri plate and then overlaid with a known volume of the test formulation for a predetermined contact time at ambient temperature. At the end of the contact time, a diluent is added to the virus-product mixture, and the test surface is scraped to resuspend the virus film. The eluates and controls are assayed for infectious virus to determine the loss in virus titer due to the test formulation's virucidal activity. Calf serum is recommended as

organic soil (except for rotaviruses), and water with a specific level of hardness is to be used if the product requires dilution in water prior to use.

The second carrier test is a part of the Canadian General Standard Board's document Assessment of Efficacy of Antimicrobial Agents for Use on Environmental Surfaces and Medical Devices (CAN/CGSB-2.161-M97). This test permits the use of glass Petri plates, glass slides, or disks of glass, metal, or plastic. The recommended test virus is the Sabin strain of poliovirus type 1 (ATCC VR-192) to permit a general virucidal claim when testing is carried out with 5% fetal bovine serum as the organic load and, when necessary, water with a hardness of 200 ppm $CaCO_3$ as diluent for the test product.

The third carrier test is the *in vivo* fingerpad method to assess the virus-eliminating activity of topicals. Full details of this method are given in the literature already cited above or in the ASTM standard itself (ASTM E-1838). The fourth method is a standard for virucidal evaluation of formulations when these are performed on the whole hand (ASTM E-2011).

The second tier of a quantitative carrier test (QCT-2) method developed by us under the sponsorship of the U.S. Environmental Protection Agency (EPA) can be applied to all major classes of microorganisms including viruses (Springthorpe and Sattar, 2003, 2005). This would allow direct comparison of results among different classes of microorganisms. The test uses flat stainless steel disk carriers (approximately 1 cm diameter) and a microbial inoculum of 10 µl. After drying of the inoculum, the contaminated carrier is exposed to approximately 50 µl of the test microbicide at 20°C for the manufacturer's recommended contact time. The reaction is terminated by neutralization of the microbicide. In most cases, neutralization is achieved by simple dilution with a physiological saline, but in some instances a chemical neutralizer is required prior to dilution. The test virus can then be titrated by standard methods. This method, which is now a standard of ASTM International (E-2197), has been used to test for virucidal activity against several types of viruses (Lloyd-Evans et al., 1986; Sattar et al., 1989, 2003; Mbithi et al., 1990).

6.0. PRACTICAL ASPECTS OF TESTING MICROBICIDES

We describe in this section the key practical considerations in evaluating microbicides against food-borne viruses. The methods described here are standards of ASTM International and have been used extensively in working with a variety of viruses (Springthorpe and Sattar, 2003; Sattar et al., 2003; Ansari and Sattar, 2002). Figure 12.1 summarizes the basic steps in the quantitative carrier test method (ASTM 2002a), and Figure 12.2 presents the basic steps in the fingerpad method (ASTM 2002c). Although no nationally or internationally accepted methods are available to assess the activity of microbicides used in the decontamination of fruit and vegetables, published studies

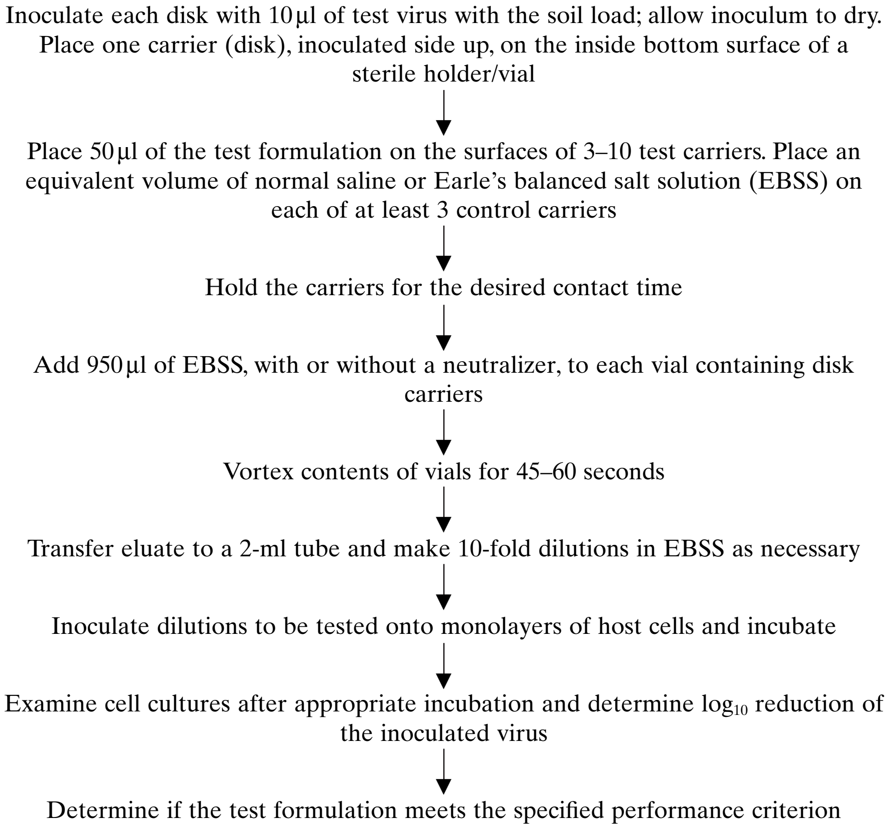

Figure 12.1 Basic steps in disk-based quantitative carrier test for virucidal activity.

(Bidawid et al., 2000, 2003) should serve as a guide in the design and performance of such testing.

The three viruses described below have been selected based on their (a) relevance as food-borne pathogens, (b) relative resistance to microbicides, (c) ability to withstand drying on environmental surfaces and human skin, (d) availability of cell culture–based infectivity assays, and (e) safety for work in experimental settings and for placement on the intact skin of adult subjects.

The need for cell cultures adds an extra layer of difficulty when working with viruses. Also, procedures that work perfectly in one laboratory do not always work in another. This may be due to even slight variations in the quality of water for making media and reagents or to procedures for the clean-up and sterilization of labware, and so forth. Each laboratory must develop and document its own standard operating procedures for each host cell type and test virus to be used. However, regardless of the methods used for cell culture, preparation of virus pools, and quantitation of virus infectivity, the procedures for testing the activity of microbicides must adhere to

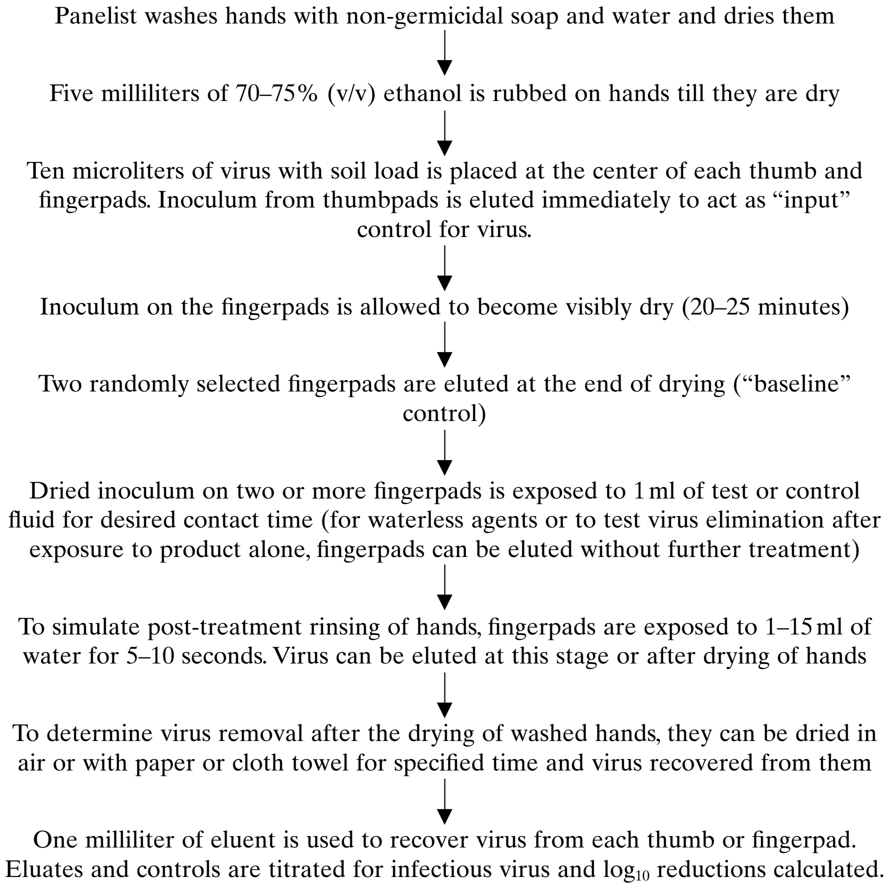

Figure 12.2 Basic steps in the fingerpad method (ASTM, 2002) for testing handwash or handrub agents against viruses.

the basic requirements as described above to ensure a sufficient level of stringency and reproducibility and to allow the comparison of the results from tests using different viruses and test formulations.

Although ultracentrifugation may sometimes be needed to increase the virus titer, the use of highly purified virus pools is not recommended for testing microbicides because such purification is likely to enhance susceptibility of the virions to microbicides. Described below are some viruses that can be used in such tests.

6.1. Strain HM-175 (ATCC VR-1402) of HAV

HAV, an important food-borne pathogen, affects the liver and is excreted in the feces of infected individuals. It is relatively resistant to drying and mechanical damage and is also generally more resistant to microbicides than other nonenveloped viruses of human origin. Immunization of lab workers

with the recently available vaccines makes the handling of this virus much safer. The recommended cell line for making HAV pools and for performing infectivity titrations is FRhK-4 (ATCC CRL-1688). No less than 7 days are needed to complete an infectivity assay due to the relatively slow rate of growth of the virus.

6.2. Strain F9 (ATCC VR-782) of FCV

FCV, pathogenic to cats but believed harmless to humans, belongs to a group of small round viruses. FCV, which is nonenveloped, is closely related to Norwalk or norovirus (NV), a major cause of acute gastroenteritis in humans and also a significant food-borne pathogen. Because NV cannot be grown *in vitro*, FCV is generally accepted as its surrogate (Doultree et al., 1999) and has been used in testing microbicides in settings where foods may be handled (Gulati et al., 2001; Bidawid et al., 2003). The cell line recommended for work with FCV is CrFK (ATCC CCL-94), and a plaque assay system based on these cells has been developed (Bidawid et al., 2002). This virus grows to high titers (~10^8 infective units/ml) within 28–36 hr and produces visible CPE or plaques in less than 36 hr. This is helpful in making test results available relatively rapidly.

6.3. Human Rotavirus

6.3.1. Wa Strain (ATCC VR-2018)

Rotaviruses, which are a common cause of acute gastroenteritis in humans, are excreted in diarrheic feces in numbers generally higher than those for other enteric viruses (Ward et al., 1991). Food-borne spread of rotaviruses has been documented (Sattar et al., 2001a). Recommended cell lines for growing rotaviruses are MA-104 (CRL-2378) and CV-1 (ATCC CCL-70; Sattar et al., 2000). Rotaviruses are safe for normal healthy adults as most adults have acquired immunity against them. The ability of rotaviruses to withstand drying also adds to their attraction as surrogates in testing microbicides. Two important factors to note when working with rotaviruses are that (a) many of them are inhibited by fetal bovine sera often used in cell culture, and (b) the presence of proteolytic enzymes such as trypsin is needed to promote rotavirus infection of host cells.

6.4. Additional Controls in Virucidal Tests

The use of cell cultures requires the incorporation of the following additional controls in tests for virucidal activity (Sattar et al., 2002, 2003) because either the test substance or the neutralizer or both could alter the susceptibility of host cells to the virus in the test. These controls must be run initially at least once and may not need to be included in subsequent tests as long as the same cell line, virus, test formulation, neutralizer, and method are used in testing.

6.4.1 Cytotoxicity Control

This control (a) determines the dilution of the test substance that causes no apparent degeneration (cytotoxicity) of the cell line to be used for measur-

ing virus infectivity and (b) assesses whether the neutralizer reduces or enhances such cytoxicity. For this control, make a 1:20 dilution, then a 1:200 dilution of the test substance in Earle's balanced salt solution (EBSS) with and without the neutralizer. Remove the culture medium from the monolayers of the host cell line(s) and put into each test monolayer separately the same volume of inoculum as used in virus titration; control monolayers receive an equivalent amount of EBSS only (without any neutralizer). Use at least three monolayers for controls as well as for each dilution of the test substance being assessed. Hold the cultures at room temperature for the same length of time used for virus adsorption, then examine under an inverted microscope for any visible cytotoxicity.

If cytotoxicity is observed, a different neutralizer or alternative approaches to the removal/reduction of cytotoxicity may be needed. It is sometimes advisable to use gel filtration to remove the disinfectant, although this procedure may lengthen the exposure time of the test organism to the disinfectant. If no cytotoxicity is observed at either dilution, the test substance and the neutralizers should be subjected to the following interference test.

6.4.2. Control for Interference with Virus Infectivity

Levels of the test substance that show no obvious cytotoxicity could still reduce or enhance the ability of the challenge virus to infect or replicate in host cells, thus interfering with the estimation of its virucidal activity (Sattar et al., 2003). An interference control must, therefore, be included to rule out such a possibility. For this purpose, remove the culture medium from monolayers of the host cell line(s) and add a 1:20 dilution, or a dilution greater than the one that demonstrated cytotoxicity, of the test substance in EBSS to each of the test monolayers with and without neutralizer, using the same volume as that of the inoculum used in virus titration. Controls receive EBSS alone (without the neutralizer). Hold the monolayers at room temperature for the same length of time as used for virus adsorption and inoculate each with a low number (approximately 10–20) of infective units of the challenge virus. Incubate the monolayers for virus adsorption, place maintenance medium in the cultures, incubate them for the time required for virus replication, and then examine for cytopathology or foci of virus infection.

Any significant difference in virus infectivity titer is indicative of the ability of the test material or the neutralizer to affect the virus susceptibility of the host cells. In such case, a different neutralizer or alternative approaches to the removal of the residues of the test product may be needed. Both the cytotoxicity and interference controls must be included even when virus infectivity is titrated using the $TCID_{50}$ method.

6.4.3. Control Carriers

The minimum number of control carriers to be used in each test is three regardless of the number of test carriers. For control carriers, add 50 µl of EBSS instead of the test formulation. The contact time and temperature for the control carriers must be the same as those for the test carriers.

7.0. MICROBICIDES IN ENVIRONMENTAL CONTROL OF FOOD-BORNE VIRUSES

Table 12.3 summarizes recent data on the activity of various concentrations of sodium hypochlorite (bleach) against FCV as tested using QCT-2 and stainless steel disks as carriers. The product effectiveness criterion was arbitrary set as $3 \log_{10}$ or greater reduction in virus infectivity. A minimum contact time of 10 min under ambient conditions was needed to meet this requirement with 500 ppm of available chlorine; when the available chlorine level was doubled, the contact time could be reduced to as low as 1 min.

The results of similar tests with other environmental surface disinfectants are presented in Table 12.4. Except for 75% ethanol, the remaining four formulations are commercially available environmental surface disinfectants; chlorine dioxide was prepared just before testing using the two solutions provided by the manufacturer. The trade names of the products tested are not given because formulations with identical types and levels of ingredients may be sold under a different trade name elsewhere. Only chlorine dioxide could meet this criterion in a contact time of 1 min, while at least 3 min were required for accelerated hydrogen peroxide and the two commercial alcohol-based sprays to do so. The quat-based formulation and 75% ethanol met the criterion after a minimum contact time of 10 and 5 min, respectively.

The findings of tests with three commercial alcohol-based hand rubs are summarized in Table 12.5. When the ASTM (2002a) fingerpad protocol was used with a contact time of 20 s, the $\log_{10}$ reduction in the infectivity titer of FCV ranged from 1.20 to 1.49. In this regard, FCV proved to be more resistant than adeno-, rota-, and rhinoviruses (Sattar et al., 2000) and less resistant than HAV (Mbithi et al., 1993).

Table 12.3 Activity of Domestic Bleach Against Feline Calicivirus[a,b]

Available Chlorine in Parts per Million	*Contact Time in Minutes*	*Log_{10} Reduction in Virus Infectivity*
100	5	0.56
500	5	1.39
500	10	>4.7
1,000	1	>4.7
1,000	2	>4.7
1,000	3	>4.7

[a] Sattar et al. unpublished data.
[b] The bleach tested was a commercially available product with 5.25% sodium hypochlorite. The metal disk-based test method is a standard ASTM method (E-2197). All testing was done at 25°C in the presence of a soil load and water with a hardness of 400 ppm as calcium carbonate (used to dilute the product).

Table 12.4 Activity of Selected Microbicides Against Feline Calicivirus

Active(s) in Microbicide	*Conc. Tested (ppm)*	*Contact Time (min)*	*Log_{10} Reduction*
0.5% accelerated H_2O_2	Undiluted (5,000)	1	1.55
	Undiluted (5,000)	3	>4.5
A mixture of four quaternary ammonium compounds (octyl decyl dimethyl NH_4Cl (4.6%); dioctyl dimethyl NH_4Cl (1.84%); didectyl dimethyl NH_4Cl (2.76%); dimethyl benzyl NH_4Cl (6.14%)	Diluted 1:62 (2,470)	5	2.3
	Diluted 1:62 (2,470)	10	4.0
79% (v/v) ethanol + 0.1% alkyl dimethyl benzyl ammonium saccharinate	Undiluted	1	2.2
	Undiluted	3	3.8
Each 100 g contains ethanol 25.92 g, 2-propanol 11.50 g, and polyhexanide 0.054 g	Undiluted	1	0.8
	Undiluted	3	3.0
	Undiluted	5	3.5
Ethanol	75% (v/v)	5	3.8
	75% (v/v)	10	4.7
Liquid chlorine dioxide	1,000	1	4.5

[a] Sattar et al. unpublished data.
[b] The metal disk-based test method used is a standard of ASTM (E-2197); all testing was done at 23 ± 2°C in the presence of a soil load and water with a hardness of 400 ppm as calcium carbonate (used to dilute the product).

Table 12.5 Activity of Commercially Available Ethanol-Based Handrub Agents Against Feline Calicivirus Using the Fingerpad Method[a,b]

Ethanol in Handrub (%)	*Log_{10} Reduction in pfu After Contact with Test Formulation*
60	1.20
70	1.42
80	1.49

pfu, plaque-forming unit.
[a] Sattar et al. unpublished data.
[b] Each fingerpad was contaminated with 10 µl of the test virus suspended in a soil load and the inoculum allowed to dry under ambient conditions. Virus from two fingerpads was eluted to determine the pfu remaining after the drying period; this figure (8.9×10^4) was used as the "baseline" to determine log_{10} reduction in virus titer after exposure to the formulation under test. Each formulation was tested on at least three adult subjects, and no less than two fingerpads were exposed to the test formulation in each test. The contact time in all experiments was 20 s.

8.0. CONCLUSIONS

Viruses continue to be important pathogens in general and as food-borne pathogens in particular, but our understanding of the actual sources of viral contamination in many food-borne outbreaks remains incomplete, making it difficult to design and apply proper strategies to prevent and control the spread of such pathogens. However, hands are universally recognized as vehicles for the spread of a number of viruses. Successful strategies to prevent virus spread through these vehicles involve a sound hand-decontamination protocol, diligently applied with a good topical agent. A lack of compliance with hand antisepsis guidelines and, perhaps, the use of ineffective agents continue to undermine the full potential of infection-control measures in this regard. The ease with which washed hands can pick up infectious viruses upon contact with contaminated environmental surfaces and objects suggests that the emphasis on hand antisepsis should be combined with an awareness of the need for proper and regular cleaning and decontamination of those surfaces and objects that come in frequent contact with decontaminated hands.

Standardization of virucide tests, nationally and internationally, will promote confidence among microbicide users and the general public. This chapter provides the basis for general understanding of the potential pitfalls in testing virucides and suggests the basic protocols and controls that should be present in generic methods. This should allow the reader to better understand this field and to be able to critique the published literature independently.

Standard tests for virucides are now available. These tests provide improved carrier design, better methods for cytotoxicity removal, a universal soil load, and other improvements. However, regulatory agencies, especially in the United States, must soon decide on accepting surrogates in tests for virucidal activity and label claims and also set product performance standards. Some jurisdictions already have one or both of these in place (CGSB, 1997). Any such discussion must consider activity against one or more carefully selected nonenveloped viruses representative of food-borne viral pathogens. Many products currently on the market list only enveloped viruses among the organisms on the label. Persons unfamiliar with virus classification can be easily misled by this, especially if the enveloped viruses listed are among those most feared.

Our current knowledge does not allow, with any degree of certainty, the determination of the desired level of reduction in virus load in a given setting to significantly reduce disease transmission. There are also obvious practical limitations to how high a level of challenge virus(es) one can present to the product under evaluation. By the same token, what would one regard as too low a level of challenge? Experience accumulated over the past two decades clearly indicates that if test viruses are chosen carefully, it is feasible to determine a 3–4 $\log_{10}$ reduction in virus infectivity titer after its exposure to a test microbicide in a proper carrier test. The viruses selected for QCT-2 are based on their (a) relative safety for the laboratory staff, (b) ability to grow to titers

sufficiently high for testing, (c) property to produce cytopathic effects or plaques, or both, in cell cultures, (d) potential to spread through contaminated environmental surfaces and (e) relatively high resistance to a variety of chemicals.

Given these considerations and the fact that enveloped viruses in general do not survive well on environmental surfaces and are relatively more susceptible to chemical microbicides, all viruses included here are nonenveloped viruses. Other strains or types of nonenveloped viruses may be substituted in the test provided they meet the preceding criteria.

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