

CONFIRMATORY VIRUCIDAL EFFECTIVENESS TEST

Using Feline Calicivirus

As Surrogate for Norovirus

**Antimicrobials Division
U.S. EPA**

OBJECTIVE:

This test is designed to validate virucidal effectiveness claims for a product to be registered as a viricide. It determines the potential of the test agent to disinfect hard, non-porous surfaces contaminated with NOROVIRUS. This test is designed to simulate consumer use, conforms to EPA Guidelines DIS/TSS-7, November 1981, and follows the general procedure outlined in the FR notice for another surrogate virus available online¹ and Virucidal Testing Format and Statistics Primer issued by EPA (March 2000).

DISCUSSION:

Norovirus was recently approved as the official genus name for the group of viruses provisionally described as "Norwalk-like viruses" (NLV). This group of viruses has also been referred to as caliciviruses (because of their virus family name) and as small round structured viruses (SRSVs; due to morphology)². Noroviruses are a group of related, single-stranded RNA, non-enveloped viruses that cause acute gastroenteritis in humans. Viral gastroenteritis, often called "stomach flu" even though it is not produced by influenza viruses, is an infection caused by a variety of viruses that result in vomiting and/or diarrhea. Many different viruses can cause gastroenteritis, including rotaviruses, adenoviruses, caliciviruses, astroviruses, Norovirus, and a group of Norwalk-like viruses^{3,4,5}. These viral infections can often be prevented by frequent hand washing, and prompt disinfection of contaminated surfaces^{6,7}. The recent outbreaks have prompted an urgent need for a surrogate for Norovirus⁸. Pending fulfillment of Koch's postulates and due to the scientific challenges Norovirus poses, its phylogenetic cousins, animal caliciviruses, have been proposed as zoonotic agents⁹.

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1. <http://www.epa.gov/fedrgstr/EPA-PEST/2000/August/Day-25/p21784.htm>
 2. <http://www.cdc.gov/ncidod/dvrd/revb/gastro/norovirus-factsheet.htm>
 3. Kapikian, AZ, MK Estes and RM Chanock. Norwalk Group of Viruses. In: Fields Virology 3rd Ed. Edited by Fields, BN., D.M. Knipe, P.M. Howley, et al. Lippincott-Raven Publishers, Philadelphia, PA, 1996
 4. Green KY, Ando T, Balayan MS, Berke T, Clarke IN, Estes MK, Matson DO, Nakata S, Neill JD, Suddert MJ, Thiel HJ. Taxonomy of the Caliciviruses. J Infect Dis. 181:S322-30, 2000
 5. Sattar SA, Springthorpe VS, Tetro J, Vashon R, Keswick B. Hygienic Hand Antiseptics: Should They Not Have Activity and Label Claims Against Viruses? Am J Infect Control 30(6): 355-72, 2002
 6. <http://www.cdc.gov/ncidod/dvrd/revb/gastro/faq.htm>
 7. <http://vm.cfsan.fda.gov/~mow/chap34.html>
 8. <http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5149a2.htm>
 9. Smith, AW, Skilling DE, Cherry N, Mead JH, Matson DO. Calicivirus Emergence From Ocean Reservoirs: Zoonotic and Interspecies Movements. Emerging Infectious Diseases 4: 13-20, 2000

There are no *in vitro* systems or *in vivo* animal models that are available for growing Norovirus, except its natural hosts humans and experimental host chimpanzees, neither of which is available for disinfectant efficacy testing on ethical grounds¹⁰. However, Feline calicivirus (FCV) has been accepted as the model for evaluation of disinfectants against Norovirus. Furthermore, critical issues on its survival and inactivation have been investigated making FCV a suitable surrogate for Norovirus, enabling the evaluation of test agents¹¹.

TESTING CONDITIONS:

One lot of the test agent will be used to inactivate the challenge virus that has been dried on a sterile glass surface (two replicates for each batch/lot of the test agents). The test agent will be tested in a manner consistent with the label directions for use of the test agent or as specified by the Sponsor.

After a timed exposure period, the test agent-virus mixture will be scraped from the glass surface, collected, neutralized, and assayed for the presence of residual infectious virus *in vitro*.

MATERIALS:

A. Test control and reference substances: supplied by the Sponsor (see last page)

The test agent will be tested as supplied by the Sponsor unless directed otherwise. The Sponsor, before the initiation of testing, must specify all operations performed on the test agent such as dilutions of the test agent, the diluent for the test agent, or specialized storage conditions.

The test agent must be tested for identity, strength, purity, stability and uniformity as applicable.

All unused test agent will be retained for a period after completion of the test, then discarded in a manner that meets the approval of the safety officer.

10. Pugh, JC, MK Ijaz, DB Suchmann. Use of Surrogate Models for Testing Efficacy of Disinfectant Against Hepatitis B Virus. Am. J. Infect. Cont., 27: 375-376, 1999

11. Doultree, JC, JD Druce, CJ Birch, DS Bowden, JA Marshall. Inactivation of Feline Calicivirus, A Norwalk Virus Surrogate. J Hosp Infect. 41:51-57, 1999

B. Materials can include, but are not limited to:

1. Challenge virus as requested by the sponsor of the study: Feline calicivirus (American Type Culture Collection, Manassas, VA; ATCC VR-782)
2. Host cell line: Crandell Reese Feline Kidney (CRFK) cell (American Type Culture Collection, Manassas, VA; ATCC CCL-94)
3. Laboratory equipment and supplies
4. Media and reagent:
 - a. Cell culture media (Eagle's Minimum Essential Media containing $\geq 5\%$ Fetal Bovine Serum)
 - b. Earle's Balanced Salt Solution (EBSS)
 - c. Fetal Bovine Serum (FBS)
 - d. Phosphate Buffered Saline (PBS)
 - e. SephadexTM/SephacrylTM columns (if necessary)
 - f. Neutralizer

TEST SYSTEM IDENTIFICATION:

All Petri dishes, dilution tube racks, and host-containing apparatus will be labeled with the following information: virus, host, test agent, and project number.

EXPERIMENTAL DESIGN:

A. Inoculum preparation:

The F-9 strain of Feline Calicivirus will be obtained from the American Type Culture Collection, Manassas, VA (ATCC VR-782). The FCV will be grown by inoculating confluent cell monolayers, no more than 24-48 hours in age, using low multiplicity of infection (MOI). Briefly, a flask of host cells grown in cell culture media containing 10% fetal bovine serum (FBS) will be used. Cells will be washed three times with phosphate buffered saline (PBS) and inoculated with virus. Post-virus adsorption, the cell monolayer will be washed once in Earle's balanced salt solution (EBSS), re-fed with cell culture media and incubated. The cytopathic effects (CPE) are described as small, rounding of the cells, with a slight granular look. The CPE starts to develop in 1-2 days following inoculation, and will be harvested when more than ninety percent cytopathic effects (CPE) are observed. Post-incubation, the cells will be disrupted, with cell debris removed by centrifugation.

Stock virus will be prepared by collecting the supernatant culture fluid from 75-100% infected culture cells. The supernatant will be removed, aliquoted, and stored in an ultra-low temperature freezer until the day of use. On the day of use an aliquot is removed, thawed and refrigerated until use in the assay.

Note: The percent FBS contained in the stock virus aliquot is adjusted to yield a

minimum of a 5% organic soil load. If the Sponsor chooses a soil load greater than 5%, the percent FBS contained in the stock virus aliquot will be adjusted to yield the percent soil load requested.

B. Carrier Preparation:

An aliquot of 0.2 ml stock virus will be spread uniformly over the bottoms of 100 X 15 mm sterile glass Petri dishes with a cell scraper. The virus will be air-dried at room temperature for 30-60 minutes (until visibly dry). The drying conditions (temperature and humidity) will be appropriate for the test virus to obtain maximum survival following drying. The actual drying time and temperature will be recorded. One carrier will be prepared for each lot of test agent and plate recovery control.

Additionally, one carrier per test agent lot will be prepared for the neutralizer effectiveness control using cell culture media in place of stock virus.

C. Test agent preparation:

The test agent will be prepared and used according to the Sponsor's directions or proposed label claims.

D. Test:

For one batch of test substance, dried virus film will be exposed to 2.0 ml of the use dilution of the test substance, or to the amount of spray released under the use conditions (spray products), for a specified exposure time and temperature. Post-contact time, the test agent will be neutralized and the mixture will be scraped from the surface of the dish. This will be considered approximately one log₁₀ dilution.

1. Sephadex™/Sephacryl™ Filtration

If columns are utilized, each sample will be loaded into individual pre-spun Sephadex™/Sephacryl™ columns. Virus-test substance mixture will be passed through individual Sephadex™/Sephacryl™ columns utilizing the syringe plunger in order to detoxify the mixture. The aseptically collected samples will be diluted as appropriate.

2. If columns are not used, serial tenfold dilutions of neutralized virus will be prepared in cell culture media.

For spray-type agents, the agent will be used as per sponsor's instructions, the volume produced by the spray product during the spraying application specified by the sponsor will be measured prior to testing and an equivalent quantity of the neutralizer will be applied post contact time. Following application of the test agent, contact time, and neutralization, the procedure for processing the samples will be the same as described earlier (see above).

E. Infection, cell maintenance and infectivity assays:

Selected dilutions of the neutralized inoculum/test agent mixture will be added to cultured cell monolayers. Four well per dilution will be added to the host cell monolayers and incubated at $37\pm2^{\circ}\text{C}$ in $5\pm1\%$ CO_2 for 5-7 days. Post incubation the infectious FCV will be scored microscopically by observing virus-specific cytopathic effects (CPE) produced by replicating infectious virus. The CPE associated with FCV is visually evidenced under the microscope by the presence of small, shrinking cells that have detached from the monolayer. These changes will be scored in comparison with the negative control (cell viability control).

F. Controls:

All controls will be performed at the same time as the test, incubated under the same conditions and assayed in the same manner as the test (see above). Neutralizer effectiveness control, Cytotoxicity control and Cytotoxicity related viral interference control will be performed for test agent(s).

1. Cell viability control

This control will demonstrate that cells remain viable throughout the course of the assay period. In addition, it will confirm sterility of the cell culture employed throughout the assay period. Four wells will receive cell culture media only.

2. Virus stock titer

The challenge virus will be titrated at the time of the test to determine the relative infectivity of the virus and to demonstrate the susceptibility of the host cells to support infection of FCV. The virus inoculum will be serially diluted tenfold in cell culture media. Selected dilutions will be inoculated into four wells per dilution and incubated under the same conditions as the test.

3. Plate recovery control (PRC)

Two ml of cell culture media will be added to the dried virus. Post-contact time, the virus/cell culture mixture will be subjected to the same neutralization procedure as the test agent. If columns are used, a portion of the virus/cell culture media/neutralizer mixture will be used for the column titer control (see below). This control will determine the relative loss in virus infectivity resulting from drying and neutralization alone.

The results from the PRC will be compared with the test results to confirm recovery of at least four \log_{10} of infectious virus following drying and neutralization. Its titer will be used to compare with the titers of the test results to reach the acceptable test criteria (see below).

4. Neutralizer effectiveness control (NEC)

The neutralization procedure will be dependent upon the active ingredient present in the test agent and in the internal control test agent. For this control, each lot of the test agent will be processed exactly as the test procedure, but instead of the viral inoculum, cell culture media will be added. Post neutralization, the sample will be divided into three portions [two for cytotoxicity related controls (see below) and one for neutralizer effectiveness].

If columns are used, each sample will be passed through individual columns and the eluate will be serially diluted as appropriate in cell culture media. If columns are not used, the neutralizer effectiveness samples will be serially diluted tenfold in cell culture media. The diluted samples will be mixed with low titer virus, held for a period equivalent to contact time and the resulting mixture of dilutions will be assayed for viral infectivity and/or cytotoxicity (see below) in order to determine the dilution of test agent at which virucidal activity, if any, is retained. Then the selected dilutions will be used to inoculate host cells as described for the test procedure. Dilutions that show virucidal activity will not be considered in determining reduction of viral infectivity by the test agent.

5. Cytotoxicity control (CT)

A CT control will be run to determine if the product is toxic to the cells. Each lot of the neutralized test agent will be run to determine cytotoxicity. The CT sample, acquired from the NEC, will be serially diluted tenfold in cell culture media, having no virus added. Selected dilutions will be inoculated and incubated in the same manner as the test and control samples.

G. Calculations

Viral and cytotoxicity titers will be expressed as $-\log_{10}$ of the 50 percent titration endpoint infectivity (TCID_{50}), respectively, as calculated by the method of Spearman Karber.

– Log of 1st dilution inoculated –[(Sum of % mortality at each dilution/100) – 0.5] x (logarithm of dilution)]

The \log_{10} reduction in infectivity will be calculated using the revised EPA approved method for calculating the Most Probable Number (MPN) as obtained from the EPA on January 4, 2001.

PRODUCT EVALUATION CRITERIA:

According to the EPA, the test agent is acceptable if a minimum of 4-log reduction in CPE of FCV (complete inactivation of the virus occurs at all dilutions tested) is demonstrated compared to PRC. When cytotoxicity is present, at least a 3-log reduction from the PRC must be demonstrated beyond the cytotoxic level with complete inactivation of the virus at all dilutions tested.

DATA PRESENTATION:

The final report will include the following information in tabular form for the test agent and appropriate control cultures:

- Virus stock titer
- Test results
- Plate recovery control
- Neutralizer system employed and effectiveness data
- Cytotoxicity control
- Cell Viability Control

TEST ACCEPTANCE CRITERIA:

The test will be acceptable for evaluation of the test results if the criteria listed below are satisfied. The study director may consider other causes that may affect test reliability and acceptance:

- The infectious virus recovered from the PRC control must be $\geq 4\text{-log}_{10}$.
- Viral-induced cytotoxicity must be distinguishable from test agent induced cytotoxic effects. When cytotoxicity is evident, at least a 3-log reduction in titer is demonstrated beyond the cytotoxic level.
- The cell viability control is negative for infectivity.

REPORT FORMAT:

Each report should provide the following information:

- Sponsor identification and test agent identification
- Type of assay and project number
- Dates of study initiation and completion
- Interpretation of results and conclusions
- Test results presented in tabular form
- Methods and evaluation criteria
- Signed Quality Assurance and Compliance Statements
- An example of a standard efficacy report format may be found at <http://www.epa.gov/oppad001/efficacystudystandards.htm>

RECORDS TO BE MAINTAINED:

All raw data, protocol, protocol modifications, test agent records, final report, and correspondence relevant to this study will be stored in the archives.

All changes or revisions to the approved protocol will be documented, signed by the study director, dated and maintained with this protocol. The sponsor will be notified of the change and resolution, and any resulting impact on the study as soon as practical.

The proposed experimental start and termination dates; additional information about the test agent; challenge virus and specific host used; media and reagent identification; and the type neutralizers employed in the test will be addressed on a project sheet issued separately for each study. The study sponsor should sign all project sheets.

PERSONNEL AND TESTING FACILITIES:

A study director will be assigned before initiation of the test. Resumes for technical personnel are maintained and are available on request.

MISCELLANEOUS INFORMATION:

The following information is to be completed by the sponsor prior to initiation of the study:

Name and address
Test Agent
Lot 1
Active Ingredient
Additional Instructions