PRE-SATURATED OR IMPREGNATED TOWELETTES
INITIAL VIRUCIDAL EFFECTIVENESS TEST

Using Feline Calicivirus

As Surrogate for Norovirus

Antimicrobials Division
US EPA

OBJECTIVE:
The purpose of this study is to evaluate the virucidal efficacy of pre-saturated or impregnated towelettes for hard, non-porous surface disinfection (single use) against Feline Calicivirus (FCV), a surrogate virus for Norovirus, according to test criteria and methods approved by the US Environmental Protection Agency for registration of a product as a viricide. The test follows the “Germicidal Spray Products as Disinfectants” test as described in the Official Methods of Analysis, 16th edition, 1995, AOAC, as required by EPA DIS/TSS 1, 2, & 3 guidelines and the EPA Notice of Efficacy Requirements for Pre-Saturated or Impregnated Towelettes for Hard Surface Disinfection. The test is designed to simulate consumer use, conforms to EPA Guidelines DIS/TSS-7, November 1981, and follows the general procedures outlined in FR notice for testing the efficacy against HBV using Duck HBV as a surrogate virus 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). 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, Noroviruses, and a group of Norwalk-like viruses. These viral infections can be prevented by frequent hand washing, and prompt disinfection of contaminated surfaces. Recent outbreaks have prompted an urgent need for a surrogate for Norovirus. Pending fulfillment of Koch’s postulates and due to the scientific challenges Norovirus poses, its phylogenetic cousins, animal caliciviruses, have been proposed as zoonotic agents.

8. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5149a2.htm
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 grounds\textsuperscript{10}. However, 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 agents\textsuperscript{11}.

**TESTING CONDITIONS:**

Two lots of the test agent will be used to inactivate the challenge virus has been dried on a sterile glass surface (ten replicates for each batch/lot of the test agent). 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.

Following the exposure period, test agent-virus mixture will be scraped from the glass surface, collected, neutralized, and assayed for viral infectivity 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.


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: Crandel 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 5% Fetal Bovine Serum)
   b. Earle’s Balanced Salt Solution (EBSS)
   c. Fetal Bovine Serum (FBS)
   d. Phosphate Buffered Saline (PBS)
   e. Sephadex™/Sephacryl™ 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 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

For the test and related controls, an aliquot of 0.1 ml of stock virus will be spread over an area of approximately 1" x 1" square that has been marked on the underside of a pre-sterilized glass Petri dish. The carriers will be allowed to dry for 30 to 60 minutes at room temperature. Both drying time and temperature will be recorded.

Ten carriers will be prepared for each lot of test agent and for the input virus control. Two carriers will be prepared for the blank towelette virus control and for each lot of test agent neutralizer effectiveness control, with the exception that the cell culture media will be used in the place of 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

When towelettes contained in a canister are used, the canister will be inverted once before removing towelettes. When individually wrapped towelettes are used the technician will observe that the towelette is moist.

Wiping of the dried contaminated carriers will be done in the following manner: each contaminated carrier will be wiped back and forth and up and down by pushing on the carrier. The carrier will be wiped from right to left three times, with one right to left motion being considered one stroke. Then the carrier will be wiped up and down three times, with one up and down motion being considered one stroke.

One towelette will be used to wipe a total of 10 carriers (see last page—MISCELLANEOUS INFORMATION)

After the contact period, the virus-test agent mixture will be neutralized with 1 ml of the appropriate neutralizer and scraped from the surface of the carrier. This will be considered a one-log \(_{10}\) dilution.

After wiping the 10th carrier, the used towelette will be placed in a sterile Petri dish and held for the remainder exposure time at the exposure temperature. Post exposure, the towelette will be squeezed to express any test agent solution remaining in the towelette.

Should no test agent be expressed from the towelette, the towelette will be soaked in 10 ml of cell culture media for 5 to 10 minutes and squeezed again to allow for expression of liquid from the towelette. Approximately 0.1 ml of the expressed liquid will be diluted in cell culture media.
If columns are used, an aliquot of the neutralized sample from each carrier will be loaded into individual pre-spun Sephadex/Sephadryl columns. Then the samples will be aseptically transferred from the columns and serial diluted as appropriate in cell culture media. In the absence of columns, serial tenfold dilutions of neutralized virus will be prepared in cell culture media.

E. Infection, cell maintenance and infectivity assays

Selected dilutions of the neutralized inoculum/test agent mixture will be added to cultured cell monolayers. Four wells per dilution will be added to the host cell monolayers and incubated at 37±2°C in 5±1% CO$_2$ for 5-7 days. Post incubation the infectious FCV will be scored microscopically by observing CPE produced by replicating infectious virus. The CPE associated with FCV is visually evident under the microscope by the presence of small, shrinking cells that have detached from the monolayer. These changes are 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 titered 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 serial 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. Input virus control (IVC)

Cell culture media will be added to the dried virus. Post-contact time, the virus/cell culture media mixture will be subjected to the same neutralization procedure as the test agent. The control virus will be scraped from the surface of the carrier to resuspend the contents. Serial dilutions will be assayed for infectious virus. 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. IVC will determine the loss of infectious virus, if any, post-drying period.
The results from this control 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. 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 serial diluted as appropriate in cell culture media. If columns are not used, the neutralizer effectiveness samples will be serial diluted tenfold in cell culture media. The diluted samples will be mixed with low titered 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 serial 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.

6. Blank towelette virus control

This control will be performed in duplicate and exactly as the test carriers, except that a sterile gauze pad (or test towelette without the active ingredient) immersed in a specified volume sterile deionized water (consistent with the test towelette) will be used in place of a towelette. Prior to application, the gauze pad will be checked for sufficient wetness. One gauze pad will be used to treat both carriers. Following the contact time, the virus-test agent mixture will be processed in the same manner as the test carriers. The sample will be serial diluted tenfold in cell culture media and selected dilutions will be inoculated into 4 wells of host cell plates. The sterile gauze pad should only be used in the absence of a test towelette without active ingredients.

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.

\[ -\log_{10} \text{_of the 1}\text{st dilution inoculated -}[(\text{Sum of } \% \text{ mortality at each dilution}/100) - 0.5) \times \text{logarithm of dilution}] \]

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 IVC. When cytotoxicity is present, at least a 3-log reduction from the IVC 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
- Blank towelette virus control
- Input virus 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 IVC control must be $\geq 4$-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 and Lot 2
Active Ingredient
Additional Instructions