

US EPA ARCHIVE DOCUMENT

VIRUCIDAL EFFECTIVENESS TEST

Using Bovine viral diarrhea virus (BVDV)

As Surrogate for human Hepatitis C virus

Prepared for

US EPA

ANTIMICROBIAL DIVISION

OBJECTIVE:

This test is designed to validate virucidal effectiveness claims for a product to be registered as a virucide. It determines the potential of the test agent to disinfect hard surfaces contaminated with human Hepatitis C virus (HCV). The 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 online at <http://www.epa.gov/fedrgstr/EPA-PEST/2000/August/Day-25/p21784.htm> and Virucidal Testing Format and Statistics Primer issued by EPA March 2000.

DISCUSSION:

There are no *in vitro* systems or suitable animal models that are available for growing HCV, other than humans and chimpanzees, neither of which is available for disinfectant testing. However, Bovine viral diarrhea virus (BVDV) is closely related to HCV based on similar genomic organization and replication and can be used as a surrogate to provide an accurate indication of human hepatitis C virucidal efficacy^{1,2,3}, meeting the acceptance (superscripts) criteria of some international regulatory agencies⁴. Furthermore, critical issues on its survival and inactivation have been investigated making BVDV a suitable surrogate for HCV enabling evaluation of test agents^{5, 6,7,8}.

1. David Zakim, M.D. *Hepatology - A Textbook of Liver Disease*, 3rd Ed. Vol. 2, W.B. Saunders Company, 1995.

2. Kenny V. Brock, Dept. of Pathobiology, Veterinary Medicine, Auburn University Office of Technology Transfer, Patent Application.

3. Next line Fields, B.N., D.M. Knipe, P.M. Howley, et al. *Fields Virology* 3rd Ed. Lippincott-Raven Publishers, Philadelphia, PA, 1996.

4. <http://www.health.gov.au/hfs/tga/docs/html/tgo/tg54.htm>

5. Sattar, SA and M.K. Ijaz. The use of surrogate viruses to study the environmental survival and chemical disinfection of Hepatitis C virus. The 1st Canadian Conference on Hepatitis C, Montreal, Canada; held during May 1-4, 2001.

6. Ijaz, MK, Suchmann, DB, Pugh, JC and Sattar, S.A. Blood borne hepatitis viruses: The use of surrogates to study the chemical disinfection of hepatitis Band C viruses. XIIth International Congress of Virology, Paris, France, July 27 - August 01, 2002.

7. Ijaz, MK, Suchmann, DB, Sattar, S.A, Chen, Z, Kang, D, Suchman, AA, and Pugh, JC. Using Viral surrogates to test chemical germicides against Human Hepatitis Band C Viruses. The 18th Annual Clinical Virology Symposium and Annual Meeting of the Pan American Society for Clinical Virology. Clearwater Beach, FL., April 28 - May 1, 2002.

8. Ijaz, MK, Suchmann, DB, Sattar, S.A. Chen, Z, Kang, D, and Pugh, JC. The use of Bovine Viral Diarrhea Virus (BVDV) as a GLP surrogate model to study the environmental survival and chemical disinfection of Hepatitis C Virus. The Chemical Specialties Manufacturer's Association, Mid Year Meeting, Chicago, Illinois, June 2002.

TESTING CONDITIONS:

Two lots of the test agents will be used to inactivate the challenge virus that has been dried on a sterile glass surface (two replicates for each batch or 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 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 dilution 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 should be retained for a period of at least three months after completion of the test, then discarded in a manner that meets the approval of the safety officer.

- B. Can include, but are not limited to:

1. Challenge virus as requested by the sponsor of the study:

Bovine viral diarrhea virus (BVDV). [American BioResearch Laboratories]

2. Host cell line:
Madin Darby bovine kidney cells (MDBK).
[American BioResearch Laboratories / ATCC CCL-22]

3. Laboratory equipment and supplies.

4. Media and reagents:
 - a. Minimum Essential Media Eagle's containing 5% horse serum (CCM)
 - b. Horse serum (HS)
 - c. Phosphate buffered saline (PBS)
 - d. pas containing 0.5% HS (PBS+)
 - e. Fluorescein isothiocyanate-conjugated (FITC)Anti-BVDV polyclonal antiserum.
 - f. Tissue culture (TC) grade alcohol.
 - g. Sephacryl S-1000 columns (if necessary)
 - h. 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:

Viral stocks are purchased from reputable sources and are propagated at testing facility. They are titered and stored in an ultra-low temperature freezer. Records are maintained that demonstrate the origin of the virus.

Frozen viral stocks will be thawed on the day of the test (freshly prepared viral stocks may also be used). The organic soil concentration will be adjusted to at least 5% for the virus.

B. Carrier preparation:

An aliquot of 0.2 mL of stock virus will be spread over an area of approximately 4 in² that has been marked on the underside of pre-sterilized Petri dishes with a cell scraper. Then the virus will be allowed to dry for 30 to 60 minutes at room temperature. The drying time and temperature will be recorded. Two carriers will be prepared for each test agent lot, data consistency concentration, and plate recovery control.

Additionally, one carrier per test agent lot and each data consistency concentration will be prepared for the neutralizer effectiveness control using CCM 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:

After the inoculum has dried, 2.0 mL of the test agent will be added, ensuring the dried virus film is completely covered. The plates will remain at the temperature and for the time specified by the sponsor.

After the contact period, the test agent will be neutralized with 2.0 mL of appropriate neutralizer and the mixture will be scraped from the surface of the dish with a cell scraper. Depending upon the test agent, the mixture can also be collected and neutralized by a method appropriate for the disinfectant being tested. This will be considered approximately one log₁₀ dilution.

If columns are utilized, 0.5 mL of each sample will be loaded into individual prespun Sephacryl columns. The columns will be spun for 4 minutes at 1000 rpm. The samples will be removed aseptically from the columns and dispensed into dilution tubes containing CCM. Ten-fold serial dilutions will be performed in CCM. If columns are not used, serial tenfold dilutions of neutralized virus will be prepared in CCM.

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:

The residual infectious virus in both test and controls will be detected by a direct immunofluorescence assay. Selected dilutions of the neutralized inoculum/test agent mixture will be added to cultured cells, four wells per dilution, and incubated at $37 \pm 2^\circ \text{C}$ in $5 \pm 1\% \text{CO}_2$ 3-5 days. Post-incubation the infectious BVDV will be assayed by immunofluorescence assay.

F. Immunofluorescence assay.

Cells will be fixed using TC grade alcohol and stained with FITC anti-BVDV polyclonal antibody. Host cells supporting active virus replication can be detected by UV-microscopy. The data thus obtained will be used to determine the $\log_{10}$ reduction (LR) of infectious BVDV fluorescent focus forming units (FFFU) using the Most Probable Number (MPN) method as described in EPA-Statistic Primer (EPA-SP). The results of test will be compared with plate recovery control (PRC) to reach acceptable test outcome criteria.

G. 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).

1. Cell viability control (CVC):

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

2. Virus stock titer (VST):

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 BVDV. The virus inoculum will be serially diluted tenfold in CCM. Selected dilutions will be inoculated into four wells per dilution and incubated under the same conditions as the test.

3. Plate recovery control (PRG):

Two mL of CCM will be added to the dried virus. Post-contact time, the virus/CCM mixture will be subjected to the identical neutralization procedure as the test agent. If columns are used, a portion of the virus/CCM/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 PRG 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 criterion (see below).

4. Column titer control (CTC)

This control will be performed only if Sephacryl columns are used. It is performed to determine any effects of Sephacryl columns on infectious virus titer while passing through the columns.

The sample for this control will be acquired from a portion of the Plate recovery control prior to passing through the columns. This sample is used to make direct ten-fold serial dilutions in CCM. Then it will be processed in the same manner as the rest of the test and controls.

5. 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 (see below) will be processed exactly as the test procedure but instead of viral inoculum, GCM will be

added. Post neutralization, a 1.0-mL sample will be divided into two portions, [one for cytotoxicity control (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 ten-fold in CCM. If columns are not used, the neutralizer effectiveness sample (0.5 mL) will be directly diluted using serial ten-fold dilutions in CCM.

Following this procedure, virus (100 uL of stock) will be added to each dilution and incubated at the contact conditions outlined for a period equivalent to or greater than the contact time. Then the selected dilutions will be used to inoculate host cells as described for the test procedure.

6. 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 and each concentration of the internal control test agent will be run to determine cytotoxicity. The CT sample, acquired from the neutralizer effectiveness control, will be serially diluted tenfold in CCM, having no virus added. Selected dilutions will be inoculated and incubated in the same manner as the rest of the test and controls.

7. Data consistency control/internal control test agent (method validation):

The dried challenge virus will be exposed to 2.0 mL/carrier of 50 ± 5 ppm and 350 ± 5 ppm Bardac 2280 (using duplicate carriers for each concentration), a quaternary ammonium compound secured from Lonza, Inc. in place of the test agent. After a contact time of ten minutes at room temperature, each carrier will be neutralized using 2.0 mL of horse serum.

Prior to use in testing, the two concentrations will be confirmed. The 350 ± 5 ppm concentration will be confirmed using the Hand-Held Titration Cell for Titrimetric Analysis for QAC from CHEMetrics, Inc. and the 50 ± 5 ppm concentration will be confirmed using the LaMotte QAC Test Kit from LaMotte Company.

The results obtained from this control will provide data to reviewers of the study

demonstrating the ability of the protocol to generate reproducible, valid data.

8. Neutralizer effectiveness for data consistency control:

For this control, each concentration of the data consistency control will be processed exactly as the control procedure but instead of viral inoculum, CCM will be added. Post neutralization, a 1.0-mL sample will be divided into two portions, [one for cytotoxicity control (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 ten-fold in CCM. If columns are not used, the neutralizer effectiveness sample (0.5 mL) will be directly diluted using serial ten-fold dilutions in CCM.

Following this procedure, virus (100 uL of stock) will be added to each dilution and incubated at the contact conditions outlined for a period equivalent to or greater than the contact time. Then the selected dilutions will be used to inoculate host cells as described for the test procedure.

9. Cytotoxicity for data consistency control:

A cytotoxicity control for the data consistency control will be run to determine if the product is toxic to the cells. Each concentration of the neutralized control agent will be run to determine cytotoxicity. The cytotoxicity samples will be acquired from the neutralizer effectiveness control performed for the data consistency controls. They will be serially diluted tenfold in CCM, having no virus added. Selected dilutions will be inoculated and incubated in the same manner as the rest of the test and controls.

H. Calculations:

The LR for each test and control culture will be determined from the dilutions plated using the MPN method as described in the EPA-SP.

PRODUCT EVALUATION CRITERIA:

According to the EPA, the test agent passes the test if a minimum of 4-LR in FFFUs of BVDV (complete inactivation of the virus occurs at all dilutions tested) is demonstrated compared to PRC. When cytotoxicity is present, at least a 3-LR 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 and appropriate control cultures:

- . The viral titration for each sample.
- . Evaluation of cytotoxicity of the test agents.

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 $\geq 4\text{-log}_{10}$
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- . Viral-induced toxicity must be distinguishable from test agent induced cytotoxic effects.

REPORT FORMAT:

Each final 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

STUDY DATES:

The anticipated date of study initiation (date when the study director signs the protocol) is within three weeks upon receipt of test agent and letter of authorization with a purchase order number and a signed protocol. The date the study director signs the final report is the study completion date.

RECORDS TO BE MAINTAINED:

All raw data, protocols, 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, resolution, and impact on the study as soon as practical.

The proposed experimental start and termination dates as well as additional information about the test agent and the type of neutralizer to be employed in the test will be addressed in 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