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**PROTOCOL FOR TESTING THE EFFICACY
OF DISINFECTANTS USED TO INACTIVATE
DUCK HEPATITIS B VIRUS AND TO SUPPORT
CORRESPONDING LABEL CLAIMS**

**Developed by MicroBioTest, Inc.
and Submitted to the
Environmental Protection Agency
August, 2000**

OBJECTIVE:

This test is designed to substantiate virucidal effectiveness claims for a product to be registered as a virucide with the Environmental Protection Agency. It determines the potential of the test agent to disinfect hard surfaces contaminated with hepatitis B virus. The test is designed to simulate consumer use, conforms to EPA Guidelines DIS/TSS-7, November 1981, and follows the procedure outlined in Virucidal Testing Format and Statistics Primer issued by EPA March 2000. The study will be conducted under EPA GLP regulations (40 CFR §160).

DISCUSSION:

There are no *in vitro* systems or suitable animal models that are available for growing human hepatitis B virus (HBV), other than humans and chimpanzees, neither of which is available for disinfectant testing. Duck hepatitis B virus (DHBV), the prototype avian hepadnavirus, can be used as a surrogate to provide an accurate indication of human hepatitis B virucidal efficacy^{1, 2}.

Experimental infection of primary duck hepatocytes (PDH) with DHBV does not result in an obvious cytopathic effect and consequently, virus replication must be demonstrated by alternative methods. Typically, DHBV replication is assayed using indirect immunofluorescence that detects virus proteins present in infected hepatocytes, or by nucleic acid hybridization which identifies intracellular DHBV DNA intermediates.^{2, 3} These methods exhibit comparable sensitivity in our laboratory⁴.

TESTING CONDITIONS:

Two lots of the test disinfectant will be used to inactivate the challenge virus that has been dried on a glass surface (two replicates for each batch or lot of the test product).

¹. Murray, SM, Freiman, JS, Vickery, K, Lim, P, Cossart, YE and Whiteley, RK, 1991 Duck Hepatitis B virus: a model to assess efficacy of disinfectants against hepadnavirus infectivity, *Epidemiol. Infect.*, Vol. 106, p. 435-443.

². Pugh, JC, Ijaz, MK, and Suchmann, DB. Use of surrogate models for testing efficacy of disinfectants against Hepatitis B virus. *Am. J. Infect. Cont.*, 27:375-376, 1999.

3. Pugh, JC, Suchmann, DB, Ijaz, MK. Hepatitis B virus Efficacy Testing: Qualification of an avian Hepadavirus *in vitro* system that uses primary duck hepatocyte cultures. 10th International Symposium on Viral Hepatitis and Liver Diseases. Atlanta, USA, April 9-13, 2000, Abstract B139.

³4. Pugh, JC and Summers, J. Infection and uptake of duck hepatitis B virus by duck hepatocytes maintained in the presence of dimethyl sulfoxide. *Virology*, 172:564-572, 1989.

The test product will be tested in a manner consistent with the label directions for use of the product or as specified by the sponsor. After a timed exposure period, the disinfectant-virus mixture will be scraped from the surface, collected, neutralized and assayed for the presence of viable viruses using primary duck hepatocyte cultures.

MATERIALS:

Substances: supplied by the sponsor (see last page).

The test substance 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 substance such as dilution or specialized storage conditions.

The sponsor assures MicroBioTest, Inc. testing facility management that the test substance has been appropriately tested for identity, strength, purity, stability, and uniformity as applicable.

MicroBioTest, Inc. will retain all unused test materials for a period of at least three months after completion of the test, then discard them in a manner that meets the approval of the safety officer.

B. Materials supplied by MicroBioTest, Inc., including, but not limited to:

1. Challenge virus: Duck Hepatitis B virus (DHBV).
2. Host: Primary duck hepatocytes (PDH).

3. Monoclonal antibody specific for DHBV envelop protein.⁵

4. Media and reagents:

- a Collagenase
- b Sephacryl S-1000 columns
- c Fetal bovine serum
- d Sterile deionized water
- e Liebovitz-15 complete tissue culture medium
- f Goat anti-mouse FITC-conjugated antibody
- g Swim's S77 medium
- h 0.2 M EGTA
- i 1 M Calcium Chloride
- j Phosphate buffered saline (PBS)
- k PBS containing 10% fetal bovine serum
- l Tissue culture (TC) grade ethanol

5. Laboratory equipment and supplies.

EXPERIMENTAL DESIGN:

A. Inoculum preparation:

Sera from ducks that are congenitally infected with DHBV are the source of DHBV used for PDH infection. Virus may be produced at MicroBioTest or purchased from a reputable source, titered and stored as aliquots at or below -70°C . Repeat freeze-thaw cycles are avoided because DHBV infectivity is adversely affected by such treatment.

B. Host cell preparation:

Ducklings will be purchased from a reputable source approximately one week after hatching. Birds will be tagged and blood will be drawn from each animal. Serum samples will be tested for the presence of DHBsAg using an enzyme immunoassay that uses a monoclonal antibody specific for the DHBV large

⁵. Pugh, JC, Di, Q, Mason, WS and Simmons, H. H. Susceptibility of duck hepatitis B virus infection is associated with the presence of cell surface receptor sites that efficiently bind viral particles. *J. Virol.* 69:4814-4822, 1995.

envelope protein. Only birds that test negative for DHBsAg will be used for PDH preparation.

Primary duck hepatocytes will be prepared by *in situ* perfusion of duckling's liver with collagenase³. Cells will be plated on TC plates and infected with DHBV within a week after plating

C Carrier preparation:

An aliquot of 0.2 mL of stock virus will be spread, with the cell scraper, over an area of approximately 28 cm² that has been marked on the underside of pre-sterilized glass Petri dishes and allowed to dry for 30 to 60 minutes at room temperature.

D. Test material preparation:

The disinfectant will be prepared and used according to the sponsor's directions or proposed label claims.

E. Test:

After the carrier preparation, two mL of test product will be added to the test surface. The plates will remain at the temperature and time specified by the sponsor. Each lot will be treated identically.

After the contact period, the virus-disinfectant will be neutralized with 2 mL of fetal bovine serum (FBS) and the mixture will be scraped from the surface of the dish with a cell scraper. This will be considered a 10⁻¹ dilution. Serial tenfold dilutions of neutralized virus will be prepared in cell culture medium (CCM).

E. Test (cont'd)

Then these samples (0.5 mL of each sample) will be loaded onto pre-spun Sephacryl columns (SCs) and spun to obtain the eluate (depending upon the test product, the mixture can also be collected and neutralized by a method appropriate for the disinfectant being tested before passing through the SCs. This eluate will be used for making serial tenfold dilutions in CCM and plated. For spray type products, the product will used as per sponsor's instructions and following

spraying and contact time, the procedure for processing the samples will be the same as described earlier (see above).

F. Infection, cell maintenance and infectivity assays:

Selected dilutions of the eluate will be inoculated into four wells of PDH for each dilution and incubated at $37 \pm 2C$ in 5% carbon dioxide and will be maintained for 7 to 14 days after infection. Post-incubation the infectious DHBV will be assayed by immunofluorescence assay (IFA) (see below).

G. Immunofluorescence assay:

Cells will be fixed in TC grade ethanol and stained using a monoclonal antibody specific for the DHBV envelope protein. Hepatocytes supporting active virus replication can be detected by UV-microscopy after incubation with fluorescein-conjugated anti-mouse immunoglobulin. The data thus obtained will be used to determine the \log_{10} reduction (LR) of infectious DHBV 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 a control comprised of a mixture of CCM and virus (also known as plate recovery) to reach an acceptable test outcome criteria (see below).

H. Controls:

1. Cell viability control (CVC):

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

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 PDH to support infection of DHBV.

3. Recovery efficiency of infectious virus after drying (plate recovery control or PRC):

The virus inoculum will be spread over the surface of a sterile glass petri dish and left to dry at ambient temperature for 30 to 60 minutes. A volume of CCM equivalent to that of the test agent will be added to the dried virus. Post-contact time, virus will be subjected to the identical neutralization procedure as the test products. This control will determine the relative loss in virus infectivity resulting from drying and neutralization alone.

When samples are required to pass through the Sephacryl columns, a column titer control (CTC) will also be performed (by assaying a portion of PRC before passing through the columns) to determine any affect on infectious virus titer while passing the columns.

The results from this control are compared with test results and provided we recover at least four log of infectious virus in this control following drying, neutralization, its titer is used to compare with the titers of test results to reach the acceptable test criteria (see below).

H. Controls (cont'd)

4. Neutralizer effectiveness control (NEC):

The neutralization procedure will be dependent upon the active compound present in the test product. Each lot will be tested. Dilutions will be prepared from the neutralized test agent in CCM and a specified amount of the virus stock, (60-100) FFFU, will be added to each dilution before inoculating PDH monolayers for virus adsorption.

Results of this test will be compared with the titer of a control mixture of CCM and virus. This control will determine the effectiveness of the neutralization procedure and therefore may be performed prior to the test.

5. Cytotoxicity control (CT):

A CT control will be run to determine if the product is toxic to the cells. Each lot of the non-neutralized test product will be run to determine their cytotoxicity. A single carrier will be processed for each batch or lot of test agent. This is be tested in duplicate microtiter wells.

6. Data consistency control (method validation):

The virus will be exposed to one lot of 175 ppm and 350 ppm BTC 835, a quaternary ammonium compound secured from Stepan Company, in place of the test agent. This will provide data to reviewers of the study demonstrating the ability of the protocol to predict reproducible, valid data.

I. 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.

TABLE PRESENTING THE MINIMUM SAMPLE NUMBERS FOR TESTING:

Experimental group	Number of carriers	Log ₁₀ dilutions per carrier	Microtiter wells per dilution	Total number of wells
Positive virus control	2	4	4	32
Test, 1 st lot	2	4	4	32
Test, 2 nd lot	2	4	4	32
1 st lot toxicity control	1	3	2	6
2 nd lot toxicity control	1	3	2	6
1 st lot neutralizer control	1	3	2	6
2 nd lot neutralizer control	1	3	2	6
Negative virus control	1	Undiluted	2	2
Total wells	NA	NA	NA	122

PRODUCT EVALUATION CRITERIA:

According to EPA, the compound passes the test if a minimum of 4-LR in FFFUs of DHBV (complete inactivation of the virus occurs at all dilutions) is demonstrated compared to PRC and NEC.

At least a 3-LR must be demonstrated beyond the cytotoxic level (from the NEC and the PRC).

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 FFFD/mL of the virus recovered from the CCM control must be at least 10^{-4} .
- Viral-induced toxicity must be distinguishable from test compound induced cytotoxic effects.

DATA PRESENTATION:

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

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

REPORT FORMAT:

MicroBioTest, Inc. employs a standard report format for each test design. Each final report provides the following information:

- Sponsor identification and test material 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 two weeks upon receipt of test material and letter of authorization with a purchase order number and a signed protocol.

The date for submission of the final report to the sponsor will be within one month of laboratory phase completion. 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 material records, final report, and correspondence relevant to this study, between MicroBioTest, Inc. and the sponsor will be stored in the archives at MicroBioTest, Inc., 105B Carpenter Drive, Sterling, VA 20164.

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; additional information about the test agent; and the type of neutralizer(s) to be employed in the test will be addressed in a project sheet issued separately for each study.

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. This study will be conducted at MicroBioTest, Inc., 105B Carpenter Drive, Sterling, VA 20164.

MISCELLANEOUS INFORMATION:

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

- A. Name and address: XXXXX
- B. Test agent: _____
Lot No 1: _____ Lot No 2: _____
Dilution to be tested: _____ Diluent: _____
Exposure time: ____min Exposure temperature : ____±2C.
Additional information: _____
- C. Precautions/storage conditions: See MSDS

REPORT HANDLING:

This information is to be:

- submitted to the EPA submitted to the FDA
- used for internal purposes only
- Other: _____

PROTOCOL APPROVAL:

Sponsor: _____ Date: _____

Study Director: _____ Date: _____