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Date of Publication (in Revised Form): May 1988

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CHAPTER 12

IDENTIFICATION OF ENTEROVIRUSES

1. INTRODUCTION

A neutralization test used to identify enteroviruses is described in this chapter. The test utilizes reference-typing sera directed against isolated waterborne viruses. This renders the viruses noninfectious when treated with a matching serotype reagent. Virus inactivation is ascertained microscopically by observing the absence of host cell destruction in the presence of the virus-antibody mixture. The procedure consists of simultaneously inoculating virus and antiserum into a microtiter plate, incubating the virus-antibody mixture for 2 hr, adding a suspension of host cells to the mixture, incubating the host cells-virus-antibody mixture for three days, then examining the cells daily for five more days for the absence of cytopathic effects (CPE).

The test utilizes the Lim Benyesh-Melnick (LB-M) antiserum pools which consist of 61 equine antisera. They include LB-M antiserum pools A-H for the identification of 41 enteroviruses, a single antiserum preparation for the identification of coxsackievirus B3 and LB-M pools J-P for the identification of 19 group A coxsackieviruses not identified by pools A-H. The A-H antiserum pools, prepared by Melnick and coworkers (1973), with instructions for rehydration and storage and with virus identification tables, had been previously available from the National Institutes of Health (NIH). The stock of these antiserum pools (including the single coxsackievirus B3

antiserum preparation) was depleted in 1983. New pools were again prepared by Melnick and Wimberly (1985) under the sponsorship of the World Health Organization (WHO) using the same bulk materials as were used to produce the earlier pools. A policy statement issued by WHO, Geneva describing conditions for their distribution is available from Dr. C. H. Mordhorst, Director, WHO Collaborating Centre for Virus Reference and Research, Statens Seruminstitut, DK-2300 Copenhagen S, Denmark. The other set of seven antiserum pools (J-P) was also prepared by Melnick and coworkers (1977) under National Institutes of Health sponsorship. Information on their distribution is available from the National Institute of Allergy and Infectious Diseases Repository, Biotech Research Laboratory, Inc., 1600 E. Gude Drive, Rockville, Maryland 20850. Single antiserum preparations are distributed by the Centers for Disease Control, Biological Products Division, Atlanta, Georgia 30333 and the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852.

The microtiter method provided herein is a modification of the method described in the literature accompanying the NIH pools. The two methods work equally well, but the microtiter method requires much less antiserum. The microtiter method was also deemed preferable to the plaque reduction neutralization test (Lennette and Schmidt, 1979) based on cost, time and supplies required for actual analyses.

2. PROCEDURE FOR TYPING VIRUSES

2.1 Apparatus and Materials

2.1.1 Microtiter plates, 96-well, flat bottom.

2.1.2 Sealing tapes for microtiter plates if plates are to be incubated in a non-CO2 incubator (recommended method), or plastic lids for microtiter plates if plates are to be incubated in a CO2 incubator.

2.1.3 Micro-pipettors or pipettes capable of dispensing volumes of 0.025 and 0.05 mL.

2.1.4 Cornwall syringe, or equivalent, capable of delivering 0.2 mL quantities.

2.1.5 Cotton-tipped applicators.

2.1.6 Magnetic stirrer and stir bars.

2.1.7 Narrow-tip felt marking pen.

2.1.8 Inverted microscope

2.2 Media and Reagents

2.2.1 ELAH--Earle's base with 0.5 percent lactalbumin hydrolysate and without NaHCO3 (Hazleton Kansas City Biological, product no. DM-303, or equivalent) supplemented with antibiotics (dihydrostreptomycin sulfate, penicillin G, tetracycline and amphotericin B; Sigma Chemical Co., or equivalent). ELAH--Earle's base solution supplemented with antibiotics is used as a dilution medium. Procure 40 mL of antibiotic supplemented ELAH--Earle's base solution for each virus to be identified. Employ stock antibiotic and ELAH--Earle's base solutions prepared for use in Chapter 10 (December, 1987 revision). Stock antibiotic solutions prepared in Chapter 9 (January, 1987 revision) may also be used. If unavailable, see Chapter 10, Section 2.1.3 for preparation of stock antibiotic solutions and Section 2.1.4 for preparation of ELAH--Earle's base solution and for supplementation of ELAH--Earle's base solution with antibiotics. Remaining reagents may be stored for subsequent use. Store antibiotic stock solutions at -20 degrees C and ELAH--Earle's base solution at 4 degrees C for periods no greater than 4 and 2 months, respectively. Reagents should be held in tightly stoppered or capped containers.

2.2.2 Antiserum pools A-H and coxsackievirus B3 antiserum are prepared as described in WHO instruction sheets. Store at -20 degrees C until used. Prepare antiserum pools J-P only when needed to type viruses not identified by pools A-H or coxsackievirus B3 antiserum. Antiserum pools J-P are prepared as described in NIH instruction sheets.

2.2.3 Growth medium supplemented with antibiotics (penicillin G, dihydrostreptomycin sulfate, tetracycline and amphotericin B; Sigma Chemical Co., or equivalent) and 5 percent fetal calf serum (GIBCO Laboratories, or equivalent) prior to addition of BGM cells. Employ MEM/L-15 growth medium and stock antibiotic solutions prepared for use in Chapter 9 (January, 1987 revision). Stock antibiotic solutions prepared in Chapter 10 (December, 1987 revision) may also be used. If unavailable, see Chapter 9, Section 3.3.3 for preparation of growth medium and Section 3.3.5 for preparation of stock antibiotic solutions. Remaining reagents may be stored for subsequent use. Store antibiotic stock solutions at -20 degrees C and growth medium at 4 degrees C for periods no greater than 4 and 2 months, respectively. Reagents and medium should be held in tightly stoppered or capped containers. Fetal calf serum used should have been heat inactivated at 56 degrees C for 30 min and certified free of animal viruses, bacteriophage and mycoplasma

by the supplier. Each liter of growth medium is supplemented with 1 mL of penicillin-dihydrostreptomycin stock, 0.5 mL of tetracycline stock and 0.2 mL of amphotericin B stock. Prepare 30 mL of medium for each microtiter plate to be used.

2.3 Procedure

2.3.1 Preparation of Microtiter Plates. Arrange each plate as indicated in Figure 1.

(a) With a narrow-tip felt marking pen, draw lines between every two columns along the length of the plate.

(b) On one end of each plate, mark identification code of samples tested. Four viruses can be identified simultaneously on one plate. Thus, number the columns 1, 2, 3, and 4 to designate duplicate wells for each virus.

(c) Mark identity of each antiserum on left side of plate next to each row of wells. (See Figure 1). Designate the first eight rows as A to H to indicate LB-M pools A-H, designate row 9 as B3 to indicate coxsackievirus B3 antiserum, designate row 10 as E to indicate virus control dilution made in antibiotic supplemented ELAH--Earle's base solution, and designate rows 11 and 12 as E1 and E2, respectively, to indicate serial 10-fold dilutions of virus control in row 10.

2.3.2 Preparation of Virus for Identification

(a) Remove virus isolate from storage in -70 degrees C freezer, thaw, and mix well. Designate the virus isolate as No. 1.

(b) Dilute thawed virus to 10-5 in antibiotic supplemented ELAH--Earle's base solution. Prepare 2 mL of 10-5 dilution of virus. The 10-5 dilution is the working dilution of virus that will be mixed with the antiserum pools in the microtiter plate wells.

(c) From the 10-5 dilution, prepare a 1:2 dilution in Earle's Balanced Salt Solution. This dilution will be transferred to row E of the microtiter plate later.

(d) From the 1:2 dilution of virus prepare two serial 10-fold dilutions (1:20 and 1:200). These dilutions will be transferred to rows E1 and E2 of microtiter plate later.

(e) Repeat Steps (a) to (d) with each virus isolate to be identified, designate these isolates 2 through 4, and proceed to Section 2.3.3.

2.3.3 Addition of Antiserum Pools to Microtiter Plate

(a) Thaw the antiserum pools, and mix each antiserum pool well.

(b) With a micro-dilutor tip or pipette, dispense 0.025 mL of antiserum from pool A into each well in row A. It is important to place tip of dilutor or pipette into the bottom of the well and to expel all of the antiserum in the pipette into the well.

(c) Repeat Section 2.3.3, Steps (a) and (b) with antiserum pools B to H and with the antiserum for coxsackievirus B3, placing antiserums into designated wells, and proceed to Section 2.3.4.

2.3.4 Addition of Virus to Microtiter Plates

(a) Add 0.025 mL of the 10-5 dilution of virus No. 1 from Section 2.3.2, Step (b) to each well in rows A to B3 of column 1. Take care to introduce the virus at the top of the wells. Do not allow tip of dilutor or pipette to touch the pooled antiserum within a well and thereby possibly cross-contaminate other antiserum pools.

(b) Into the two wells marked E in column 1, add 0.05 mL of the 1:2 dilution of virus No. 1 from Section 2.3.2, Step (c).

(c) Into the two wells marked E1 in column 1, add 0.05 mL of the 1:20 dilution of virus No. 1 from Section 2.3.2, Step (d).

(d) Into the two wells marked E2 in column 1, add 0.05 mL of the 1:200 dilution of virus No. 1 from Section 2.3.2, Step (d).

(e) Repeat steps (a) to (d) with viruses No. 2 through 4, adding the appropriate dilutions of the viruses to the appropriate wells (See Figure 1).

(f) Gently tap the sides of the microtiter plate with index finger to mix the contents of the wells.

(g) Cover microtiter plates with lids or with a loose sterile cover, and incubate plates at 36.5 plus or minus 1 degree C for two hours.

2.3.5 Preparation of Cell Suspension and Completion of Microtiter Test. Many host cell types, primary and continuous, are available for propagating viruses. Usually, the host cell type in which a virus is recovered from the environment is suitable for identifying that virus by the microtiter neutralization test. See Chapter 9 for procedures and media

for culturing Buffalo green monkey (BGM) kidney cells. See Lennette, E. H. and Schmidt, N. J., Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections, American Public Health Association, Washington, D. C., 1979, for methods for preparation of primary and other continuous cell types, for suckling mouse procedures necessary for identifying most Group A coxsackieviruses, and for methods for identifying viruses other than enteroviruses.

(a) Trypsinize sufficient cells to yield a final cell count appropriate for the cells used in the test. For BGM cells a count of 30,000 to 50,000 cells per 0.2 mL of cell culture medium is appropriate. The number of cells required for this test differs with different cell types.

(b) Add cells to appropriate volume of cell culture medium.

(c) Mix cells on a magnetic stirrer and stir for at least 15 minutes at speed sufficient to develop vortex. A longer period of mixing will generally not injure cells.

(d) Dispense 0.2 mL of cell suspension into each well with a Cornwall syringe after completing the two hour incubation of virus-antiserum mixtures in Section 2.3.4, Step (g). Do not allow tip of syringe to touch contents of a well and thereby possibly cross-contaminate the contents of other wells. With cotton-tipped applicators, wipe up spilled cells on the top of plates between and around wells.

(e) Seal each plate with sealing tape and incubate plates at 36.5 plus or minus 1 degree C. If plates are to be incubated in a CO2 incubator, do not seal plates.

(f) After three days of incubation, examine cells in wells daily for five or more days for the apprearance of CPE. Use an inverted microscope to examine cells.

(g) When CPE develops, use identification tables provided with antiserum pools to identify viruses. Virus identification is based upon the absence of CPE in those wells containing antiserum and virus. If all wells evidence CPE, titrate virus and repeat entire test with a virus dilution calculated to add 100 infective doses to each well in Row E. Follow this same procedure if all virus control wells in Rows E1 and E2 are negative and the pattern of results does not allow identification with identification tables. Where an appropriate quantity of viral infective doses has been used in test and cells in only one of the duplicate wells containing antiserum show no CPE, repeat tests with antiserum pools A-H and B3. If CPE still appears in all wells containing virus and

antiserum, repeat test but with antiserum pools J-P instead of A-H and B3. If CPE continues to be observed in all wells, one must take into consideration the possibility that more than one virus was present in the original test sample. To eliminate the possibility of a mixture of viruses being present, dilute the original test sample with ELAH--Earle's base solution to a viral concentration of between 5 and 10 plaque forming units per mL. Plaque purify the virus using the cell monolayer plaque assay technique described in Chapter 10 (December, 1987 revision). Pick at least three well-separated plaques and prepare sufficient virus quantities from each plaque in accordance with the procedures given in Chapter 11 (March, 1988 revision). Retest each of the new viral stocks utilizing the Lim Benyesh-Melnick antiserum pools. If the viral isolates are still not neutralized by known antisera, record the virus in question as unidentified. If it is essential that all isolates be identified in a monitoring program, store the virus at -70 degrees C for later studies. The virus in question may either be further tested against single antiserum preparations of serotypes not included in the pools or used to prepare antiserum to characterize the isolate against known enteric viruses.

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FIGURES

Figure 1. Representation of Microtiter Plate Preparation.

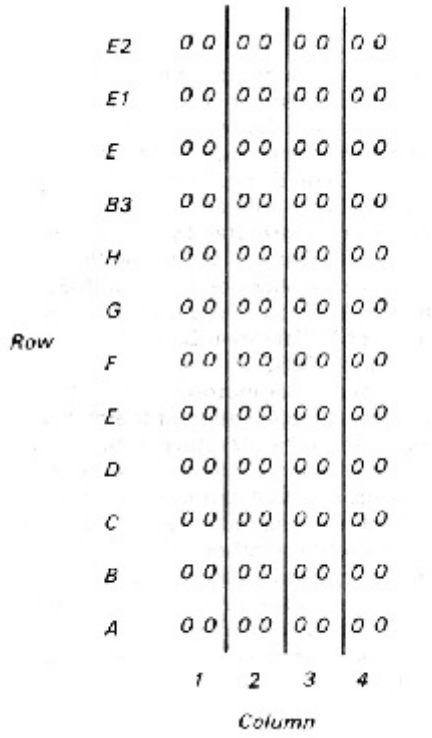
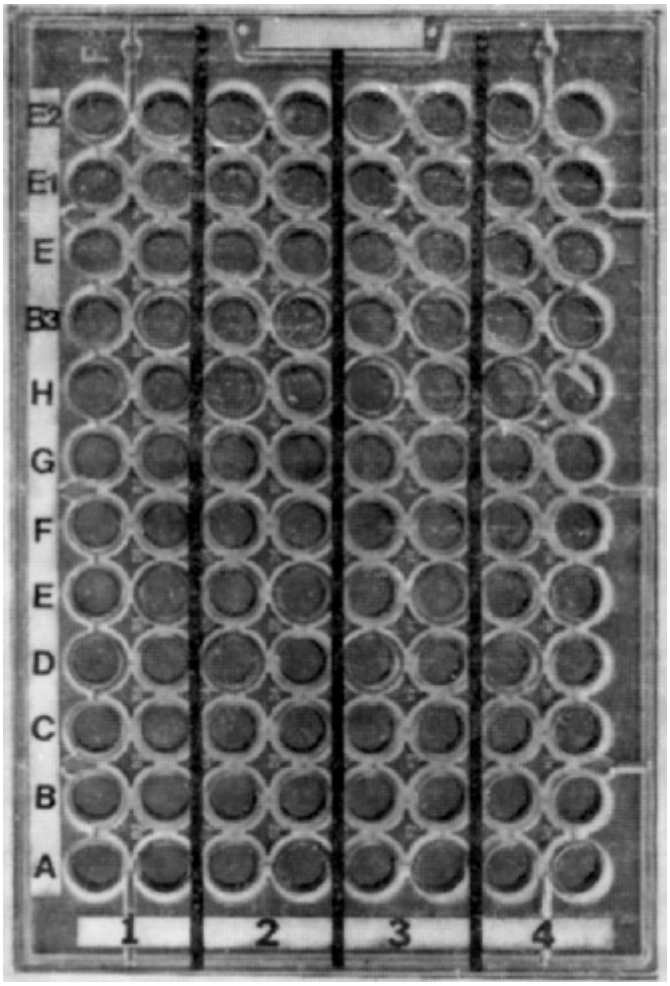


Figure 2. Photographic representation of microtiter plate preparation.



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