A SIMPLIFIED PROCEDURE FOR THE CULTURE OF VIRUSES

George W. Beran

INTRODUCTION

THE CULTURING of viruses in the laboratory poses difficulties not encountered in the culturing of the larger micro-organisms, namely the bacteria, the fungi and the protozoa. As the smallest of the organisms classified as micro-organisms, most individual viral particles can be visualized only with the aid of the electron microscope. The inability of viruses to replicate apart from the living cells in which they exist as obligate parasite precludes against their being cultured on artificial media.

The problem of the minute size of viruses can be resolved in many cases by visualizing the effects of viral activity rather than observing the viral particles themselves. Gross and microscopic observation of lesions produced in infected tissues enables the pathologist to characterize and classify many viral agents without seeing the actual viral particles.

The second problem of the obligate parasitism of viruses is less easily resolved in the laboratory. Although certain viruses may be cultured in experimental laboratory animals or embryonating hens' eggs, suitable laboratory animals have not been found for many viruses. Laboratory animals, being complex living hosts, often show variable responses to viral infection. They are frequently costly, difficult to maintain, and may even be dangerous to the experimenter.

In the last two decades, the cell culture method of supporting viral repiclation has been developed into an efficient and sensitive

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GEORGE W. BERAN *is Acting Head of the Agriculture Department. A microbiologist, Dr. Beran has been an epidemic intelligence officer of the U. S. Public Health Service, a research fellow and instructor in virology at Kansas University Medical Center. He is planning a study of viruses affecting the population of Negros Oriental.*

laboratory tool for the recognition, identification and characterization of viruses. The principles of the cell culture technique are simple. Sterile, living tissues are collected from avian, mammalian, or human hosts and cultured *in vitro*, usually in closed test tubes. Media are employed which provide essential nutrients to the living cells for reproduction and maintenance. Viruses are introduced into the cell cultures and the effects of their infections are observed with the aid of an ordinary light microscope, usually at a magnification of 40-100X.

Cell cultures may be derived from a wide variety of small laboratory animals and large wild or domestic animals and even from human tissues, providing a large host range for the culture of viruses. They may be maintained in a small space and are convenient to use, silent and safe. Their high degree of uniformity reduces the variability of response inate in the use of whole laboratory animals. Viruses cultured in cell cultures are readily recovered for further study or storage. Certain cell cultures, particularly those originating from malignant tissues may be subcultured from one culture tube to others. More frequently employed, however, are primary cultures which utilize fresh tissues from the host animals each time new sets of cell cultures are prepared.

Although the principles of the cell culture technique are simple, the procedures involved are specialized and sensitive. Recent developments in the technique have been toward the use of increasingly complex media and of more costly equipment. These refinements have led to more precise results and wider applicability of the cell culture method for viral studies. Many laboratories, however, which desire to use cell cultures and which have personnel who have received some training in their use, find the costs and technical difficulties too great to surmount.

These difficulties are especially marked in those countries where equipment and media must be imported, frequently at high cost and with long delays. Deterioration of imported items is often rapid and the lack of a repair part or a single chemical may cause the loss of valuable time or of a continuously maintained line of cells which may be difficult to replace.

Studies in the Silliman University Mission Hospital laboratory have been directed toward the development of cell culture techniques which incorporate as many as possible of the benefits of recent advances with a maximum simplication of the procedure. Primary emphasis has been on the development of primary cultures of monkey renal cells for the recognition of polioviruses, ECHO viruses, and influenza viruses in clinical specimens, the last by the aid of the hemadsorption technique, and for the preliminary classification of unidentifiable agents. The performance of more complex studies, particularly metabolic studies, may be more readily performed in the larger, better equipped laboratories. The procedures described here have utilized the simplest equipment, materials and techniques which were found to give satisfactory results. Economy of equipment was given preference over the reduction of labor whenever both could not be mutually achieved.

Material and Methods

The following minimum required items were available from local importers as standard items for hospital and clinical laboratory use.

Equipment and supplies purchased.

Balance, analytical, with weights, one only;
Refrigerator, household, one only;
Incubator, bacteriological, small, one only;
Centrifuge, laboratory, angle head, one only;
Burner, gas, pressure; or hot plate, electric, one only;
Cooker, pressure, household, ten liter size, one only;
Microscope, student type, one only;
Filter, Seitz, Model EK, 30 or 100 ml. capacity, one only;
Pads, filter, Seitz, Model ST, Size B or D, one package;
Aspirator, water, with rubber tubing, one unit;
Lamp, alcohol; or burner, Bunsen, one only;
Thermometer, laboratory, one only;
Flasks, Erlenmeyer, 250 ml., two or more;
Flask, suction, 250 or 1000 ml., one only;
Flask, boiling, flat bottom, 250 ml., one or more;
Beads, glass, solid, 5 mm., 50-100;
Cylinders, graduated, 100 ml., two or more;
Pipettes, 10 ml., six or more;
Pipettes, 1 ml., six or more;
Funnel, glass, one or more;
Plates, Petri, glass, two or more;
Tubes, test, 16 x 150-mm., 100-200;
Tubes, test, 13 x 100 mm., 50 or more;
Tubes, centrifuge, conical, calibrated, 12-15 ml., two or more;

Syringes, glass, 10 ml., two or more;
Needles hypodermic, 20 gauge 1-1/2 inch, two or more;
Scalpel, surgical, one or more;
Scissors, surgical, two or more;
Forceps, hemostatic, rat-nosed, two or more;
Forceps, thumb, rat-nosed, two or more;
Miscellaneous items: wrapping paper, rubber bands, string, gauze, cotton or kapok, waxed paper, household detergent, glass marking pencils, assorted bottles for chemicals and solutions, assorted cans to hold equipment for sterilization or storage.

Equipment and supplies made in laboratory.

Pipettes capillary: Drawn from glass tubing. Etched at 0.1, 0.2, and 1.0 ml. contents;
Racks, test tube, vertical: Wooden blocks with holes drilled to fit tubes;
Racks, cell culture tubes 6° from horizontal; Wood and fiber board construction with holes drilled to fit tubes;
Holder, cell culture tubes 6° from horizontal; microscope: Wooden block with center hole for passage of light through tube, groove to hold tube;
Pans, washing, and racks, glassware: Cut from 19 liter kerosene cans, painted with white enamel.

Solutions and media.

Hank's Basic Salt Solution (BSS), modified[1]

Distilled water	1000 ml.
NaCl	8.0 gm.
KCl	400 mg.
$MgSO_4.7H_2O$	200 mg.
Na_2HPO_4	60 mg.
KH_2PO_4	60 mg.
Glucose	1.0 gm.
Penicillin	100,000 u.
Streptomycin	100 mg.
Mycostatin	10,000 u.
$CaCl_2$	140 mg.
$NaHCO_3$	350 mg.

Add components in order listed, Sterilize by Seitz filtration. For sterilization by autoclaving, solution A $=$ $NaHCO_3$ in 50 ml. water, solution B $=$ other components in 950 ml. water. Autoclave 10 lbs. pressure for 10 min. Store not over 12 hours at room temperature or 24 hours in refrigerator. Mix solutions A and B before using.

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[1] Hanks, J. H., and Wallace, R. E., "Relation of Oxygen and Temperature in the Preservation of Tissues by Refrigeration," Proceedings of the Society for Experimental Biology and Medicine, Vol. 71, 1949, p. 196-200.

Phosphate Buffered Saline (PBS), modified[2]

Distilled water	1000 ml.
NaCl	8.0 mg.
KCl	200 mg.
Na_2HPO_4	1.15 gm.
KH_2PO_4	200 mg.
Penicillin	100,000 u.
Streptomycin	100 mg.
Mycostatin	10,000 u.
$MgCl_2.6H_2O$	100 mg.
$CaCl_2$	100 mg.

Add components in order listed. Sterilize by Seitz filtration. For sterilization by autoclaving, solution A $=$ $MgCl_2.6H_2O + CaCl_2$ in 100 ml. water, solution B$=$other components in 900 ml. water. Autoclave 10 lbs. pressure for 10 min. Store not over 12 hours at room temperature or 24 hours in refrigerator. Mix solutions A and B before using.

Chick Embryo Extract, aqueous (CEE)

9-10 day chick embryos, eyes removed, and adjuncted fluids	50.0 ml.
Phosphate buffered saline, sterile	50.0 ml.
Penicillin	10,000 u.
Streptomycin	10 mg.
Mycostatin	1,000 u.

Employ sterile equipment and aseptic precautions throughout.

Homogenize embryos and adjuncted fluids in mortar with sterilized washed sea sand. Add PBS and antibiotics. Stand at room temperature 30 min. Stand frozen in freezing compartment of refrigerator overnight. Thaw and refreeze two additional times. Clarify by centrifugation 3000 rpm. for 20 min. and decant supernatant fluids, or by Seitz filtration. Store in refrigerator not over 6 months.

Ascitic Fluid (AF)

Obtain by aseptic paracentesis from patients with cirrhosis of the liver. Culture to ensure bacterial sterility. Store in sterile bottles in refrigerator not over 6 months.

Monkey Serum

Obtain by cardiac puncture of anesthetitized monkeys. Aseptically separate serum from retracted clot and store in sterile bottles in regrigérator not over 6 months.

Trypsin Solution[3]

Trypsin 1:250	2.50 gm.
Distilled water	1000 ml.

[2]Dulbecco, R., and Vogt, M., "Plaque Formation and Isolation of Pure Lines of Poliomyelitis Viruses," *Journal of Experimental Medicine*, Vol. 99, 1954, p. 167-182.

[3] Dulbecco, R. and Vogt, M., *op. cit.*

Penicillin	100,000 u.
Streptomycin	100 mg.
Mycostatin	10,000 u.

Sterilize by Seitz filtration. Store in refrigerator not over 6 months.

Distilled Water

Single distilled in Barnstead(R) still. Boil 1/2 hours before use in cell culture solutions.

Pomerat's Ascitic Fluid Medium, modified[4]

BSS	450 ml.
CEE	50 ml.
AF	500 ml.

Adjust to pH 6.8-7.0 with NaOH or HCl as needed, using Fisher's Gramercy Universal Indicator(R)

Melnick's Lactalbumin Medium, modified[5]

BSS	980 ml.
Lactalbumin Enzymatic Hydrolysate (LEH)	2.5 gm.
Monkey Serum	20 ml.

Pancreatic Digest of Casein (PDC) may be subsituted for LEH

Adjust pH to 6.8-7.0

Gramercy Universal Indicator(R)

BSS	950 ml.
LEH or PDC	2.5 gm.
Yeastolate	1.0 gm.
CEE	50 ml.

Adjust to pH 7.0-7.2

RESULTS

Handling of equipment. All glassware and instruments were washed in tapwater with the aid of household detergent. Pipettes were washed by bubbling detergent solution through them with the aid of a water aspirator. All items were rinsed for one minute under flowing tap water. Cell culture tubes were rinsed four times in flowing distilled water. Other items were rinsed twice by serial dipping in pans of distilled water. (*See Figure* 1.)

Test tubes, flasks, and cylinders were closed with kapok plugs wrapped with gauze and tied with string. Kapok was locally available and naturally water repellent, making it a satisfactory subs-

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[4] Pomerat, C. M. "Motion Picture Studies of Living Papilloma of the Breast and Breast Cancer," *Texas Reports on Biology and Medicine*, Vol. 10, 1952, p. 217-227.

[5] Melnick, J. L. "Tissue Culture Techniques and their Application to Original Isolation, Growth, and Assay of Poliomyelitis and Orphan Viruses", *Annals of the New York Academy of Science*, Vol. 61, 1955, p. 754-773.

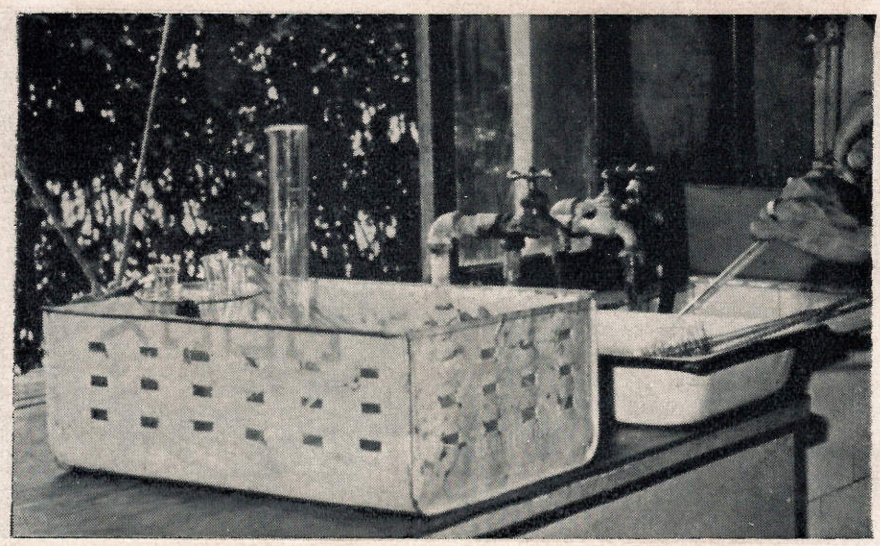

FIGURE 1: WASHING GLASSWARE. Glassware rack made from 19-liter kerosene can. Water aspirator on faucet draws detergent solution through pipette.

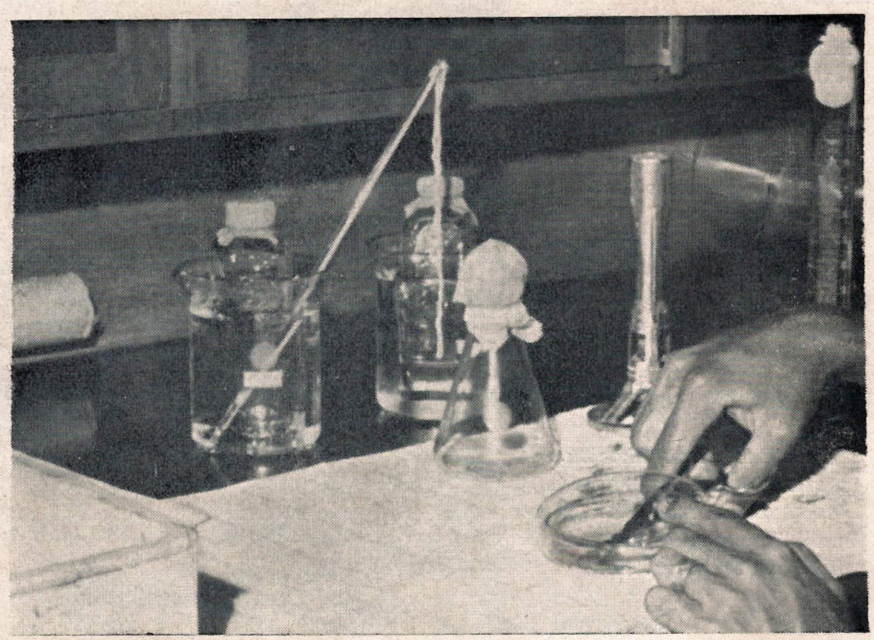

FIGURE 2: MINCING OF KIDNEY CORTEX with scissors. Bottles in water in beakers contain PBS and trypsin solutions at 37°C. Plugs are gauze-covered kapok.

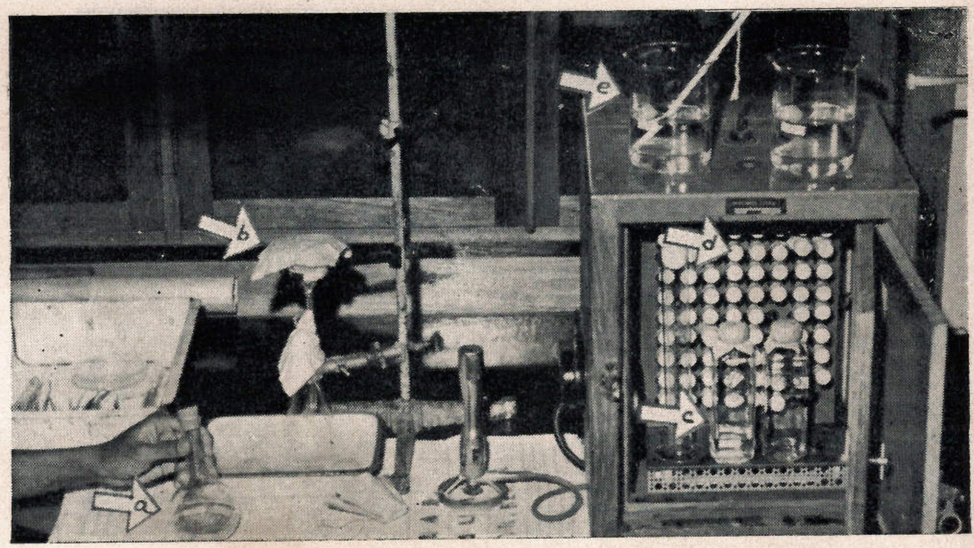

FIGURE 3: Trypsinization of Minced Kidneys. (a.) Spherical flask with glass beads for enzymatic extraction. (b.) Gauze-covered funnel and flask in ice bath for dispersed cells. (c.) Bottles of solutions in 37° incubator. (d.) Cell cultures in rack in incubator. (e.) Beakers of water used to heat solutions to 37°C.

FIGURE 4: Microscopic Examination of Cell Cultures. (a.) Wood rack to hold tubes. Natural light is used to illumine field under observation.

titute for nonabsorbent cotton. The plugs were reused until worn. Wrapping paper was used for hoods over kapok-plugged vessels and for wrapping instruments, mortars and pestles, Petri plates, pipettes, funnels, syringes and needles.

Autoclaving was performed in a household pressure cooker, being careful to maintain a pressure of 15 lbs. for 20 minutes. Sterilization indicators were not employed. Each item was marked "Sterile" with a wax pencil as it was taken from the cooker. Moisture remaining in the glassware was allowed to evaporate during storage or was removed by placing the items in the incubator set at 60-70°C.

Contaminated equipment was boiled for 20 minutes immersed in tapwater with one teaspoon of detergent per four liters of water before being washed.

Preparation of renal cells for culture. The method employed was a modification of that described by Youngner.[6] Healthy, immature monkeys (*Macaque cynamologus*) were anesthetized with ether and exsanguinated by cardiac puncture. The blood was preserved to supply serum for media or alternately the whole blood was utilized to prepare blood agar plates for bacteriological studies.

Immediately following death of the monkeys, the kidneys were aseptically removed and placed in sterile Petri plates. The capsule and medulla were dissected from the cortex of each kidney and the cortical area was minced into segments 4-6 mm. in diameter with scissors. Careful aseptic technique was employed throughout the procedure. (*See Figure* 2.)

Before proceeding further, trypsin and PBS solutions were warmed to 37°C in beakers, or a pan of water, with the aid of a gas burner or hot plate. The solutions were then placed in the incubator at 37°C to maintain this temperature during the remainder of the operation, affording an effective substitute for the constant temperature water bath.

The minced tissues were then placed in a sterile Erlenmeyer flask and washed three times with warm PBS, using 20 ml. of the salt solution per kidney for each washing. Following decanting of the last wash solution, the PBS was replaced with 20 ml. per kidney

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[6] Youngner, J. S. "Monolayer Tissue Cultures. I. Preparation and Standardization of Suspensions of Trypsin-Dispersed Monkey Kidney Cells," *Proceedings of the Society for Experimental Biology and Medicine,* Vol. 85, 1954, p. 202-205.

of warmed trypsin solution. After five minutes of gentle agitation, the trypsin was removed by decanting and discarded. These four washings were performed to remove blood, urine, and fragmented cells remaining in the mass of minced kidneys.

Trypsin solution was again added to the minced kidneys; the mass was swirled; and the suspension of kidney segments was transferred aseptically to a sterile boiling type flask containing approximately 50 glass beads. The flask was shaken to agitate the kidney segments in the trypsin solution by the action of the glass beads. Tryptic digestion of the intracellular cement of the kidney tissue was allowed to procede under agitation for seven to ten minutes until the solution became cloudy. The shaking was then stopped to allow the large particles to settle from the trypsin solution. The trypsin with its suspended cells were decanted into a glass funnel covered with four layers of sterile gauze and into a sterile flask placed in an ice water bath. The gauze pad acted to remove tissue fragments that remained suspended. Further tryptic action on the isolated cells was prevented by cooling in the ice bath. (*See Figure* 3.)

Five to eight further extractions were performed in the flask with glass beads until the majority of the epithelial cells were removed and the exhausted cortical fragments remained as white threads of connective tissue. The chilled suspensions of epithelial cells in trypsin were transferred into sterile 16 x 150 mm. test tubes and separated by centrifugation in the angle head centrifuge at 800 rpm for 5 minutes or by standing in the refrigerator for two hours. The supernatent trypsin solution was removed from the sedimented cells by decanting or by aspiration with a 10 ml. pipette fitted with a rubber bulb. The cells were resuspended in an estimated 20 volumes of sterile chilled BSS by aspiration into and expulsion from a 10 ml. pipette. They were resedimented by centrifugation or standing as before. Two to three washings were considered adequate to remove all remaining trypsin from the cells. The last washing was performed in sterile conical centrifuge tubes to facilitate measurement of the sedimented cell volume. After removal of the last wash fluid, the sedimented cells were resuspended in a small volume of cell culture growth medium and diluted to form a 1:200 suspension by volume in a flask of growth medium. This cell suspension was distributed in one ml. aliquots into 16 x 150 mm. culture tubes with the aid of a 10 ml. pipette. Before each additional 10 ml. sample was withdrawn from the flask, the cells were resuspended by aspiration and expulsion with a pipette. The culture tubes were closed with gauze-wrapped kapok plugs and covered with two thicknesses of waxed paper secured with rubber bands to reduce the escape of carbon dioxide from the tubes. In later studies, white rubber stoppers were available and proved to be more convenient, but no more effective in closing the culture tubes.

Culture media employed. The relatively simple culture medium described by Pomerat in 1952[7] supported satisfactory growth of the cells. The lactalbumin medium[8] or the digest of casein medium were also simply compounded and proved to be satisfactory growth media. Since these media contained ascitic fluid or serum, the danger existed that they might contain antibodies that would interfere with viral replication, so the maintenance medium was formulated to contain neither of these components.

Care of cell cultures. Culture tubes containing cells suspended in growth medium were marked with a wax pencil by lines near the openings parallel to the long axis of the tubes. They were placed in the incubator at 37°C in racks 6° from horizontal and rotated until the wax pencil lines were at the tops of the tubes. The cultures were left undisturbed for three days to allow the cells to settle from the growth medium and attach to the inside walls of the tubes. After the third day, a sampling of the culture tubes was examined microscopically each day to observe the growth of the cells into a monolayer. At the time the cell sheets had developed over approximately one half of the area covered by medium when the tubes were in the incubating positions, the spent medium was decanted from the tubes and replaced with newly prepared growth medium, observing strict aseptic precautions. At the time of the replenishment of the growth medium, usually on the fourth to sixth day of culture, the pH of the spent medium was found to be 5.5-6.0. The tubes were returned to the incubator as before.

Daily microscopic observations of the cultures were made until the cell sheets were developed into confluent monolayers on the inside walls of the tubes. At this time the growth medium was removed. One ml. aliquots of sterile BSS were placed into each tube and de-

7 Pomerat, C. M., *op. cit.*

8 Melnick, J. L., *op. cit.*

canted so that the fluid flowed over the cell layer and out of the tube. The culture tubes were then replenished with sterile maintenance medium and returned to the incubator for one to four days. At this time, the tubes were examined for the presence of contaminating bacteria by observing for cloudiness of the medium, and tubes showing no evidence of contamination were inoculated with specimens for viral study. Tubes kept for more than four days were replenished with newly prepared maintenance medium before inoculation. Satisfactory maintenance of the cells was usually obtained for ten days without further replenishment of the maintenance medium following inoculation.

Handling of specimens. Throat swabs, fecal specimens, and tissues or body fluids collected at necropsy were stored in the freezing compartment of the refrigerator until processed for viral assay. Particulate matter and absorbed fluids collected on throat swabs were suspended in 2 ml. each of sterile PBS in sterile 13 x 100 mm. test tubes by swirling the swabs in the fluid and expressing them against the inside walls of the tubes. Fecal samples or tissue specimens were homogenized in sterile mortars to make 10% suspensions in sterile PBS. Body fluids were diluted to make a 10% solution in PBS. All suspensions were clarified by centrifugation at 3000 rpm for 30 minutes or by standing in the refrigerator overnight and the supernatent fluids were decanted or aspirated into sterile 13 x 100 mm. test tubes. Antibiotics were added at the rate of 50,000 u. of penicillin, 50 mg. of streptomycin, and 5,000 u. of Mycostatin per ml. of fluid. The tubes were plugged, hooded with waxed paper, and stored in the frozen state. Blood samples collected by venipuncture were kept at room temperature until the blood clotted and then placed in the refrigerator until the clots retracted. The sera were decanted into 13 x 100 mm. test tubes, closed and stored as described above.

The saline extracts of the specimens were assayed for viruses by placing 0.1 ml. aliquots of each extract into 2-3 cell cultures each, with the aid of capillary pipettes. The inoculated cultures were maintained in the incubator and observed microscopically every 1-2 days. Cultures showing microscopic evidence of viral activity were harvested at the time that cytopathic degeneration of the cell sheet appeared to be maximal. Cultures were harvested by vigorously shaking the culture tubes and pouring the fluids into sterile 13 x100 mm. test tubes. These tubes are closed and stored in the freezing compartment of the refrigerator. Inoculated cultures which failed to show evidence of viral activity within ten days of observation were either recorded as negative or the fluids were subcultured into newly prepared cell cultures and observed for an additional ten days.

Discussion

The cell culture method employed in this study was found to support the replication of enteroviruses and influence viruses of human origin for isolation and serologic studies. The glass bead method for trypsin extraction of renal cells was found by comparative studies with the tissue blendor method to give equal or higher yields of viable cells. The lack of an indicator dye in the growth and maintenance media was found to be no handicap if the medium changes were scheduled by the development of the cell sheet as outlined.

This description of a simplified procedure for the production and use of cell cultures for viral study is intended for the microbiologist or medical technologist who has a general knowledge of the cell culture method but may not have access to the literature on the subject. For this reason, the principles underlying each phase of the operation are not discussed in as much detail as is allotted to the mechanics of the procedures and the formulation of the media involved.

Summary

Laboratory aid in the diagnosis of viral diseases is becoming an urgent need in hospitals throughout the world. The economic and technical difficulties involved in the utilization of modern methods of virus culture preclude their use in medium size or even large hospitals or research laboratories in many countries. By the utilization of the simplified procedures described in this report, the required equipment and chemicals for the production of cell cultures which satisfactorily support the replication of enteroviruses and influenza viruses from human patients can be limited to items of standard supply for clinical laboratories.

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This study is aided in part by a gift from Dr. C. D. Van Houweling, Ames, Iowa, U.S.A., and a grant from the Chapman Research Foundation, Silliman University.