

Appendix A. Viral Isolation Protocol (for Cell Culture)

Introduction

The optimal method for determining specific etiology of an arbovirus infection requires isolation of the virus from a specimen obtained from the patient during the acute stage of the disease and the demonstration of a rise in titer of an antibody to the isolate during convalescence. For a number of reasons successful isolation of most arboviruses from specimens from patients is the exception, reasons being that the specimen to be examined is not collected soon enough, is not properly handled, or is not expeditiously transmitted to the virus laboratory for inoculation. The viremia for many arbovirus infections in humans, if detectable at any stage, ceases by the time of or soon after onset of symptoms-- a stage when antibody is often demonstrable. Because some circulating virus may be recoverable and the antibody may be absent, or present in low titer, the acute-phase blood specimen should be collected immediately upon suspicion of a viral etiology. Delay of an hour or so can compromise the chance of virus isolation; the allowable time depends upon the type of viruses involved.

Certain arboviruses produce a viremia of sufficient magnitude and duration that the viruses can be isolated from blood during the acute phase of illness, e.g., 0 to 5 days after onset. Examples of these viruses include the agents of yellow fever, dengue, chikungunya, Venezuelan equine encephalitis (VEE), and sandfly, Ross River, and Oropouche fevers. The viremia in Colorado tick fever is unique because it can extend for weeks or months, and infection has been transmitted by transfusions. Many encephalitogenic arboviruses can be recovered from cerebrospinal fluid (CSF) obtained during the acute phase of illness and brain; rarely St. Louis encephalitis (SLE), Japanese encephalitis (JE), western equine encephalitis (WEE), and eastern equine encephalitis (EEE) viruses have been recovered from blood. Viral isolates can be recovered by biopsy or at autopsy from the viscera of patients with acute yellow fever, dengue hemorrhagic fever or other viscerotropic arboviral infections. For isolation from brain, samples should be taken from several areas including the cortex, brain nuclei, cerebellum and brain stem. Neurotropic arboviruses sometimes can be isolated from CSF obtained by lumbar puncture during the acute stages of encephalitis or aseptic meningitis. Alphaviruses have been isolated from joint fluid of patients with acute polyarthritis; and from the upper respiratory tract of patients with acute VEE. Under certain circumstances arboviruses have been recovered from urine, milk, semen, and vitreous fluid.

Principle

Susceptible cell culture systems are available for the attempted isolation of the presumed etiologic agent of an illness or disease. Following successful isolation, the isolate may be positively identified and an antigen prepared from this isolate or the virus itself may be used to test the patient's serum for the presence of antibodies to the viral isolate. If antibodies are detected, this entire exercise confirms that the isolate was the causative agent of the illness or disease. In certain instances, serum from a patient may not be available. Under those circumstances, one relies on reisolation of the causative virus from the same original specimen. However, reisolation is always attempted whether serum is available from the patient or not.

Materials and Reagents

Vero cell culture monolayers or other suitably susceptible cell cultures
C6/36 cell cultures-cloned *Aedes albopictus* mosquito cells

Procedure

Available tissues or fluids should be divided for viral isolation, electron microscopy, and for immunohistochemical examination. Tissues should be collected aseptically and rapidly transported to the laboratory in viral transport. The aliquot for viral isolation should be immediately frozen at -70 C in a mechanical freezer or stored on dry ice. Samples for viral isolation should be kept frozen continuously, avoiding freeze-thaw cycles that inactivate virus. The aliquot for electron microscopy should be minced and placed directly in glutaraldehyde. Autolytic changes occur rapidly and tissues should be fixed as quickly as possible. A portion of the sample should be fixed in buffered formalin or preferably, embedded in freeze-media and frozen, to prepare sections for immunohistochemical examination.

Procedure con't Processed specimens should be inoculated into cell cultures with a minimum of delay. Sera from patients with acute febrile illnesses can be used undiluted for virus isolation or at dilutions of 1:10 and 1:100 in a protein-containing diluent. It is important to inoculate unknown specimens at two or preferably more dilutions (undiluted to 10^{-2}). Shell vial cultures or 25cm² flask cultures of Vero are inoculated and observed for the production of cytopathic effects (CPE) during 10-14 days. For shell vials, a total volume of 400 ul is inoculated followed by centrifugation at 100 x g for 1 hour at 37degrees. A portion of the cell supernatant can be collected and tested for the presence of virus by either targeted RT-PCR or consensus RT-PCR assays. Alternatively cells are harvested and spot slides are prepared for IFA examination using monoclonal dengue type-specific antibodies.

Controls Uninoculated Vero and C6/36 cells

Interpretation Positive virus isolation, reisolation and definitive identification define the etiologic agent of the patient's illness. If paired sera or a convalescent serum from that patient are available, the identified viral isolate is tested serologically with the patient's sera to verify antibody response to that virus.

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