Current Practice

Diagnosis of viral fever

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The spectrum of syndromes that fall under the domain of 'viral fevers' is both diverse and extensive. It is also a fact that members of different families of viruses could give rise to a similar clinical syndrome as well as a given member of a virus family could give rise to different viral syndromes. Furthermore, the clinician is often faced with the absence of tell-tale signs in the febrile patient. Thus, the demand for rapid and accurate diagnosis of viral diseases has increased dramatically over the past decade. This article is not intended to be a comprehensive manual of diagnostic methods for specific viral diseases, but rather a general description of the principles behind established assays and an introduction to the newer methods now employed in diagnostic virology.

Before attempting to describe the methods, which will continue to change, it is important to emphasise several principles that are important to both the clinicians and the diagnostic laboratory. First, no assay is perfect; thus, it is always dangerous to base all patient management procedures on a single result. The clinician should remember that all assays can give false negative and false positive results. He/she must ensure that specimens are obtained, transported and stored properly prior to arrival at the diagnostic laboratory.While the molecular biology of viruses and their interactions with cells are highly complex, virtually all diagnostic techniques are based on three principles. First, viruses are biologic machines dedicated to the generation of progeny viruses. In vitro virus isolation techniques remain the most widely used and perhaps the best diagnostic method available. However, the maintenance of tissue cultures is an expensive and labour intensive procedure and is not available in all laboratories. The second principle is that viruses are composed of two distinctive components, nucleic acid and proteins. The detection of these virus specific components in clinical specimens is the basis for diagnostic techniques that examine clinical specimens for viruses without isolating them in cell culture. The final principle is based on the immune response of the host. Viral infectious elicit virus specific immune responses that may be detected in the patients serum or cerebrospinal fluid (CSF).

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Specimens

Perhaps the best place to begin any consideration of viral diagnostics is with the specimen itself. The proper interpretation of results from any assay depends on obtaining, transporting, and processing an adequate specimen in terms of both quantity and quality.

Virus isolation in tissue culture

Viruses such as poliovirus, coxsackie virus, echovirus, herpes virus, cytomegalovirus, varicella virus, adenovirus, influenza virus, parainfluenza virus and dengue virus can be relatively easily grown in cell culture. Of these the Medical Research Institute (MRI) carries out isolations of polio, coxsackie and echo viruses, herpes simplex virus and dengue virus. In case of polio, coxsackie and echo viruses the sample is 2 -5 g of stools (size of 2 tamarind seeds) collected into a suitable container such as a used yoghurt cup and properly sealed. The container need not be sterile. However, it should he held in ice and transported as soon as possible to MRI. The timing of the sample should be early as possible in the disease, although samples could be sent up to 14 days from the onset of the disease. The number of samples should be two, collected 24-48 hours apart.

Sample for isolation of herpes simplex virus is ideally the vesicular fluid in case intact vesicles are seen. It could be done with a sterile scalpel blade, by puncturing the vesicle and collecting the released fluid on to the blunt side of the blade. The fluid should ideally he transported in viral transport medium (VTM) available at MRI. In the absence of VTM, normal saline could be substituted. The container and the fluid should be sterile before introducing the sample. The collected specimen should be transported in ice as soon as possible. When only ulcers are seen a sterile swab should he

gently rubbed on the base of the ulcer and the distal end of the swab transported in VTM.

For isolation of dengue virus, 5 ml of blood is collected within the first week of fever into an anticoagulated vial (preferably citrate anticoagulant) and sent in ice with minimal delay. Bone marrow is also useful, if sent anticoagulated.

Virus detection directly from clinical specimens

Due to low sensitivity and specificity, histochemical and light microscopy has largely been replaced by more sophisticated techniques. Perhaps the only exception is the Seller's stain done to detect rabies virus in specimens which is carried out at MRI.

Electron microscopy and immune electron microscopy, because of the expense of the instrument and the high degree of technical expertise that is required, is not available in Sri Lanka for routine diagnosis. However it is useful in detecting viruses that cannot be cultured in normal cell culture, particularly rotaviruses and other gastroenteritis agents such as norwalk and astrovirus.

lmmunofluorescence and immunochemical staining

Immunofluorescence tests are most useful for the detection of respiratory viruses and viruses causing lesions of the skin or mucous membranes. In the case of respiratory infections the sample is a nasopharyngeal aspirate collected into a sterile vial. After processing the sample, it is spotted on to a special slide and stained with the direct or indirect technique. When such slides are illuminated by a fluorescent microscope a characteristic pattern of either cytoplasmic or nuclear staining can be observed. MRI carries out immunoflurescent tests for influenzae A and B, respiratory syncitial virus, adenovirus, rabies virus and hantavirus.

The term immunochemical staining refers to the use of an enzyme as the indicator instead of a fluorescent substance. Once an appropriate substrate containing a chromogen is added, a change of colour occurs at sites where viral antigen is present. This can be observed under a light microscope.

Solid phase immunoassay

Direct antigen detection is also performed using solid phase immunoassays. Some assays use filtration of specimen into a membrane that non specifically binds antigen. This type of assay uses an enzyme indicator and a substrate that causes the substrate to precipitate on to the membrane and is read visually. The second type of solid phase assay uses capture antibody. These assays rely on the binding of a capture antibody to plastic microtitre plate or polystryrene beads. When viral antigen is present in the clinical specimen it is bound or captured by the immobolised antibody. This captured antigen is then detected with a second antibody labelled with an enzyme. The enzyme causes a colour change when substrate is added. Results are read on a spectrophotometer of plate reader. Such ELISAs are used in the detection of p24 antigen of HIV, antigens of HSVL and HSV2.

Radioimmunoassays (RIAs) use radioactive labelled detector antibodies and results are determined in a beta or gamma counter. Although initially RIA was more sensitive than ELISA, the gap has narrowed with improved enzyme labelling methods and the use of fluorogenic and chemiluminescent substrates. Viral antigen can also be detected by agglutination assays. These assays are based on specific antibody immobilised on latex particles that form visible clumps in the presence of antigen. Such kits for detecting rotavirus and HSV are available commercially and have the advantage of a long shelf life.

Nucleic acid hybridisation

This test requires a sample containing an adequate number of cells infected by a given virus. e.g. nasopharymgeal aspirate in case of respiratory viruses, a section of a wart in case of human papilloma virus, buffy coat in case of Epstein-Barr virus etc. The test essentially involves digestion of cells together with viral surface proteins and extraction of the nucleic acids which is hybridised with a specific probe. This probe is a piece of single stranded RNA or a synthesised DNA oligonucleotide which is fashioned to have the exact reverse sequence of a given portion of the viral genome. As such, when a specific match is found with the extracted nucleic acid, hybridisation occurs. As the probe has been prior labelled, the hybridised complex can he detected by standard procedures.

Hybridisation techniques are becoming more standardised and some are now available as commercial kits. However extracting nucleic acid from specimens and labelling and detection methods are somewhat complex and require specialised equipment. Thus this method is not routinely used in small laboratories.

Serodiagnosis of viral disease

Detection of virus specific antibody in a single serum specimen is useful to diagnose infection by persistent pathogens like HIV, herpes viruses and hepatitis viruses or to detect prior infection with infrequent viruses like viruses from animal reservoirs. It is also used to determine the immune status of patients at high risk for acquiring viral infections or in need of vaccination. Pregnant women are often screened to determine if they are immune to CMV, rubella, mumps and varicella zoster virus. Blood donors, transplant donors and recipients are also screened for antibody to CMV.

However, most diagnostic methods are based on the demonstration of a significant rise in antibody titres between an acute serum, taken at or very near the onset of symptoms and a convalescent serum obtained 14 days later. Diagnosis based on the detection of IgG antibodies in convalescent sera are, by definition, retrospective. However assays that detect IgM in acute sera are more useful to the clinician in patient management. This is by virtue of the fact that IgM is raised only during the early phase of an infection and does not usually persist beyond 6-8 weeks. This would mean that if IgM is detected it's an ongoing infection or a recent infection.

There are two main types of antibody assay that rely on the inhibition of biologic function of viruses viz. neutralisation of infectivity and haemagglutination inhibition. The end point of the neutralisation assay is the failure of a standard virus to grow in tissue culture after exposure to serum. Thus, the time required can be quite long depending on the rate of viral replication in vitro. The major advantage of this assay is that neutralising antibody is most often indicative of protective immunity.

The haemagglutination inhibition test is dependent on the ability of certain viruses to agglutinate red blood cells from some animal species. Thus, like the neutralisation of infectivity assay, the end point is failure of antibody bound virus to haemagglutinate. These assays are still considered to be the gold standard for some viruses e.g. dengue virus. However, most laboratories rely on ELISA methods for serologic diagnosis.

The ELISA method for IgG antibody detection uses viral antigen immobilised onto a solid surface. Specific antibody in the test serum binds to the antigen. After washing away unbound antibody, the specific antibody bound to viral antigen is detected with a labelled antihuman indicator antibody followed by a substrate. The ELISA method has the advantage of being sensitive, versatile and amenable to automation. The ELISA for the detection of IgM follows the same format as that of a IgG ELISA except the second labelled antibody specifically binds human IgM. The major disadvantage of this type of IgM ELISA is that high concentrations of specific IgG or rheumatoid factor can interfere with the test causing false positives to occur. This has been overcome by development of a IgM capture assay which makes use of anti-human IgM antibody anchored to the solid phase. This antibody binds the IgM in the sample regardless of antigen specificity. In the subsequent washes all IgG is washed away. The captured IgM is then incubated with the specific viral antigen which can be directly labelled. Alternatively, to detect the viral antigen a second viral-antigen specific detection antibody is used. Although the presence of [gM is indicative of an acute infection, in certain instances they should be interpreted with caution. For example in patients with rubella, herpes virus infections and certain arbovirus infections, there is persistence of IgM making interpretation of IgM assays difficult. In general, the use of on [gM assay for hepatitis A, CMV, EBV, herpes simplex virus, mumps, measles, rubella, JE, dengue is reliable. These tests are presently available at IU MRI. The sample for serology is usually 3-5 ml of blood obtained by venepuncture collected into a sterile container without anticoagulants or preservatives. The specimen is allowed to clot at room temperature and the serum is separated by centrifugation. The serum is then collected to a separate sterile vial. It is particularly important that separated serum is sent if a lengthy transit is expected. For longer storage, serum is usually frozen to -20^0C or below. Whole blood should never be frozen as it causes complete haemolysis and may render the specimen unsuitable for serologic testing. In case of paired sera, each specimen could be submitted separately, carefully identifying it as 'acute' or 'convalescent' or store the acute phase specimen to be submitted for testing with the convalescent specimen. Paired acute and convalescent phase sera from a patient should always be tested simultaneously in one laboratory, since results obtained from two laboratories cannot be accurately compared for changes in antiibody titre. If the specimen is a random sample for determination of immunity, it should be identified as 'for immune status'.

CSF collected from patients with neurologic disease is sometimes submitted for serologic testing. These specimens should be tested in parallel with serum specimens collected at the same time in order for the

results to be interpreted accurately. CSF contaminated with blood is not usable, as it is not possible to tell what proportion of antibodies detected is from the blood and what is produced locally in the CNS. CSF is normally well isolated from the blood, so that CSF titres of antibodies derived from blood are less than 2-3 % of those of antibodies in serum of the same person and are usually not detectable. CSF antibody titres that are higher than 5% of serum titres are often regarded as evidence for CNS infection, leading to local antibody production.

Western blotting or immunoblotting technique is another method of diagnosis of viral infections. This assay has been used as the confirmatory assay for HIV and hepatitis C virus serology. All blotting techniques share a common principle in that large macromolecules of proteins or nucleic acid separate into component parts based on size by electrophoresis. The components are then transferred to a membrane that is a mirror image of the original gel. The individual components can then be identified by the specific binding of an antibody or probe to the membrane.

Nucleic acid amplification

Since the technique was first described polymerase chain reaction (PCR) has revolutionalized diagnostic virology. However, most PCR assays are 'home brewed' and there is no realistic quality control for such assays at this time. This is to be expected as the laboratories that perform PCR use different methods of specimen preparation, different primers and different contamination control measures. As with any new technology, it will take time to become standardised and routinely available. For the present, PCR assays for viral agents are primarily performed in academic institutions and larger reference laboratories. Valuable experience is being gained in the use and application of this technique in the research setting. PCR has been invaluable in the diagnosis of HSV encephalitis and CNS involvement in neonatal herpes infections, hepatitis C, CMV etc. In more recent times it has played an important role in assessing viral load in persistent infections such as HIV, HBV, HCV, enabling accurate measurement of response to antiviral therapy in these infections.

Thus, while PCR and other amplification techniques are fast becoming the diagnostic tests of choice, the clinician should remember that these methods are still experimental and should be used in conjunction with other clinical and diagnostic information in the acute care of patients. Presently, PCR for the detection of myobacterium tuberculosis is carried out at the Department of Microbiology, Faculty of Medicine, Colombo. In case of pulmonary TB, 10-15 ml of a bronchial wash collected into a sterile container is recommended. If delays in transport are expected the sample should be held on 4^0C. In case of suspected tuberculous meningitis a sample of CSF should be promptly submitted.