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MINIREVIEW

Advances in Dengue Diagnosis

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INTRODUCTION

Despite improvements in health, epidemics of infectious diseases continue to occur, and new diseases emerge and old diseases reemerge (113). Mosquito-borne flavivirus diseases are currently considered reemerging infections because of the increase in the incidences of yellow fever and, mainly, dengue fever and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) observed in the last few years (30, 86).

The dengue syndrome is an acute febrile viral exanthem, accompanied by headache, myalgia, anorexia, gastrointestinal disturbances, and postration, caused by viruses transmitted by mosquitoes (43). DHF is a severe febrile disease characterized by abnormalities of hemostasis and increased vascular permeability. DSS is the result of a hypovolemic shock observed in some DHF cases. DHF/DSS represents the severe form of dengue fever (52).

The disease is caused by any one of the four distinct serotypes (1 to 4) of the dengue virus (52, 114). These viruses are members of the family *Flaviviridae*; they have a common morphology and genomic structure, and all members share common antigenic determinants. The four dengue virus serotypes are classified as a complex on the basis of clinical, biological, and immunological criteria. Dengue virus complex-specific antigenic determinants have been demonstrated by using neutralization assays, which also can differentiate the dengue virus complex into four antigenically distinct dengue virus serotypes, since each serotype presents a type-specific determinant (49, 52).

The flaviviruses are transmitted by mosquitoes of the *Stegomia* family, mainly *Aedes aegypti*, a domestic, day-biting mosquito that prefers to feed on humans (52, 99). This is a highly urbanized mosquito, breeding in water stored for domestic use or collected rainwater. A jungle cycle has been proposed to exist in Southeast Asia, since there is a high rate of dengue transmission among different species of monkeys (52, 105). The genomic RNA of dengue viruses is single stranded and approximately 11 kb in length. The RNA is infectious and, as in the rest of the flaviviruses, it has a single open reading frame (103). The order of proteins encoded in the long open reading frame is 5′-C-prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′. The mature virion contains three structural proteins: C, the nucleocapside or core protein of 13.5 kDa; M, a membrane-associated protein of 8 kDa; and the E (envelope) protein of 51 kDa. The E protein has the sites for viral attachment to and transport through host cell plasma membranes. Functional domains responsible for the neutralization and hemagglutination of goose erythrocytes are associated with the E protein. It contains epitopes specific for serotype, dengue complex, and group (6, 48, 103, 115). Considering the technology currently used for the diagnosis of dengue viruses, a case definition in which laboratory confirmation is emphasized has been proposed. The laboratory criteria for confirmation of the infection and the disease include the isolation of dengue virus from serum and/or autopsy samples, the demonstration of a fourfold or greater increase in the titer of immunoglobulin G (IgG) or IgM antibody to one or more dengue virus antigens in paired serum samples, or the demonstration of dengue virus antigen in autopsy tissue or serum samples by immunohistochemistry, by immunofluorescence, or by the detection of the viral nucleic acid (98).

EPIDEMIOLOGY

Different factors, such as population growth, uncontrolled urbanization, high densities of the domestic mosquito vector, a rise in commerce and travel, and the breakdown of vector control programs, have facilitated the emergence of dengue fever in the American region (30). The tropical world is in a dengue pandemic, with 80 million persons affected annually (attack rate of 4%) (86). Epidemics have occurred in Southeast Asia (58), South America (29), East Africa (17), and China and Australia (76). *A. aegypti* eradication campaigns in the Americas deteriorated during the 1970s and 1980s (29), and as a consequence the mosquitoes proliferated and the dengue incidence increased. Countries that had been free of dengue for many years or that had never reported dengue activity, such as Bolivia, Brazil, Ecuador, Paraguay, and Peru, have recently reported dengue outbreaks (29, 97). Moreover, in 1993 the last two Central American countries that had been free of dengue (Costa Rica and Panama) reported indigenous dengue transmission, and finally, at the end of 1994 the reintroduction of dengue virus serotype 3 in Nicaragua and Panama after an absence of 17 years was reported (89). By 1995 almost all Central American countries and Mexico reported the circulation of this serotype.

A severe situation is observed in the Americas with regard to DHF/DSS. In 1981 Cuba reported the first American DHF/DSS outbreak, with reports of 344,203 dengue and DHF/DSS cases, including 10,312 severe cases and 158 fatal cases (65). A successful vector control program was implemented, and the country is still virtually free of *A. aegypti*. This severe event was followed by another serious DHF/DSS epidemic in Venezuela in 1989 and 1990 (29, 97, 98). Smaller DHF/DSS outbreaks have also been observed, and annually there is an increased frequency of DHF/DSS case reports (29). The Americas might face a situation similar to that in Southeast Asia, where since the 1950s DHF/DSS has been a serious health problem in terms of morbidity and mortality (29, 30, 98).

In order to prevent these diseases and control the severe situation that the American region now has, an expert committee of the Pan American Health Organization has proposed guidelines that include the establishment of a laboratory-based active surveillance system for dengue fever and DHF/DSS in order to provide early and precise information to public health

authorities (98). Thus, the diagnosis of dengue becomes an important aim for any laboratory (98).

DETECTION AND LABORATORY DIAGNOSIS

Although dengue is one of the most important viral diseases in humans, the four serotypes are among the most difficult to detect and propagate in the laboratory.

Viral isolation in mice. The two traditional methods for primary isolation of dengue virus are the inoculation of newborn mice and of cell cultures. Dengue viruses may infect mice by a number of routes, but the intracerebral route is the most sensitive, especially in 1- to 2-day-old suckling mice (84), producing paralysis or other signs indicative of pathological involvement of the central nervous system (52, 61, 84, 108, 109, 127). Unlike most other arthropod-borne viruses, the dengue viruses are not very pathogenic when inoculated into the brain of a newborn mouse, probably since they are not neurotropic. The intracerebral inoculation of newborn mice is currently considered the least sensitive isolation system.

Viral isolation in tissue culture and mosquitoes. The application of cell culture techniques to the detection of dengue viruses has led to improvements in isolation sensitivity. However, no mammalian or insect cell culture system in which all dengue virus strains produce a cytopathic effect has yet been found (104). Several mammalian cell cultures have been used for the study of dengue viruses. The LLCMK2 (monkey kidney) line is the most sensitive, although these cells vary in sensitivity to different dengue virus types and strains, and they are insensitive to certain strains (37, 136). Besides the LL CMK2 cell line, Vero (monkey kidney) and BHK21 (baby hamster kidney) cells have also been used (21, 37, 46, 54, 55, 85, 104, 121, 136). In general, the virus requires an adaptation period after the inoculation of cell cultures.

Singh and Paul (117) first succeeded in the maintainence of the four dengue virus serotypes in a mosquito cell line established from larvae of *Aedes albopictus*. Since then, several other mosquito cell lines have also been used or recommended for dengue virus isolation, such as the AP61 (*Aedes pseudoscutellaris*) (47, 127, 128), Tra-284 (*Toxorynchites amboinensis*) (71), C636 (*A. albopictus*) (56, 126), AP64 (clone of an *A. pseudoscutellaris* cell line) (90), and CLA-1 (clone of an *A. pseudoscutellaris* cell line) (91, 92) cell lines.

In general, the advantages of the mosquito cells are that (i) they are more sensitive than vertebrate culture systems for the recovery of dengue viruses (102), (ii) they are relatively easy to maintain and grow at room temperature (56, 71, 102, 126), and (iii) they can be kept for at least 14 days without a change of medium. Further, mosquito cell cultures can be carried into the field and inoculated directly with human sera from patients (101).

Although some reports record the presence of a cytopathic effect (syncytium formation, the presence of multinucleated giant cells, and the phagocytocis of affected cells), induced by all four serotypes, the cytopathic effect produced in mosquito cell lines by many dengue virus field strains is difficult to detect and can be variable (126). Currently, the continuous mosquito cell lines are the most sensitive and the most used for dengue virus isolation.

Because of its higher sensitivity (74, 104), the mosquito inoculation technique is still the method of choice for attempting dengue virus isolation from important specimens and, especially, in fatal cases. *A. albopictus* (27, 68, 69) and *Toxorhynchites splendens* (133) have been shown to be useful for dengue virus recovery. *A. albopictus* mosquitoes have been found to be more sensitive for the detection of dengue viruses than LLCMK2 (104). The use of *T. splendens* larvae is a more rapid and sensitive method for isolation (133). However, the high isolation rate obtained with mosquito cell cultures, plus the ability to economically process large numbers of samples, more than makes up for the lower sensitivity of the cell culture system. Additionally, mosquito inoculation requires special facilities to establish the mosquito colonies and a certain degree of technical training.

For viral isolation, blood should be obtained during the febrile period, preferably before the fifth day after the onset of illness. The acute-phase serum or plasma may be frozen at −70°C. Homogenized tissues, especially liver, spleen, lymph nodes, and thymus, from fatal cases can be used (98). However, most tissues obtained at autopsy have not yielded virus when tested in tissue culture systems. This is probably due to the relative lateness of death after illness onset and the high concentration of neutralizing antibodies in serum and tissues (36, 44, 95).

In general, major factors favoring successful isolation are (i) obtaining the specimen early in the course of the disease and (ii) delivering it promptly to the virus laboratory.

For short periods of storage (up to 24 h), materials for virus isolation are usually kept at 4 to 8°C; for longer storage, the material should be frozen at −70°C (98). It is important to avoid repeated freeze-thawing of the samples.

The presence of large quantities of antibodies in patients with a secondary infection may interfere with viral isolation because of immune complex (virus-antibody) formation (95). Cocultivation of leukocytes (from washed buffy coat) with mammalian cells has been one of the most sensitive isolation methods with patients with a high dengue virus antibody titer (116), although this method is not commonly used.

Currently, inoculation of the C636 cell line with acute sera from patients is the method of choice for dengue virus isolation.

Viral identification. The development of hybridomas that produce serotype-specific monoclonal antibodies for dengue virus provided a simple, economical, reliable, and rapid method for the identification of dengue viruses by the immunofluorescence assay (IFA) independent of the biological system used for dengue virus isolation (26, 49). Henchal et al. (49) produced monoclonal antibodies that were flavivirus group specific, dengue virus complex specific, dengue virus subcomplex specific, and dengue virus type specific. These four kinds of specific monoclonal antibodies were used to identify dengue virus isolates from different geographical areas by the immunofluorescence assay or the plaque reduction neutralization test (49).

The monoclonal antibodies have proved to be effective in identifying dengue viruses of all four serotypes (39, 67, 119, 120), although some evidence suggests that not all serotype 1 and 3 dengue viruses are easily identified with the monoclonal antibodies (120). In general, the major problem associated with monoclonal antibody identification of dengue viruses in culture is poor replication with a resulting low viral concentration in the cells. For that reason, identification in primary cultures is sometimes impossible, and one or two passages through the cell system are necessary to increase the viral concentration.

Serological diagnosis. Two patterns of serological response can be observed in acute dengue infection: primary and secondary. A primary response is seen in individuals who are not immune to flaviviruses. A secondary seroresponse pattern occurs in an individual with an acute dengue virus infection who has had a previous flavivirus infection. An individual infected

with one serotype can never become infected with the same serotype (43).

Nonimmune populations suffer outbreaks of dengue fever. DHF/DSS is currently observed in areas where multiple dengue virus serotypes are endemic and occurs in two immunological settings: primary infections in infants born from dengue-immune mothers (with dengue virus antibodies passively acquired) (42, 62, 83, 134) and second dengue infections in children and adults (actively acquired) (3, 20, 41). The circulation of infection-enhancing antibody, passively or actively acquired, is the proposed pathogenic mechanism of the severe clinical form of the disease. DHF/DSS occurs in individuals with infection-enhancing antibodies in whom neutralizing antibodies are not present.

The hypothesis of the immune enhancement of infection is based on the assumption that the severity of the disease is related to the number of infected cells. It is hypothesized that antibody-dependent immune enhancement is the etiopathogenic mechanism of DHF/DSS. These enhancing antibodies form immune complexes with dengue viruses which efficiently infect mononuclear phagocytes via the Fc receptor; this efficient viral infection produces a high concentration of virus and consequently increases the number of newly infected cells. The severe form of the disease (DHF/DSS) is observed in this case (41–45).

It seems that the pathogenesis of the disease is the result of both virus- and host-dependent factors. Differences in the frequency of DHF/DSS are also related to the ethnic group, sex, age, chronic diseases, immune response, lapse between first and second dengue infections, and nutritional status of the host (3, 34, 35, 44, 63, 65, 93). In primary dengue infection, the antibody titer rises slowly and is relatively serotype specific, although convalescent-phase sera usually contain detectable cross-reactive antibodies in low titer. In secondary infections, the antibody titer rises rapidly to high levels. Frequently, even acute-phase sera show high antibody titers (52).

The serological diagnosis of dengue viruses is complicated by the existence of cross-reactive antigenic determinants shared by all four dengue virus serotypes and some other flaviviruses (52).

The capacity of dengue viruses to agglutinate goose erythrocytes permitted the wide application of the hemagglutination inhibition (HI) assay with pairs of sera to the serological diagnosis of dengue viruses (16). A fourfold or greater increase in antibody titer is diagnostic for a recent flavivirus infection but not for any specific agent. Although an HI antibody titer of $\geq$1/2,560 is the criterion widely accepted to classify a case as a secondary infection, different criteria also have been applied (64, 127).

Considering the broadly reactive determinants among flaviviruses and the high antibody titers observed in individuals with secondary infections, the study of early-convalescent-phase sera from dengue patients can be useful for a presumptive rapid diagnosis (66, 135).

The plaque reduction neutralization test is a sensitive and specific serological assay for detection of anti-dengue virus antibodies (106, 107). Neutralizing antibodies are very stable with time. Some authors have reported that in an individual with a secondary infection, the neutralization titer against the dengue virus serotype responsible for the first infection is anamnestically greater than the neutralization titer against the dengue virus serotype responsible for the second infection, indicating the first infecting serotype ("original antigenic sin") (45, 110). Recently, Kuno et al. (70) have reported that the theory of original antigenic sin cannot be applied reliably in serodiagnosis, because discrepant results were obtained when neutralization results were compared with those of viral isolation. Because of its specificity, the plaque reduction neutralization test is a useful tool for seroepidemiological studies (33, 34). It has also been used for viral identification (2).

The HI and neutralization tests require paired serum samples from suspected cases, and the use of these tests involves long delays before laboratory confirmation can be made.

Enzyme-linked immunosorbent assays (ELISAs) for dengue virus antibody detection have been developed during the past several years. ELISA is inexpensive and is quick and simple to perform. It has many of the properties needed for a good screening test, including broad cross-reactivity and high sensitivity. Several ELISAs for detection of flavivirus total immunoglobulin have been described as being useful for seroepidemiological studies and serological diagnosis (14, 22, 24, 57, 123, 124, 129, 130).

The detection of IgM antibody to dengue virus by ELISA has become one of the most important and useful methods for dengue diagnosis (4, 22, 28, 75). Anti-dengue virus IgM antibody is produced transiently during primary and secondary infections. The detection of anti-dengue virus IgM antibodies indicates an active or recent infection. The antibodies develop rapidly, and by day 5 of illness most patients have detectable anti-dengue virus IgM. On average, IgM antibodies fall to undetectable levels between 30 and 60 days after the onset of illness (98).

The use of IgM for detection of dengue viruses is an invaluable tool for the surveillance of dengue fever and DHF/DSS and is the serological test of choice for most laboratories (28, 98). A kit for the detection of anti-dengue virus IgM antibody based on detecting dengue virus-specific IgM antibodies in the test serum by capturing them with an anti-human IgM has been developed (100). This system has a 92% sensitivity, 100% specificity, and 94% coincidence in single acute-phase serum samples as compared with results for sera from the same patients tested by HI. This indicates a false-negative rate of 8% for the DENGUE IgM* kit compared with HI (87).

Laferté et al. (72) reported the standardization and evaluation of a 10-μl ultramicro-ELISA for anti-dengue virus IgM detection. Compared with HI, the system showed 85.7% sensitivity and 100% specificity. Compared with the IgM ELISA, it had 100% sensitivity and 98.6% specificity. Both the DENGUE IgM* and ultramicro-ELISA kits are currently used by some laboratories in Central and South America.

Several test systems to demonstrate anti-dengue virus antibodies have been developed, with special emphasis on rapidity, simplicity, and specificity (5, 8, 9, 31, 38, 81, 96). Hemolysis in gel (38, 127), a hemadsorption immunosorbent technique (31), and a staphylococcal agglutination-inhibition reaction (9, 81) are examples of some systems used in a few laboratories. Complement fixation has also been used (53, 129).

Molecular detection. Nucleic acid hybridization, specifically, a dot blot nucleic acid hybridization test (50) using RNA extracted from dengue virus-infected cell culture supernatants and pools of infected *A. albopictus* with biotinylated probes (59) or ^{32}P-labelled probes, is a sensitive method that has been applied in both diagnostic and epidemiological studies. The detection method using biotinylated probes is less sensitive than the test using radiolabelled probes and is not very useful for direct virus identification in clinical samples unless the genetic material has been previously amplified (50).

PCR is increasingly being applied to the diagnosis of flaviviruses and specifically dengue viruses. By using PCR, dengue viruses have been detected directly in sera, in dengue virus-infected mosquito cell culture supernatants, and in infected mosquito larvae (7, 18, 23, 25, 51, 77, 94, 122, 125). In addition,

a dual viremia resulting from naturally acquired dengue virus 1 and serotype 3 infections has been demonstrated with PCR (73).

Dengue virus type-specific primers and dengue virus and flavivirus consensus sequences located in different genes, such as those for E, NS1, NS3, and NS5, have been widely used for the detection and identification of dengue viruses (7, 15, 18, 25, 122, 125).

Lanciotti et al. (77) developed a rapid PCR assay using dengue virus consensus primers located in the C and prM genes that amplify a 511-bp product in a reverse transcriptase PCR followed by a nested PCR with primers specific to each dengue serotype. This assay has shown to be very useful in dengue diagnosis, with a limit of sensitivity of 10^3 50% tissue culture infective doses for viremic sera and infected mosquitoes.

The sensitivity, specificity, and rapid detection of minute quantities of genetic material in patient samples make PCR a very useful diagnostic tool for this disease. False-positive results that have been reported for PCR-based assays generally have been due to improper sample manipulation (111), which can be circumvented by precautionary measures.

Besides the utility of PCR as a method for rapid diagnosis, it can also be used for the genomic study of dengue virus strains, allowing restriction enzyme (131, 132) or nucleotide sequence (1, 10, 12, 13, 19, 32, 78, 79, 80) analysis of the genetic material.

Deubel et al. (18, 19) have demonstrated the usefulness of the nucleotide sequence analysis of an E gene fragment previously amplified by PCR as a rapid method of genetic classification of dengue virus strains. Other authors (131, 132) have applied PCR and restriction enzyme analysis to develop a rapid and simple procedure for identifying geographic subgroups of dengue virus types 2 and 3. Chow et al. reported a comparative analysis of the NS3 sequences of dengue virus serotype 3 strains by using a combination of PCR and direct-cycle sequencing (11).

Dengue diagnosis with tissues from fatal cases is still a problem, although some immunohistochemical studies have been developed (40, 112). RNA-RNA hybridization is a sensitive technique which can be applied in direct or retrospective analysis with fixed samples (87). Dengue virus detection by in situ hybridization and PCR have been reported to be useful for dengue diagnosis and also for the study of viral pathogenesis and can be an alternative to immunohistochemical analysis (60, 82).

CONCLUSIONS

Currently, dengue diagnosis is based on viral isolation, serology, and RNA detection. Viral antigen detection has been difficult because of the presence of virus-antibody immunocomplexes in patients with a secondary infection (88), although it has been useful for detecting dengue virus antigen in mosquitoes (118). The inoculations of mosquito cell cultures and adult or larval mosquitoes are the most sensitive systems for viral isolation. The use of specific monoclonal antibodies for isolate identification has simplified this process. ELISA and HI are still the tests most used for serological diagnosis with paired sera, although detection by IgM ELISA with single sera is widely applied for dengue surveillance. During the last few years, PCR has been applied to nucleic acid detection in sera, tissues, and mosquitoes, and different methodologies have been used. However, it is necessary to standardize the reverse transcriptase PCR protocols for use as a routine diagnostic method.

Despite the huge advances in dengue diagnosis that have been made since the first dengue virus isolations, new technologies are required. During the International Dengue and Dengue Hemorrhagic Fever training course held in Havana, Cuba, in August 1995, the participants recommended looking for new technologies that allow a rapid, early, and sensitive diagnosis. More research with such an aim is needed.

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