

Principle of Laboratory Diagnosis and Epidemiological Surveillance on Dengue and Japanese Encephalitis Viruses

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Abstract: Laboratory diagnoses on viral infection are indispensable in order to obtain precise information on the disease agents, including dengue and Japanese encephalitis viruses. Such information is indispensable for proper clinical case management, epidemiology, and strategy to control viral diseases.

Key words: Laboratory diagnosis, dengue virus, Japanese encephalitis virus

1. Taxonomy of the viruses and arboviruses

Modern taxonomy of the viruses depends on the molecular characteristics of the viruses, such as virion morphology, type of viral nucleic acid, and replication strategy. On the other hand, quite a number of viruses have been grouped based on their mode of transmission, typical example of which is arboviruses, that is the arthropod–borne vertebrate viruses. Arboviruses are defined as the group of viruses which have been maintained in nature, mainly or to their great extent, by alternative transmission between susceptible vertebrate by hematophagous arthropods, such as mosquitoes and ticks. In the infected vertebrate hosts, the arboviruses multiply and after intrinsic incubation, appear in the blood stream to cause viremia.

Arthropod vectors biting on such viremic vertebrates take up the virus with blood meal. Then, the virus multiply in the arthropod tissues, and after extrinsic incubation period, appears in the salivary gland. Such arthropods are then able to transmit the viruses to other susceptible vertebrate hosts (Fig. 1). Approximately 400 species of arboviruses have been listed in the Catalogue of Arbovirus (Berge, 1975), and many of them have been identified to cause human diseases. Among multiple species of arboviruses, majority belong to the Family *Bunyaviridae*, and *Togaviridae* (Fig 2). The latter was then classified into *Togaviridae* and *Flaviviridae* (Westaway *et al.*, 1985) after genomic structure of yellow fever (YF) virus was analyzed (Rice *et al.*, 1985).

Among a number of arboviral diseases, those caused by mosquito–borne flaviviruses, such as YF, dengue fever (DF)/ dengue hemorrhagic fever (DHF), and Japanese encephalitis (JE) have been regarded as major health problems in the tropics, because of their clinical severity, number of patients, and epidemic areas. Recent epidemiological data and significance of DF/DHF and JE have been documented (Halstead, 1992; Igarashi, 1992).

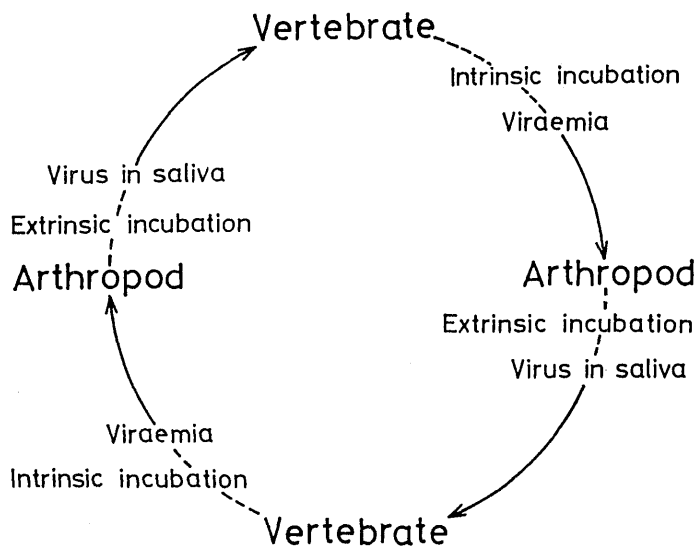


Fig. 1 Transmission cycle of arboviruses

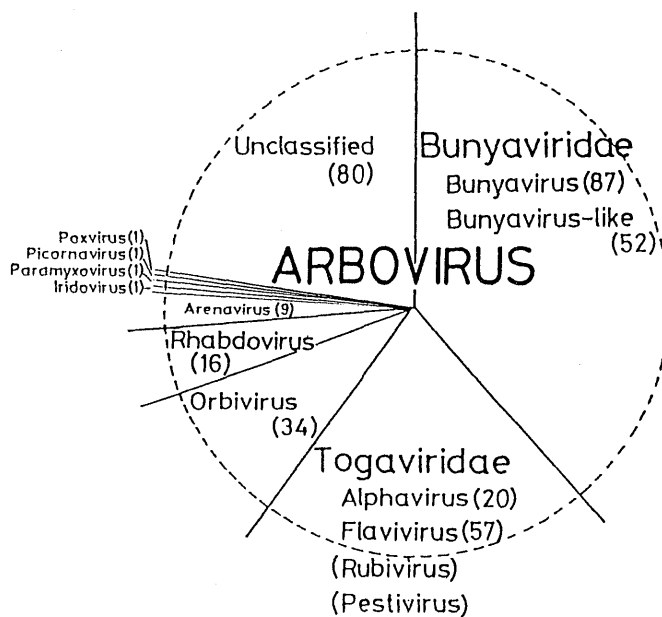


Fig. 2. Classification of arboviruses

2. Clinical manifestations of arbovirus infections

The clinical manifestation of arbovirus infection is not uniform even for the same species of the viruses. The mildest form is undifferentiated fever, which is accompanied by nonspecific constitutional symptoms. The next is dengue fever or dengue syndrome, with triad of fever, pain and rash. These 2 manifestations seldom lead to fatal outcome. While, the 3rd manifestation of arboviral infection is hemorrhagic fever, characterized by a triad of fever, hemorrhage, and circulatory failure. Viral hemorrhagic fevers, for example YF and DHF, can lead to fatal outcome if not properly treated. The 4th manifestation of arboviral infection is encephalitis, which is characterized by high fever headache and impaired consciousness, accompanied by high fatality and grave sequelae, as typically shown by JE.

Since multiple arboviruses can cause similar clinical manifestations, and the infection with the same virus can lead to broad spectrum of diseases, it is absolutely important to rely on laboratory diagnosis in order to identify causative agent of the disease.

3. Basic virology of flaviviruses

Viruses of the family *Flaviviridae*, genus flavivirus, is represented by YF virus. The virion is an enveloped spherical particle of 40–50 nm diameter. There are 3 structural proteins: core protein (C), membrane protein (M) and envelope glycoprotein (E). The E protein which exists on the surface of the virion plays an important role in the initial steps of virus–cell interaction. There are multiple epitopes on the E protein, including flavivirus cross–reacting, subgroup specific, as well as species–specific epitopes. The flaviviral genome is a single–stranded positive–sense RNA molecule, consisting of approximately 11 Kb nucleotides (nt). There are approximately 100 nt and 500 nt untranslated regions (UTR) in its 5' and the 3' ends, respectively. While, the remaining central part of the viral RNA forms a long open reading frame (ORF) of approximately 10 Kb nt. The 5'–1/4 of the ORF corresponds to the structural protein genes in the order of C, PreM (precursor of M), and E. While remaining 3/4 of the ORF corresponds to nonstructural protein genes: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. The function of these nonstructural proteins have not well been characterized yet. Recent studies revealed that NS1 is a soluble complement–fixing antigen (SCF) and could be another protective antigen besides E protein. NS3 is a bi–functional protein, with helicase activity in its N–terminal and protease activity in C–terminal domains, respectively, and could also be a target of cytotoxic T lymphocytes. While, NS5 is considered to be an RNA–dependent RNA–polymerase based on the consensus sequence.

4. Laboratory diagnosis on viral infection

Laboratory diagnosis on viral diseases depends on 2 principles: (1) virus isolation and (2) serology, which can be applicable also for the mosquito–borne arbovirus diseases such as DF/DHF and JE. Time course of the events in typical acute viral diseases is described below. Following the viremic period corresponding to the acute febrile period, antibody is produced and viremia is cleared, leading to the recovery from the diseases. Therefore, the

virus isolation can be applicable during the acute viremic period, while serology can provide the answer only after antibody production.

Virus isolation is the most direct way in the laboratory diagnosis on viral diseases by demonstrating the presence of infectious agent(s) in the patient's specimens. But it requires skillful techniques and relatively long time to get the final results. Historically, the virus isolation was carried out by inoculating test materials into susceptible host animals, for example intracerebral inoculation into mice. When abnormal clinical symptoms or pathological changes were observed in these animals, the infectious agent was assumed to have multiplied and isolated. The whole animal was then replaced by embryonated eggs, and cell cultures for easiness as well as saving space and cost of the experiment. In the case of dengue viruses which were relatively difficult to isolate by inoculation into mouse brains or vertebrate cell cultures, application of mosquito cell culture was a breakthrough. The *Aedes albopictus* clone C6/36 cell line was established primarily to isolate dengue viruses (Igarashi, 1978). Its applicability was examined under the field setting by Igarashi *et al.* (1982). Tesh (1979) compared sensitivity of the C6/36 and Ap61 (Varma *et al.*, 1974/1975) cell lines to various dengue virus isolates with mosquito inoculation technique (Rosen and Gubler, 1974), which was considered as the most sensitive method at that time. His result indicated that although mosquito inoculation is more sensitive than mosquito cell culture inoculation, almost comparable results can be expected in the isolation of dengue viruses by both method because of the larger volume of the materials to be inoculated to cell cultures than mosquitoes. The time required in the virus isolation procedures depends not only on the incubation for the progeny virus to multiply, but also on the identification of the isolated agents. Classical identification was carried out by the neutralization (N) test, and the procedure was greatly simplified by the development of type-specific monoclonal antibodies (MAb) for dengue viruses (Henchal *et al.*, 1982). In some cases, demonstration of viral antigen can be used as an alternative to virus isolation, for example JE antigen in postmortem brain specimens by the immunofluorescent staining.

Recent revolutionary advance was achieved by the introduction of viral genome detection by using polymerase chain reaction (PCR). Its principle and detailed procedures will be explained by another lecturer. Further analysis on the nt and deduced amino acid sequences of viral genome provided more detailed information on the genotype of the virus strains. Such molecular epidemiological studies have provided valuable information on the route of disease transmission. Studies on the molecular structure of viral genome will elucidate the disease mechanisms at a molecular level, for example, by identifying the virulent viral gene in the future. These informations will ultimately be utilized for the development of genetically engineered viral vaccines.

The second method of serology in the diagnostic virology depends on the principle of immunology, particularly its specificity. Three conventional methods have been utilized in the viral serology for long time; namely complement-fixation (CF), hemagglutination-inhibition (HI), and N-tests. Among these 3 methods, the HI was most widely used because it is relatively sensitive, simple and rapid (World Health Organization, 1986). One of the disadvan-

tages in the HI test is the crossreaction, which is highest among the 3 classical serological methods particularly in sequential infections with related but different flaviviruses. Although the N-test is most specific among classical serological methods, it has not been used so frequently because of the required time and skill similar to virus isolation. The CF test is not sensitive enough and relatively complicated in its procedure, therefore was not so widely used either. All these 3 classical serological methods require paired sera which were collected at acute and convalescent phase with appropriate interval in order to demonstrate significant rise in the antibodies. Since 19S or IgM-class antibodies are produced earlier and more specific than the 7S or IgG antibodies and persists transiently, the assay on IgM-antibodies was introduced to the viral serology with a potentiality to provide results even with a single serum specimen. The application of radioimmunoassay (Burke and Nisalak, 1982), followed by enzyme-linked immunosorbent assay (ELISA) on IgM-antibodies (Bundo and Igarashi, 1985; Lam *et al.*, 1987; Innis *et al.*, 1989) has gradually replaced or supplemented classical HI test. In addition to these quantitative antibody assay methods, qualitative or semi-quantitative antibody detection by dotblot tests were recently introduced as a simple test. In the standard HI or the ELISA on dengue or JE, assay antigen was prepared from infected mouse brains by sucrose-acetone or acetone-ether extraction. Recently, such antigen can also be prepared from infected cells cultures. It should be pointed out that serological method is not able to tell the infecting serotype of the virus, which can be identified only by virus isolation or viral genome detection. One of the future directions in viral serology is to develop genetically engineered or synthetic assay antigens in place of current antigens.

5. Virological methods in the epidemiological surveillance

Principle of (1) virus isolation, demonstration of viral antigen or viral genome detection by PCR, and (2) assay of antiviral antibodies was also applicable to the epidemiological surveillance on dengue and JE virus infections. In these mosquito-borne diseases, entomological surveillance on disease vectors should be supplemented with virological detection of viral agents in the vectors, in order to obtain more precise and useful information. Isolation of JE virus from principal vector *Culex tritaeniorhynchus* was carried out by classical mouse brain inoculation and C6/36 cell culture inoculation (Igarashi *et al.*, 1981a). The result showed that C6/36 cell culture possessed comparable to or better sensitivity than mouse brains to isolate JE virus from field-caught mosquitoes. The same study group (Igarashi *et al.*, 1981b) also demonstrated that C6/36 cell culture inoculation was able to isolate such mutant viruses that were not detected by mouse brain inoculation.

JE virus isolation from vector mosquitoes was coupled to the antibody surveillance among swine, the most important amplifier vertebrate. The results of our study (Igarashi *et al.*, 1981c) clearly showed the first introduction of JE virus in vector mosquitoes on 27 July, which was followed by swine viremia on 4 August, then amplification of JE virus infection among vectors on 11 August (Fig. 3).

Seroepidemiological surveillance on anti-viral antibodies in healthy human population can provide information on the past exposure or prevalence of viral agents in nature, or even

recent exposure to the disease agents by IgM-antibody assay (Burke *et al.*, 1984). These informations were required to get an idea on the levels of natural infection, and are indispensable to work out national control strategy on the viral diseases in general.

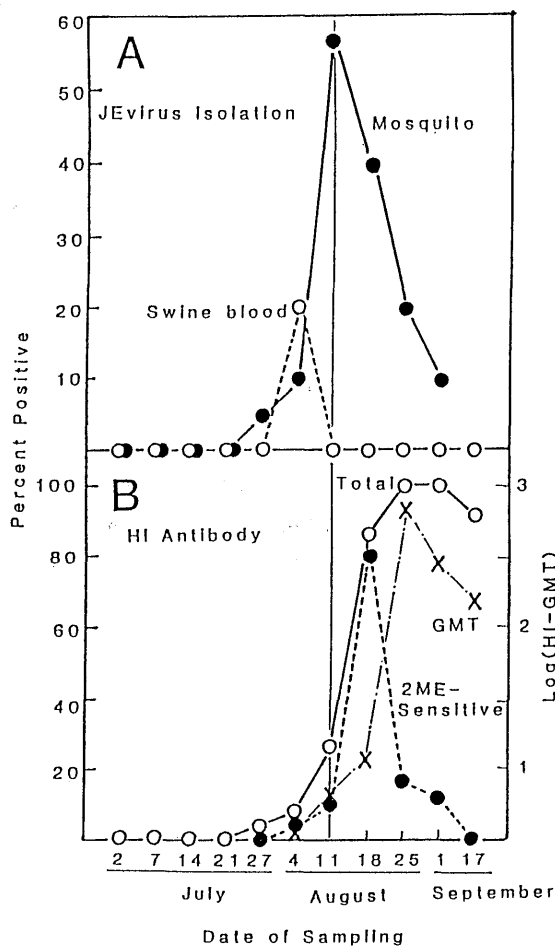


Fig. 3. Isolation of JE virus from *C. tritaeniorhynchus* and swine blood and appearance of anti-JE antibodies in sera of slaughtered swine (A) Isolation of JE virus from *C. tritaeniorhynchus* (●—●) and blood specimens from slaughtered swines (○—○) according to the sampling date as shown by the percent positives. (B) Appearance of anti-JE antibodies in sera of slaughtered swines as shown by percent positives of total (○—○) and 2ME-sensitive (●—●) HI antibodies, as well as by the geometrical mean titer (GMT) in logarithmic scale (×—•—•—×).
Reproduced from Igarashi *et al.* (1981c)

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