Togaviridae and Flaviviridae: The Alphaviruses and Flaviviruses

CHARLES H. CALISHER and THOMAS P. MONATH

Diseases: Yellow fever, dengue, St. Louis encephalitis, Japanese encephalitis, Wesselsbron, tick-borne encephalitis, louping ill, Kyasanur Forest disease, other tick-borne hemorrhagic fevers, Murray Valley encephalitis, Rocio encephalitis, equine encephalitides (eastern, western, Venezuelan), chikungunya, o'nyong-nyong, Ross River, Mayaro, Sindbis, Ockelbo.

Etiologic Agents: Yellow fever, dengue-1, dengue-2, dengue-3, dengue-4, Central European encephalitis, St. Louis encephalitis, Japanese encephalitis, West Nile, Murray Valley encephalitis, Wesselbron, Ilheus, Rocio, Russian spring-summer encephalitis, Omsk hemorrhagic fever, louping ill, Kyasanur Forest disease, Powassan, eastern equine encephalitis, western equine encephalitis, Venezuelan equine encephalitis, Ross River, Mayaro, Sindbis, Ockelbo.

Source: Mosquitoes, ticks; Omsk hemorrhagic fever may be water-borne.

Clinical Manifestations: Fever, fever with rash, fever with rash and polyarthritis, fever with rash, myalgia, and arthralgia, hemorrhagic fever with shock, abortion, encephalitis.

Pathology: Disturbance of the integrity of microcirculation, with leakage of plasma and plasma proteins into extravascular spaces (hemorrhagic fevers); typical viral encephalitis.

Laboratory Diagnosis: Virus isolation, fourfold or greater increase or decrease in antibody in infected individuals, antigen detection or IgM antibody capture ELISA, neutralization, hemagglutination-inhibition, complement-fixation, indirect fluorescent antibody tests.

Epidemiology: Focally or widespread worldwide, dependent on distribution of virus, vectors, and vertebrate hosts.

Treatment: Symptomatic, immune plasma.

Prevention and Control: Prevention of bite by infected arthropod, insecticide spraying, vaccination.

Abbreviations for virus names: yellow fever (YF), louping ill (LI), West Nile (WN), Japanese encephalitis (JE), Russian spring-summer encephalitis (RSSE), St. Louis encephalitis (SLE), western equine encephalitis (WEE), eastern equine encephalitis (EEE), Venezuelan equine encephalitis (VEE), Sindbis (SIN), Semliki Forest (SF), Ilheus (ILH), Uganda S (UGS), Ntaya (NTA), dengue-1 (DEN-1), dengue-2 (DEN-2), Murray Valley encephalitis (MVE), Rocio (ROC), Central European encephalitis (CEE), Omsk hemorrhagic fever (OHF), Kyasanur Forest disease (KFD), Powassan (POW), chikungunya (CHIK), Ross River (RR), Mayaro (MAY), Getah (GET), Sagiyama (SAG), Bebaru (BEB).

Introduction

At the beginning of this century it was reported that YF was transmitted to humans by the bite of *Aedes aegypti* mosquitoes infected with that virus (Reed et al., 1983). That seminal work led to a concentration of efforts to eradicate this disease, which at the time was a considerable public health peril. In turn, this not only led to a greater understanding of YF and YF virus, but effected a significant expansion of our knowledge of other arthropod-borne viruses. Loup-

ing Ill, WN, JE, RSSE, and SLE viruses were all isolated prior to 1940 (Karabatsos, 1985). A worldwide effort was begun to isolate viruses from arthropods and to determine the role of these viruses in human and animal diseases; there was a need to determine whether the viruses being isolated were unique or were pieces in a larger epidemiologic puzzle. First, SLE, JE, and LI viruses were reported to be distinct from one another (Webster et al., 1935), but then it was shown that sera from patients who had recovered from JE neutralized not only the homologous virus but SLE virus as well, though not to the same extent (Webster, 1938). This indicated an antigenic relationship between the two viruses.

Smorodintseff (1940) showed by neutralization tests that RSSE and LI viruses were related to each other, but that JE virus, although related to both, was sufficiently distinct from them to be considered a "less complex" virus. Then Smithburn (1942), investigating the reactivities of antibodies produced in humans and monkeys infected with WN, SLE, and JE viruses, discovered even more complex serologic interrelationships.

It was not until the mid-1940s that a simpler tool, the complement-fixation (CF) test, was applied by Casals (1944) to the problem of antigenic interrelationships. He found that RSSE and LI viruses were more closely related to each other than they were to the similarly closely related JE, WN, and SLE viruses and suggested that each of these sets of viruses should be considered separate antigenic "complexes". Further, he demonstrated by CF that WEE virus was not related to any of these. Later, Sabin (1950) showed that two types of dengue viruses elicited the production of CF antibodies to YF, WN, and JE viruses in monkeys and that experimental infections of human volunteers with one of the dengue viruses elicited the production of antibody not only to the homologous virus but to YF, WN, and JE viruses as well.

In the years that followed, more viruses related to YF, JE, SLE, WN, RSSE, and LI viruses were isolated from mosquitoes, ticks, humans, and other mammals, and serologic surveys provided further evidence for cross-reactivity between antibodies to some but not other viruses. Development and application of the hemagglutination-inhibition (HI) test (Sabin and Buescher, 1950; Casals and Brown, 1954; Clarke and Casals, 1958) led to the concept of "serogroups" (sets of viruses that are antigenically related to one another). First, Casals and Brown (1954) demonstrated that 16 of the 21 viruses they studied belonged to one of two serogroups, Group A or Group B. Those in Group A included WEE, EEE, VEE, SIN, and SF viruses, whereas YF, JE, SLE, WN, RSSE, LI, ILH, UGS, NTA, DEN-1, and DEN-2 viruses belonged to the Group B viruses.

The other five viruses were poliovirus, rabies virus, or arboviruses not belonging to either Group A or Group B. It was later reported that 7 viruses belonged in Group A and 17 in Group B and that others either belonged to other serogroups or were not at that time classifiable on the basis of serologic testing (Casals, 1957). By the mid-1960s, 19 Group A viruses and 39 Group B viruses had been recognized (World Health Organization, 1967).

The International Committee on the Taxonomy of Viruses (ICTV), an organ of the International Union of Microbiology Societies, has been attempting to classify viruses (Matthews, 1982). The ICTV originally placed Group A and Group B arboviruses in the family *Togaviridae*, which also included rubella and hog cholera and related viruses, on the basis of molecular and morphologic characteristics. The ICTV has now recognized a reorganization of this family, based on additional molecular data regarding strategy of replication, and has separated viruses formerly placed in the family *Togaviridae* into two families, *Togaviridae* (genera *Alphavirus*, *Rubivirus*, *Pestivirus*, and *Arterivirus*) and *Flaviviridae* (genus *Flavivirus*) (Westaway et al., 1985a,b). Invertebrate hosts are not known to carry viruses of the genera *Rubivirus* (rubella virus), *Pestivirus* (mucosal disease-bovine virus diarrhea virus, hog cholera virus, and border disease virus), or *Arterivirus* (equine arteritis virus); viruses belonging to the latter two genera are not human pathogens. Because rubella is covered in Chapter 23 of this book, none of the viruses of these three genera will be addressed in this chapter.

The term "arbovirus", a contraction of the term "*arthropod-borne virus,* denotes a virus maintained in nature in a biological transmission cycle between susceptible vertebrate hosts (in which they replicate) and hematophagous arthropods (in which they also replicate). The generic term "arbovirus" generally has an ecologic connotation. In this chapter, mention of these viruses will employ universal taxonomic descriptions. Differences in antigenic, morphologic, biochemical, and genetic characteristics are used to separate the arboviruses into families, genera, serogroups, complexes, viruses, subtypes, and varieties, in an increasing order of relatedness. Most recently, molecular analyses have substantiated previous antigenic classification schemes, and a clearer view of the taxonomy of these viruses has emerged. The foresightedness of Casals was remarkable!

Family *Flaviviridae,* Genus *Flavivirus*

Flavivirus virions are spherical, 40 to 50 nm in diameter. They contain one molecule of single-stranded, positive-sense RNA, three structural proteins, and

have a molecular weight about 4×10^6. One protein is the core protein (C) with a molecular weight of 13 to 16×10^3, another is a membrane-associated protein (M) with a molecular weight of 7 to 9×10^3, and the third is an envelope glycoprotein (E) with a molecular weight of 51 to 59×10^3. At present more than 70 viruses, subtypes, and varieties have been assigned to this genus (the former Group B arboviruses). Antigenic classification schemes have been proposed for the flaviviruses (de Madrid and Porterfield, 1974; Varelas-Wesley and Calisher, 1982). Simian hemorrhagic fever virus has been assigned to the family *Flaviviridae,* but information regarding it is insufficient, and it has not been placed in a genus. One antigenic classification for the flaviviruses is presented in Tables 1 and 2 (for definitions of the antigenic categories serogroup, complex, virus, subtype, and variety see Chapter 32, *Bunyaviridae*). Some of these viruses are transmitted principally by mosquitoes, some by ticks, and some have not been associated with an arthropod vector. When segregated by vector associations, flaviviruses generally exhibit distinct antigenic differences, pos-

sibly as a consequence of such associations (Chamberlain, 1980). Among the mosquito-borne flaviviruses are YF, WN, SLE, MVE, JE, ROC, and the four dengue viruses. The viruses that cause encephalitides are more closely related to one another antigenically than they are to the viruses that cause YF or dengue. So, too, YF and dengue viruses cause diseases distinct from each other and are antigenically quite distant from each other.

The tick-borne flaviviruses, including RSSE, CEE, OHF, and KFD viruses, are of considerable medical importance and concern from central Europe to eastern Siberia. The antigenic relationships of these viruses to the mosquito-borne flaviviruses are distant, but that relationship is significant phylogenetically. It may be that evolutionary pressures (e.g., geographic isolation, environmental selection, survival of most genetically fit) have created a divergence of virus–vector relationships, perhaps from a common origin. For example, WN virus replicates nearly as well in ticks as in its usual arthropod vectors, *Culex* species mosquitoes. Powassan, a tick-borne flavivirus isolated first and principally in Can-

TABLE 1. Classification of viruses of the family *Flaviviridae,* genus *Flavivirus* (Group B arboviruses)

Complex	Virus (subtype) ⟨variety⟩
Mosquito-borne viruses	
St. Louis encephalitis	St. Louis enc. ⟨3⟩, Alfuy, Japanese enc., Kokobera, Koutango,[a] Kunjin, Murray Valley enc., Stratford, Usutu (Usutu) (Yaounde), West Nile
Uganda S	Uganda S, Banzi, Bouboui, Edge Hill
Dengue	Dengue-1, dengue-2, dengue-3, dengue-4
Ntaya	Ntaya, Tembusu (Tembusu) (Yokose), Israel turkey meningoenc. (Israel turkey meningoenc.) (Bagaza)
Tick-borne viruses	
Russian spring-summer encephalitis	Russian spring-summer enc.[b] (Russian spring-summer enc.) (Omsk hemorrhagic fever) (Central European enc.) (Kumlinge) (Kyasanur Forest disease) (louping ill) (Langat) ⟨Langat⟩ ⟨Negishi⟩ ⟨Carey Island⟩, Powassan, Karshi, Royal Farm
Tyuleniy	Tyuleniy, Saumarez Reef, Meaban
Vector-unassociated viruses	
Modoc	Modoc, Cowbone Ridge, Jutiapa, Sal Vieja, San Perlita (San Perlita) ⟨San Perlita⟩ ⟨MA387-72⟩
Rio Bravo	Rio Bravo, Apoi, Bukalasa bat, Dakar bat, Entebbe bat, Saboya

Flaviviruses not assigned to an antigenic complex

Aroa	Phnom-Penh bat
Bussuquara	Rocio
Cacipacore	Sepik
Gadgets Gulley	Sokuluk
Ilheus	Spondweni
Jugra	Tamana bat
Kadam	Wesslesbron
Kedougou	yellow fever
Montana Myotis leukoencephalitis	Zika
Naranjal	

[a] Koutango virus has not been isolated from naturally infected mosquitoes.
[b] enc. = Encephalitis.

TABLE 2. Viruses of the family *Togaviridae,* genus *Alphavirus* (Group A arboviruses)

Complex	Virus (subtype) ⟨variety⟩
Eastern equine encephalitis	Eastern equine encephalitis
Middelburg	Middelburg
Ndumu	Ndumu
Semliki Forest	Semliki Forest, chikungunya (chikungunya) ⟨several⟩ (o'nyong-nyong), Getah (Getah) (Sagiyama) (Bebaru) (Ross River), Mayaro (Mayaro) (Una)
Venezuelan equine enc.	Venezuelan equine enc. (Venezuelan equine enc.[a]) ⟨A-B⟩ ⟨C⟩ ⟨D⟩ ⟨E⟩ ⟨F⟩, (Everglades), (Mucambo) ⟨Tonate⟩ ⟨71D-1252⟩, (Pixuna), (Cabassou), (AG80-663)
Western equine enc.	Western equine enc. (several), Y 62-33, Highlands J, Fort Morgan, Sindbis (Sindbis) (Babanki) (Ockelbo) (Whataroa) (Kyzylagach), Aura
Barmah Forest	Barmah Forest
(No complex assigned)	Zingilamo

[a] enc. = Encephalitis.

ada but also isolated in the northern United States and the Soviet Union, is widely divergent antigenically from the mosquito-borne flaviviruses, but has been isolated from adult *Anopheles hyrcanus* mosquitoes and from larvae of *Aedes togoi* mosquitoes in the Soviet Union.

The flaviviruses that have not been associated with an arthropod vector are not closely related antigenically to other flaviviruses, but they are connected to each other by virtue of host and geography (*Rodentia* in the New World; *Chiroptera* in the Old World). This set of viruses represents divergent evolution, possibly indicating relatively restricted spread (vertebrate to vertebrate), whereas the mosquito- and tick-borne viruses represent more moderate divergent evolution and relatively unlimited geographic spread. Given the rather discrete geographic foci and econiches in which the vector-unassociated flaviviruses are found, phylogenetic divergence reflected by antigenic dissimilarities is not surprising.

Relationships between the flaviviruses may be summarized as follows: a) viruses that are mosquito-borne are far more antigenically similar to one another than they are to the tick-borne or vector-unassociated flaviviruses, whereas b) viruses that are vector-unassociated are nearly as antigenically dissimilar from one another as they are from mosquito- and tick-borne flaviviruses. As an example, Koutango virus has not been associated with an arthropod vector in nature but has been transmitted to vertebrates by mosquitoes and passed transovarially in laboratory studies. This virus is closely related antigenically to the mosquito-borne flaviviruses, suggesting common ancestry.

Family *Togaviridae,* Genus *Alphavirus*

Alphavirus virions are spherical, 60 to 70 nm in diameter. They contain one molecule of single-stranded, positive-sense RNA, three structural proteins, and have a molecular weight 4×10^6. Two of the proteins

are cell membrane-derived envelope glycoproteins (El and E2) with molecular weights of 50 to 59×10^3, and one is a nonglycosylated capsid protein with a molecular weight of 30 to 34×10^3 (Semliki Forest virus possesses three envelope glycoproteins). At present, more than 37 viruses, subtypes, and varieties have been assigned to this genus (Table 2); a classification scheme for these viruses has been published (Calisher et al., 1980). Alphaviruses have been isolated on six continents. All but Fort Morgan virus have been isolated from mosquitoes. This virus, which has been isolated only from the bird-nest bug *Oeciacus vicarius* and from passerine birds, is an example of a virus whose distribution is restricted by its vector. Alphaviruses of the EEE and WEE antigenic complexes appear to employ birds as principal vertebrate hosts in their maintenance cycles in nature; this probably accounts for their widespread distribution. Alternatively, subtypes of VEE virus appear to be restricted to small mammals in discrete enzootic foci and have been found only in the Americas. Semliki Forest complex members SF, CHIK, RR, and MAY viruses also are widely distributed; MAY virus has been associated with avian hosts. Two of the four antigenic subtypes of GET virus, SAG and BEB, appear to be geographically isolated, but little is known of their natural histories. Triniti virus may be a member of the family *Togaviridae,* but insufficient information regarding it is available, and it has not been placed in a genus.

Clinical Features

Flaviviruses

Several clinical syndromes are caused by infections with flaviviruses, including uncomplicated fever; fever with rash; fever with rash, myalgia, and arthralgia; hemorrhagic fever with shock; stillbirth and abortion (in domestic livestock); and encephalitis.

Representative examples of flaviviruses that have been associated with each disease category are presented in Table 3. Evidence from serologic surveys indicates that the ratio of inapparent to apparent infections is quite high, so that these viruses, even in situations in which the incidence of infection is high, only rarely cause disease. The flaviviruses that cause encephalitis (Table 3), for example, usually cause abortive infection characterized by fever with headache or other relatively benign signs. However, in those few individuals who develop full-blown infection, disease may be severe or fatal. The case-fatality rate may be 20% or more in the encephalitides, YF, and dengue shock syndrome.

YF, the dengue viruses, and two tick-borne flaviviruses (OHF and KFD) are associated with hemorrhagic fever syndrome. The common denominator in the clinical features of these infections is hemorrhagic diathesis and circulatory failure (hypotension, shock). Hepatic necrosis occurs, but only in YF to an extent that is clinicopathologically significant; patients with YF develop signs of severe hepatic dysfunction. The central event in the pathophysiology of dengue hemorrhagic fever/shock syndrome is a disturbance of the integrity of the microcirculation, with leakage of plasma and plasma proteins into the extravascular space, shock, and hemorrhage. Kyasanur Forest disease virus, in addition to causing hemorrhagic fever, frequently produces meningoencephalitis and thus displays some pathobiological similarities to its antigenic relative, RSSE virus.

Alphaviruses

As with the flaviviruses, certain alphaviruses can cause a variety of clinical syndromes (Table 4). The most severe, and therefore the most extensively studied, are the encephalitides, caused by EEE, WEE, and VEE viruses. Eastern equine encephalitis

and VEE viruses and their antigenic relatives occur only in the Americas, but antigenic relatives of WEE virus also occur in Europe, Africa, Asia, and the South Pacific (Table 2). Humans and horses are clinically affected; humans are dead-end hosts for these three viruses, whereas horses are the principal viremic host for VEE virus. Horses may serve only occasionally as a source of mosquito infection by EEE virus. Human infections are most often self-limiting or subclinical, and the severe manifestations of encephalitis occur only infrequently in infected individuals. Rates of inapparent to apparent infections are age- and perhaps strain-dependent. Children appear to be at greater risk of encephalitis.

Encephalitis, whether caused by flaviviruses or alphaviruses, is an infection of the brain parenchyma and results in either localized or diffuse signs of cerebral dysfunction. Signs of meningeal irritation (meningoencephalitis) are also nearly always present, but may not be obvious in the very young, the very old, or the comatose patient. Inflammation of the leptomeninges may occur without evidence of brain dysfunction; this is termed aseptic meningitis. Onset of neurologic disease may be insidious, preceded by a period in which the patient has an influenza-like illness. Encephalitis may follow quite soon after the onset of this rather mild stage, or it may occur days or weeks later. Humans infected with tick-borne encephalitis viruses (flaviviruses) may experience a bimodal fever curve, with encephalitis appearing only after occurrence of the second febrile episode, days to weeks after the first.

All individuals with encephalitis experience some alteration in degree of consciousness. Generalized convulsions may occur, and damage to corticospinal tracts causes paresis, paralysis, hyperactive reflexes, and plantar extensor responses. In encephalitis due to SLE, JE, and other viruses, there is involvement of extrapyramidal structures, causing tremors and muscular rigidity. Cerebellar dysfunction leads to incoordination, dysmetria, and ataxic speech. Damage

TABLE 3. Flaviviruses associated with various clinical syndromes

Syndrome	Flavivirus
Fever	Dengue 1–4, West Nile, St. Louis encephalitis, Banzi, Bussuquara, Ilheus, Kunjin, Japanese enc., Murray Valley enc., Rio Bravo, Rocio, Sepik, Spondweni, Wesselsbron, yellow fever, Zika, tick-borne enc.,[a] Kyasanur Forest Disease, Omsk hemorrhagic fever, Powassan, Usutu
Fever with rash	Dengue 1–4, West Nile
Fever with rash, myalgia, and arthralgia	Dengue 1–4
Hemorrhagic fever with shock	Dengue 1–4, yellow fever, Kyasanur Forest disease, Omsk hemorrhagic fever
Abortion	Japanese enc. (pigs), Wesselsbron (sheep)
Encephalitis	St. Louis enc., West Nile, Ilheus, Japanese enc., Murray Valley enc., Rocio, Apoi, Rio Bravo, tick-borne enc., louping ill, Powassan, Negishi

[a] enc. = Encephalitis.

TABLE 4. Alphaviruses associated with illness in humans or equines

Syndrome	Alpha virus
Fever	Eastern equine enc.,[a] western equine enc., Venezuelan equine enc., Mucambo, Everglades, chikungunya, o'nyong-nyong, Mayaro, Ross River, Sindbis, Ockelbo, Semliki Forest
Fever with rash	Sindbis, Ockelbo
Fever with rash and poly-arthritis	Ross River
Fever with myalgia, arthralgia, and rash	Chikungunya, o'nyong-nyong, Mayaro
Encephalitis	Eastern equine enc., western equine enc., Venezuelan equine enc., Semliki Forest

[a] enc. = Encephalitis.

to brainstem nuclei or supranuclear tracts results in cranial nerve palsies; cardiovascular irregularity, urinary retention, and sialorrhea reflect autonomic disturbances. Confusion, memory defects, changes in speech, personality, and behavior, as well as pathologic reflexes indicate damage to the cerebral cortex, hypothalamus, thalamus, or temporal lobes. Inappropriate antidiuretic hormone secretion and hyperthermia indicate disturbance of the pituitary–hypothalamic axis. Spinal cord involvement may be indicated by lower motor neuron and sensory deficits, hyperreflexia, and paralysis of the bladder. Interference with respiratory functions, laryngeal paralysis, cardiac arrhythmia, and cerebral edema are potentially life-threatening complications. Surviving patients may suffer permanent neuropsychiatric or other damage.

Clinical laboratory findings are rather nonspecific but usually include a leukopenia. The cerebrospinal fluid contains predominantly polymorphonuclear cells early in the course of infection and lymphocytes later, with the cell count usually less than 500/mm^3. An exception to this is EEE virus infection, in which a more fluid polymorphonuclear reaction occurs. There may be a moderate elevation in cerebrospinal fluid protein, but glucose and lactate concentrations usually are normal. Changes in serum-enzyme levels indicate pathologic processes in the myocardium, liver, and skeletal muscle.

Diagnosis

Viremia and Antibody Responses

It is important to determine the specific etiologic agent quickly and accurately in instances of any arboviral disease, not only so that the patient can be treated appropriately, but so that public health officials can institute vector-control operations to limit further spread of the virus. Laboratory confirmation of a clinical diagnosis depends upon direct detection

of antigen, virus isolation, or serologic tests, such as HI, CF, neutralization, fluorescent antibody and, more recently, enzyme-linked immunosorbent assays (ELISA). It is not often that one isolates flaviviruses that cause encephalitis from blood or spinal fluid taken in the acute stage of illness of infected individuals. Often the viremic stage has passed before the individual becomes ill. This is not the case with a few human pathogens, for example, dengue viruses, YF, KFD, and WN viruses, which may be consistently isolated during the first few days to a week after onset of disease. St. Louis encephalitis and JE viruses and antigens may be more often detected in brain collected postmortem. Venezuelan equine encephalitis complex viruses can be isolated from the blood of febrile individuals, but EEE and WEE viruses usually are isolated only from brain.

Antibody generally is not detectable until the end of the viremic phase. Virus–antibody complexes have been demonstrated in YF (Sarthou and Lhuillier, 1984) and dengue (Theofilopolous et al. 1976). In the case of YF, Sarthou and Lhuillier reported the dissociation of IgM antibody–YF virus complexes by use of a mild reducing agent (dithiothreitol), with subsequent isolation of virus that had been rendered noninfectious by antibody.

The IgM fraction of serum contains both neutralizing and HI antibodies appearing soon after onset, and its presence can serve as an indicator of recent infection (Burke et al., 1982; Monath et al., 1984b). IgG antibody appears later, containing both neutralizing and HI as well as CF antibodies. IgG antibodies to the flaviviruses probably persist for life, as evidenced by the persistence of HI and neutralizing antibodies and the absence of detectable IgM antibodies in late convalescent-phase serum. However, IgM antibodies to flaviviruses have been detected for long periods (Monath, 1971), and it appears that a minority subset of patients have prolonged IgM antibody responses, limiting somewhat the value of these assays as a measure of very recent infection. IgM antibodies in serum appear to be relatively type-specific (Westaway et al., 1974; Lhuillier and Sarthou, 1983;

Monath et al., 1984b), but complex- and serogroup-reactivity also are observed (Monath et al., 1984b). In the only study of sera from humans infected with various alphaviruses, IgM antibodies were antigenic complex-specific (Calisher et al., 1986b).

Measurement of IgM antibody in cerebrospinal fluid is an extremely useful method of serodiagnosis. Because IgM antibodies do not cross the blood-brain barrier, finding them in cerebrospinal fluid implies local antibody synthesis in response to central nervous system infection. Antibody-producing cells have been found in the cerebrospinal fluids of JE patients (Burke et al., 1982). Moreover, the titer of IgM antibody in cerebrospinal fluid is a prognostic indicator of JE (Burke et al., 1982) and possibly other flaviviral encephalitides.

HI antibody is broadly reactive among viruses of a serogroup, making this a useful test for preliminary screening. CF antibody is both more complex-specific and short-lived than HI antibody, and therefore is useful (as is IgM) for determining recent infections. Neither HI, CF, nor IgM antibody capture (MAC) ELISA are virus-specific. However, MAC ELISA is, at present and for the foreseeable future, the test of choice for making provisional serodiagnoses with single serum samples or with cerebrospinal fluids and is of greater or equal value to HI and CF tests when paired acute- and convalescent-phase serum samples are available.

Specimen Collection

After collection, whole blood, serum, cerebrospinal fluid, and tissue samples should be processed immediately or placed on dry ice (−70°C) or other suitable deep-freezing agent if virus isolation is to be attempted. Although this may not be such a critical issue for antigen detection, it appears sensible to ship and store specimens at low temperatures to prevent degradation of proteins. When serum is to be tested only for antibody, it can be shipped at ambient temperatures for brief periods, provided it has been collected aseptically and not subsequently contaminated with microorganisms. If transit time to the laboratory is longer than several days, refrigeration or addition of a small quantity of antibiotics is necessary for prevention of deterioration of the specimen; other chemical sterilants can be used, provided they will not interfere with the tests.

Virus Isolation and Identification

No single virus-isolation system is adequate for all flaviviruses and alphaviruses. As newer, more sensitive isolation systems (inoculation of mosquitoes in vivo, inoculation of arthropod cells in vitro) are increasingly employed, it becomes apparent that many virus strains and viruses have not been revealed because of the bias incurred by use of "traditional" systems, such as suckling mice and vertebrate cell cultures. For example, in initial isolation attempts, strains of yellow fever virus may cause cytopathic effects in C6/36 (Aedes albopictus mosquito) cell cultures, but may not kill suckling mice inoculated intracranially (Mendez et al., 1984).

Traditional methods of virus isolation are still firmly entrenched in many laboratories. Suckling mice have been used as laboratory hosts for amplifying virus in diagnostic specimens and from field-collected mosquitoes, ticks, and animal tissues. Mice are inoculated intracranially with clarified suspensions of clinical specimens or with macerated and clarified arthropod pools or animal tissues. Because suckling mice are available to almost all laboratories, this system holds certain advantages over others.

Nevertheless, mosquito cell cultures, particularly C6/36 (Aedes albopictus), AP-61 (Aedes pseudoscutellaris), TR-248 (Toxorhynchites amboinensis), increasingly are being used for virus isolation. These and other cell culture systems have the additional advantage of ease of containment and reduction of aerosols. Because they are highly stable and have growth optima at lower temperatures than do mammalian cells, cultures and mosquitoes may be taken to the field, inoculated with specimens, and returned to the laboratory weeks later, after amplification of the virus being sought.

For several viruses, for example, YF, dengue, and vesicular stomatitis, mosquito cell cultures are more sensitive than mice or mammalian cell culture systems for virus isolation. However, they have the disadvantage in some cases of not producing cytopathic effects, and thus require specific, secondary steps for recognition of the presence of virus in the culture. Intrathoracic inoculation of Toxorhynchites mosquitoes, which do not take blood meals but in which dengue and other viruses replicate, has also been used with sensitivity and safety for virus isolation (Rosen, 1981).

The classical procedure for isolating and identifying an arbovirus begins with inoculation of suckling mice or a cell culture system in which cytopathic effects or plaques develop. The isolate is characterized by testing its ability to pass through a filter that excludes bacteria and its sensitivity to lipid solvents such as ether, chloroform, or sodium deoxycholate (Theiler 1957). It is often useful to determine the pathogenicity of the agent and to determine end point titers in various laboratory animals and cell cultures. A crude alkaline extract or partially purified (sucrose–acetone-extracted) antigen is prepared for use in serologic tests (Clarke and Casals, 1958). The antigen is tested for its ability to agglutinate the erythro-

cytes of male domestic geese (*Anser cinereus*) and to react in CF tests (Clarke and Casals, 1958; Casey, 1965) with homologous antibody preparations. One then tests the antigen by HI or CF with a battery of antibody preparations, including a) those against viruses of various serogroups, b) those against viruses suspected as the etiologic agent of the disease, and c) those known to be present in the area in which the specimen was collected or in which the patient contracted the illness.

The best method for identifying a flavivirus or an alphavirus is one that is rapid, specific, and inexpensive. In some laboratories, electron microscopy can be used as an early step to identify the virus family to which the isolate belongs, and this can greatly facilitate subsequent characterization. The application of direct or indirect fluorescent microscopy (Gardner and McQuillan, 1980) with polyclonal or monoclonal (Henchel et al., 1983) antibodies can provide a rapid and simple means of virus identification. Because a complete battery of reagents is not yet available, this method is now used only for identifying certain viruses. Direct and indirect fluorescent antibody tests have been used to detect viral antigens in clinical specimens. Once the isolate is characterized to the level of serogroup or antigenic complex, neutralization tests are performed with antisera against individual viruses to confirm the identification. If necessary, antiserum is also prepared against the isolate and cross-tested against antigens of viruses in the serogroup to which it belongs. Most of the data regarding antigenic characterization of arboviruses have been generated with these tests, so that they will remain the standards by which newly isolated viruses are identified and against which new methods of characterization are to be judged. Newly developed reagents (such as monoclonal antibodies) and procedures (such as ELISA) will add significantly to our diagnostic ability and enable us to more fully characterize the epitopes and other antigenic moieties of viruses.

For early detection of flavivirus antigens in mosquito cell cultures inoculated with clinical materials, fluorescent antibody can provide identification of YF (Saluzzo et al., 1985), dengue (Halstead et al., 1983), and other viruses in fewer than 72 to 96 h, long before the appearance of cytopathic effects in these mosquito cells or mammalian cells or before the appearance of signs of illness in suckling mice. The indirect immunofluorescence test is also used to identify viral antigens in intrathoracically inoculated *Toxorhynchites* (Rosen and Gubler, 1974).

Monoclonal antibodies are available with group-specificity against all flaviviruses and all alphaviruses. This allows rapid assignment of an arbovirus isolate to a serogroup. In addition, monoclonal antibodies have been characterized which show com-plex-reactive as well as type-specific and even strain-specific reactivities against YF, JE, Kunjin, WN, MVE, DEN-1, DEN-2, DEN-3, DEN-4, CEE, both North and South American varieties of EEE, various subtypes (variants) of VEE including a vaccine strain (TC-83), SF, WEE, Highlands J, and SIN viruses. These monoclonal antibodies have been prepared against the viruses considered important either for epidemiologic purposes or for research. In the future, monoclonal antibodies undoubtedly will be prepared against other flaviviruses and alphaviruses, and a more extensive battery of reagents will be available for use in diagnostic laboratories. Given an appropriate battery of serogroup-specific, antigenic complex-specific, and type- and subtype-specific antibodies, a virus isolate can be quickly and specifically identified.

Direct Antigen Detection

Direct detection of viruses and antigens in clinical specimens can provide a rapid diagnosis but is less sensitive than methods that require replication of the virus and thus amplify the antigenic components. Rapid, early diagnosis has obvious benefits both in formulating a therapeutic plan for the individual patient and in formulating appropriate public health measures in the instances of a potentially epidemic disease. The more rapid, sensitive, simple, and specific the test, the better. For many years respiratory viruses have been identified directly in nasopharyngeal aspirates by detection of their antigens via immunofluorescence (Gardner and McQuillan, 1980) or by ELISA (Sarkkinen et al., 1981). More recently, enteric viruses have been used to develop rapid identification techniques, such as nucleic acid hybridization (Hyypia et al., 1984). With fluorescent antibody or immunoperoxidase techniques, both flaviviruses and alphaviruses may be detected directly in tissues from infected humans or animals. Frozen sections or impressions smears (as for rabies) are suitable for such tests. Sections (6 μm) are cut on a cryostat–microtome, placed on clean slides, and fixed for 15 min in cold ($-20°C$) acetone. Indirect fluorescent antibody (IFA) or immunoperoxidase tests are then performed with the most sensitive and specific immune reagents available. In the case of IFA tests, primary (antiviral) antibody is added to the section or smear and incubated at 37°C for 30 min in a humidified chamber; the slides are washed in phosphate-buffered saline (PBS), pH 7.2 to 7.4, air-dried, and stained with fluorescein-conjugated antibody to the species in which the primary antibody was made. Trypan blue may be added to the conjugate at a concentration of 1:2,000, as a counterstain. Optimal concentrations of both the first and second (conju-

gated) antibodies are determined by prior titrations with control antigens (virus-infected cell cultures or tissues). Controls include infected and uninfected tissues and cell cultures as well as infected tissues or cell cultures incubated with normal mouse ascitic fluid or other appropriate reagent. Stained slides are examined with a fluorescence microscope fitted with appropriate filters and objective. Test results are scored semiquantitatively according to the intensity of fluorescence; the percentage of fluorescing cells per field may be determined as well. Nonspecific reactivity may be reduced by prior adsorption of the primary and, if necessary, secondary antisera with normal tissue.

The IFA technique has been used for primary detection of JE (Kusano, 1966) and SLE (Gardner and Reyes, 1980) antigens in human brain tissue and of EEE antigen in horse brain tissue (Monath et al., 1981b). Immunocytochemical staining may also be applied to formalin-fixed tissues. For IFA staining of fixed tissues, tissues must first be treated with trypsin or protease to uncover antigenic determinants. The immunoperoxidase technique appears to be more satisfactory for direct antigen detection in fixed materials and has been used for the diagnosis of YF in liver tissue (de la Monte et al., 1983). The immunofluorescence technique requires skilled technicians, as well as expensive microscopes. These needs, the subjectivity of the results, and the lack of sensitivity of the method are additional drawbacks. For these reasons, ELISA and nucleic acid hybridization are increasingly used for direct detection of viral antigens in clinical specimens, in tissues from

wild vertebrates, and in mosquitoes, ticks, and other arthropods.

Detection of antigen in clinical specimens requires construction of a sensitive and specific immunoassay utilizing the most appropriate combination of capture and detecting antibodies, enzyme, and substrate, as well as optimal conditions of incubation (Fig. 1a,b,c,d). Development of such assays requires a research study, first with laboratory-cultivated virus and then with clinical samples. Capture antibodies generally are characterized by high avidity for antigen and may be polyclonal (Halonen et al., 1980; Hildreth et al., 1984), physically separated IgM antibodies of human (or other species) origin (Sarthou and Lhuillier, 1984), or monoclonal (Monath and Nystrom, 1984a). Capture antibody is added at optimal dilution (determined by titration, usually 1 : 500 to 1 : 2,000) in carbonate–bicarbonate buffer, pH 9.6, to the solid-phase support (for example, a polystyrene microtiter plate) and allowed to bind for 3 h at 22°C or 37°C, or overnight at 4°C. The plate is washed repeatedly with PBS containing 0.05% Tween 20, and the clinical sample is added; virus-positive and -negative controls are included in each assay. After optimal incubation (usually 16 h at 22 to 45°C, depending on the virus system) followed by washing, detecting antibody is added (double-sandwich method); detecting antibody may be polyclonal or monoclonal. When polyclonal or monoclonal antibody of the same species is used for both capture and detection, the detecting antibody must be conjugated to an enzyme (or radionuclide); if antibody prepared in another species is used, it should be followed

Y   Capture (anti-viral) antibody
    (prepared in species A)

Ⓤ   Antigen

✶   Detecting (anti-viral) antibody
Ⅎ   conjugated to an enzyme

○   Detecting (anti-viral) antibody not conjugated
⅄   to an enzyme (prepared in species B)

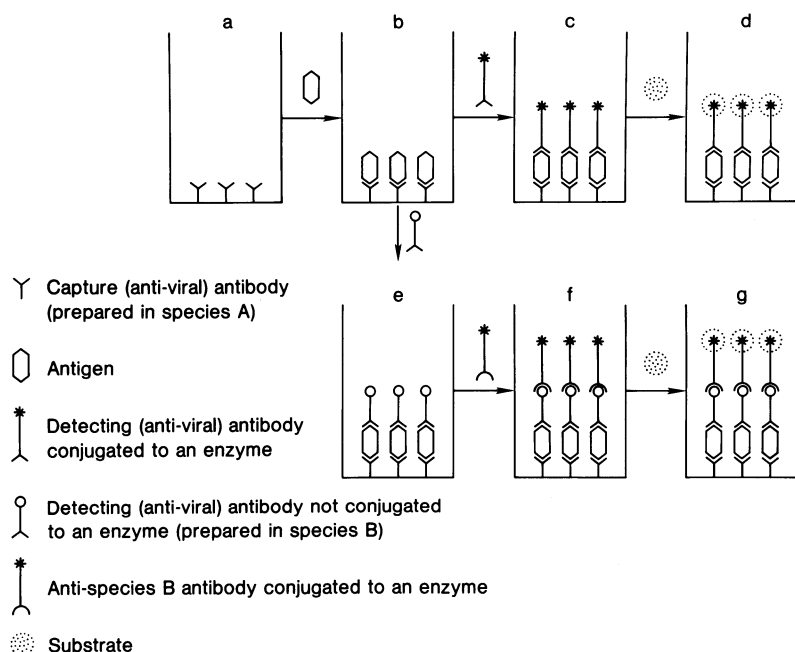✶   Anti-species B antibody conjugated to an enzyme
∧

⬚   Substrate

FIG. 1. Double-sandwich antigen detection enzyme-linked immunosorbent assay.

by conjugated anti-species-detecting antibody (Fig. 1a,b,e,f,g). The color change is then recorded as optical density with a spectrophotometer. If incubations and washings between the additions of reagents were adequate, the results can be read as the ratios of test to control; usually, ratios greater than or equal to 2.0 are considered positive for IgM antibody. Should the optical density of the control (background) be exceedingly low, such ratios will be artificially high; common sense should be applied in these situations.

An advantage of this system is that there is no need for enzyme-conjugated antibodies for all the secondary antibodies used, and that for secondary antibodies one can use hyperimmune mouse ascitic fluids (Tikasingh et al., 1966), available in almost all arbovirus diagnostic laboratories and from the Centers for Disease Control (Atlanta, Georgia, or Ft. Collins, Colorado). The principal disadvantage of the double-sandwich ELISA is the need for an additional step and the possible increase in attendant nonspecific reactivity. We have not found the latter to be a problem, because the high titers of hyperimmune mouse ascitic fluids used as secondary antibodies allow the use of high dilutions, thereby diluting any nonspecifically reacting substances as well.

The double-sandwich method is generally not as sensitive, however, as the simultaneous-sandwich technique (Fig. 2). In this single incubation assay, the virus-containing sample and detecting antibody conjugate are added and incubated simultaneously (Monath et al., 1986a,b). If the simultaneous-sandwich method is used for enzyme immunoassays, the detecting antibody must be linked to biotin, because the horseradish peroxidase molecule is so large that it sterically interferes with virus–capture-antibody interaction. Following interaction of virus and biotinylated detecting antibody, the plate is washed,

and avidin-peroxidase is added at optimal dilution. The final step in either the double-sandwich or simultaneous-incubation assay is addition of enzyme substrate. Many substrates are available, and some show considerable promise, but we prefer 2.2'-azino-di[3-ethyl] benzthiazoline sulfonate. The color reaction may be stopped with 5% sodium dodecylsulfate after an appropriate incubation at room temperature in the dark. Usually this requires 10 to 20 min, the time necessary for maximal color generation in positive controls without appearance of color in negative controls.

Antibody Assays

Classical techniques for serologic diagnosis include the HI (Clarke and Casals, 1958), CF (Casey, 1965), neutralization (Lindsey et al., 1976), and IFA (Monath et al., 1981a) tests. The acceptable standard has been a fourfold or greater increase or decrease in antibody titers determined by these methods. For confirmation, these tests require multiple specimens, collected days to weeks apart. When rapidity of diagnosis is not critical or when infections with closely related viruses complicate interpretation of results, these tests are often invaluable. However, their lack of specificity creates a variety of problems in interpretation. For example, in areas hyperendemic for flaviviruses, broad cross-reactions may occur in HI, CF, and even neutralization tests, limiting their usefulness in defining the infecting agent (Monath et al., 1980). Moreover, prior infection results in a specific rise in antibodies to the heterologous (related) virus responsible for a remote infection, rather than in antibodies to the current infecting virus (Halstead, et al., 1983). Antibody cross-reactivity to closely related viruses also tends to broaden with time after

Y   Capture (anti-viral) antibody

〇   Antigen

B   Detecting (anti-viral) antibody linked to biotin
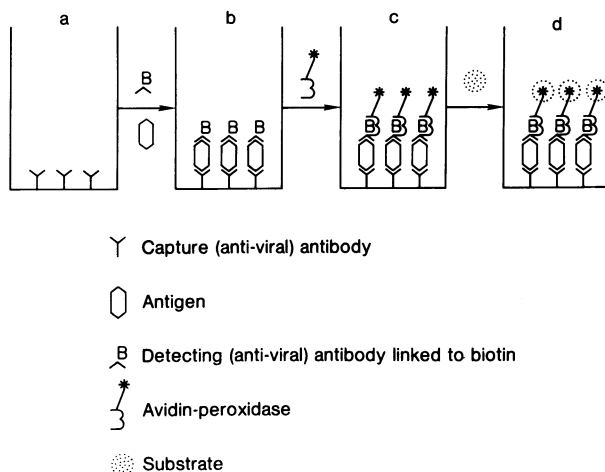
⚹   Avidin-peroxidase

⠿   Substrate

FIG. 2. Simultaneous-sandwich antigen detection enzyme-linked immunosorbent assay.

infection. In the case of primary infection, however, any one of the classical serologic methods applied to paired acute- and early convalescent-phase serum samples can provide a specific diagnosis.

The relatively recent adaptation and application of IgM antibody capture–enzyme-linked immunosorbent assay (MAC ELISA) to arbovirus serology has provided a tool for more rapid serodiagnostic testing and has been extensively studied in the case of flavivirus and alphavirus infections (Heinz et al., 1981; Burke et al.,1982; Lhuillier and Sarthou, 1983; Monath et al., 1984b; Calisher et al., 1985, 1986a,b,c). Unlike IgG antibody, which is detected later in infections and persists for years after a primary infection, IgM antibody is detected in serum soon after infection with arboviruses and, in most cases, does not persist in high titer. Presence of IgM antibodies, therefore, indicates recent infection in most instances. IgM antibodies are also quite specific, although cross-reactions remain an interpretive problem in some cases, particularly in distinguishing between infections with dengue serotypes. IgM antibodies may also have prognostic value, as shown by Burke et al. (1985) in the case of patients infected with JE virus. Patients who died had low levels of IgM antibody in the cerebrospinal fluid. The presence of IgM antibody in cerebrospinal fluid indicates neuroinvasion by the virus in question and provides important evidence in the etiology of encephalitis.

Briefly, MAC ELISA is performed as follows (Fig. 3a,b,c,d,e): wells of a microtiter plate are coated with (commercially available) antibody to human IgM. Patient serum or cerebrospinal fluid is then introduced, usually at a dilution of 1 : 100 for serum and 1 : 10 for cerebrospinal fluid. Density-gradient-purified virus, supernatant fluid from virus-infected cell cultures, or other viral antigen (such as sucrose–acetone-extracted antigen prepared from the brains of virus-infected suckling mice) and antiviral (secondary) antibody are then added. If the detecting antibody has been conjugated to an enzyme, the (commercially available) substrate is then introduced, and the intensity of the color change is recorded as optical density. Optical densities of positive serum samples are proportional to antibody titer (Monath et al., 1984b). Therefore, a single dilution of patient serum can be used to estimate changes in titers between paired acute- and convalescent-phase serum samples. Alternatively, positive serum can be diluted serially twofold and each dilution tested for positivity. In this way, titers comparable to those developed in HI, CF, and neutralization tests can be obtained. However, the presence of IgM antibody in a single serum or cerebrospinal fluid sample can be taken as presumptive evidence of recent infection by the virus used in the test.

A double-sandwich ELISA, with anti-species IgG third (detecting) antibody conjugated to an enzyme that reacts with the secondary (antiviral) antibody, also has been used with considerable success (Fig. 3,a,b,c,f,g,h). The inconvenience caused by having to take the extra step is largely overcome by being able to use a single anti-species conjugate, rather than one for each antiviral reagent. For alphaviruses and flaviviruses, this is accomplished by using serogroup-reactive, enzyme-conjugated, monoclonal an-

Y Capture (anti-human IgM) antibody

♡ Human IgM (anti-viral) antibody

▯ Antigen

⊥ Detecting (anti-viral) antibody conjugated
   to an enzyme

○⊥ Detecting (anti-viral) antibody not conjugated to an enzyme
   (prepared in species B)

⊥ Anti-species B antibody conjugated to an enzyme
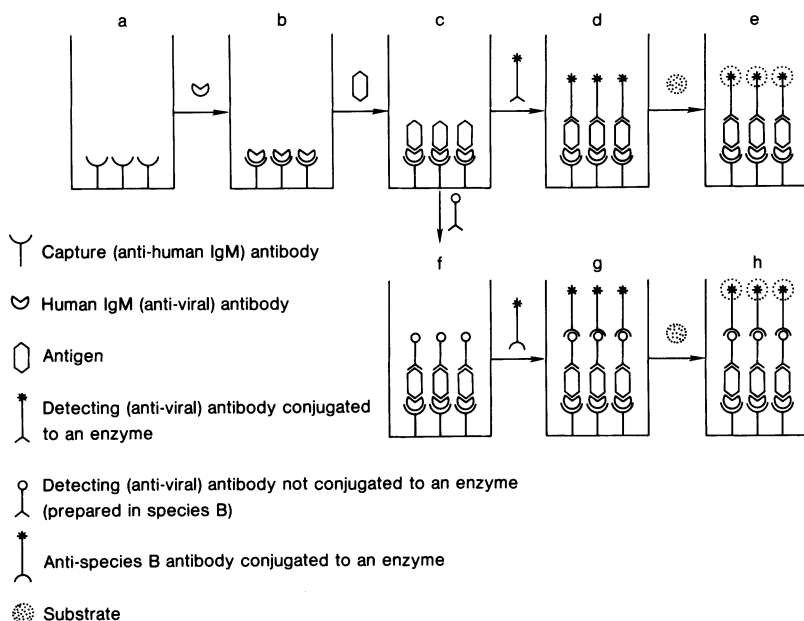
▒ Substrate

FIG. 3. IgM antibody capture enzyme-linked immunosorbent assay.

tibodies. The individual needs of given situations and laboratories warrant tailoring the technique to specific purposes.

Rheumatoid factor, an IgM antibody directed against IgG, can cause false-positive results in immunoassays for IgM. In MAC ELISAs, all IgM antibodies, including rheumatoid factor, are captured. Rheumatoid factor captured in this way may result in binding of a) human IgG antibodies against the viral antigen used in the test or b) mouse (or other species) antiviral IgG antibodies used to detect antigen. Interference by rheumatoid factor cannot be controlled by use of control antigens, but specific tests for rheumatoid factor are commercially available. Additionally, rheumatoid factor can be removed from serum by various methods, which have been reviewed by Meurman (1983).

IgG antibody produced after infections with arboviruses persists for months, years, or even for the life of the individual. Therefore, as with HI and neutralizing antibodies, the presence of IgG antibody does not necessarily denote an active or recent arboviral infection, unlike IgM antibody. The fetus or neonate produces IgM, but not IgG, in response to infections in utero or shortly after birth. Thus, the presence of IgM in the fetus or neonate indicates active infection of the fetus or neonate; the relevance of this observation to diagnosing alphavirus and flavivirus infections is limited.

IgG antibody in serum or in cerebrospinal fluids may be determined as follows (Fig. 4): a) coat microtiter plate wells with about 350 to 500 ng of gradient-purified virus (Fig. 4a) or coat the wells with antibody (prepared in a nonhuman species) and then add virus or sucrose-acetone-extracted antigen or supernatant fluid from virus-infected cells (Fig. 4aa); b)

introduce the serum or cerebrospinal fluid suspected to contain IgG antibody, testing only a single dilution of serum or the first of a series of dilutions beginning with about 1 : 40; c) add anti-human IgG (which does not contain antibody to species (A) conjugated with an enzyme; d) add the substrate and record the optical density of the test and control sera. Again, ratios of test to control greater than or equal to 2.0 are considered positive. This IgG assay is performed relatively quickly, has the advantages of allowing precoating and storage of plates, and requires only a few steps for completion. It is somewhat less sensitive and specific than neutralization tests, but because it is performed relatively easily, it is used to rapidly detect antibody to a variety of viruses. A distinct disadvantage of this assay is the need for either purified virus or separate coating antibodies for each virus included in a panel. Also, because there is a need for paired acute- and convalescent-phase serum samples, it holds little advantage over the HI test.

In summary, we suggest using MAC ELISA for rapidly detecting antibody in situations of immediate importance, and neutralization, HI, IFA, or IgG assays for determining antibody in paired serum samples and for serosurveys. In individual instances in which IgM antibody has been shown to be present, HI and CF can be used as accessory tests, and neutralization tests can be used for definitive and confirmatory determinations of the infecting agent.

Interpretation of Serologic Data

All viruses of the genus *Flavivirus* are antigenically related to one another, as are all viruses of the genus *Alphavirus*. Antigen sharing provides a practical ad-
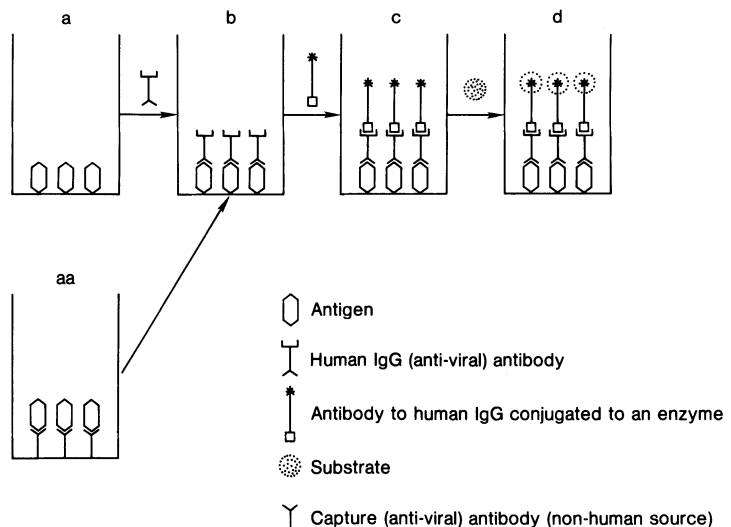

FIG. 4. IgG antibody detection enzyme-linked immunosorbent assay.

vantage, in that evidence for the presence of shared antigens can be used to place an isolate within a serogroup, thereby eliminating the possibility that the virus belongs to one of the many other serogroups in which arboviruses are found. Biological characterization cannot be used to place an isolate within a serogroup, and extensive and complex biochemical and electron microscopic methods are expensive and time-consuming. Therefore, detecting group-reactive antigen can be of value in serologic tests for antibodies to flaviviruses and alphaviruses.

When a human or other animal is infected with a virus, that animal produces type-specific, complex-specific, and group-specific antibodies, which are detected as a mixture in serum taken from the animal. Therefore, identification of group-specific antibody in a patient indicates infection with a member of that serogroup, identification of complex-specific antibody indicates infection with a member of a particular antigenic complex within that serogroup, and identification of type-specific antibody indicates infection with a particular virus (type).

The flavivirus hemagglutinin is contained in the envelope (E) glycoprotein (Trent, 1977), whereas the hemagglutinins of alphaviruses are contained in one of the two glycoproteins (E1 and E2); for example, for SIN virus in E1 and for VEE virus in E2 (reviewed by Roehrig, 1986). Irrespective of site, antibodies to all flaviviruses and alphaviruses inhibit hemagglutination by other members of those serogroups, so that the HI test can be used to place a virus in a serogroup.

The nucleocapsid antigen is responsible for the broad group- and antigenic complex-reactivity of both the flaviviruses and the alphaviruses (Dalrymple, 1972; Trent, 1977); this is one of the antigens that could participate in the CF reaction. Finding CF antibody to a particular virus in an individual indicates that that individual was recently infected with that virus or with one closely related to it. After dengue virus infections, individuals produce CF antibodies that persist for as many as 50 years (Halstead, 1974), or for life, as does neutralizing antibody. Certain individuals infected with other flaviviruses and alphaviruses never produce CF antibody or produce it too late to be of diagnostic value (Calisher and Poland, 1980). Nevertheless, the presence of CF antibody to a particular virus in a patient can be used as presumptive evidence of recent infection with that virus and, as with HI or other tests, a fourfold rise in titer between paired acute- and convalescent-phase serum samples confirms infection with that or a closely related virus.

Reactivity of a patient's serum with a given antigen by any test, be it ELISA, HI, CF, direct or indirect fluorescent antibody, or neutralization, is useful in the serodiagnosis of arbovirus infections. Because of the significant cross-reactivity of the flaviviruses, patient serum can be tested with the suspected etiologic agent of the infection and a few other viruses (flaviviruses and others). If the test detects antibody, it can be used to make a presumptive diagnosis of infection with that virus or a member of that serogroup, with some notable exceptions. First, antibody in a single serum merely indicates infection at some time in the past and, therefore, finding antibody in such a serum cannot be used to indicate any more than a presumptive diagnosis. Second, antibody in a single serum could be due to vaccination with YF, JE, or other flavivirus. IgM antibody detected by ELISA (as described above) usually confirms infection with the virus for which the specimen was tested or with a closely related virus. In epidemic situations ELISA-positive serum samples can be considered presumptive evidence of recent infections, but in all instances adequate epidemiologic information must be obtained to be secure in one's interpretation of results of serologic tests. Examples of the variety of serodiagnostic test results that can be obtained with flavivirus and alphavirus infections of humans are presented in Tables 5 and 6, respectively.

Case Reports

Patient F-1 (Table 5) was a 65-year-old female from Mississippi. Serum, collected on the 4th day after onset of encephalitic signs, was positive in MAC ELISA, HI, and neutralization tests for antibody to SLE virus. Only the MAC ELISA was specific, however, and the CF test was negative. Based on these results, a presumptive (and epidemiologically conclusive) serodiagnosis of SLE virus infection could be made. No advantage was conferred by performing similar tests with convalescent-phase serum, collected 18 days after onset. The MAC ELISA was no less specific, the HI test was no more specific, the CF test was uninterpretable (except to say that the patient had recently been infected with a flavivirus), and the neutralization test, although it revealed higher titers to SLE virus than to the other flaviviruses with which the serum was tested, nevertheless was not type-specific. Both HI and neutralization tests provided confirmatory evidence (fourfold rises in titer between paired serum samples) of recent infection with a flavivirus, most likely SLE virus.

Some areas outside the United States are hyperendemic for multiple flaviviruses. Residents of these areas, besides being at risk of exposure to more flaviviruses and to the possible complications brought about by sequential infections with such viruses, may respond to the latest in a series of flavivirus infections by producing broadly cross-re-

TABLE 5. Examples of serodiagnostic results obtained with serum samples from patients infected with flaviviruses

Patient	Days after onset	Test[b]	Virus[a]					
			SLE	JE	WN	MVE	YF	LGT
F-1	4	MAC ELISA	3.15	1.22	1.60	1.79	1.10	1.59
		HI	160	80	160	80	20	10
		CF	—	—	—	—	—	—
		N	160	20	NT	20	—	—
	18	MAC ELISA	2.98	1.15	1.21	1.37	1.38	1.01
		HI	1,280	320	320	1,280	40	40
		CF	16	8	8	8	8	—
		N	1,280	160	NT	160	40	10

Patient	Days after onset	Test[b]	LGT	POW	SLE	YF	LAC
F-2	14	HI	20	—	20	20	—
		CF	32	—	—	—	—
	18	HI	20	—	20	20	—
		CF	32	—	—	—	—
	35	HI	80	10	40	20	—
		CF	256	16	—	—	—

Patient	Days after onset	Test[b]	YF	ZIKA	WN	UGS	USU
F-3	14	IgM IFA	64	—	—	—	—
		IgG IFA	128	—	8	—	—
		HI	1,280	10	10	40	40
		CF	8	—	—	—	—
		N	640	—	—	10	—
	42	IgM IFA	64	—	—	NT	NT
		IgG IFA	128	—	—	—	—
		HI	640	10	—	20	20
		CF	32	—	—	—	—
		N	640	—	—	—	—
F-4	28	IgM IFA	128	—	—	8	8
		IgG IFA	256	256	256	128	256
		HI	1,280	1,280	640	1,280	1,280
		CF	32	—	64	—	—
		N	320	320	NT	160	320
	77	IgM IFA	—	—	—	—	—
		IgG IFA	512	128	128	16	32
		HI	640	1,280	640	1,280	1,280
		CF	512	—	8	—	—
		N	80	320	—	10	80

[a] SLE = St. Louis encephalitis; JE = Japanese encephalitis; WN = West Nile; MVE = Murray Valley encephalitis; POW = Powassan; YF = yellow fever; LGT = Langat; LAC = LaCrosse; ZIKA = Zika; UGS = Uganda S; USU = Usutu.

[b] IgM antibody capture–enzyme-linked immunosorbent assay (MAC ELISA) results given as ratio of optical densities with test and control antigens and serum at the lowest test dilution, in this instance 1 : 100 (greater than or equal to 2.0 considered positive). Results of other tests given as titers; − indicates <10 HI (hemagglutination-inhibition) or N (neutralization), <8 CF (complement-fixation), <8 IFA (indirect fluorescent antibody); NT indicates not tested.

active HI, CF, and neutralizing antibodies (Monath et al., 1980; Patient F-4, Table 5). It has not yet been proven, but it is unlikely that antibody detected under such circumstances by MAC ELISA is less specific than in the example provided here; thus, MAC ELISA is the test of choice in any circumstance in which a flavivirus is the suspected etiologic agent of human infection:

Patient F-2 (Table 5), a 4-year-old female from Ohio, became febrile while in central Europe. An engorged tick was found on her scalp; it was removed and her symptoms disappeared. Two weeks

later, she returned to the United States and became ill again, this time with obvious signs of central nervous system disease. A tick-borne encephalitis virus was suspected as the etiologic agent of her illness, and a serum sample was serologically tested. Rather than using several tick-borne viruses known to be hazardous to laboratory workers, we used Langat virus as antigen. This virus occurs in southeast Asia, but not in Europe. It is related to CEE, POW of North America, and other viruses of the tick-borne complex of flaviviruses (Table 1). Results of HI and CF tests revealed that the patient had HI antibody to the four flaviviruses, but not to LaCrosse, a California serogroup bunyavirus. Serologic conversion, demonstrated to Langat virus, and stable titers to the other flaviviruses suggested recent infection by a flavivirus belonging to the tick-borne encephalitis complex. CF test results confirmed this; an eightfold rise in antibody to Langat and a lesser rise to POW virus was demonstrated between paired serum samples. Neutralization tests were not performed because of the hazard to laboratory workers. Extensive studies of IgM antibodies by Heinz et al. (1981) now have shown that ELISA for antibodies to CEE virus is a rapid and sensitive method for detecting such antibodies in serum samples and in cerebrospinal fluids from humans infected with this and related viruses.

Patient F-3 (Table 5), from The Gambia, West Africa, had a history of febrile illness with jaundice clinically compatible with YF. Diagnosis was made on the basis of fourfold rise in CF antibody between paired serum samples; antibody titers determined by other tests were stable, albeit high, and heterologous flavivirus reactivity was minimal or not detected. YF could be readily diagnosed from the fluorescent antibody test results, because high IgM and IgG antibody titers were present in the absence of heterologous cross-reactions. This is an example of primary YF virus infection, rather than infection by one flavivirus at some time after another.

Patient F-4 (Table 5), also from The Gambia, had a clinical history similar to that of patient F-3. Diagnosis was made on the basis of a 16-fold decrease in IgM antibody, 16-fold increase in CF antibody, fourfold decrease in neutralizing antibody, and elevated HI and IgG titers to YF virus. Extensive cross-reactions with other flaviviruses indicated that this was a clear instance of flaviviral superinfection, that is, infection with YF virus at some time after infection with another flavivirus.

Patient A-1 (Table 6) was a 2-year-old female from Florida. As has been shown previously (Calisher et al., 1986b), MAC ELISA was type-specific for infection with EEE virus, as were CF test results. HI and neutralization test results with both single and paired serum samples could be used as presumptive and confirmatory evidence, respectively, for recent infection with EEE virus.

Patient A-2 (Table 6), a 35-year-old female from Minnesota, was ill with fever and headache during an outbreak of WEE in that state. Again, the MAC ELISA was not type-specific for WEE virus; it reacted with SIN virus, a member of the same antigenic complex. Neutralization test results could be used to make an accurate serodiagnosis.

Patient A-3 (Table 6) was a 30-year-old male from Texas who became ill with fever and headache during the temporary incursion of VEE into that state in 1971. Serum samples from this patient and patient A-2 were stored at −20°C and tested by MAC ELISA more than 10 years after collection. Acute-phase serum, collected on the day of onset, did not react in any of the four tests. However, the convalescent-phase serum, collected 31 days after onset, was positive in MAC ELISA and in HI, CF, and neutralization tests for both VEE virus and the closely related Everglades virus. Titers were considerably higher to the former than to the latter. The MAC ELISA was the only test that did not provide evidence for reactivity with heterologous alphaviruses.

Patient A-4 (Table 6), was a male in Indonesia with fever during an outbreak of chikungunya there. MAC ELISA was positive to both CHIK virus and RR virus, a closely related alphavirus; however, as with MAC ELISA of the convalescent-phase serum of Patient A-3 with VEE and Everglades viruses, the serum of Patient A-4 reacted to much higher titer with the homologous (infecting) virus. That this was, in fact, an infection caused by CHIK virus could be determined from the neutralization test results and by the fact that the HI and CF tests were positive to CHIK and not to RR virus. Results of similar tests with sera from individuals infected with RR virus have given reciprocally similar results to these.

Epidemiology and Ecology

Because of the large number of alphaviruses and flaviviruses that have been implicated in diseases of humans and domesticated animals, selection of test methods for virus isolation and of antigens for use in diagnostic tests should be based on a thorough understanding of the clinical features and epidemiology of these viruses. The disease syndrome associated with infection can narrow the etiologic possibilities (Tables 3, 4, 7), but it should be emphasized that viruses that cause encephalitis or hemorrhagic fever also produce mild, nonspecific, systemic febrile illnesses. The geographic location of the patient's exposure should be considered in terms of virus distribution (Table 7), and diagnostic tests should be chosen and designed accordingly.

TABLE 6. Examples of serodiagnostic results obtained with serum samples from patients infected with alphaviruses

Patient	Days after onset	Test[b]	Virus[a]						
			EEE	WEE	SIN	VEE	EVE	CHIK	RR
A-1	1	MAC ELISA	4.21	1.51	1.31	0.89	1.14	NT	NT
		HI	40	—	—	—	—	—	—
		CF	—	—	NT	—	—	NT	NT
		N	20	—	NT	—	NT	NT	NT
	22	MAC ELISA	3.10	1.26	1.00	1.44	1.71	1.37	1.47
		HI	320	10	—	—	—	—	—
		CF	32	—	—	—	—	—	—
		N	10,240	20	NT	20	NT	NT	NT
A-2	3	MAC ELISA	1.39	1.79	1.82	1.03	1.61	1.45	1.63
		HI	—	—	—	—	—	—	—
		CF	—	—	—	—	NT	NT	NT
		N	—	—	—	—	NT	NT	NT
	10	MAC ELISA	1.57	3.89	2.15	1.28	1.46	1.23	1.08
		HI	—	40	20	—	—	—	—
		CF	—	8	—	—	NT	NT	NT
		N	—	160	20	—	NT	NT	NT
A-3	0	MAC ELISA	1.28	0.90	1.41	1.28	1.36	1.04	1.61
		HI	—	—	—	—	—	—	—
		CF	—	—	—	—	—	—	—
		N	—	—	NT	—	—	NT	—
	31	MAC ELISA	1.12	1.35	1.49	3.33	3.27	1.60	1.02
		HI	10	40	—	1,280	320	40	80
		CF	—	—	8	128	32	—	—
		N	—	—	—	2,560	160	—	10
A-4	5	MAC ELISA	1.82	1.61	1.20	1.47	1.18	5.52	3.39
		HI	—	—	—	—	—	40	—
		CF	—	—	NT	—	NT	8	—
		N	—	—	NT	—	NT	40	—
	19	MAC ELISA	1.72	1.34	1.05	1.22	1.66	4.62	2.82
		HI	—	—	NT	—	—	80	—
		CF	—	—	NT	—	—	16	—
		N	—	—	NT	—	NT	640	40

[a] EEE = eastern equine encephalitis; WEE = western equine encephalitis; SIN = Sindbis; VEE = Venezuelan equine encephalitis (variety IAB); EVE = Everglades (VEE type II); CHIK = chikungunya; RR = Ross River.
[b] See footnote [b], Table 5.

Season of the year also is important; alphaviruses and flaviviruses transmitted by mosquitoes in temperate areas cause human infections in the summer and early fall, during the period of vector activity. Tick-borne flavivirus infections usually occur during the spring and early summer. In tropical areas, infections generally coincide with the rainy season, the monthly distribution thus varying with latitude. Information about the occurrence of multiple cases in the same place and time, as well as association with disease in domesticated animals, will greatly assist in design of diagnostic studies. For example, an outbreak of fever, arthralgia, and rash in an African city is more likely to be due to dengue or chikungunya virus than to other flaviviruses or alphaviruses that have been associated only with individual or sporadic cases (for example, Zika, Spondweni, Banzi, or Wesselsbron viruses). The concurrence of cases of encephalitis in horses and humans suggests, depending on geographic area, a diagnosis of EEE, WEE, VEE, or JE.

Age, sex, and occupation are also significant risk factors for alphavirus and flavivirus infection. For example, an outbreak of encephalitis in the western United States could be due to WEE or SLE viruses, or both. Because children are more susceptible to severe encephalitis caused by WEE virus, cases in infants are more likely to be due to that virus; in contrast, the elderly are more prone to develop SLE.

Most alphaviruses and flaviviruses have unique transmission cycles involving specific vector arthropods and vertebrate hosts. The principal elements of these cycles are listed in Table 7.

TABLE 7. Clinical, epidemiologic, and ecological features of alphaviruses and flaviviruses

Genus	Virus	Clinical features	Geographic distribution
Alphavirus	Chikungunya	Fever, arthralgia, myalgia, rash	Africa, southern Asia, Philippines
	O'nyong nyong	Fever, arthralgia, myalgia, rash	East Africa
	Mayaro	Fever, arthralgia, myalgia, rash	Tropical South America
	Ross River	Fever, arthralgia, myalgia, rash	Australia, South Pacific
	Sindbis and related agents (e.g., Ockelbo)	Fever, arthralgia, myalgia, rash	Africa, Asia, Europe
	Eastern equine enc.	Encephalitis	North and South Americas, Caribbean
	Western equine enc.	Encephalitis	North and South Americas
	Venezuelan equine enc.[a]	Encephalitis	South America, Central Americas (enzootic subtype in Florida)
Flavivirus	Dengue-1, -2, -3, -4	Fever, arthralgia, myalgia, rash	Tropics worldwide
	West Nile	Fever, arthralgia, myalgia, rash	Africa, tropical Asia, Mediterranean region
	Banzi	Fever, arthralgia, myalgia, rash	Africa
	Bussuquara	Fever, arthralgia, myalgia, rash	Tropical South America
	Ilheus	Fever, arthralgia, myalgia, rash	Tropical South America
	Sepik	Fever, arthralgia, myalgia, rash	New Guinea
	Spondweni	Fever, arthralgia, myalgia, rash	Africa
	Wesselsbron	Fever, arthralgia, myalgia, rash	Africa
	Zika	Fever, arthralgia, myalgia, rash	Africa
	Usutu	Fever, arthralgia, myalgia, rash	Africa
	St. Louis enc.	Encephalitis	North, Central, South America
	Japanese enc.	Encephalitis	Asia
	Murray Valley enc. and Kunjin	Encephalitis	Australia and New Guinea
	Rocio	Encephalitis	Southeastern Brazil
	Yellow fever	Fever, jaundice, hemorrhage, and renal failure	Tropical Africa and Americas
	Kyasanur Forest disease	Fever, hemorrhage, encephalitis	Southwest India
	Omsk hemorrhagic fever	Fever, hemorrhage	Central U.S.S.R.
	Tick-borne enc.[b]	Encephalitis	U.S.S.R., Eastern Europe, Scandinavia, Finland
	Louping ill	Encephalitis	British Isles
	Powassan	Encephalitis	Canada, United States, U.S.S.R.

[a] Multiple antigenic subtypes and varieties, of which two (IAB, IC) are responsible for epizootic/epidemic disease; other (enzootic) viruses (e.g., subtypes ID, IE, II, IIIA, IIIB, IV) cause sporadic human infections.
[b] Multiple antigenic types and subtypes, including Russian spring-summer (Far Eastern) enc., Central European enc., and Kumlinge viruses.

TABLE 7. *Continued*

Epidemiologic features	Principal vector(s)	Principal hosts
Urban epidemics similar to dengue; enzootic cycle similar to yellow fever	*Aedes aegypti, Ae. africanus, Ae. furcifer-taylori*	Humans, monkeys
Single large rural outbreak in 1950s	*Anopheles* sp.	Humans
Sporadic cases, limited outbreaks associated with deforestation	*Haemagogus* sp.	Monkeys, ? rodents, birds
Annual epidemics (Australia), occasional introductions and epidemic spread (South Pacific)	*Ae. vigilax, Culex annulirostris, Ae. polynesiensis*	Large marsupials, humans
Sporadic causes, epidemics in nonendemic areas	*Culex* sp., *Culiseta* sp.	Wild birds
Periodic small equine epizootics and epidemics	*Cu. melanura, Cq. perturbans*	Wild birds
Periodic equine epizootics and epidemics	*Cx. tarsalis*	Wild birds
Periodic equine epizootics and epidemics	*Aedes, Mansonia, Culex* spp. (epizootic subtypes) *Cx. (Melanoconion)* (enzootic subtypes)	Equines (epizootic) Rodents (enzootic)
Urban epidemics	*Ae. aegypti,* other *Aedes* spp.	Humans
Endemic (tropics) with sporadic cases; summertime epidemics (Mediterranean, temperate South Africa)	*Culex* spp.	Wild birds
Sporadic cases	*Culex* spp.	Rodents
Sporadic cases	*Culex (Melanoconion)* spp.	Rodents
Sporadic cases	*Psorophora* sp., other species	Wild birds
Sporadic cases	*Armigeres, Ficalbia* sp.	?
Sporadic cases	*Aedes* sp., other species	?
Sporadic cases	*Aedes* sp.	? Sheep
Sporadic cases	*Ae. africanus*	Monkeys
Sporadic cases	*Culex* sp.	Wild birds
Periodic epidemics, North America; sporadic disease, tropical South America	*Culex tarsalis* (western U.S.); *Culex pipiens* (eastern U.S.); Multiple *Culex* sp (tropics)	Wild birds
Endemic (S.E. Asia); summertime epidemics (northern and central Asia)	*Culex tritaeniorhynchus*	Swine, birds
Periodic summertime epidemics	*Cx. annulirostris*	Mammals, birds
Singular epidemic	? *Psorophora, Aedes* sp.	Wild birds
Periodic epidemics (Africa); sporadic cases related to jungle exposure	*Ae. aegypti,* other *Aedes* sp., *Haemagogus* sp.	Monkeys, humans
Sporadic cases; occasional outbreaks related to deforestation	*Haemaphysalis* ticks	Rodents
Sporadic cases; winter outbreaks associated with trapping muskrats	*Dermacentor* ticks	Rodents
Sporadic cases with periodic high incidence; outbreaks associated with ingestion of infected raw milk	*Ixodes* ticks	Rodents, birds
Sporadic cases, especially acquired by direct contact with infected sheep or by tick bite	*Ixodes* ticks	Rodents, birds
Sporadic cases	*Ixodes* ticks	Small mammals

Prevention and Control

Identification of even one patient with an arthropod-borne disease known to undergo epidemic spread is extremely important in developing an appropriate prevention and control strategy, because such a patient serves as an indicator of a larger public health problem. Surveillance systems designed to detect virus transmission between vectors, wild and sentinel vertebrate hosts, and humans early in the season require collection and testing of samples. Positive cases should be reported immediately to the responsible public health agency.

Prevention and control of epidemics of mosquito-borne diseases generally is accomplished by reducing vector populations. Preventive strategies involve long-term programs aimed at limiting breeding of mosquitoes through source reduction and use of larvicides. The control of active virus transmission in preepidemic and epidemic situations requires use of space sprays to kill infected adult mosquitoes. Public education programs aimed at protecting against mosquito bites emphasize use of protective clothing, repellents, window screens, and bed nets.

Vaccines are available for a few viruses. Human immunization against YF, JE, and tick-borne encephalitis is relatively common; veterinary vaccines against EEE, WEE, VEE, and JE are available to protect horses. Use of VEE vaccine in horses, which are the principal natural viremic hosts implicated in infecting mosquito vectors, therefore provides protection against human disease. Similarly, immunization of swine is used to prevent human outbreaks of JE in Asia.

Vaccines against the equine encephalitides also are used to protect certain high-risk groups of humans, including laboratory workers. Indeed, in diagnostic laboratories in which work with EEE, WEE, VEE, YF, or JE viruses is anticipated, laboratory workers should be immunized, and then immunity should be ascertained by testing for neutralizing antibodies. For assistance in obtaining information regarding vaccines not otherwise available, contact the Division of Vector-Borne Viral Diseases, Centers for Disease Control, P.O. Box 2087, Fort Collins, Colorado 80522. Information regarding experimental vaccines for DEN-2, CHIK, KFD, and OHF also is available from this source.

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