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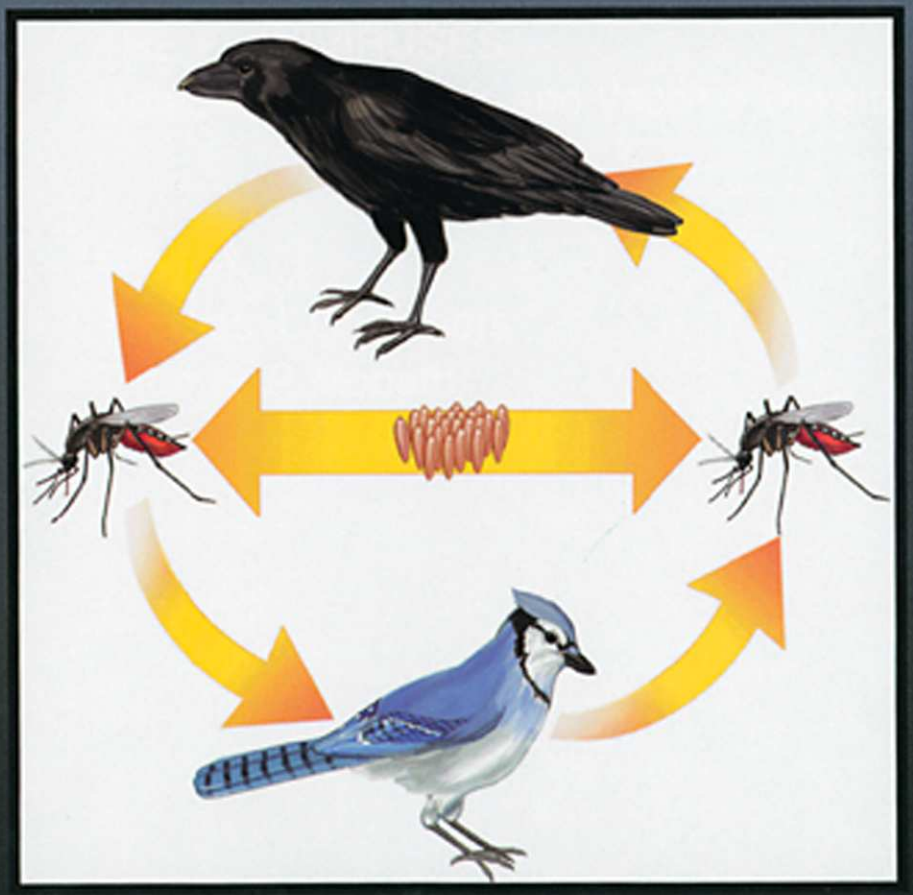
Volume 61

THE FLAVIVIRUSES:

DETECTION, DIAGNOSIS AND VACCINE DEVELOPMENT



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VOLUME 61

The Flaviviruses: Detection, Diagnosis, and Vaccine Development

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CONTENTS

Epidemiology of St. Louis Encephalitis Virus

WILLIAM K. REISEN

West Nile Virus: Epidemiology and Ecology in North America

NICHOLAS KOMAR

Dengue Epidemiology: Virus Epidemiology, Ecology, and Emergence

STEPHEN J. THOMAS, DANIEL STRICKMAN, AND DAVID. W. VAUGHN

Epidemiology and Ecology of Yellow Fever Virus

ALAN D. T. BARRETT AND THOMAS P. MONATH

Tick-Borne Flaviviruses

T. S. GRITSUN, P. A. NUTTALL, AND ERNEST A. GOULD

Epidemiology of Other Arthropod-Borne Flaviviruses Infecting Humans

ROBERT E. SHOPE

Chimeric Flaviviruses: Novel Vaccines against Dengue Fever, Tick-Borne Encephalitis, and Japanese Encephalitis

CHING-JUH LAI AND THOMAS P. MONATH

Prospects for Antiviral Therapy

P. LEYSSEN, N. CHARLIER, J. PAESHUYSE, E. DE CLERCQ, AND J. NEYTS

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PREFACE TO THE FLAVIVIRUSES

The Flavivirus family continues to provide great fascination for virologists, immunologists, entomologists, epidemiologists, and scientists in various other disciplines. Research over the past few decades has yielded considerable progress in many of these areas, but there remain a number of challenges surrounding our understanding of the behavior of flaviviruses in natural conditions and in the laboratory. At a time when continued global emergence of flaviviruses calls for the development and improvement of vaccines and antiviral agents, it is appropriate that a broad compendium of knowledge be made available that presents recent conceptual advances and reviews current information on the many different facets of these viruses. Certainly there have been some noteworthy scientific achievements. For instance, the molecular details of virus structure have been greatly advanced as a result of high-resolution analysis of the envelope protein and its organization at the level of the virion particle, which, together with functional studies, have revealed the uniqueness of this viral protein during replication and pathogenesis. The characterization of an increasing number of flavivirus strains at the sequence level has led to an improved taxonomic classification of the genus, enhanced our understanding of evolution, geographic variation, and epidemiology, and stimulated research on variation in viral virulence. Use of molecular clone technology has advanced from basic studies that have that have identified the functions and properties of viral proteins during RNA replication and virus assembly to the evaluation of candidate virulence determinants, engineering of live attenuated vaccines, and related applications.

Studies on the immunobiology of flaviviruses have led to the realization that these viruses interact with the host immune system in ways that differ from other small RNA viruses. The importance of neutralizing antibody responses for immunity continues to be an area of focus, and the basis for this protection at the epitope-specific level is gradually being dissected. However, there remain enigmatic aspects, such as the wide cross-reactivity elicited by these viruses and the phenomenon of antibody-dependent enhancement, both of which have important implications for pathogenesis and vaccine development, and

require better molecular characterization. It is becoming clear that T-cell responses to flavivirus infections also have unusual properties that may contribute to pathogenesis through immunopathologic and/or immune-subverting events. Further characterization of these responses and their relationship to immune protection are avenues of research needed to optimize the use of the increasing range of vaccine modalities that are being pursued.

In conjunction with advances in flavivirus molecular virology and immunology, more and more attention is being directed to investigation of the pathogenesis of flavivirus diseases. Progress in this area has been heralded by the long-awaited identification of the molecular basis for genetic susceptibility of mice to flaviviruses. This will undoubtedly increase interest in the role of innate defenses in these infections and promote research into the genetic basis of flavivirus susceptibility in humans. Together with the use of modern techniques to identify critical target cells of infection, research in this area will expand our understanding of the cellular and molecular basis for flavivirus tropism. In this regard, the cell-surface molecules that interact with these viruses during entry have yet to be fully characterized, but progress continues to be made on this front. It remains somewhat frustrating that suitable animal models for some flavivirus diseases, particularly dengue hemorrhagic fever, are not available. However, data accumulated from human clinical studies are yielding insight into the pathogenesis of this disease, and similar studies with other pathogenic flaviviruses are anticipated in the future.

The interactions between flaviviruses and their arthropod hosts have been the subject of many classical studies that have now progressed to the molecular level as well. There are many secrets to these interactions that must be discovered to understand the process of virus persistence in molecular terms. These will be forthcoming with the use of modern technologies by creative investigators interested in vector biology. The improvement in molecular technologies has had concomitant impact on the ability to conduct molecular epidemiology at the “macro” and “micro” levels. In response to progressive emergence in recent years of dengue, Japanese encephalitis, West Nile, and tick-borne viruses, the application of such technologies for detection and surveillance in arthropod and vertebrate reservoirs has provided insight into the factors that support the global movements of flaviviruses. Yet, there is a tremendous amount of such data concerning virus evolution in the natural environment that is still needed to understand this process and possibly predict future

trends. Additional molecular studies of these viruses as they are transmitted among vectors, reservoirs, and humans are needed to further our conceptual understanding of virus emergence.

The development of vaccines for flaviviruses has also benefited greatly from the availability of modern technologies, and new as well as next-generation vaccines for some viruses are on the horizon. As better understanding of the immune responses to these viruses in the context of disease as well as vaccine-induced protection becomes available, the ability to control the growing worldwide burden of disease from these agents will likely be improved.

Clearly a comprehensive research approach in many scientific disciplines is needed to unravel the complexities of the virus-host interactions that these viruses have had the benefit of manipulating for centuries. In this three-volume edition on the flaviviruses, our goal has been to assemble a base of knowledge that encompasses these complexities, describes technologies that have contributed to this knowledge, and identifies the major problems faced in attempting to further understand the virus-host interactions that result in disease, and in using vaccine strategies for preventing them. We are grateful to the many contributors who have generously assisted in the preparation of this book series. We must also acknowledge that there are many other colleagues who are active in the field whose expertise has not been represented here.

Thomas J. Chambers,
St. Louis, Missouri, 2003

Thomas P. Monath
Cambridge, Massachusetts, 2003

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PREFACE TO VOLUME 61
THE FLAVIVIRUSES:
DETECTION, DIAGNOSIS, AND VACCINE DEVELOPMENT

Emergence of flaviviruses is a continuing reality and a threat to public health on a worldwide scale. Movement of many of these viruses both regionally and globally has been occurring progressively for the last half-century, resulting in new distributions of viruses, new vector associations, and new human disease syndromes. Japanese encephalitis virus has penetrated many areas in Asia, including the Australasian region to the east and parts of India and Pakistan to the west. Dengue has established itself as a pandemic virus. West Nile virus has both evolved and emerged as the most important cause of arbovirus encephalitis in the Western hemisphere. Growing burdens of tick-borne flavivirus diseases in their endemic zones also pose concerns for regional public health as well as intercontinental spread. Proliferation and dissemination of arthropod vectors, long-range human travel and bird migration, human incursions into vector and reservoir habitats, and environmental disturbances are major factors in the ongoing emergence of these viruses that deserve consideration for control efforts. However, better knowledge of actual vector-host cycles and how they evolve into epidemic outbreaks in conjunction with sensitive molecular and serological assays for detection and diagnosis of these viruses will also contribute to the ability to assess their potential for future transmission.

Despite many possible avenues toward prevention and control of flavivirus diseases, vaccine development still offers the most promising approach. Although yellow fever 17D serves as a paragon for live-attenuated viral vaccines, adopting this modality for other serious flavivirus pathogens has not been very straightforward, with the multiple serotypes of dengue virus being the most flagrant example. Achieving a suitable balance of attenuation and immunogenicity remains a difficult proposition with live viral vaccines, and safety issues continue to be prominent, even for YF 17D. Inactivated vaccines have traditionally also been effective against some flaviviruses, but seem destined for replacement by subunit vaccines designed to have better immunogenicity and fewer side effects. The use of alternative

technologies, including DNA vaccines for flavivirus, is under active investigation, with an as yet undefined role in the next generation products. Demand for flavivirus vaccines encompasses military, civilian, and veterinary realms, and has increased because of the threat of bioterrorism. Development of new vaccines will exploit innovations that arise from continued basic research on flavivirus evolution, biology, and pathogenesis. The challenge of keeping pace with these viruses in the future will certainly remain, despite advances in these many different areas.

Thomas J. Chambers
Thomas P. Monath

DIAGNOSIS AND SURVEILLANCE

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ADVANCES IN VIRUS RESEARCH, VOL 61

SERODIAGNOSIS OF FLAVIVIRAL INFECTIONS AND VACCINATIONS IN HUMANS

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I. Introduction

In the past three decades, we have witnessed a considerable technological shift and a dramatic proliferation of commercially available reagents, diagnostic kits, and testing services for serodiagnosis of flaviviral infections or vaccinations. As more diagnostic data were

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produced, ironically the inadequate levels of our understanding of the variation in antibody response, cross reactivity of antibody, and of other associated complications involved in diagnostic practice became more evident. In addition, new molecular and enzyme-linked immunosorbent assay (ELISA) techniques revealed that some of the cases previously classified as negative or not current infections based on the conventional serologic criteria were found to be actually current infections. In this chapter, the principles and applications of both traditional and new techniques are first critically re-evaluated, followed by an examination of the qualities of specimens. Variations of human antibody response kinetics are briefly summarized, because a clear understanding of the subject is indispensable for a better serodiagnosis. Then, miscellaneous diagnostic complications and the qualities of the criteria used in common serologic techniques are examined. Finally, a list of commercial reagents, diagnostic kits, and services is presented.

Basically, serodiagnosis is performed for one of the three major objectives: laboratory diagnosis of an ill patient, a seroprevalence study in an epidemiologic investigation, and to evaluate an immune response in humans and animals, including vaccine efficacy trials and animal experiments. As more vaccines are introduced, for those in public health, epidemiology, veterinary medicine, and agriculture, it has become critical to be able to differentiate the antibodies induced upon vaccination from those acquired in response to natural infection and homologous *in vivo* protective antibodies from heterologous, nonprotective *in vitro* neutralizing antibodies, for designing an efficient immunization strategy for a particular human (or animal) population and for determining the safety of importing or exporting animals.

II. Serodiagnostic Tests

The techniques based on specific agglutination of blood cells, bacteria, or particles had been developed early in the history of flaviviral serodiagnosis. With the advent of ELISA, some of the traditional techniques, in particular, the complement fixation test (CF), lost popularity, and radioimmunoassay is no longer practiced in arbovirology. Nevertheless, in terms of the overall advantages and other benefits, the hemagglutination-inhibition (HI) test still remains invaluable and the neutralization test (NT) most specific for primary infections.

A. Standardization and Multicenter Evaluation of Serologic Tests

Unquestionably, standardization of key serologic tests at global or at least at the regional level for those viral diseases affecting many countries (such as DEN, JE, TBE, WNF, and YF) is highly desirable, although past such attempts have met a variety of difficulties. With the increasing trends toward the use of commercial reagents, kits, and services (Table I), occasional multicenter evaluation of selected tests is another approach for reducing variation in diagnostic quality among institutions. In all these activities, participation of governmental laboratories, research institutions, and industry is of critical importance.

B. Validation and Quality Assurance

For each serologic test, at least a pair of negative and positive control human serum specimens must be obtained from a reliable reference laboratory or other sources, if not readily available. Preferably, they are pooled, laboratory-confirmed specimens when many tests are planned or expected. When no source of such control specimens exists or it is difficult to obtain, at least internal controls must be prepared in each laboratory from the specimens of laboratory-confirmed cases. In the highly *Flavivirus*-endemic locations where negative control specimens are not easily available, acquisition of serum specimens from the residents in the non-endemic areas should be arranged. These control specimens must be tested independently in each laboratory at first using the same technique used in the source laboratory, and necessary adjustments are made until the results in the two institutions are comparable. If a different technique is used, a comparative test between the two methods must be performed to evaluate the qualities of the results obtained with the different techniques. In each test, routinely this pair of control specimens is included and their titers monitored within a test or between tests for quality control. If available, additional internal control specimens may be included. For interpretation of results, first, a set of diagnostic criteria are established, including the acceptable range of variation from the optimal results with negative or positive control specimen. For any specimens of special importance, it is ideal to perform more than one diagnostic test, even including non-serologic tests, for improved reliability of the diagnostics. In addition, it is strongly recommended that all diagnostic laboratories or institutions, regardless of the depth of experience, arrange a periodic proficiency test program in collaboration with an unaffiliated,

TABLE I
LIST OF COMMERCIALLY AVAILABLE REAGENTS, DIAGNOSTIC KITS, AND TESTING SERVICES FOR FLAVIVIRAL INFECTIONS[a]

Virus	Product[b]	Source[c]
DEN	Ag DEN 1-4	ANS/FII/MIB/USB
	DEN-1	ANS/BID/BGN/IMC/VNT
	DEN-1 fENV	VNT
	DEN-1 rENV	HWB
	DEN-2	ANS/BGN/BID/FII/IMC/MIB/VNT
	DEN-2 (16681)	BID/MIB
	DEN-2 fENV	VNT
	DEN-2 rENV	HWB
	DEN-3	ANS/BID/BGN/IMC/VNT
	DEN-3 fENV	VNT
	DEN-3 rENV	HWB
	DEN-4	ANS/BID/BGN/IMC/VNT
	DEN-4 fENV	VNT
	DEN-4 rENV	HWB
	DPS AgC	GLB/MAS/PNB
	ELISA IgMC	AMQ/CBT/CHM/FCT/GLY/GVT/IAC/OMD/PNB
	IgGC	AMQ/CBT/CHM/FCT/GLY/GVT/IAC/OMD/PNB
	IgGI	PNB
	IgGT	PNB
	HYB DEN complex-specific (2H2-9-21)	ATC
	DEN-1 (15F3-1)	ATC
	DEN-2 (3H5-1)	ATC
	DEN-3 (5D4-11)	ATC
	DEN-4 (1H10-6)	ATC
	IFA IgG	AMR/PGB
	IgM	AMR/PGB
	IMB AgC	GLB
	IgG	GLD/VNT
	IgG/IgM	PNB
	IgM	GLD/VNT
	IMC Strip IgG	AMT/GVT/RBP
	IgM	AMT/GVT/RBP
	Cassette IgG/IgM	PNB
	IgG	AMT/CDI/GLY
	IgM	AMT/CDI/GLY

(*continues*)

TABLE I (*continued*)

Virus	Product[b]	Source[c]
	MAb DEN complex reactive	BGN/BID/CHM/USB/VNT/VRS
	DEN 1-4 (IgG & IgM)	IMC
	DEN 1-4 (BD1419)	ACS
	DEN 1-4 (9F14)	BGN
	DEN 1-4 (M125)	FII
	DEN-1	ANS/CGM/ECB/IMC/USB
	DEN-1 (9F10)	BGN
	DEN-1 (M121)	FII/USB
	DEN-1 (15F3)	CHM/MIB
	DEN 1+2 (biotin-labeled)	CMI
	DEN-2	ANS/BID/CHM/ECB/IMC/USB/VNT
	DEN-2 (9F11)	BGN
	DEN-2 (M122)	FII
	DEN-2 (3H5)	CHM/MIB
	DEN-2 (Env-specific)	VNT
	DEN-2 (NS1-specific)	VNT
	DEN-3	ANS/CHM/ECB/IMC/USB
	DEN-3 (9F12)	BGN
	DEN-3 (M123)	FII
	DEN-3 (5D4)	BID/CHM/MIB
	DEN-4	ANS/BGN/CHM/ECB/IMC/USB
	DEN-4 (9F13)	BGN
	DEN-4 (M124)	FII
	DEN-4 (1H10)	BID/CHM/MIB
	PAb DEN-complex reactive	
	(positive control)	PNB
	(mouse)	ATC
	(rabbit)	BGN/BID
	DEN-complex reactive	BID
	IgG (human)	BID/IMC
	IgG (rabbit)	IMC
	IgM (human)	IMC
	anti-DEN-1 (human)	ANS
	anti-DEN-1 (mouse)	ANS/ATC
	anti-DEN-1 (rat)	ANS
	anti-DEN-2 (human)	ANS
	anti-DEN-2 (mouse)	ANS/ATC
	anti-DEN-3 (human)	ANS
	anti-DEN-3 (mouse)	ANS/ATC

(*continues*)

TABLE I (*continued*)

Virus	Product[b]	Source[c]
	anti-DEN-3 (rat)	ANS
	anti-DEN-4 (human)	ANS
	anti-DEN-4 (mouse)	ANS/ATC
	anti-DEN-4 (rat)	ANS
	Service DEN IgG ELISA	ARUP/FCT
	DEN IgM ELISA	ARUP/FCT/SPL
	DEN total Ab (CSF)	SPL
	Virus[d] (live & inactivated)	
	DEN-1,2,3,4	ANS/ATC
	DEN-2	BID/USB
Flavi-viruses	HYB Group reactive (4G2)	ATC
	IFA Arbovirus screening Including Flaviviruses	PNB
	MAb Group reactive (4G2/6B6C-1)	CHM/HRA/MIB
	Service Diagnostic–unspecified	QST
	Custom MAb production	CMI
JE	Ag rEnv	HWB
	rPrME	MIB
	CF	ACS/DSC
	HI	ACS/DSC/KYB
	ELISA IgM	VNT
	IMB IgM	VNT
	MAb JE-specific (995)	MIB
	6B4A-10 (also SLE, MVE, WN-reactive)	CHM
	Group-reactive	VNT
	Envelope-specific	VNT
	NS1-specific	VNT
	PAb (mouse)	ATC
	CF (mouse)	ACS/DSC
	HI (mouse)	ACS/DSC
	Service IgM (serum)	SPL
	IgM (CSF)	SPL
	Virus[d]	ATC
KUN	PAb	ATC
(also see WN)	MAb (10A1)	CHM
MVE	MAb (4B6C-2)	CHM

(*continues*)

TABLE I (*continued*)

Virus	Product[b]	Source[c]
RSSE	PAb (mouse)	ATC
	(sheep)	NIBSC
SLE	DPS AgC	MAS
	MAb (1B5D-1/6B5A-2)	CHM
	PAb (mouse)	ATC
	Service IgG/IgM IFA	ARUP/CNI/FCT/QST/SPL/VRM
	Virus[d]	ATC
TBE	Ag (inactivated)	ANS (permit required)/SID
	ELISA IgG (human)	DBM/EUI/GLY/GVT/PGB/SID
	IgM (human)	DBM/EUI/GLY/GVT/PGB/SID
	(for animals)	PGB
	PAb (human)	ANS
	PAb	
	Int. Ref. Reagents for TBE against louping ill strain	NIBSC
	against Sofjin and Absettarov strains (sheep)	NIBSC
WN	Ag (gamma irradiated)	
	(for avian serology only)	BRC
	(for equine serology only)	BRC
	rPrME	FCT/HRA
	DPS AgC	MAS/PNB
	ELISA IgG and IgM	IBS/PNB
	IFA IgG (slide)	PNB
	MAb (WN-A or H546)	BID/MIB
	Env	CHM
	Nt Ab to Env	BRC
	Non Nt to Env.	BRC
	(for IFA and IHC)	BRC
	PAb (mouse)	ATC/BRC
	(positive control)	PNB
	(avian spp. IgG)	BRC
	(equine IgG+IgM)	BRC
	(rabbit IgG for IFA & IHC)	BRC
	Service IgG/IgM ELISA or IFA	ARUP/FCT/QST/SPL/VRM
	Virus[d]	ATC
YF	HYB (2D12)	ATC/CHM/EUR
	MAb (2D12A)	CHM
	(OG5)	BGN
	(2031–13)	BGN

(*continues*)

TABLE I (*continued*)

Virus	Product[b]	Source[c]
	17D-specific (864)	MIB
	Wild-strain specific (117)	MIB
	PAb (mouse)	ATC
	(monkey) [WHO International Reference]	NIBSC
	Virus[d]	ATC

Other viruses (and their corresponding mouse ascitic fluids-AF) at ATC: Banzi; Bukalasa Bat; Bussuquara (AF); Cowbone Ridge (AF); Dakar bat; Edge Hill; Entebbe bat; Ilhéus (AF); Kokobera; Modoc (AF); Montana myotis meningoencephalitis (AF); Murray Valley encephalitis; Ntaya; Powassan (AF); Rio Bravo (AF); Sepik; Stratford; Tembusu (AF); Uganda S; Zika.

[a] Mention of trade names and sources is for identification only and does not imply the endorsement by the Centers for Disease Control and Prevention or U.S. Dept. Health and Human Services. The list includes, in addition to the original manufacturers, the corporations that only market the products made by the others. Product availability is valid as of April 2003 but subject to rapid change depending on market conditions.

[b] Product abbreviations. Ag: antigen; AgC: antigen capture; Cassette: horizontal flow card test; CF: complement fixation; DPS: dipstick; EIA: enzyme immunoassay (unspecified); ELISA: enzyme-linked immunosorbent assay; ENV: envelope protein; fEnv: envelope protein as fusion protein; HI: hemagglutination inhibition; HYB: hybridoma cell; IFA: immunofluorescence; IgGC: IgG capture; IgGI: IgG indirect; IgGT: IgG total; IgMC: IgM capture; IHC: immunohistochemistry; IMB: immunoblot/immunodot; IMC: immunochromatographic test; MAb: monoclonal antibody; Nt: neutralizing; PAb: polyclonal antibody; rEnv: recombinant envelope protein; rNS1: recombinant NS1 protein; rPrME: recombinant PrM-E protein; Service: testing or custom service.

[c] Product sources. (E: e-mail address; F: fax number; W: website [after http://www.])

ACS: Accurate Chemical & Scientific (Westbury, NY, USA)
E: info@accuratechemical.com F: +1 516-997-4948
W: accuratechemical.com

AMQ: American Qualex Antibodies, Inc. (San Clemente, CA, USA)
E: info@americanqualex.com F: +1 949-492-6790

AMR: American Research Products (Belmon, MA, USA)
E: staff@arp1.com F:+1 617-489-5120 W: arp1.com

AMT: AmeriTek, Inc. (Seattle, WA, USA)
E: info@ameritek.org F:+1 206 528-8107
W: ameritek.org

ANS: Antibody Systems, Inc. (Bedford, TX, USA)
E: asitmf@airmail.net F:+1 817-498-8277 W: antibodysystems.com

ARUP: ARUP Laboratories (Salt Lake City, UT, USA)
F: +1 801-583-2712 W: aruplab.com

ATC: American Type Culture Collection (Manassas, VA, USA)
E: sales@atcc.org F: +1 703-365-2750 W: atcc.org

BGN: Biogenesis Ltd. (Poole, UK)
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W: biogenesis.co.uk

(*continues*)

TABLE I (*continued*)

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BID: Biodesign International (Saco, ME, USA)
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BRC: BioReliance Corp. (Rockville, MD, USA)
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TABLE I (*continued*)

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[d] Restricted to the qualified institutions in the United States only. Also, in the United States, domestic or international shipment of infectious agents is subject to the latest regulations of the Department of Commerce.

qualified institution or organization. By such an arrangement, a set of coded serum specimens representing negative, low, intermediate, and high titers of antibody received from a collaborating laboratory are tested and the results returned to the sending laboratory for performance evaluation. It is important to include a disproportionately larger number of confirmed positive specimens with low antibody titers in this set of coded specimens, because the quality of diagnostic performance is more accurately judged on those specimens than on the specimens with high titers. This is based on the frequent observations that, using a set of specimens with predominantly only two contrasting titers (negative specimens and positive specimens with high antibody concentrations), the difference in the quality of diagnostic test among laboratories becomes much less evident even if a considerable difference exists (Kuno *et al*., 1998).

1. Tests Based on Agglutination of Blood Cells or Particles

a. Hemagglutination-Inhibition Test The procedure adopted for microtitration (Sever, 1962) of the original protocol (Clarke and Casals, 1958) has been widely used for a variety of objectives, ranging from case diagnosis to serosurvey.

The principle of this test is based on the propensity of most arboviruses to aggregate erythrocytes of certain animals. If, however, virus is mixed with a serum specimen containing an antibody against the virus, hemagglutination is abrogated, the highest serum dilution causing the inhibition corresponding to the antibody concentration in the specimen. Because hemagglutination is pH dependent, selection of an optimal pH is critical.

The major advantages of the this test are (i) it does not require expensive equipment or instruments and (ii) it is highly useful to initially screen etiologic agents at the major group level because of its extensive and exclusive cross-reaction to all members of one virus group (antigenic complex, genus, or family) and excellent ability to segregate that group from others.

Although it has been sometimes erroneously believed to be an IgG assay, actually it measures other immunoglobulins, such as IgM and IgA, as well. Kaolin treatment of serum specimens for removal of nonspecific inhibitors still leaves a considerable amount of HI-reactive IgM (Granström *et al*., 1978; Wiemers and Stallman, 1975), although it was once thought to remove it (Mann *et al*., 1967).

For the visualization of agglutination, goose erythrocytes have been used in most laboratories. Other investigators have found trypsinized human type "O" blood cells or goose cells preserved with formalin

treatment useful in laboratories where fresh goose blood cells are difficult to procure (Ahandrik *et al*., 1986). As for antigen, sucrose-acetone extracts of infected suckling mouse brains were popularly used in the past, but some of them have been replaced with antigens prepared from infected cell cultures. More recently, recombinant antigens, such as Japanese encephalitis (JE) viral antigen expressed as extracellular subviral particles, became available. However, although some recombinants had a hemagglutination activity (Heinz *et al*., 1995; Hunt *et al*., 2001; Konishi *et al*., 1996), other recombinant antigens either have not been evaluated for utility in the HI test or were found to be nonreactive (Davis *et al*., 2001; Konishi *et al*., 2001). Availability of a good HI-reactive recombinant antigen for the diagnoses of West Nile fever (WNF) and other viral infections in wildlife is important, because an HI test with such a safe antigen obviates development of antispecies antibodies necessary in the popular ELISA but currently unavailable commercially.

Although HI antibodies in neurotropic flaviviral infections, compared with those of non-neurotropic infections, are sometimes detectable within 3 to 5 days after the onset of illness because of longer intervals between infection and development of symptoms; generally, a disadvantage of this test is that for case diagnosis it is essentially a retrospective diagnostic test because both acute phase and convalescent phase specimens must be obtained to determine a significant change in antibody titer. Many recovered former patients do not feel a strong need to return to clinics for second blood samples; thus, unless convalescent phase specimens are actively sought by physicians or diagnostic laboratories, many cases with only acute phase specimens would remain inconclusive. Furthermore, it is one of the most cross-reactive tests to flavivirus. It should also be remembered that in the microHI test, which is the standard today, titers obtained are often lower compared with those by the macroHI test (Akov, 1976).

b. Hemadsorption Immunosorbent Test In this test, first, a solid phase (multi-well plate) is sensitized with a capture antibody (such as anti-human IgM antibody). Serum specimen and antigen are added in that order, with washing between steps. When goose erythrocytes are added, hemagglutination develops only in the wells with bound antigen. The hemadsorption immunosorbent test (HIT) has been used for IgM assay for dengue (DEN) and Wesselsbron viral infections (Baba *et al*., 1999; Gunasegaran *et al*., 1986). Although, like the HI test, no expensive equipment is necessary for the test, it is not as

sensitive as IgM capture ELISA. Furthermore, a prozone tends to develop in antibody- or antigen-excess regions.

c. Complement Fixation Test The original protocol developed for the serologic study of yellow fever (YF) virus infections in the 1920s was further improved in the early 1930s, laying the foundation for this classic technique (Davis, 1931; Frobisher, 1931). The protocol for microtitration (Casey, 1965) has been most commonly used. Like the HI test, it is not useful as a rapid test during the acute phase of illness due to the requirement of convalescent phase specimens.

The Complement Fixation (CF) test exploits the unique affinity of complement for antigen-antibody complexes. In this test, cellular antigens on the membrane of erythrocytes are complexed with an antibody prepared against the blood cells, and those sensitized cells serve as indicator. In practice, two sets of reagent mixes are prepared. In one set, virus and serum specimen are mixed, to which complement is added later. If the serum had antibody to the virus, complement is fixed to the virus-antibody complex, and little complement remains unbound. In the second set, erythrocytes bearing complement receptors are coated with an anti-erythrocyte antibody (hemolysin). When the two sets of reagents thus prepared are mixed, lysis of erythrocytes does not occur with a positive serum specimen because of little unbound complement. On the other hand, the reaction with negative specimen will result in hemolysis because of a large amount of unbound complement. The relatively short half-lives of CF antibodies are useful markers of recent infection. However, the many disadvantages of CF outnumber the advantages. In addition to the slow rise in titer after infection, CF antibody is not induced in some individuals in any sizable population (Buescher *et al*., 1959; Doherty *et al*., 1976). In an SLE outbreak, between 20% and 22% of the patients with confirmed cases did not demonstrate CF antibody 3–8 weeks after onset (Calisher and Poland, 1980). Similarly, the lack of CF antibody response among YF (17D) vaccinees has been well recognized (Monath *et al*., 1980). Furthermore, contrary to the general belief, reports of persistence of CF antibody for longer than 5 years have not been rare for some flaviviral infections (Buescher *et al*., 1959; Fujita *et al*., 1979; Halstead, 1974). Also, some serum specimens are anticomplementary, and hemolyzed blood specimens cannot be used. Most importantly, the complexity of the procedure, which requires titrations of at least three reagents (antigen, complement, and hemolysin) for optimization, is technically demanding and requires time-consuming training of diagnosticians.

d. Immune Adhesion Hemagglutination Test Immune adhesion is an adherence of erythrocytes to tripartite immune complexes of virus antigen-antivirus antibody-complement (C1 or $C3_b$) via C3b receptors on erythrocytes. Addition of complement ($C1_{qrs}$) into antigen-antibody immune complex (IC) initiates transformation of C1 to $C3_b$. The conversion of $C3_b$ in the complement pathway is interrupted by the addition of dithiothreitol. Introduction of type "O" erythrocytes bearing $C3_b$ receptors completes the agglutination of tripartites.

The advantages of IAHA over the CF test are that it is more sensitive and consumes less complement. However, limiting the source to type "O" blood cells poses a supply problem, depending on location. Furthermore, serum specimens taken early (<2 weeks after onset of illness) may not be sufficiently reactive because of their lower sensitivity (Inouye *et al*., 1980).

e. Reverse Passive Hemagglutination Test When the reverse passive hemagglutination (RPH) test is used for detecting antibody, antiviral antibody is chemically bound to erythrocytes. Separately, the serum specimen and virus antigen are mixed, to which is added the antibody-sensitized erythrocyte suspension. The specimen that does not generate hemagglutination is interpreted as positive. The test has been used for the diagnosis of WNV fever (Estival *et al*., 2001).

f. Single Radial Hemolysis Test In the single radial hemolysis (SRH) test, virus antigen is bound to erythrocytes. The virus-coated erythrocytes and complement are mixed in melted agar and mixed agar solidified in a mold that produces wells in agar. When a serum specimen containing antiviral antibody is introduced to a well in the gel, the antibody radially diffuses into gel. As antibodies diffuse, they meet and form immune complexes with the antigen bound on erythrocytes. Complement immediately adjacent to the complex interacts with the complexes, lysing the cell membrane, which produces a zone of hemolysis. The test has been used for the diagnoses of DEN, JE, and WNF (Chan, 1985; Duca *et al*., 1979; George and Pavri, 1986; Guzmán *et al*., 1985).

g. Indirect Hemagglutination Test This test is a simple modification of hemagglutination test. Antigen-sensitized sheep blood cells are reacted with a serum specimen. After proper mixing and incubation, if hemagglutination is observed, the specimen is scored positive. Although the test is very simple, it suffers from variation in the quality of the sheep erythrocytes used (Gupta *et al*., 1990).

h. Other Tests Using Synthetic, Natural, or Bacterial Particles Many other techniques based on agglutination use either synthetic or natural particles (Latex, silica, gelatin). The principles of those techniques are the modifications of the aforementioned blood cell agglutination tests and are designed either as direct or indirect (passive) tests, depending on the kinds (antigen, antibody) of ligands bound to particles. The key to successful application lies in optimal preparation of sensitized particles with minimum distortion of ligands while maintaining good reactivity. Particle agglutination tests have been developed for the diagnoses of many human viral diseases but have not been popular for experimental studies of arboviral diseases, except for a small number of studies (Jia *et al*., 2002; Likar *et al*., 1971; Yamamoto *et al*., 2000, 2002).

Bacterial agglutination test takes an advantage of certain strains of bacterial cells bearing immunoglobulin (i.e., IgG) receptors. Because not all antibodies captured are virus-specific, the quality and utility of the test are largely determined by the reagents used and the subsequent steps in the test format. It has been rarely used except for an agglutination inhibition test for DEN (Chan *et al*., 1975).

2. Neutralization Tests

The neutralization test (NT) measures all neutralizing immunoglobulins, including IgG and IgM (Ishii *et al*., 1968). The excellent specificity for virus identification is well recognized. However, when used for serodiagnosis *in vitro*, its superior specificity primarily applies to the diagnosis of primary but not secondary infections. The three kinds of NT (constant virus–constant serum dilution, variable virus dilution–constant serum dilution, constant virus dilution–variable serum dilution) are generally performed *in vitro* using cell culture. The first test is useful when a large number of specimens must be processed economically, as in a serosurvey or when the neutralizing antibody (Nt) titer can be reasonably extrapolated based on plaque count at a fixed serum dilution (Sangkawibha *et al*., 1984). The second test, assayed in laboratory animals, and all passive immunity tests using surrogate animals are useful for a small number of specimens, but are expensive, laborious, and impractical for processing of a large number of specimens.

The most popular method today is the third test, known as plaque-reduction serum dilution neutralization test (or PRNT) (Russell *et al*., 1967a). To economize, laboratories most often perform microPRNT using multi-well plates (DeFraites *et al*., 1999; Fujita *et al*., 1975). Despite its importance in evaluation of vaccine efficacy and as a more

definitive, confirmatory test in serodiagnostics, neither the procedure nor diagnostic criteria have been standardized, given the numerous variations among laboratories, for example, in qualities of reagents, virus, cell culture, and protocols. Some of those problems and concerns are discussed in following sections.

Accurate determination of the proportion of a human population with a protective antibody is critical for planning a vaccination program. As described subsequently in Section V, development of flavivirus cross-reactive, heterologous antibodies demonstrating *in vitro* neutralization presents a serious problem. Currently, *in vitro* assays for Nt antibody often cannot adequately determine if heterologous, *in vitro* Nt antibodies are protective *in vivo* against the respective heterologous viruses based on the specimens demonstrating a pattern of secondary infection, when the history of flavivirus exposure of the subjects is poorly known.

a. Plaque Reduction Neutralization Test In this test, virus is pretitrated by plaque assay, and the heat-inactivated serum specimen is serially diluted. A known amount of infectious virus is mixed with an equal volume of each serum dilution and incubated. If the serum had a Nt antibody to the virus, reduction in the amount of infectious virus in the mixture occurs. The mixture is then inoculated into a susceptible cell monolayer and incubated for virus adsorption. After an incubation period, the monolayer is overlaid with a solid or semi-solid overlay medium. After an optimal incubation period, plaques are visualized with a dye. Using the mean plaque count of the virus dose mixed with an equal volume of a normal serum and applying a selected criterion for significant plaque reduction, the highest serum dilution demonstrating a significant plaque reduction is determined as the Nt titer. Alternatively, it is determined by probit analysis.

Basically, three kinds of overlay medium have been used: solid single overlay (Barnes and Rosen, 1974), solid double overlay (Russell *et al*., 1967a; Yuill *et al*., 1968), and semi-liquid overlay (De Madrid *et al*., 1969). Although a particular overlay medium has been selected in most laboratories on the basis of personal preference or expertise available, generally, for the flaviviruses that grow more slowly (such as some strains of DEN-3 virus) a double overlay method is superior because an overlaid monolayer can be kept alive for several days or longer under the optimal condition favorable for plaque development, before a second solid medium containing a dye is applied for visualization of plaques.

As for virus, it is important to use a well-preserved stock because noninfectious virions react with Nt antibodies in the specimen, distorting the results (Schlesinger *et al*., 1956). Usually, prototype viruses have been used. However, substantial differences in antigenicity exist among geographic strains of some flaviviruses, such as Southeast Asian vs pre-1990 Caribbean strains of DEN-3 virus (Russell and McCown, 1972), the Taiwan strains vs the Nakayama strain of JE virus (Susilowati *et al*., 1981), and South African vs Indian strains of WN virus (Blackburn *et al*., 1987). For specimens from those locations, use of local strains would provide more relevant data (Ku *et al*., 1994).

The accuracy of plaque reduction first depends on the accuracy of plaque counts, in particular, the denominator, which is the virus dose that survives after being mixed with an equal volume of normal reference serum. The optimal range of the amount of virus for the plaque reduction neutralization test (PRNT) (plaque-forming units, or PFUs) depends on the mean diameter of the plaque of the virus strain used, the cell culture surface space available for plaque development per vessel, the susceptibility of the cell culture to plaque development, and the efficacy of the plaquing procedure. As demonstrated early, when vessels of a larger surface area are used, unless the plaque size is unusually large, a higher dose of virus can be inoculated without compromising the accuracy of the plaque count (Russell *et al*., 1967a). However, in many laboratories, microPRNT with multi-well plates is used to process a large number of specimens economically. A proportional relationship between plaque count and serial dilution of virus exists only in a narrow range of virus dilution when a small surface area is used (Sukhavachana *et al*., 1969). This is an important consideration for an accurate back titration. Thus, unless plaque size is very small, virus quantities much less than 50 PFUs have been found optimal for 24-well plates (Graham *et al*., 1999), rather than nearly 100 PFUs (Lang *et al*., 1999). Generally, whenever possible, large wells (i.e., 9.6 cm^2 of 6-well plates) or at least wells of intermediate size (4.5 cm^2 of 12-well plates) are much preferred. Despite its popularity, PRNT may not be the best NT for all flaviviruses, as reported for tick-borne encephalitis (TBE) virus (Vene *et al*., 1998).

b. Focus, Cytopathic Effect, or Other Infectious Titer Reduction Tests As variants of PRNT, a few NTs were developed on the basis of reduction of infectious foci or cytopathic effects (CPEs) rather than of plaques. The principles of these tests are basically identical to those of PRNT, the only difference being the method of demonstrating

evidence of neutralization. Thus, in contrast to visual counting of plaques, infected foci or CPEs in cell culture that develop after inoculation of virus-serum mixture are counted microscopically (RFFIT and PAP) or macroscopically (CPE test). The same definition of Nt titer as that for PRNT is used in the former tests, while in the latter test, the highest serum dilution that inhibited CPE development is the Nt titer.

In the rapid fluorescent focus inhibition test (RFFIT) (Thacker *et al*., 1978; Vene *et al*., 1998) and peroxidase-anti-peroxidase (PAP) test (Ishimine *et al*., 1987; Jirakanjanakit *et al*., 1997; Okuno *et al*., 1985), foci are counted with a fluorescence microscope or with a regular compound microscope. When results are compared with those obtained with PRNT, comparable results have been obtained by these focus reduction tests. Moreover, the RFFIT for TBE was reported to be faster and more reproducible than PRNT (Vene *et al*., 1998). Two major disadvantages common to focus reduction methods are tedious, time-consuming counting of foci with a microscope and error in scoring by less experienced operators. The CPE reduction test suffers not only from subjectivity of scoring due to variation in the definition or visual perception of CPE among operators but also from variation in susceptibility of the cell culture used.

In another modification of the NT protocol, after a known amount of infectious virus was mixed with a serum specimen, the mixture was inoculated into a suspension of susceptible cells in the wells of multi-well tissue culture plates. Infectious virions that survived neutralization and replicated were titrated by ELISA to deduce Nt antibody titer in serum (Holzmann *et al*., 1996; Vorndam and Beltran, 2002). Although they may be useful for primary infections, the frequent problems in all similar tests, including quantification of replicated virus with reverse-transcriptase polymerase chain reaction (Ting *et al*., 2001), were the difficulty of regulating viral growth and establishing a reliable correlation between the amount of infectious virus and Nt antibody titer due to a rapid change of the slope of the relationship over a short period and of obtaining reproducible differences in titer in paired specimens, particularly in secondary infections.

c. Metabolic Inhibition Neutralization Test In this test, like PRNT, a known amount of infectious virus is added to each set of serial dilutions of serum. The diluent for both virus and serum dilutions is a metabolic medium containing a higher concentration of glucose. After incubation, first the serum-virus mixture and then an aliquot of cell suspension are dropped in each well of a microtiter plate. Cell controls

include serial twofold dilutions of cell suspension. Wells are then sealed and incubated at 37 °C for a desired period. Wells are scored Nt antibody–positive (lower pH) or Nt antibody–negative (higher pH) with respect to neutralization, using a pre-determined cut-off pH value in cell culture, such as 7.4. Adding the correct amount of cells and incubating for an optimal length of period are critical because adding too many cells accelerates metabolism and reduces the pH quickly, while the opposite occurs if too few cells are added. This test has been used for detecting antibodies to a few tick-borne viruses (louping ill and tick-borne encephalitis viruses) (Kääriäinen, 1965) and for serosurveys of SLE virus infection in wildlife in North America.

3. Enzyme Immunoassay

The application of enzyme immunoassays (EIAs), in particular, the ELISA that began in late 1960s, dramatically changed serologic practices by the late 1980s and spawned numerous procedural modifications and commercial diagnostic kits. Provided that basic equipment are available in all laboratories, the selection of a format depends on (i) the targeted molecules (antibody or antigen) for assay, (ii) availability of necessary reagents, (iii) the specificity and sensitivity desired, (iv) speed of test, and (v) expertise available or personal preference.

a. Types of Protocols Among many available protocols and modifications, the antibody capture format has been used for most flavivirus diagnoses. Before reaction with the specimen, a solid phase is sensitized either with virus antigen, anti-virus antibody, or anti-human IgG or IgM antibody. When the solid phase is sensitized with density gradient-purified DEN antigen and serial serum dilutions are used, the test is fast and the color pattern that develops by IgG-ELISA is very much similar to the HI pattern obtained (Fig. 1A) (Feinstein *et al*., 1985; Kuno *et al*., 1991). On the other hand, in other IgG capture ELISA protocols, the solid phase was sensitized with a polyclonal antiserum (hyperimmune mouse ascitic fluid or HIMAF) (Fig. 1B), which yielded good sensitivities (Chungue *et al*., 1989; Miagostovich *et al*., 1999). For IgG capture, the solid phase may be sensitized with anti-human IgG antibody (Burke *et al*., 1985b; Innis *et al*., 1989), but the sensitivity has been found to be generally inferior because a small amount of virus-specific IgG has to compete for binding sites with much higher concentrations of nonspecific IgGs. IgG-ELISA could be made more broadly reactive to a spectrum of flaviviruses, like the HI test, by sensitizing the solid phase with a *Flavivirus* group-reactive monoclonal antibody (MAb) (Johnson *et al*., 2000).

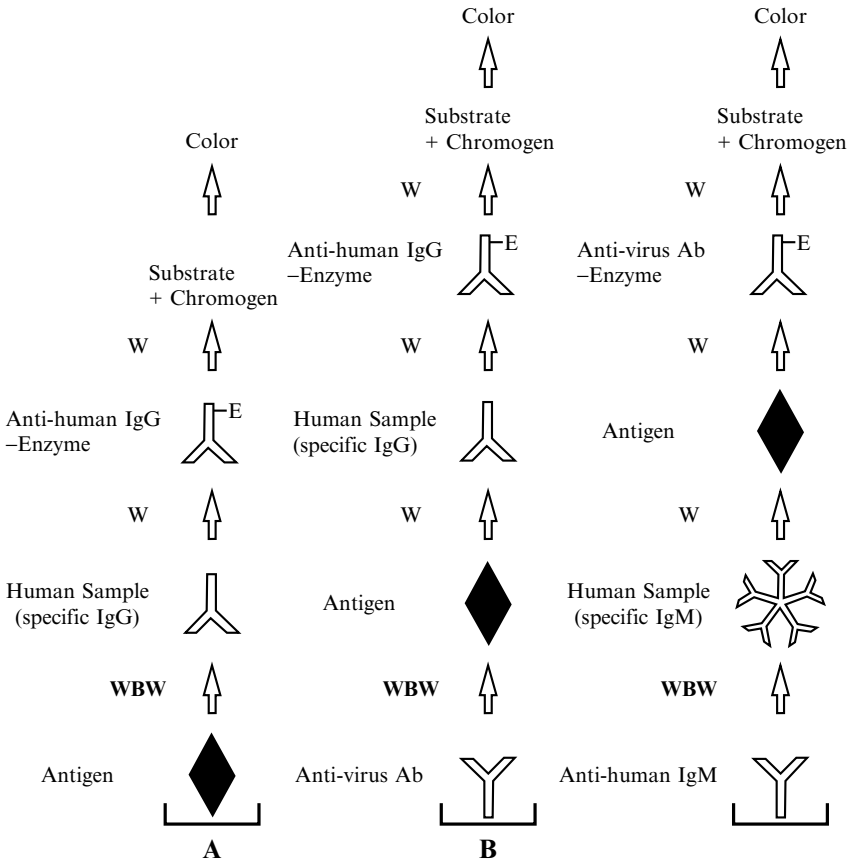

FIG 1. Examples showing variation of indirect ELISA format for antibody assay using horseradish peroxidase. (A) Antibody capture by a specific antigen. (B) Antibody capture by a specific antibody. (C) IgM capture by an antibody against human IgM. E, enzyme; WBW, washing-blocking-washing.

For investigating the immune responses among IgG subclasses, viral antigen-sensitized plates are reacted first with serum sample and then with mouse anti-human IgG1, 2, 3, or 4 antibody before the final reaction with an enzyme-conjugated antibody against mouse IgG (Thein *et al*., 1993).

For IgM assay, although an antigen-sensitized solid phase could be used (Dittmar *et al*., 1979), solid phase sensitized with anti-human IgM (Fig. 1C) (Burke and Nisalak, 1982; Gadkari *et al*., 1984; Heinz

et al., 1981; Kuno *et al*., 1987; Roggendorf *et al*., 1981) has proven to be quite useful for nearly all medically important flaviviruses assayed thus far. As one other modification of ELISA, antigen and enzyme-conjugated detector antibody were incubated simultaneously to speed up the test apparently without compromising sensitivity (Chong *et al*., 1994). Use of biotin-labeled anti-*Flavivirus* IgG, followed by streptavidin-peroxidase conjugate, was reported to have increased sensitivity of IgM-ELISA in acute phase specimens (Kittigul *et al*., 1998).

i. Antigen or Immune Complex Capture ELISA Antigens in early acute phase specimens may be detected by antigen capture (AgC) ELISA. Most often specificity is enhanced by using a specific MAb as capture or detector antibody (Heinz *et al*., 1986; Kuno *et al*., 1985; Monath and Nystrom, 1984). ELISA procedures in which an anti-DEN NS1 polyclonal antibody was used as capture antibody revealed a higher level of NS1 in the acute phase of DEN infections (Alcon *et al*., 2002; Young *et al*., 2000).

A biotin-streptavidin amplification step was applied to improve sensitivity and specificity in another protocol (Malergue and Chungue, 1995).

DEN immune complex (IC) was investigated intensely with regard to the pathogenesis of DHF/DSS. The validity of early investigations was not entirely certain because the techniques used were not antigen-specific (Agnello, 1980). By a simple modification of IgM capture ELISA, the IgM IC of DEN virus or JE IgG- or IgM-IC also could be detected (Desai *et al*., 1994; Kuno *et al*., 1987).

ii. Blocking ELISA Because IgG ELISA is generally cross-reactive, to make the assay more virus-specific or strain-specific, modified, competitive protocols have been developed. In blocking ELISA, competition is allowed to proceed in sequence, first with test specimens, followed by the introduction of a competitive antibody without washing the plates. If optical density (OD) is significantly reduced as a result of blocking or inhibition, the presence of specific antibody in the samples is assumed. Thus, in such blocking tests, JE could be distinguished from DEN (Burke *et al*., 1987) and Murray Valley encephalitis (MVE) from other Australian *Flavivirus* (Alfuy and Kunjin) infections (Hall *et al*., 1995; Hawkes *et al*., 1990). Inhibition ELISA is a simple modification of blocking ELISA developed for dengue diagnosis (Balmaseda *et al*., 2003; Vázquez-Ramudo and Fernández-Lianes, 1989). In this test, the solid phase, which is coated with anti-DEN antibody, is first reacted with DEN antigen and then with a human serum dilution specimen. If the specimen had anti-DEN antibody, it will

coat the DEN antigen captured in the previous step, thus blocking ("inhibiting") it from binding an enzyme-conjugated anti-DEN human antibody to be introduced in a subsequent step. In contrast to blocking ELISA, however, the plates are washed between the serum specimen and enzyme conjugate steps. The highest serum dilution demonstrating $\geq$50% inhibition of absorbance (compared with that of a negative control serum) is used to determine antibody titer. *Flavivirus* cross-reactivity is a problem with this test.

b. Reagents and Procedural Modifications

i. Antigens and Enzyme-Conjugated Antibodies Sucrose-acetone extract of infected suckling mouse brain used to be the most common source of antigens for ELISA (Roggendorf *et al*., 1981). Later, virus grown in cell culture became an important source of viral antigen (Besselaar *et al*., 1989; Cardosa *et al*., 1992). Also, for simplifying the assay of antibodies to DEN complex viruses, tetravalent antigen has been used. It has been recognized, however, that some specimens have positive results with monovalent antigen rather than with tetravalent antigen, and vice versa (Igarashi and Antonio, 1997). Recombinant antigens are now considered not only as a viable but necessary alternative to the mouse brain or cell culture antigens. Recombinant antigens, consisting of premembrane and envelope (E) proteins or E protein alone, have been found to be useful for diagnosis of DEN (Cuzzubbo *et al*., 2001; Konishi and Fujii, 2002; Makino *et al*., 1991), JE (Hunt *et al*., 2001; Konishi *et al*., 1996, 2001), TBE (Heinz *et al*., 1995; Marx *et al*., 2001; Yoshii *et al*., 2003), and WN viruses (Davis *et al*., 2001). Viral proteins expressed as fusion proteins of *Escherichia coli* for DEN viruses (Fonseca *et al*., 1991; Makino *et al*., 1991) are also useful, but their applications require special care because of the need to subtract the high background caused by the reactions of anti–*E. coli* protein antibodies present in nearly all human serum specimens and because of the lack of reaction in early specimens (Simmons *et al*., 1998). Also, for vaccinia constructs, such as that for JE virus, minor contamination of vaccinia virus antigen in the recombinant antigen preparation affects the results of serum specimens from smallpox vaccinees (Konishi *et al*., 1996).

Infected cells fixed on a solid phase are also useful in ELISA. Cell-associated antigen has been used in ELISA of DEN, WN, and YF viruses (Ansari *et al*., 1993; Figueiredo and Shope, 1987; Soliman *et al*., 1997). Although most antigens used in ELISA consist of envelope protein, DEN and JE virus NS1 antigens either affinity-purified from infected cell culture or expressed in eukaryotic cells by recombinant

plasmid were also found to be useful (Huang *et al*., 2001; Konishi and Suzuki, 2002; Shu *et al*., 2000).

Regarding the quality of antibodies, MAbs are more advantageous for reducing specificity variation than polyclonal antibodies. For enzyme-conjugated antibodies, broadly flavivirus cross-reactive MAbs, such as 4G2 (Gentry *et al*., 1982) and 6B6C-1 (Roehrig, 1982), have been most popularly used as detector antibodies.

ii. Removal of IgG from Specimens To improve the specificity of IgM ELISA, removing IgG from specimens has often been recommended, particularly when samples contain too much specific IgG. In one study, the use of an anti-human IgG antibody was reported to have yielded improved results (Reinhardt *et al*., 1998). However, in another study in which the efficacies of three IgG absorbents (RF adsorbent, protein G adsorbent, and *Streptococcus pyrogenes*) were evaluated, no improvement was observed for DEN (Kheong *et al*., 1993). Similar negative results were also obtained using several commercial IgG adsorbents elsewhere (Kuno, unpublished).

iii. Reducing Background Sensitized plates are routinely blocked with a blocking material, such as non-fat dry milk, animal serum protein, or Tween 20, before the reaction with the specimen is begun. Diluting antigen and/or enzyme-conjugated detector antibody in acetone-extracted normal human serum (NHS) has been shown to greatly reduce background (Innis *et al*., 1989).

4. Immunoblot and Immunochromatographic Tests

Instead of a multi-well plate used in ELISA, a membrane strip is used in a variation of the EIA as a dipstick or in an immunochromatographic assay. Strips may be held upright for vertical diffusion of reagents or horizontally as in cassette kits. A plain membrane strip may be used, but membrane impregnated with reactant(s), such as capture antibody, and pre-blocked is more useful for a rapid test. In some diagnostic kits for DEN, a recombinant envelope protein is used (Ludolfs *et al*., 2002; Cuzzubbo *et al*., 2001). Both IgM and IgG capture protocols have been developed (Cardosa and Tio, 1991; Cardosa *et al*., 1995; Devine *et al*., 1997; Wu *et al*., 1997).

5. Immunofluorescent Antibody Test and Western Blot

Indirect immunofluorescent antibody tests (IFA) have been used for serodiagnosis of DEN (Boonpucknavig *et al*., 1975), WNF (Besselaar *et al*., 1989), and YF infections (Monath *et al*., 1981; Niedrig *et al*., 1999). Cross-reaction among flaviviruses was observed in a JE study (Yamagishi *et al*., 1977). Two other problems with the method are that

it involves a tedious and time-consuming examination of fluorescence and that reading fluorescence accurately depends on the competence of the operator. Thus, a survey of the literature clearly shows that IFA has yielded more reports of false results than other major serologic methods. Also, an IgM assay with IFA is generally less specific than an IgM ELISA. Additionally, the residual infectivity of virus, such as SLE virus, in acetone-fixed slides is a biosafety concern (Yabrov *et al*., 1978).

Western blot is useful for analyzing antibody responses to all viral proteins. One of the major interests of the use of Western blot for dengue has been to determine if it can distinguish dengue fever (DF) from dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) (Churdboonchart *et al*., 1990; Kuno *et al*., 1990; Shu *et al*., 2000; Valdes *et al*., 2000). Also, it was reported that detection of antibodies to nonstructural proteins (NS) of DEN virus depended on the type of cell culture used for preparation of viral antigens (Se-Thoe *et al*., 1999). Detection of the antibody to prM protein was reported to be useful for distinguishing DEN from JE and WNF (Cardosa *et al*., 2002). When this technique was used for CSF specimens of JE, the antibody profile was found to be different from that of serum specimens (Patarapotikul *et al*., 1993).

III. Specimens

A. Specimen Collection

1. Source of Specimen and Timing of Collection

Blood specimens constitute most diagnostic samples. Virus-specific IgM is detectable even in blood collected shortly after birth from infants congenitally infected with DEN virus (Boussemart *et al*., 2001; Poli *et al*., 1991). Cerebrospinal fluid (CSF) specimens from patients demonstrating central nervous system (CNS) syndrome are also sources of IgG, IgM, and IgA (Ehrenkranz *et al*., 1974; Günther *et al*., 1997; Han *et al*., 1988). In JE, SLE, TBE, and WN, specific IgM is often detectable on admission when viral RNA in CSF and in blood may be no longer detectable (Ehrenkranz *et al*., 1974; Fine *et al*., 2000; Günther *et al*., 1997; Morita and Igarashi, 1992).

As for IgM in blood, specimens collected too early (within a few days after onset) in nonneurotropic flaviviral infections, such as DEN, often do not demonstrate measurable titers, because IgM becomes detectable usually 3–10 days after onset, depending on virus and host.

In those cases, which tend to give false-negative results, most ideally, additional acute phase specimens need to be obtained. Blood specimens from patients who received blood transfusion within a few months before sampling should be examined carefully, since anti-flavivirus antibodies, such as anti-WN virus antibody, are sometimes detected in blood used for transfusion (Charrel *et al*., 2001) and because the half-life of injected anti-flavivirus IgG was at least 26 days in adults (Adner *et al*., 2001).

In addition, saliva in the acute phase has been found to be a source of specific IgM, IgG, and IgA in DEN (Artimos de Oliveira *et al*., 1999; Balmaseda *et al*., 2003; Cuzzubbo *et al*., 1998).

2. Filter Paper

Most blood specimens are intravenously collected in tubes, but filter paper strips or discs also have been found useful to save money, to obtain blood from infants via finger or foot pricking, and to facilitate the shipment of samples through the postal service in a surveillance program over a large territory (Bond *et al*., 1969; Burke *et al*., 1985a; Sangkawibha *et al*., 1984; Top *et al*., 1975; Vázquez *et al*., 1998).

B. Physicochemical Factors Adversely Affecting the Qualities of Specimens and Interfering Molecules

Heat inactivation at 56 °C for 30 minutes is a standard procedure in diagnostic laboratories to perform Nt or to prevent inadvertent laboratory infection with bloodborne agents. Although adverse effects on arboviral serology have not been widely recognized, elsewhere heat treatment has been identified as the cause of distorted results in serologic tests of other viruses, including HIV and arenavirus (CDC, 1989; Tomori *et al*., 1987), and in the loss of Nt enhancing factors (Chappell *et al*., 1971; Lehmann-Grube, 1978; Porterfield, 1980). Accordingly, in PRNT of flaviviral infections sometimes the reaction is supplemented with complement or normal serum to compensate for the loss of "accessory factor" caused by heat inactivation and improve sensitivity (but not specificity) (Halstead, 1974; Lang *et al*., 1999; Study Group, 1961; Vaughn *et al*., 1996; Westaway, 1965; Wisseman *et al*., 1962). Conversely, storage at −20 °C for long periods or repeated cycles of freezing and thawing are known to reduce CF and Nt titers of many specimens (Goldblum *et al*., 1957; Porterfield, 1980). The IgM titer in dried specimens on filter paper declines rapidly at any temperature, when kept for a long period (Cohen *et al*., 1969), whereas the IgG decay in dried specimens is much slower when stored at 4 °C for 4–6 months (Chungue *et al*.,

1989; Cohen *et al*., 1969). Accordingly, it was recommended that IgM test of filter paper specimens be performed within a relatively short period, such as 1 month after sample collection (Ruangturakit *et al*., 1994; Vázquez *et al*., 1998). However, the advice not to use diluted IgM-positive serum specimens kept at 4 °C for more than 10 days (Martin *et al*., 2000) was not supported by others (Wong and Seligman, 2001). In the HI test, natural hemagglutinins are removed with erythrocytes and non-specific inhibitors with acetone or kaolin. Rheumatoid factor in serum was found to interfere with IgM ELISA in TBE and DEN diagnoses (Jelinek *et al*., 2000; Roggendorf *et al*., 1981).

IV. Variations in Antibody Responses and Antibody Kinetics

A. Introduction

During the early studies of HI and CF antibody responses to natural cases of YF, it became apparent that most immune responses could be classified into two patterns, primary and secondary infections (Theiler and Casals, 1958). This and other observations laid the foundation for serologic characterization of flaviviral infections. When analyzed for each immunoglobulin class, as described in the previous section, antibody response, in terms of temporal and quantitative dynamics, is different between different kinds of immunogen (i.e., wild versus vaccine strain), primary and secondary infections, or between sources of specimen, such as serum and CSF. Full understanding of human antibody responses to flaviviral infections is essential for a better serodiagnosis.

B. Primary Infections

1. IgG

In blood, the IgG titer begins to rise shortly after the IgM titer in the acute phase, but peak titers are generally lower than those in secondary infections. In many YF patients, the antibody begins to appear towards the end of the first week in the acute phase of illness, and the titer gradually rises thereafter (Lhuillier and Sarthou, 1983). The long-term persistence of Nt (and even CF) antibodies has been well recognized (Fujita and Yoshida, 1979; Halstead, 1974; Niedrig *et al*., 1999; Poland *et al*., 1981; Sawyer, 1931).

According to the classification of DEN, when paired specimens are collected more than 7 days apart and showing $\geq$ four-fold rise in HI titers, with the highest titer $\leq$1280, they are considered to be the cases