Guidelines for Plaque-Reduction Neutralization Testing of Human Antibodies to Dengue Viruses

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ABSTRACT

Through the Advisory Committee on Dengue and other Flavivirus Vaccines, the World Health Organization (WHO) has had a long-standing commitment to facilitate and to guide research and development of vaccines for medically important flaviviruses. Recently, the Paediatric Dengue Vaccine Initiative (PDVI) was formed to accelerate the development, testing, and introduction of dengue (DEN) vaccines worldwide, partnering with WHO in this important public health effort. There are now a variety of DEN vaccines in various stages of the developmental pipeline. In an attempt to make interlaboratory information more directly comparable, WHO with the support of PDVI initiated a program to coordinate the procedures used for the plaque-reduction neutralization test (PRNT). The PRNT is the most common assay used to measure neutralizing antibody. The presence of antibody is believed to be most relevant means of determining protective anti-DEN virus (DENV) immunity. While other neutralizing antibody assays are being considered for use in large-scale vaccine field trials, the PRNT is still considered to be the laboratory standard against which other neutralizing antibody assays should be compared. The need for PRNT coordination has been identified at several consultations between the WHO and PDVI. A more complete version of these guidelines is available on the WHO website: http://www.who.int/immunization/documents/date/en/index.html.

INTRODUCTION

THE DENGUE VIRUSES (DENVs) ARE MEMBERS OF THE FAMILY FLAVIVIRIDAE, genus *Flavivirus*, are mosquito-borne, and represent a major public health problem throughout the tropical world. The DENVs are a set of four different serotypes of viruses (DENV 1–4). Each of these DENVs is similar to one another, but serologically distinct enough that infection with one serotype will not protect against infection with another serotype. For this and other reasons to be discussed later, an effective DENV vaccine should induce an immune response against the four serotypes simultaneously. Although now somewhat dated, the review book on DEN compiled by Gubler and Kuno still serves as an excellent source for information on DEN (16).

Additionally, there are no laboratory animal models that reliably mimic clinical human dengue disease. The lack of a suitable animal model makes it difficult to assess protective capacities of vaccine candidates and correlates of protection *in vivo*. In the absence of correlates, the protective capacity of any vaccine candidate will be

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finally defined as its ability to protect humans from DENV infection. Laboratory studies with DENVs and other flaviviruses have indicated, however, that protection of small animals from virus infection is best correlated to levels of virus-neutralizing antibodies (1,24,25,27). Similar studies with DENV in sub-human primate models have confirmed these observations (2,17,41,53). Sub-human primate studies have been used to identify and down-select candidate dengue vaccines with levels of dengue-neutralizing antibody (as measured by the PRNT), serving as the correlate of vaccine immunogenicity and reduced viremia following challenge with virulent wild-type virus used as a measure of vaccine efficacy. While the correlation of the presence of virus-neutralizing antibody to protection from infection is not absolute, the studies suggest that the PRNT is the best and most widely accepted approach to measuring virus-neutralizing and protective antibodies. Newer assays measuring virus-neutralizing antibodies are being developed and will be briefly discussed later.

All flaviviruses are simple positive-sense, single-stranded, RNA viruses, approximately 55 nm in diameter. The genome is approximately 11,000 nucleotides long, with 5′ capped and 3′-end usually not polyadenylated. The genome encodes 10 proteins in a single open reading frame (Fig. 1). There are three structural proteins encoded in the 5′-one quarter of the viral genome: the capsid (C) protein forms the nucleocapsid shell protecting the viral genome, and the premembrane (prM), and envelope (E) proteins, both virion surface proteins embedded in the virion envelope. Seven non-structural (NS) proteins are encoded in the 3′-two thirds of the viral genome: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. Each of the NS proteins has specific viral functions, but for the purpose of these guidelines, will not be discussed in further detail.

FLAVIVIRUS IMMUNOCHEMISTRY

Antibody-mediated virus neutralization is defined as the interaction of virus and antibody resulting in inacti-
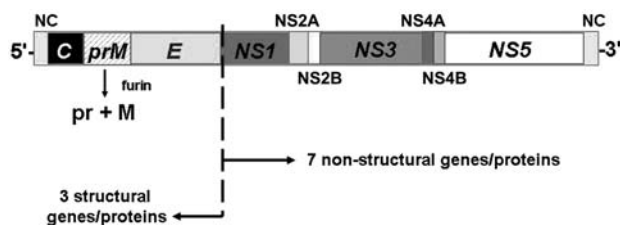

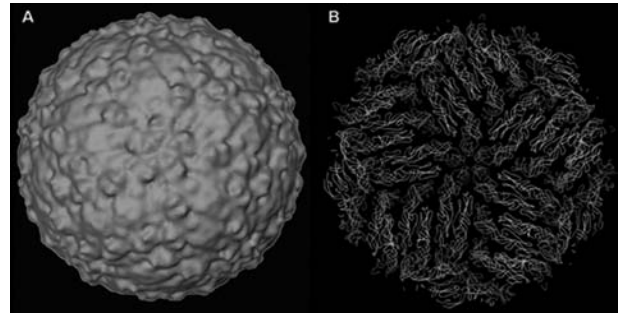**FIG. 1.** Organization of the flaviviral genome.

FIG. 2. Structure of the flavivirion. Cryoelectron microscopic structure of the West Nile virus. (**A**) Surface shaded view of the West Nile virion looking down the fivefold axis. (**B**) C-alpha trace showing the fitted E protein heterodimers looking down the fivefold axis. Courtesy of Buerbel Kaufmann and Richard Kuhn, Purdue University.

vation of virus such that it is no longer able to infect and replicate in cell cultures or animals. Virus-neutralizing antibody is the primary protective antibody type elicited by flaviviral vaccines that result in protection from disease. The flaviviral E glycoprotein is a class II fusion protein and is responsible for viral attachment to host-cell receptors, and virus-mediated cell membrane fusion (49). As such, it is the most important viral protein with regard to viral infectivity, and therefore elicits essentially all virus-specific neutralizing antibody. It is primarily anti-E glycoprotein antibody that is measured in the current PRNT. Other non-E glycoprotein-specific antibodies (e.g., anti-NS1 antibody) can demonstrate virus protective effects in vivo in small animal models; however, these effects are not mediated by virion-antibody interactions (6,8,9,46,57).

Great progress in understanding the structure and function of the flaviviral E glycoprotein has recently been made (33,35,37,54,55). The E glycoprotein exists as 90 "head-to-tail" homodimers on the virion surface (Fig. 2). The E glycoprotein monomer can be divided into three structural domains: DI, DII, and DIII (Fig. 3). DII (also known as the dimerization domain) is a long finger-like structure that contains the hydrophobic membrane-fusion sequence at its tip. In the homodimer, the fusion tip is protected during replication by a combination of DIII of the associated monomer, E protein glycosylation, and the prM protein (40). DIII has been shown with DENV to be involved in virus attachment to Vero cells in culture (11). These binding characteristics have been confirmed using expressed DIII (7). The DI contains the E glycoprotein molecular hinge. As a class II fusion protein, the E glycoprotein can undergo an acid-catalyzed oligomeric reorganization into a fusogenic homotrimer (3,36,48,56). It is believed that this
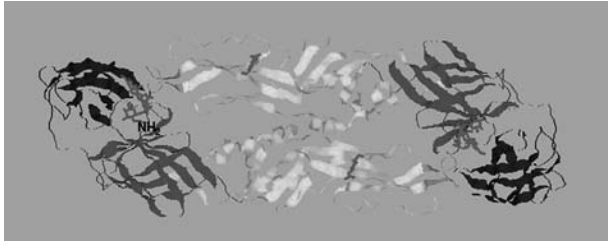
FIG. 3. Structure of the E glycoprotein homodimer.

event occurs in the endosome, allowing the viral nucleocapsid to escape into the cytoplasm and initiate RNA and protein synthesis. Antibodies to both DII and DIII have been shown to neutralize virus. Anti-DIII antibodies tend to be powerful neutralizing antibodies, and are more virus type–specific. Anti-DII antibodies are more virus cross-reactive, and while they can neutralize virus infectivity, they are usually less potent than anti-DIII antibodies (10). Two mechanisms of flaviviral neutralization (blocking attachment of virus to cells, and blocking the virus fusion process) have been identified (11,13). It is not known at this time how many antibody molecules are needed to neutralize the infectivity of a single virion. However, there is evidence that for some monoclonal antibodies it is not necessary for antibodies to bind to all 180 copies of the E protein to mediate neutralization (15,39).

DENV IMMUNOLOGY

The adaptive, protective immune response to DENV infection is driven by the presence or absence of virus-neutralizing antibodies and T-cell responses involved in helping antibody synthesis. Upon infection DENV elicits IgM, IgG, and IgA antibody responses. The IgM response begins early, frequently before onset of symptoms. IgM is usually detectable in serologic assays by 7–8 d after onset of symptoms (44). IgA antibodies are also detectable and have half-lives similar to those of IgM (14). IgG antibodies are detectable soon after infection and are maintained for years (19). Infection with any given DENV serotype results in immunity to that particular serotype; however, there is no long-term protection (>6 mo) against infection with any of the other three DEN serotype viruses (45).

Re-infection of individuals with a distinct second or third serotype of DENV may result in dengue hemorrhagic fever (DHF), or a more severe infection resulting in dengue hemorrhagic fever (DHF) and/or dengue shock syndrome (DSS). There have been a number of hypotheses presented that might explain the more severe

manifestation of disease following secondary DENV infections. Detailed discussions on these mechanisms are beyond the scope of this paper. Regardless of the hypothesized mechanism, pre-existing DENV-reactive antibody appears to be one factor involved in mediating DHF/DSS (5,18,20–23,28–30,32). The antibodies that are most likely involved are those that cross-react with, but do not neutralize, multiple serotypes of DENV. Nonneutralized DENV-antibody complexes can be ushered into DENV-susceptible cells via surface expressed Fc receptors (4,34,42,47). This phenomenon is known as antibody-dependent enhancement (ADE) of DENV replication.

The concept of ADE of DENV infections has been studied for a number of years, and remains a concern for vaccine developers. While the biological relevance of *in vitro* ADE remains ill-defined, our understanding of the antibody specificities that lead to protection or enhancement is improving. It is known that antibodies elicited by DIII of the E glycoprotein are more virus-type specific and neutralizing. Because of their high virus-neutralizing potential, these antibodies are highly protective in animal models of infection. On the other hand, antibodies elicited by either DI or DII are more cross-reactive among viruses, and demonstrate lower or no virus-neutralizing capacity. Recent evidence from West Nile virus (WNV) primary-infected humans, whose lymphocytes were used to prepare human monoclonal antibodies, indicates that the early antibody response may be directed towards DII (51). If the early antibody response to DENV is similar, then the primary humoral response will likely consist of cross-reactive, non-neutralizing antibodies. It is also possible that upon secondary infection with a different DENV serotype, there will be a rapid memory response that consists of DENV cross-reactive antibodies due to epitopes shared between DENV serotypes and the abundance of memory B cells specific for DI/DII cross-reactive epitopes. The theoretical possibility of ADE in postvaccinal DENV infections resulting in DHF/DSS dictates that any vaccine candidate should best elicit only serotype-specific virus-neutralizing antibodies against all four serotypes, and therefore should be tetravalent, and include viruses from all four DENV serotypes. Because of this vaccine developers are creating tetravalent DENV vaccines (2,17,26,31,50).

THE PLAQUE-REDUCTION NEUTRALIZATION TEST

The PRNT measures the biological parameter of *in vitro* virus neutralization and is the most virus-specific serologic test among flaviviruses, and serotype-specific

test among DEN viruses, correlating well with serum levels of protection from viral infection. Newer tests measuring virus neutralization are being developed, but PRNT remains the laboratory standard against which these tests will need to be validated.

The virus PRNT assay was first described in the 1950s, and was later adapted to DENV (43). The basic design of the PRNT allows for virus-antibody interaction to occur in a test tube or microtiter plate, and then antibody effects on viral infectivity are measured by plating the mixture on virus-susceptible cells. The cells are overlaid with a semi-solid medium that restricts spread of progeny virus. Each virus that initiates a productive infection produces a localized area of infection (a plaque), that can be detected in a variety of ways. Plaques are counted and compared with the starting concentration of virus to determine the percentage reduction in total virus infectivity. In the PRNT, the serum specimen being tested is usually subjected to serial dilutions prior to mixing with a standardized amount of virus. The concentration of virus is held constant such that, when added to susceptible cells and overlaid with semi-solid medium, individual plaques can be discerned and counted. In this way, PRNT endpoint titers can be calculated for each serum specimen at any selected percentage reduction of virus activity. A disadvantage of the PRNT is that it is labor intensive and therefore not readily amenable to high throughput, making it difficult to use for large-scale surveillance and vaccine trials.

TEST CONDITIONS FOR THE PLAQUE-REDUCTION NEUTRALIZATION TEST WITH DENGUE VIRUS

Cell lines and growth of viral stocks

DENVs grow in many different cell lines derived from both vertebrate and invertebrate sources. The cell line used for virus amplification needs to be coordinated between laboratories. Because DENV vaccines are targeted for humans, and because the processing of the prM protein is altered in C6/36 mosquito cell–grown DENV, a cell line of mammalian origin, such as the continuous African green monkey–derived Vero cells is recommended by the WHO to produce seed viruses and for use in the PRNT for DENV. Because different passages and clones of Vero cells may be phenotypically different, a WHO-certified cell line is available. To avoid the problems of cell-culture adaptation of virus, low-passage virus stock banks should be developed and employed for viral growth. While the number of virus passages deemed to be acceptable in the PRNT has never been experimentally determined, using viruses with no more than 5–10

cell-culture passages beyond the banking passage should be attainable if the master and working seed stock approach is used.

The conditions for virus amplification/preparation should be standardized with the use of an appropriate multiplicity of infection (around 10^{-2} to 10^{-3}). Virus should be harvested during the middle to end of the exponential phase of growth, to avoid high concentrations of inactivated particles in viral preparations. The supernatant from infected cell cultures should be clarified by low-speed centrifugation and stabilized with a cryo-protective agent (e.g., fetal calf serum >20%) before aliquoting and storage of virus at $-70°C$. Lyophilization is an alternative for long-term storage. Virus working banks should be appropriately qualified for sterility (absence of bacteria, fungi, and mycoplasma), potency (virus titration on the cell line used for PRNT), and if possible identity (using serotype-specific monoclonal antibodies).

Cell line for plaquing viruses

DENVs will plaque in a variety of cell lines. Currently two mammalian cell lines are used most widely, Vero cells or rhesus monkey kidney–derived LLC-MK$_2$ cells. Each of these cell types has advantages; however, a specific derivation of Vero cells has been evaluated and certified by the WHO for production of live-attenuated vaccines and for use in the PRNT. Master and working cell banks should be prepared (as done for vaccine production). Doing so will limit the number of passages and prevent any drift of sensitivity to virus infection. Quality control protocols for the cell banks that monitor sterility (bacteria, mycoplasma, and fungi) and susceptibility of cells to viral infection should be included.

Vero cell monolayers should be prepared 2–3 d before use. Sub-confluent or just-confluent monolayers should be used to avoid any alteration or loss of cells during the course of the assay. Quality of the cell monolayer is critical for plaque development and therefore to generate accurate results.

Viruses used in the test

Currently there are a variety of DENV strains on which vaccine development or diagnostic testing is based. Table 1 lists the representative viruses from each serotype being used in tetravalent formulations by five different vaccine developers or diagnostic laboratories. Because the well-established dengue laboratory strains used for PRNT implementation were isolated many years ago and have been amplified by several passages in mosquito or various other cell lines, there is no clear rationale to choose one set of viruses over another.

Plaquing efficiency is an important parameter in determining PRNT results. Using virus stocks containing

TABLE 1. VIRUSES CURRENTLY USED IN DENV VACCINE DEVELOPMENT OR REFERENCE LABS

Virus serotype	Vaccine developer or reference lab				
	1	2	3	4	5
DEN1	Hawaii	16007	16007/PUO-359	West Pacific or PR94	West Pacific
DEN2	New Guinea C	16681	16681/PUO-218	New Guinea C or Tonga 74	S16803
DEN3	H87	16562	16562/PaH881/88	Sleman 78	CH53489
DEN4	H241	1036	1036/1228	814669 (Dominica/81)	TVP360 or 341750

large amounts of inactive virions could result in falsely low PRNT titers. There are a variety of ways to analyze the contents of a viral preparation. These approaches include, but are not limited to: the quantization of viral genomic copies by RT-PCR; the quantization of the total envelope protein mass (by ELISA or other methods); or direct particle counting in an electron microscope. Each of these techniques has limitations. The best practical approach to developing a virus stock with high ratio of infectivity to particles is to carefully monitor the viral growth conditions and harvest at the appropriate time post-infection.

In the context of assessing vaccine immunogenicity (i.e., serological surrogates of protection), it is advisable to determine PRNT activity against a variety of virus strains such as other wild-type or virulent viruses (low passage virus or viruses isolated from human cases of dengue fever or DHF), or recent viral isolates from different DEN-endemic areas (12, 31). These results should be compared to PRNT results using the prototype laboratory strain(s). This being said, there is little evidence of antigenic drift within a given DENV serotype that would result in a strain resistant to a post-vaccinal response to that serotype. Ongoing comparisons of the DEN E protein structure and cross-reaction analyses between laboratory strains and recent field isolates should yield helpful data to develop future recommendations. Regulatory authorities may also choose to specify other strains that might be used in assays measuring vaccine-induced immunity, in addition to the strains used currently by vaccine developers.

Media

Fetal calf or bovine serum should be used for virus and cell growth, and for virus and sample dilutions should be heat-inactivated at 56°C for 30 min, and used at a low final concentration (2–5%) in the PRNT. The medium used for cell growth should be compatible with the cell type used. For Vero cells, minimal essential medium (MEM), Dulbecco's modified MEM (DMEM), M199, or equivalent media are generally sufficient.

PFU target and vessel size

The challenge virus dose and the number of repeats tested for each dilution (serum or virus) are key factors for achieving accurate measurements. The challenge dose should be modified based on the surface area of the cell monolayer (e.g., 6-well versus 24-well plates), to get readily discernable plaques and minimize plaque overlap. The target number of plaques per well can vary by virus strain, however 40–60 PFU per 35-mm dish should permit accurate titrations while minimizing plaque overlap. Plaque overlap results from crowding of plaques in an individual well. Comparing plaque counts in the test system versus input virus that has been "back-titrated" is the most acceptable way to rule out plaque overlap. In order to reach an acceptable precision for the plaque counts, it is recommended that at least three repeat wells for a challenge dose of 50 PFU/reaction or less be used.

Specimen handling

The technique of heat inactivation at 56°C for 30 min of serum specimens targeted for serological evaluation was introduced to limit the effects that complement or adventitious virus may have on the final results. This practice is routine in most laboratories and should be employed. Filtration of serum specimens to remove particulates is not necessary. Neutralization of DENVs does not require complement, so addition of exogenous sources of complement to the PRNT is not necessary.

In functional assays intended to assess vaccinal immunogenicity, the serum sample dilution series for antibody titration should ideally start below the "seroprotective" threshold titer. Regarding DENV-neutralizing antibodies, the seroprotection threshold remains unknown, but numerous laboratories in the context of vaccine immunogenicity assessment consider a seropositivity threshold to be 1:10. A 1:5 starting dilution would be preferred over 1:10 in this context; however, that should be balanced with the increased sample volume required for the test.

The number of dilutions can vary according to the objective of the testing. For screening purposes, sera can be

processed using a dilution corresponding to the seropositive threshold titer; however, the use of at least three dilutions is recommended due to possible cell toxicity or presence of non-specific inhibitors of virus replication in the first serum dilution. For end-point titration, two- to fourfold serum dilutions should be used. These dilution series lead to a more precise estimate of the end-point titer than higher dilution factors. The number of dilutions used depends on the dilution series and what is sufficient to establish the end-point titer in post-vaccinal samples. The appropriate dilution range can be previously determined by preliminary titration from 10-fold serum dilutions.

Virus-antibody incubation periods

Antigen-antibody reactions are quite rapid, with a period of 1–2 h at 37°C sufficient for the PRNT. Extending this period could result in partial virus inactivation. An overnight incubation at 4°C is also acceptable; however, switching between a variety of incubation periods within a given laboratory should be avoided.

Virus adsorption to cells and incubation of plates

Virus adsorption to Vero cells occurs rapidly at 37°C, with 90% of the infectious virus attaching within the first 30 mins (11). Longer adsorption periods, while not necessarily detrimental to infection, are not needed for maximal viral adsorption. It is important to ensure coverage of the cell monolayer with media during viral adsorption. This is accomplished by tilting the plates at recurring intervals to ensure consistent media coverage of each well. The days of incubation for the plaquing plates will depend on the growth characteristics of the virus strains used. Typically DENV requires 4–7 d for plaques to be visible. Temperature of incubation for the plaquing plates should be compatible with the cells used. For Vero and LLC-MK2 cells, an incubation temperature of 37°C is appropriate, although lower temperatures (e.g., 35°C) are acceptable.

Overlay

In the case of conventional PRNT an overlay is added onto the cell monolayer to limit the virus diffusion within the plate and promotes plaque formation. The overlay can be added to the cell monolayer either after aspiration of the serum/virus mixtures, or without elimination of the mixtures (e.g., yellow fever PRNT). The approach used should be consistent and documented within each laboratory. Semi-solid media such as carboxymethyl cellulose (CMC), methylcellulose (MC), and agarose are acceptable. Lower-grade agar solutions may contain charged inhibitors that may inhibit plaque formation. If

these types of chemicals are used, they should first be tested to determine the presence of such inhibitors. Since the quality of the overlay medium is critical to ensure data reliability, a new batch of commercial reagent should be qualified by comparison with a previous batch for plaque-forming efficacy. Selection of the overlay medium is based on the techniques used for plaque visualization, and whether or not the overlay must be removed for staining. In the latter case, CMC is used at 2–3% and MC is used at 0.8–1.5% final concentration. Agarose solutions are typically used at 1–2% final concentrations, and are primarily used when plaques are visualized with a vital dye such as neutral red. For flaviviruses, a two-overlay approach is preferred. The first overlay is added after the virus adsorption period, and does not contain neutral red. After an appropriate growth period, a second overlay containing 0.5% neutral red is added to visualize plaques.

Plaque visualization

Direct staining of cells. There are a variety of ways to reveal plaques for enumeration in the conventional PRNT. Cell coloration with vital dyes added in the first or second overlay (such as neutral red) permits monitoring of the development of viral plaques as uncolored holes in the cell monolayer. However, this method has some limitations. Neutral red is cytotoxic at high concentrations and light sensitive, therefore the dye concentration in the overlay is necessarily limited, and plates stained with neutral red should be kept in light-tight containers or incubators. This is especially important for the single-overlay technique. Because of this the contrast between the colored cell monolayer and uncolored plaques may be weak, and thus affects the accuracy of plaque enumeration. On the other hand, keeping cells alive with a neutral red overlay makes it possible to introduce flexibility into incubation time that may sharpen plaques or even bring out new plaques. Other dyes (such as amido-black or crystal violet) can be used to improve the cell monolayer staining and to allow possible automation for counting plaques. These stains cannot be added directly to the overlay and require overlay removal for staining. Even though dying the cell monolayers requires an additional coloration step after plaque development and before plaque counting, this approach has some other advantages: (1) the area of the cell monolayer required to reach the minimal recommended plaque count can be reduced because of the increased capacity to visualize smaller plaques; (2) the strong contrast between the cell sheet and the clear plaque permits photographic capture of plate images, and storage of images as raw data records for compliance purposes; (3) chemical fixation of cells inactivates DENV so the staining and counting steps can be implemented under the biological safety level (BSL)

1 containment (instead of BSL2 or BSL3 for dengue vaccine or wild-type viruses); and (4) plaques do not have to be counted immediately.

Direct staining of viral plaques. Viral plaques can also be immunostained using DENV-reactive polyclonal antisera or monoclonal antibodies. Staining plaques with DENV serotype-specific monoclonal antibodies allows an additional identity test of the viral serotype used in the assay. Immunostaining also permits the detection of viruses that plaque poorly, or the use of cell lines in which the virus does not demonstrate cytophathic effects (e.g., C6/36 cells). As with staining of the cell sheet with amido-black or crystal violet, immunostaining requires chemical fixation of cells that inactivates DENV. Subsequent counting can be implemented under BSL1 containment. Plaques do not have to be counted immediately, and the strong contrast between the cell sheet and the stained plaque permits photographic capture of plate images and storage of images as raw data records for compliance purposes.

Counting plaques

Regardless of the method of visualization, plaques are usually counted manually. Depending on the visualization method, plaques can be counted immediately, or later, as in the case of immunostaining or cell monolayer coloration.

Data analysis

The criteria needed to validate an individual test are: (1) integrity of uninfected cell monolayer control; (2) appropriate plaque counts per well as determined by back-titration of input virus; (3) little or no reduction in plaque counts with negative serum control; (4) appropriate PRNT titer of positive control sera; and (5) no serum toxicity observed with low serum dilutions.

PRNT end-point titers are expressed as the reciprocal of the last serum dilution showing the desired percentage reduction in plaque counts. A serum standard has been evaluated by the WHO and is available; however, it has never been characterized as a precise international standard, and at best serves as an internal positive control specimen. Currently no international reference sera are available for routine testing. The PRNT titer should be calculated based on a 50% or greater reduction in plaque counts ($PRNT_{50}$). A $PRNT_{50}$ titer is preferred over titers using higher cut-offs (e.g., $PRNT_{90}$) for vaccinee sera, providing more accurate results from the linear portion of the titration curve. However, $PRNT_{50}$ titers are more variable. The more stringent $PRNT_{90}$ titers are more useful in DENV-endemic areas for epidemiological studies

or diagnostic purposes, by decreasing the background serum cross-reactivities among flaviviruses.

There are several ways to calculate PRNT titers. The simplest and most widely used way to calculate titers is to count plaques and report the titer as the reciprocal of the last serum dilution to show $\geq 50\%$ reduction of the input plaque count as based on the back-titration of input plaques (see above). One area for titer variability between labs is whether or not the dilution made by mixing virus and antibody dilution (usually in a 1:1 volume ratio, resulting in a further 1:2 dilution of antibody) is included in the final titer calculations. Because there is no consistency in whether the 1:2 antigen:antibody dilution is included in the final titer, reporting of the data should explicitly state how this dilution was handled. Use of curve-fitting methods from several serum dilutions may permit calculation of a more precise result. There are a variety of computer analysis programs available for this (e.g., SPSS or GraphPad Prism). That being said, consistency in the interpretation method is as important as the method chosen for analysis.

NEW TESTS TO MEASURE VIRUS NEUTRALIZATION

Modifications in the standard PRNT assays are being investigated to shorten the incubation period required to see plaques, stain infected cell cultures to enhance plaque visibility, eliminate the plaque enumeration step, or reduce or eliminate the reliance on cell culture. Any new approach to assessing virus-neutralizing antibodies will have to be validated against the standard PRNT, as outlined in this document, so that the relationship and equivalence of the new and old test is fully understood. The first modification in the PRNT is the reduction of the size of vessel used for plaquing; 24-well plates seem to be the smallest reasonably sized vessel for plaquing when conventional cell staining techniques are used.

Immunostaining of virus-infected cells, instead of directly staining cells with histochemical stains or vital dyes, offers some advantages over these standard procedures. Immunostaining relies on adding enzyme-conjugated antiviral antibody (direct test) or non-conjugated antiviral antibody (later detected with an enzyme-conjugated anti-species antibody indirect test) to virus-infected cell cultures. The binding of the enzyme conjugate to the cell sheet is detected with an immunohistochemical stain specific for the enzyme used (usually horseradish peroxidase or alkaline phosphatase). The availability of DEN virus serotype-specific monoclonal antibodies permits enumeration of plaques within complex mixtures of serotypes using this approach. While using mixtures of DEN viruses in this way may reduce the total number of

plates needed to quantitate virus plaques, issues of plaque overlap still need to be considered. Use of immuno-chemical staining, which results in signal amplification because of the presence of the enzyme-conjugate, may also facilitate identification of plaques at an earlier stage post-infection, resulting in reduced plaquing incubation times.

Perhaps the most promising technique is to quantitate infectious virus using small numbers of virions and a 96-well plate format (micro-neutralization assay) (52). In this assay, individual plaques are not enumerated, but rather the viral growth measured in any given well is related to the optical density observed in that well using a soluble enzyme substrate. The most difficult parameters to control using this assay design are determining the appropriate amount of input virus and the incubation times between virus infection and ELISA detection. Since viral spread is not limited by semisolid overlays, waiting too long before staining can result in overgrowth of virus. Since not all viruses grow at the same rate, the incubation periods will be virus-specific. Standardizing this assay for four virus serotypes is possible; however, if cross-reactivities need to be measured against a variety of viral isolates, the task becomes more difficult. It is also possible to convert this assay from an immunochemical assay to a nucleic acid detection assay. In this way, viral RNA produced by non-neutralized virus can be detected in a quantitative assay (from culture supernatants and/or cell monolayers). The issues of input virus and time of incubations are still relevant for this process.

Another new approach to measuring virus-neutralizing antibodies is to dispense with virus completely and use an "infectious" vector that expresses the E protein or E/prM proteins with or without genes that encode "reporter" molecules (38). These assays are based on the observation that essentially all virus-neutralizing antibodies are elicited by the E protein. A variety of reporter molecules (e.g., green fluorescent protein) can be used to detect residual "virus" activity. However, such approaches suffer the same drawbacks as the micro-neutralization assays. In addition, this system requires the construction of a new vector for each strain to be tested.

SUMMARY

The levels of flavivirus-neutralizing antibody titers in the serum of vaccinated or infection-immune individuals correlates best with protection from subsequent viral infection. However, the lack of a standardized PRNT poses a hurdle for comparing data between vaccine trials and testing laboratories, and defining a threshold value to use as a true serological correlate of protection. The purpose of these guidelines is to: (1) provide scientific insight into the biol-ogy of the flavivirus neutralization test; (2) provide guidelines for test coordination; and (3) provide minimal recommendations for a test protocol for laboratories that might be interested in establishing the test. It must be remembered that the PRNT is above all a biological assay, and as such will always have a certain degree of inter-laboratory variation. Due to this and other lab-to-lab variability, such as specific vaccine design approaches, strains used for vaccine development, and other unique manufacturing requirements, these guidelines will not mandate the use of a single standardized protocol. Ultimately, the definition of a protective level of vaccine-induced neutralizing antibody by whatever suitable assay will need to be validated in a vaccine efficacy trial. Standardization of the procedure using appropriate reference material, followed by each laboratory's own qualification and validation, may result in better inter-laboratory comparisons of results. These guidelines outline the variables that are important to consider in performing this biological assay, and suggest a procedure that can be used by those laboratories interested in using the PRNT. It is important to note that virus-neutralizing antibody titer, as determined by the PRNT, is considered to be the best immune correlate of protection for flaviviral infections. The fact remains, however, that a true definition of protective levels of vaccine-induced DENV-neutralizing antibody will require vaccine-efficacy trials using fully validated assays.

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