

POINT OF CARE TEST FOR THE DETECTION OF EXPOSURE OR IMMUNITY TO DENGUE VIRUS

FIELD

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[0001] The present invention relates generally to the field of diagnostic assays. More particularly, the present invention provides a point of care test (POCT) to detect for recent exposure of a subject to a dengue virus or an immunologically related virus or to a dengue virus vaccine. Point of care (POC) diagnostic and sero-evaluation kits for dengue virus or
10 dengue virus-specific antibodies also form part of the present invention.

BACKGROUND

[0002] Bibliographic details of the publications referred to by author in this specification
15 are collected alphabetically at the end of the description.

[0003] Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

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[0004] Dengue fever is one of the most prevalent mosquito-borne diseases in humans. The disease is best controlled by regular routine reduction activities of the mosquito, *Aedes aegypti*. Dengue virus control requires prompt response to viral infection clusters and early and reliable diagnosis of dengue infection or exposure is critical to this effort, which aims
25 to halt the dengue virus transmission. There has been good progress in recent years in the development of dengue diagnostic tools, resulting in the availability of suitable tests for each stage of the disease. In addition to anti-dengue virus IgM/IgG antibody assays and real time RT-PCR, the recent development of NS1 antigen detection in the ELISA and rapid lateral flow platform had offered clinical laboratories a promising tool for early
30 diagnosis (Kumarsamy *et al*, *J Virol Methods* 40:75-79, 2007).

[0005] Unfortunately, all these techniques require more than one platform and are not

sensitive enough to detect the majority of dengue cases. This is because none of these techniques covers two diagnostic windows [antigen/virus (early stage) and antiviral signature molecules (late stage)]. This deficiency results in misdiagnoses of many dengue patients. Furthermore, this type of approach is expensive and not readily available at all
5 levels of health care systems.

[0006] Dengue virus contains a positive-stranded RNA of approximately 11kb in length and is composed of three structural protein genes that encode the nucleocapsid or core protein (C), a membrane-associated protein (M), an envelope protein (E), and seven
10 nonstructural (NS) protein genes.

[0007] The gene order for dengue virus, as well as other flaviviruses, is 5'-C-prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3'. There are four distinct serotypes, serotypes 1 to 4 (referred to as DEN1, DEN2, DEN3 and DEN4). Infection induces a life-long
15 protective immunity to the homologous serotype but confers only partial and transient protection against subsequent infections by the other three serotypes. Instead, it has generally been accepted that secondary infection or infection with secondary or multiple infections with various dengue virus serotypes is a major risk factor for dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) due to antibody-dependent
20 enhancement. Other factors which have been postulated to be important in the pathogenesis of DHF include viral virulence, host genetic background, immune-cell activation, the viral burden and auto-antibodies. As attempts to eradicate *Aedes aegypti*, the most efficient mosquito vector of dengue virus, are not successful in countries where dengue is endemic, the control of dengue will be possible only after an efficient vaccine
25 has been developed. At present, no effective dengue vaccine has achieved wide usage.

[0008] The laboratory diagnosis of dengue virus infection can be made by the detection of specific virus, viral antigen, genomic sequence, and/or antibodies. At present, the three basic methods used by most laboratories for the diagnosis of dengue virus infection are
30 viral isolation and characterization, detection of the genomic sequence by a nucleic acid amplification technology assay and detection of dengue virus-specific antibodies. After the onset of illness, the virus is found in serum or plasma, circulating blood cells, and selected tissues, especially those of the immune system, for approximately 2 to 7 days, roughly

corresponding to the period of fever.

[0009] Two patterns of serological response can be observed in patients with dengue virus infection: primary and secondary antibody responses, depending on the immunological status of the infected individuals. A primary antibody response is seen in individuals who are not immune to dengue or other member of dengue viruses. A secondary antibody response is seen in individuals who have had a previous dengue or dengue virus infection. For acute- and convalescent-phase sera, serological detection of antibodies based on capture immunoglobulin M (IgM) and IgG enzyme-linked immunosorbent assay (ELISA) has become the new standard for the detection and differentiation of primary and secondary dengue virus infections. This is important, since a sensitive and reliable assay for the detection and differentiation of primary versus secondary or multiple dengue virus infection is critical for the analysis of data for epidemiological, pathological, clinical, and immunological studies.

[0010] Progress toward the detection of antigen in acute-phase serum samples by serology has been slow due to the low sensitivity of the assay for patients with secondary infections, as such patients have pre-existing virus-IgG antibody immunocomplexes. However, recent studies that used ELISA and dot blot assays directed to the envelop membrane (E/M) antigen (the denKEY kit; Globio Co., Beverly, Mass.) and the NS1 antigen demonstrated that high concentrations of the E/M and NS1 antigens in the forms of an immune complex could be detected in the acute-phase sera of both patients with primary dengue virus infections and patients with secondary dengue virus infections up to 9 days after the onset of illness. Koraka *et al*, *J. Clin. Microbiol.* 41:4154-4159, 2003, recently reported on the detection by a dot blot immunoassay of immune complex-dissociated NS1 antigen in patients with acute dengue virus infections and concluded that NS1 antigen detection by dot blot immunoassay in both non-dissociated and dissociated serum and plasma samples from patients with primary and secondary dengue virus infections results in the highest number of dengue antigen-positive patients compared with the numbers obtained by RT-PCR and with the denKEY kit. The recent development of NS1 protein based ELISA showed high level of detection of dengue cases particularly during early stage of infection but remained low sensitive during early convalescence (*Blacksell et al.*, 2007).

[0011] The serological diagnosis of dengue virus infection is rather complicated for the following reasons: (i) patients may have multiple and sequential infections with the four dengue virus serotypes due to a lack of cross-protective neutralization antibodies; (ii) multiple and sequential dengue virus infections make differential diagnosis difficult due to the presence of pre-existing antibodies and original antigenic sin (many B-cell clones responding to the first dengue virus infection are re-stimulated to synthesize early antibody with a greater affinity for the first infecting virus than for the present infecting virus in every subsequent dengue virus infection) in regions where two or more dengue viruses are co-circulating; (iii) IgG antibodies have high degrees of cross-reactivity to homologous and heterologous dengue virus antigens; and (iv) the serodiagnosis of past, recent, and present dengue virus infections is difficult due to the long persistence of IgG antibodies (6 months, as measured by E/M-specific capture IgM ELISA, or life long, as measured by E/M antigen-coated indirect IgG ELISA) in many dengue patients with secondary infections. Thus, among the viral infections that can be diagnosed by serology, dengue virus infection is most challenging.

[0012] Several methods have been described for the serological detection of dengue virus-specific antibodies, including the hemagglutination inhibition (HI) test, the neutralization test, the indirect immunofluorescent-antibody test, ELISA, complement fixation, dot blotting, Western blotting, and the rapid immunochromatography test. Among these, capture IgM and/or IgG ELISA, antigen-coated indirect IgM and/or IgG ELISA, and the HI test are the most commonly used serological techniques for the routine diagnosis of dengue virus infections. Traditionally, the HI test was used to detect and differentiate primary and secondary dengue virus infections due to its simplicity, sensitivity, and reproducibility. Patients are classified as having secondary dengue virus infections when the HI test titer in their sera is greater than or equal to 1:2,560 and are classified as having primary dengue virus infection if the HI test titer is less than 1:2,560. The HI test has recently become less popular and has gradually been replaced by the E/M-specific capture IgM and IgG ELISA due to the inherent disadvantages of the HI test.

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[0013] Many rapid test kits that use the principle of immunochromatography are commercially available. Most of these kits can simultaneously detect IgM and IgG antibodies to dengue virus in human whole blood, serum or plasma within 15 to 30

minutes. Some of these kits claim that it is possible to differentiate primary and secondary dengue virus infections, although this is not always reliable. The results showed that these kits generally have higher sensitivities for IgG detection but lower sensitivities for IgM detection and various specificities compared to the results of the E/M-specific capture IgM and IgG ELISA. Although the rapid test kits (RDT) have the advantages of easy performance and rapid provision of results, they should at best serve as a screening test for clinicians in hospitals. The accuracy of RDTs is highly variable, particularly during acute phase of infection with sensitivities ranging from 6.4% to 65.3% and specificities from 69.1% to 100% (Blackwell *et.al.* 2006).

[0014] There is a need for an effective and sensitive point of care test (POCT) for the detection of exposure of subjects to dengue virus to an acceptable level of performance during both acute and convalescent phases of infection and to monitor the efficacy of dengue virus immunity.

SUMMARY

[0015] The present invention provides a "point of care test" (POCT) for detecting a subject who has had exposure to a dengue virus or immunological relative thereof or who has achieved some level of immunity against a dengue virus antigen. The method is predicted in part on the detection of a binding partner (generally IgA antibodies) which is specific for a dengue virus or an immunological relative thereof. The POCT comprises contacting a biological sample from the subject with a mixture of dengue virus specific immunogenic components; and determining the presence of a complex that forms between a binding partner (e.g. IgA antibodies) in the biological sample and a dengue virus specific immunogenic component; and characterizing the binding partner in the complex with an anti-binding partner antibody (e.g. anti-human IgA) conjugated with gold colloid using reverse flow technology.

[0016] In an embodiment, the POCT relies on the identification of IgA antibodies specific for a dengue virus or its immunological relative in the biological sample.

[0017] The POCT is a low cost, rapid and straightforward assay to determine present or prior recent exposure to dengue virus. In an embodiment, the POCT utilizes nitrocellulose membrane to hold dengue virus immunogen, which subsequently captures binding partners identified as anti-dengue IgA from a biological sample such as whole blood, serum,
5 plasma, saliva, urine or tissue fluid of subjects to be tested for exposure to dengue virus and finally visualized by anti-IgA antibody conjugated with gold particle.

[0018] Accordingly, the POCT of the present invention shows greater specificity and sensitivity compared to other conventional and most currently used dengue capture IgM
10 ELISA by providing a reverse flow rapid platform that can specifically identify antibody (e.g. IgA) produced against dengue virus or immunological relatives thereof at an early stage of dengue infection. In a particular embodiment, the POCT identifies current (acute) dengue virus infection.

[0019] In another aspect of the present invention there is provided a solid support for use in a POCT for detecting exposure of a subject to a dengue virus or immunological relative thereof, the support comprising dengue virus specific immunogenic components immobilized on the support. In use, the solid support is used in a method which comprises contacting a biological sample from the subject with a mixture of dengue virus specific
20 immunogenic components or an immunogenic equivalent thereof; determining the presence of a complex that forms between a binding partner present in the biological sample and a dengue virus specific immunogenic component; and optionally characterizing the binding partner in the complex to correlate the binding partner to exposure to the dengue virus.

25 [0020] In an embodiment, the biological sample is applied to a nitrocellulose membrane previously loaded with dengue virus. Conveniently, the antigen or immunogenic component is derived from dengue virus infected cells. The complex formed by an immunogenic component of the dengue virus antigen and the binding partner then be
30 detected using a detection agent that contains a reporter group, wherein the detection agent specifically binds to the component/binding partner complex.

[0021] More particularly, the binding partner is IgA and the detection agent is an anti-IgA antibody.

[0022] Yet another aspect of the present invention provides a POCT kit in a cassette form
5 for detecting a binding partner in a subject that is specific for a dengue virus or an immunological relative thereof or for detecting dengue virus exposure, the kit comprising a solid support comprising a dengue virus specific immunogenic component or immunological relative thereof; or a solid support comprising a dengue virus specific immunogenic component or immunological relative thereof attached to a second support;
10 at least one detection agent conjugated to a reporter group for detecting a binding partner in a biological sample that forms a complex with the dengue virus specific immunogenic component; and optionally instructions for using the kit to further identify the binding partner of the complex. In an embodiment, the binding partner is IgA. In that aspect, the reporter group generally comprises anti-IgA antibodies conjugated with gold particles and
15 the platform is reverse flow technology.

[0023] The present invention also provides individual components of the POCT kit for use in the method of the present invention. The present invention also serves as a method of assessing the relative risk of one or more subjects being exposed to dengue virus or an
20 immunological relative thereof within a defined location (e.g. geographical area, housing estate, means of transport or center for medical treatment or assessment), comprising; obtaining samples from a representative population within a defined location; and assessing evidence of exposure of individual members of a sample population to a dengue virus or immunological relative thereof by the method comprising the steps of –contacting
25 a biological sample from the subject with a mixture of dengue virus specific immunogenic components; and determining the presence of a complex that forms between a binding partner in the biological sample and a dengue virus specific immunogenic component and wherein the presence of the complex is indicative of exposure of the subject to a dengue virus or equivalent thereof; and assessing the relative risk of exposure within the defined
30 location by characterizing the binding partner in the complex. In an embodiment, the binding partner is IgA.

[0024] Risk analysis may be conducted using software in a computer readable form. Consequently, the present invention further relates to a computer readable program and computer comprising suitable for analyzing exposure of subjects or group of subjects or a risk of exposure of subject or group of subjects to a dengue virus or immunological relative thereof.

[0025] The present invention is further directed to the use of IgA antibodies specific for dengue virus or an immunological relative thereof or a dengue virus antigen or an immunogenic component thereof in the manufacture of a POCT to detect current or recent prior infection or exposure to dengue virus or an immunological relative thereof.

BRIEF DESCRIPTION OF THE FIGURES

[0026] Some figures contain color representations or entities. Color photographs are available from the Patentee upon request or from an appropriate Patent Office. A fee may be imposed if obtained from a Patent Office.

[0027] Figure 1 shows the optimization of dengue antigen on the reverse flow platform (nitrocellulose membrane) at different dilutions ranged from 0.223mg/ml to 0.175mg/ml. The antigen dilution at 0.185mg/ml showed highest level of intensity (1.35) and considered as optimum concentration. (Optimization of dengue antigen using Reverse Flow Technique).

[0028] Figure 2 shows the optimization of protein-L as control line on nitrocellulose membrane with various concentrations ranging from 0.02mg/ml to 0.04mg/ml. The concentration of protein-L 0.03mg/ml is considered as optimum dilution used for the development of Den-IgA POCT. (Optimization of Protein-L (C-line) against anti-human IgA gold-conjugate).

[0029] Figure 3 shows the optimization of optimal dilution (OD) of anti-human IgA gold conjugate against protein-L. Gold-conjugate of various OD dilutions ranging from OD-6 to OD-10 were used against protein L (0.03mg/ml) and OD-9 was found as an optimal

dilution and used for the development of Den-IgA POCT. (Optimization of OD of anti-human IgA gold conjugate against Protein-L (C-line)).

5 [0030] Figure-4 shows the optimization of protein-L against viral antigen (0.185mg/ml) and 0.03mg/ml of protein-L was found optimum in terms of intensity of T-line (1.35) and used as an optimal dilution for the development of Den-IgA POCT. (Optimization of OD anti-human IgA gold-conjugate against C-line (Protein-L)).

10 [0031] Figure 5 shows the comparative performance of Den-IgA POCT against Den-IgA, Den-NS1 Ag and Den-Cap-IgM ELISAs. Den-IgA POCT detected more dengue positive cases compared to other dengue tests. (Comparative performance of Den-IgA POCT against Den-IgA, Cap-IgM and NS1 antigen ELISAs).

15 [0032] Figure 6 shows the performance of Den-IgA POCT using spiked whole blood (W/B), saliva and serum with 25 dengue IgA positive serum samples. All spiked samples even diluted at 1:3 dilution showed positive reactions (>0.5) in Den-IgA POCT. (Performance of Den-IgA POCT using spiked with whole blood and saliva from dengue antibody negative person).

20 [0033] Figure 7 shows the comparative performance of Den-IgA POCT against other dengue rapid tests (Dengue combo IgM and IgG) currently available in the market and Den-IgA POCT showed highest level of detection of dengue positive cases (80.59%). (Comparative Performance of Den-IgA against other dengue diagnostics (Den-Cap IgM ELISA, NS1 antigen ELISA, Uni-dipstick, Panbio Cassette, and Uni-check Cassette)).

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[0034] Figure 8 shows the kinetics of anti-dengue IgA produced at different point of sample collection after onset of fever. The comparative performance of Den-IgA POCT with Den-NS1 Ag and Den-cap IgM ELISAs based on the day of illness. Den-IgA POCT showed higher level of detection of dengue positive cases than Den-Cap IgM ELISA but 30 lower than Den-NS1 Ag ELISA. Den-NS1 Ag ELISA is considered to be test of early dengue diagnostics.

DETAILED DESCRIPTION

[0035] All scientific citations, patents, patent applications and manufacturer's technical specifications referred to hereinafter are incorporated herein by reference in their entirety.

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[0036] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

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[0037] It is to be understood that unless otherwise indicated, the subject invention is not limited to specific assay components, manufacturing methods, diagnostic protocols, or the like, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

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[0038] It must be noted that, as used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a dengue virus" includes a single dengue virus, as well as two or more dengue viruses; reference to "an antibody" includes a single antibody, as well as two or more antibodies; reference to "the invention" includes a single or multiple aspects of an invention; and so forth.

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[0039] The present invention provides an assay to detect dengue virus infection or exposure. The "assay" may also be referred to as a "method" or "protocol" and is a point of care test or "POCT".

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[0040] Reference to "dengue virus" includes an immunological relative thereof. The latter term encompasses variants of one of the serotypes of dengue virus. An immunological relative includes another genus within the Flaviviridae family or a species of Flavivirus. Potential relatives include members of the mammalian tick-born virus group, the seabird tick-born virus group, the mosquito-born virus group, the Kokobera virus group, the Ntaya virus group, the Spondiveni virus group and the yellow fever virus group. Reference to "dengue virus" includes any or all of the Den1, Den2, Den3 and Den4 serotypes as well as

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variants thereof. Reference to "exposure" includes current or recent prior infection as well as an infection which does not lead to a disease condition. In one embodiment, the POCT screens for a binding partner such as IgA in a subject that is specific for a dengue virus or immunological thereof and the test comprises contacting a biological sample from the
5 subject with a mixture of dengue virus specific immunogenic components; and determining the presence of a complex that forms between a binding partner in the biological sample and a dengue virus specific immunogenic component; and characterizing the binding partner in the complex with an anti-IgA antibody conjugated with gold colloid.

10 [0041] This method is specific for the identification of dengue virus-specific binding partner molecules. In a particular embodiment, the binding partner is IgA. This method may be further enhanced by the use of immunogenic components that have been isolated using a dengue virus specific IgA. Accordingly, the immunogenic components may be dengue-specific IgA immunogenic components. The use of IgA reactive immunogenic
15 components renders the POCT specific for IgA antibodies in the biological sample which are specific for dengue virus or its immunological relative. However, other binding partners may also be employed.

[0042] Accordingly, one aspect of the present invention provides a method for detecting a
20 binding partner in a subject that is specific for dengue virus the method comprising contacting a biological sample from the subject with a mixture of dengue virus reactive immunogenic components; and determining the presence of a complex that forms between the binding partner in the biological sample and a dengue specific immunogenic component; and characterizing the binding partner in the complex with an antibody
25 specific for the binding partner conjugated with gold colloid.

[0043] In another embodiment, the present invention provides a method for detecting IgA in a subject that is specific for dengue virus thereof said method comprising contacting a biological sample from the subject with a mixture of dengue virus reactive immunogenic
30 components; and determining the presence of a complex that forms between a binding partner in the biological sample and a dengue specific immunogenic component; and characterizing the binding partner in the complex with an anti-IgA antibody conjugated with gold colloid.

[0044] In a further aspect, the present invention provides a POCT using reverse flow technique for detecting exposure of a subject to a dengue virus or immunological relative thereof the method comprising contacting a biological sample from the subject with a mixture of dengue virus specific immunogenic components; determining the presence of a complex that forms between a binding partner in the biological sample and a dengue virus specific immunogenic component; characterizing the binding partner in the complex; and correlating the binding partner to exposure to the dengue virus.

10 [0045] In a particular embodiment, the binding partner is IgA.

[0046] Hence, in a particular aspect, the present invention provides a POCT reverse flow rapid test for dengue reactive IgA which in one particular embodiment, uses serum with the aim to use saliva from dengue infected patient as the biological sample. Saliva contains high levels of IgA (19.9mg/100ml) compared to IgG and IgM (1.4mg/100ml and 0.2mg/100ml respectively). The technique shows a high level of performance in terms of detecting anti-dengue IgA antibodies compared to the detection of dengue-specific antibodies using ELISA techniques. The POCT of the present invention is compatible to diagnostics at the primary health care level where molecular and ELISA based dengue diagnostic assays are not feasible.

[0047] The POCT is rapid (approximately 10-20 minutes, average time 15 minutes), low cost and straightforward without needing laboratory facilities and special technical expertise to determine present or prior recent exposure to dengue virus. In accordance with the present invention, the subjects including animals such as mammals and in particular humans are screened for the presence of binding partners and in particular IgA to dengue or an immunological relative thereof using reverse flow technology. The binding partners are generally subject-derived binding partners such as, but not limited to, immunointeractive molecules. The most useful immuno-interactive molecules are antibodies particularly IgA. The identification of such binding partners is then used as evidence of present or prior recent exposure of the subject to dengue virus or an immunological relative thereof.

[0048] The present invention specifically utilizes reverse flow technique, where dengue virus antigen is sprayed or loaded onto the nitrocellulose for capturing of dengue reactive IgA from the biological samples of the subject exposed to dengue infection. The resultant complex is later detected by anti-IgA antibody conjugated with gold particle and the technique can be useful at any level of health care system which is called point of care (POC) testing.

[0049] Accordingly, the present invention shows greater sensitivity and specificity compared to other conventional and most currently used antibody and antigen capture IgA (AAC and AAC-ELISAs) by providing a platform that can identify antibody produced against dengue virus or an immunological relative thereof at an early stage of the infection by reverse flow technique.

[0050] Therefore, the present invention provides a specific, rapid and economical POCT using dengue virus antigen, such as dengue envelope protein immunogenic components of the virus, which permit the specific detection of dengue virus binding partners such as IgA that may be present in the test samples (whole blood, serum, plasma, saliva, urine or tissue fluid). The test is conducted at room temperature (RT). It is a convenient technique for specific antibody detection. The POCT utilizes reverse flow platform in a cassette format and anti-IgA antibodies conjugated with gold for the detection of anti-dengue virus IgA from saliva, whole blood, serum, plasma, urine or tissue fluid. Furthermore, the POCT is used for testing a single sample with high sensitivity.

[0051] As indicated above, the term "dengue virus" as used herein refers to all dengue serotypes (Den-1, Den-2, Den-3 and Den-4) associated with a dengue infection or symptoms of dengue infection. The present invention is applicable to detecting dengue virus infection or exposure in any subjects including human, non-human animals and laboratory animals. Human subjects, however, are the most useful subjects in accordance with the present invention. Notwithstanding, the present invention includes any subject that can respond to an infection or immunization by the dengue virus or an immunological relative thereof.

[0052] However, the term "infection" includes exposure to dengue virus but without

necessarily causing disease symptoms. The exposure may also be in the form of an experimental vaccine.

[0053] A dengue virus is defined as a group of RNA human virus consisting of enveloped particles of about 40-50nm in diameter. The viral genome is approximately 11kb (Stollar *et al*, *Am J Trop Med Hyg.* 47(6):709-720, 1966). Mature virion consists of a positive sense RNA genome enclosed by an isometric nucleocapsid. The genome encodes a single open reading frame of about 11000 nucleotides, coding for the three structural (C-Capsid, M-Membrane and E-Envelope) and seven non-structural (NS1, NS2a and NS2b, NS3, NS4a and NS4b, NS5) proteins.

[0054] Dengue virus is transmitted to humans through the bites of infected female *Aedes* mosquitoes, principally the *Aedes aegypti* mosquito. This is a small, black and white, highly domesticated tropical mosquito that prefers to lay its eggs in artificial containers found in and around homes that may hold water such as buckets, flower vases and other water containers. The adult mosquitoes are rarely noticed outside; they usually rest in dark indoor sites, are unobtrusive and prefer to feed on humans or animals during the day light hours, with most biting activity occurring in the early morning or late afternoon. The female mosquitoes are nervous feeders, disrupting the feeding process at the slightest movement of the host, thus returning to the same or different host to continue feeding. Because of this behavior the mosquito often feeds on several persons during a single blood meal and if infective may transmit the virus to multiple persons (Platt *et al*, *Am J Trop Med Hyg.* 57(2):119-25, 1997; Scott *et al*, *am J Trop Med Hyg.* 57(2):235-239, 1997). Such behavior has been used to explain the epidemiological observation that dengue diseases occur mainly in children although in certain places, like Singapore, this may have changed due to adaptation to vector control measures (Ooi *et al*, *Lancet.* 357(9257):685-686, 2001).

[0055] Following the bite of an infective female mosquito, the virus undergoes an intrinsic incubation period of 3 to 12 days (average 4 to 7 days) after which the person may experience acute onset of fever accompanied by other non-specific signs and symptoms. During this viraemic period (which may be between 2 to 7 days) the virus circulates in the blood of infected humans. If an uninfected *Aedes* mosquito feeds on the host during this viraemic period, this mosquito will become infected after an obligatory extrinsic

incubation period of 10 to 12 days. It would subsequently be able to transmit the virus to other uninfected hosts. In this transmission cycle, humans are the main amplifying hosts for the virus although studies show that monkeys can get infected and perhaps serve as a means of amplification for the virus.

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[0056] Dengue virus infection causes a spectrum of illness in humans depending on the infecting virus, the host's age and immunological conditions. It may result in asymptomatic illness or ranges from an undifferentiated flu-like illness (Viral syndrome) to dengue fever (DF), to dengue hemorrhagic fever (DHF), and the severe and fatal dengue shock syndrome (DSS) (World Health Organization, *Dengue haemorrhagic fever: diagnosis, treatment, prevention and control*, 2nd ed., 1997).

[0057] The World Health Organization has set up standards for the grading of the severity of DHF. There are four grades of severity of which grade III and grade IV are considered to be DSS.

[0058] Grade I: Fever with non-specific constitutional symptoms, and the only haemorrhagic manifestation is a positive tourniquet test and or easy bruising.

[0059] Grade II: In addition to the manifestation in grade I one spontaneous bleeding in the forms of skin or other haemorrhages.

[0060] Grade III: Circulatory failure manifested by a rapid weak pulse, narrowing of pulse pressure or hypotension with the presence of cold, clammy skin and restlessness.

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[0061] Grade IV: Profound shock with undetectable blood pressure or pulse (WHO, 1997).

[0062] Classical DF is more common in older children, adolescents and adults, and they are less likely to be asymptomatic. The fever is abrupt in its onset with high fever, headache, incapacitating myalgias and arthralgias, nausea vomiting and macular or maculopapular rash. The fever usually lasts for 5-7 days and sometimes can follow a biphasic course (Saddle back appearance).

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[0063] DHF is primarily a disease of the younger children below 15 years although it may also occur in adults and is mainly associated with secondary dengue infections. The critical stage of DHF is at the time of defervescence, when the temperature becomes normal. The major factors that determine the severity of the illness at the time are plasma leakage due to the increased vascular permeability and abnormal homeostasis and other common hemorrhagic manifestations like petechiae, purpuric lesions, and ecchymoses. These symptoms plus a positive tourniquet test are helpful for accurate diagnosis of DHF (Gubler, *Clin. Microbiol. Rev.* 11:480-496, 1998).

10 [0064] DSS is the terminal stage of DHF and is manifested by hypovolemic shock due to plasma leakage (World Health Organization, 1997 *supra*). There are four warning signs of DSS: sustained abdominal pain, persistent vomiting, restlessness or lethargy, and a sudden change from fever to hypothermia with sweating and prostration. Early recognition and appropriate treatment by experienced hospital staff can decrease the case fatality rate of DSS to 0.2% but once shock sets in the mortality rate can be over 40%.

[0065] The E-protein, the largest and the only structural protein exposed on the surface of the virus, is the major protein involved in immunological reactions such as receptor binding, haemagglutination and neutralization. Infection in humans by one of the serotypes provides life long immunity to that serotype but only temporary protection against other serotypes.

[0066] The nucleocapsid is in turn surrounded by lipid containing the envelope and membrane proteins. In addition to envelope and capsid proteins, dengue virus has seven non-structural proteins NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5.

[0067] As indicated above, the term "immunological relative" as used herein and applied to the dengue virus is intended to include similar molecules that can elicit the same or similar response that the dengue virus or a structural or non-structural protein of the dengue virus could elicit. For instance, various antigens expressed by the dengue virus at various stages of infection or various virus particles or fragments may cause similar effects that the whole virus causes. The response may be an immunological response (non-clinical response) or it may be an infectious response (clinical response) or due to vaccination.

[0068] The present invention is applicable to detecting exposure to the dengue virus or an immunological relative thereof. Exposure may be present or prior exposure to the dengue virus or an immunological relative thereof. Generally, the exposure is sufficient to elicit an immune reaction or response in the body so as to induce a binding partner in response to the dengue virus or immunological thereof. Once the subject is exposed, the method of the present invention may be applied at any stage of exposure as described above. Generally, the method is used to detect exposure where there are no signs and symptoms that are obvious of a dengue virus infection. The POCT herein detects exposure of the subject at a phase of dengue virus infection at an early acute phase for secondary infection or late convalescence stage of exposure to dengue virus or equivalent thereof for the primary infection or vaccination. The exposure may not always manifest in a dengue virus infection or notable signs or symptoms but it will cause a response so as to induce a binding partner (e.g. IgA). The response is particularly an immunological response.

[0069] The subject may have been exposed to dengue virus but need not show visual symptoms of the infection. The present method detects exposure that may lead to infection or may indicate prior exposure with no symptoms manifested.

[0070] An "immune response" or "immunological response" is understood to be a selective response mounted by the immune system of vertebrates in which specific antibodies or fragments of antibodies and/or cytotoxic cells are produced against invading pathogens and antigens which are recognized as foreign in the body.

[0071] The binding partner is any molecule or cell that is produced against the foreign dengue virus or immunological relative thereof. The binding partner is generally an antibody or immunologically active fragment thereof, or a cytotoxic cell. The binding partner includes an immuno-interactive molecule that can interact with dengue virus antigen or equivalent and is most particularly an IgA molecule.

[0072] As indicated herein, the binding partner is generally an immuno-interactive molecule, which refers to any molecule comprising an antigen binding portion or a derivative thereof. Particularly, the immuno-interactive molecule is an antibody against

any portion of dengue virus proteins or epitopes thereon produced during a humoral response in the subject of a dengue virus infection or exposure.

5 [0073] As indicated herein, a particular binding partner is an antibody produced in the subject to a dengue virus or related virus components. However, a binding partner of the targeted antibody may also be used. An example of such a binding partner is an anti-idiotypic antibody or an antibody specific for and discriminatory of a subject antibody specific for a dengue virus or related virus components.

10 [0074] As used herein, an "anti-idiotypic antibody" is an antibody which binds to the specific antigen binding site of another antibody generated in response to exposure to a component derived from dengue virus or immunological relative thereof.

15 [0075] As used herein, the terms "antibody" or "antibodies" include the entire antibody and antibody fragments containing functional portions thereof. The term "antibody" includes any monospecific or bispecific compound comprised of a sufficient portion of the light chain variable region and/or the heavy chain variable region to effect binding to the epitope to which the whole antibody has binding specificity. The fragments can include the variable region of at least one heavy or light chain immunoglobulin polypeptide, and
20 include, but are not limited to, Fab fragments, F(ab')₂ fragments, and Fc fragments.

[0076] In a particular embodiment, the binding partner is an antibody and more particularly is a dengue virus-specific IgA molecule or a dengue antigen-specific IgA molecule.

25

[0077] The method of the present invention detects exposure to the dengue virus or immunological relative thereof *via* the use of a biological sample obtained from a subject having been potentially exposed to the virus. The biological sample may be any sample from the body that may contain a binding partner. Such biological samples may be selected
30 from the group including blood, saliva, cord fluid, B cells, T-cells, plasma, serum, urine and amniotic fluid. Conveniently, the biological sample is serum or plasma. Most conveniently, the biological sample is serum or saliva.

[0078] It is also useful that the biological sample be obtained from subjects suspected of exposure to a dengue virus. A biological sample may also be modified prior to use, such as by centrifugation, dilution, and the like. Accordingly, a biological sample may refer to a homogenate, lysate or extract prepared from a whole organism or a subset of its tissues, cells or component parts, or a fraction or portion thereof.

[0079] It should be noted that a biological sample might also be devoid of a binding partner that can interact with dengue virus or an immunological relative thereof. This occurs when the subject has not been exposed to dengue virus or an immunological relative thereof. Hence "determining the presence of a complex that forms between a binding partner in the biological sample and a dengue virus specific immunogenic component" may yield a zero result, as a complex cannot form in the absence of binding partners. A control may be performed with dengue virus specific immunological agent such as a monoclonal antibody designed to compete with binding partners in the biological sample.

[0080] Reference to a biological sample being placed in contact with a component, such as an immunogenic component or its immunological relative thereof should be understood as a reference to any method to facilitating the interaction of one or more immuno-interactive molecules of the biological sample with a component of dengue antigen derived from infection of cells with dengue virus or an immunological relative thereof. The interaction should be such that coupling or binding or otherwise association between the immuno-interactive molecule and a specific immunogenic component of dengue virus antigen derived from cells infected with the dengue virus or an immunological relative thereof can occur.

[0081] The biological sample is contacted with a mixture of dengue virus specific immunogenic components preferably derived from cells infected with dengue virus or an immunological relative thereof. The dengue virus provides viral immunogenic components that may be provided by the dengue virus at any stage of its development. In the early convalescent stages of dengue virus infection, antibody, particularly IgA developed or derived from previous dengue infection is one of the indications of either primary or secondary dengue virus infection and this may be detected by the formation of a complex

between it and a dengue virus specific immunogenic component, particularly an immunological component of the virus.

5 [0082] Dengue virus infected vero cells may be cultured for any period, generally for a period that allows the dengue virus to establish and infect the cell. More particularly, the cells are cultured until a cytopathic effect is apparent in the cell culture thereby indicating active infection of the virus in the cells.

10 [0083] At this point, dengue virus is collected from the infected cells either by lysed infected cells or from the supernatant of the infected cells by any other method available. Later the viral antigen is purified and inactivated using standard protocol and concentrated at 1mg/ml.

15 [0084] Conveniently, the dengue virus antigen is envelope protein.

[0085] Particularly, the dengue specific immunogenic component is a protein of the dengue virus particle. More particularly, the structural protein is E-Envelope protein, which may be more immunogenic for anti-dengue IgA antibodies, it is exposed on the envelope of the virus.

20 [0086] However, the present invention is not to be limited solely to use E-protein antigens. A mixture of other dengue virus molecules/immunogens (present in the dengue virus infected cells, which may contain dengue virus particles and other immunological components, generally structural and non-structural proteins) against which antibodies
25 develop during the course of dengue virus exposure/infection may also be used.

[0087] The dengue virus specific immunogenic component is generally from lysate and culture supernatant. The biological sample is contacted so that a complex may form between the viral immunogenic components of the lysate and the binding partner contained
30 within the biological sample. Particularly, immunogens of the dengue virus particles, including but not limited to those of the structural and non-structural proteins captured by anti-dengue IgA, will form complexes with binding partners. Preferably, the specific binding partners are antibodies or fragments thereof derived from the biological sample.

These will only be present when the subject has been exposed/infected or immunized to the dengue virus.

5 [0088] A complex forms between an antibody, particularly an IgA reactive with dengue virus or immunological relative thereof.

[0089] The methods and kits of the present invention seek to detect components and binding partners which form complexes and are indicative of a dengue virus infection. These components and binding partners are generated in the course of a dengue virus
10 infection/immunization.

[0090] The complex may comprise one or more binding partners bound to one or more components derived from dengue virus or an equivalent thereof. However, not all will be dengue virus specific IgA. Other immunological molecules such as IgG and IgM may also
15 bind.

[0091] A second binding molecule is also loaded on the nitrocellulose membrane which binds all immunoglobulins (IgA, IgG, and IgM) called Protein-L and this protein is used as a control line to indicate the validity of the assay.

20

[0092] The biological sample is left in contact with the component derived from dengue virus or an equivalent thereof for a period of time sufficient and conditions, which allow the stable formation of the complex.

25 [0093] The dengue virus specific immunogenic components and the biological sample are contacted so that a complex may form between the components and a binding partner present within the biological sample. Conveniently, immunogens of the dengue virus particles, including but not limited to those of the structural and non- structural proteins particularly captured by anti-dengue virus IgA having a binding site specific to dengue
30 virus antigen, will form complexes with either binding partners or a competing dengue virus specific immunological agent such as a specific IgA.

[0094] Particularly, the specific binding partners are antibodies or fragments thereof

present in the biological sample. These will only be present when the subject has been exposed/immunized to the dengue virus.

5 [0095] Particularly, the complex forms between an antibody, particularly an IgA specific for the member of the dengue virus antigen or equivalent thereof and a anti-dengue virus IgA captured dengue viral component. This is then indicative of dengue virus specific IgA in the sample and hence recent or prior exposure.

10 [0096] In one embodiment, the present invention relies upon the detection of dengue virus specific binding partners particularly IgA present in the biological sample that are specific for a component of the dengue virus antigen present in cell lysate derived from a cell infected with the dengue virus or an immunological relative thereof which has been captured using anti-dengue virus IgA. The complex may comprise one or more binding partners bound to one or more components derived from dengue virus or an equivalent
15 thereof. However, it is the identification of an IgA bound to the complex that is indicative of prior exposure in this present invention.

[0097] A non-immunological complex is formed with biological samples (saliva, serum) along the line loaded with protein-L containing all three types of immunoglobulins (IgA,
20 IgG and IgM). This complex does not indicate the exposure of dengue virus to the subject but does provide the validity of the assay.

[0098] Another aspect of the present invention provides a solid support for use in a method for detecting exposure of a subject to a dengue virus or equivalent thereof, the method
25 comprising contacting a biological sample from the subject with a mixture of dengue virus specific immunogenic components or an immunological relative thereof; determining the presence of a complex that forms between a binding partner present in the biological sample and a dengue virus specific immunogenic component; and optionally characterizing the binding partner in the complex to correlate the binding partner to
30 exposure to the dengue virus; the support comprising dengue virus specific immunogenic components immobilized on the support.

[0099] The solid support may be any material known to those of ordinary skill in the art to

which a binding partner or dengue virus specific immunogenic component may be attached. For example, the solid support may be a test well in a microtitre plate or a nitrocellulose or other suitable membrane. Alternatively, the support may be a bead or disc, such as glass, fiberglass, latex or a plastic material such as polystyrene or polyvinylchloride.

[00100] The binding partner or the dengue virus specific immunogenic component may be immobilized on the solid support using a variety of techniques known to those of skill in the art, which are amply described in the patent and scientific literature. In the context of the present invention, the term "immobilization" refers adsorption of antigen on the nitrocellulose membrane following spraying by BioDot machine. Immobilization by adsorption of antigen is onto the membrane (prima-85). In such cases, adsorption may be achieved by spraying of antigen at 7" from the bottom of membrane using BioDot machine in a suitable buffer. Two more lines are sprayed on the same membrane at 14" and 12" using crystal violet and Protein-L. Following spraying membrane is allowed dry in a dryer and finally is blocked by blocking buffer.

[00101] The dengue virus specific immunogenic component derived from a dengue virus or an equivalent thereof loaded/sprayed onto the membrane is allowed to contact with biological samples which is passed over the membrane once added onto the sample pad, which allow the stable formation of a complex. Once the biological sample meets the indicator line (crystal violet) a detection system which is anti-IgA antibodies conjugated with gold particle is allowed to flow over the membrane from other side of the cassette which then detect the specific immunocomplex through the specific binding with IgA molecule.

[00102] Detecting the complex between the components derived from dengue virus or an equivalent thereof and a subject-derived binding partner such as immunoreactive molecules, may be based on any convenient method, which will be known to those of the skill of the art.

[00103] It is contemplated that procedures useful for detecting components and binding partners which form complexes and are indicative of a dengue virus infection in a

biological sample include, but are not limited to, immunological assays, such as immunoblotting, immunocytochemistry, immunohistochemistry or antibody-affinity chromatography, Western blot analysis, lateral flow or variations or combinations of these or other techniques such as are known in the art.

5

[00104] In general, components and binding partners, which form complexes and are indicative of a dengue virus infection, may be detected in a biological sample obtained from a subject by any means available to the skilled addressee. In an embodiment, the method of detection employs a further detection agent such as specific anti-body
10 conjugated with enzyme, gold particle/colloid which permits detection of the complexes with the binding partners.

[00105] In an embodiment, the methods as herein described involve the use of a dengue virus antigen onto nitrocellulose membrane to which a binding partner of a
15 biological sample may absorb/bind. The complex formed by a component of the dengue virus antigen and the binding partner may then be detected using a detection agent that contains a reporter group and specifically binds to the component/binding partner complex. Such detection agent may comprise, for example, an anti-antibody or other agent that specifically binds to the binding partner, such as an antiimmunoglobulin (i.e. antibody),
20 protein G, protein A, protein-L or a lectin.

[00106] In an embodiment, the detection reagent is an antibody or secondary antibody or an antigen-binding fragment thereof, capable of binding to the binding partner of the biological sample. Antibodies may be prepared by any of a variety of techniques
25 known to those of ordinary skill in the art (See, for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988).

[00107] The secondary antibody which may be conjugated to a label, can be added to the complex to facilitate detection. A range of labels providing a detectable signal may
30 be employed. The label may be selected from a group including chromogen, an enzyme, a catalyst, a fluorophore, gold colloid and a direct visual label. In the case of a direct visual label, use may be made of a colloidal metallic or non-metallic particle, a dye particle, an enzyme or a substrate, an organic polymer, or a latex particle. A large number of enzymes

suitable for use as labels are disclosed in United States Patent Nos. 4,366,241; 4,843,000 and 4,849,338. Suitable enzyme labels in the present invention include alkaline phosphates, horseradish peroxidase, preferably horseradish peroxidase. The enzyme label may be used alone or in combination with a second enzyme, which is in solution. In the present invention a secondary antibody attached with horseradish peroxidase, which then reacts with its substrate DAB and produces a visually detectable colour change, preferably achieves the detection of the complex.

[00108] Generally, the antibody is an anti-IgA antibody conjugated with gold colloid and therefore detects IgA binding partners that have bound to the dengue virus specific immunogenic components.

[00109] This assay may be performed by first contacting a binding partner of a biological sample that has been immobilized on a solid support, the nitrocellulose membrane, with the dengue virus specific immunogenic components as herein described, such that a component is allowed to bind to the immobilized binding partner such as an antibody. Alternatively, the dengue virus specific immunogenic components may be bound to the solid support such that binding partners are allowed to bind to the immobilized component and then detection reagent is added which may be specific reporter group. Unbound sample is then removed from the immobilized complex through reverse flow technique.

[00110] More specifically, once the binding partner or a dengue virus specific immunogenic component is immobilized on the support as described above, the remaining binding sites on the support are typically blocked. Any suitable blocking agent known to those of ordinary skill in the art, such as bovine serum albumin or milk casein with either Triton X 100 or Tween 20 (Trademark) [Sigma Chemical Co., St. Louis, Mo.]. The component or binding partner is used undiluted form during contact with dengue virus immunogenic component. In general, an appropriate contact time is 5-10 minutes that is sufficient to allow a dengue virus specific immunological component presence in the biological samples to bind to the immobilized binding partner, or vice versa. Preferably, the contact time is sufficient to achieve a level of binding to the target epitope on the attached dengue virus specific immunogenic component that is at least about 95% of that

achieved at equilibrium between the bound and unbound binding partner or dengue virus specific immunogenic component. Those of ordinary skill in the art will recognize that the time necessary to achieve equilibrium may be readily determined by assaying the level of binding that occurs over a period of time.

5

[00111] The detection agent is generally an anti-IgA antibody. Preferred reporter groups include those groups recited herein. The detection agent is then released from the gold-pad over the membrane where immunocomplex is formed with dengue virus antigen and anti-dengue IgA from biological samples. An appropriate amount of time may
10 generally be determined by assaying the level of binding that occurs over a period of time. Unbound detection agent is then removed and bound detection agent is detected using the reporter group. The method employed for detecting the reporter group depends upon the nature of the reporter group. For radioactive groups, scintillation counting or autoradiographic methods are generally appropriate. Spectroscopic methods may be used
15 to detect dyes, luminescent groups, chromogenic enzymes and fluorescent groups. Chromogenic enzymes include, but are not limited to, peroxidase and alkaline phosphatase. Fluorescent groups include, but are not limited to, fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC) 1 rhodamine, Texas Red, Gold colloid and phycoerythrin. Biotin may be detected using avidin, coupled to a different
20 reporter group (commonly a radioactive or fluorescent group or an enzyme).

[00112] As used herein, "binding" refers to a non-covalent association between two separate molecules such that a complex is formed. The ability to bind may be evaluated by, for example, determining a binding constant for the formation of the complex. The binding
25 constant is the value obtained when the concentration of the complex is divided by the product of the component concentrations. In general, two compounds are said to "bind," in the context of the present invention, when the binding constant for complex formation exceeds about 10^3 L/mol. The binding constant may be determined using methods well known in the art.

30

[00113] Membranes contemplated by the method and kits of the present invention include any membrane to which either the binding partner or components derived from the flavivirus or equivalent thereof can bind. Examples of membranes include without being

limited to, nitrocellulose membranes, polytetrafluorethylene membrane filters, cellulose acetate membrane filters and cellulose nitrate membrane filters with filter paper carriers. Most preferably, the membrane is a nitrocellulose membrane.

5 **[00114]** Alternatively, the diagnostic methods of the present invention may adopt an automated analytic method using a biological microchip. For instance, a diagnostic kit can be structured to perform immuno-blotting using a glass slide coated with the component of the cell lysate. This diagnostic kit may comprise a biological microchip onto the surface of which a flavivirus specific immunogenic component is immobilized, an appropriate buffer,
10 a standardised sample comprising a detectable level of binding agent, and a secondary detection reagent, as herein described.

[00115] The method and kits of the present invention can detect specific exposure of human or animals to a dengue virus or any specific member of the family or equivalent
15 thereof either during acute infection or in convalescent phase. As used therein "acute infection" refers to the period of time when a virus has infected a host and is actively replicating and/or causing symptoms associated with infection like fever, rash, joint pain and or abdominal pain. The "convalescent phase" refers to the stage of dengue virus infection cycle when dengue virus is no longer multiplying or remains in the host blood
20 and has developed binding partners such as, but not limited to antibodies. Using the method and kit of the present invention, exposure can be detected at any time after generation of a binding partner in the infected patient or patient derived it from his/her previous infection/infections.

25 **[00116]** In another aspect of the present invention there is provided a POCT kit for detecting IgA in a subject that is specific for a dengue virus or equivalent thereof or for detecting dengue virus exposure comprising: a solid membrane support including a dengue virus specific immunogenic component or equivalent thereof; or a solid support including a dengue virus specific immunogenic component or equivalent thereof attached to a second
30 support; at least one detection agent conjugated to a reporter group for detecting a binding partner in a biological sample that forms a complex with the dengue virus specific immunogenic component; and optionally instructions for using said kit to further identify the binding partner of the complex.

[00117] Optionally, the kit will also include additional parts such as plastic dropper, chase buffers, cassette and instructions as are necessary for conducting the method.

5 [00118] Accordingly the present invention provides a kit for detecting exposure of a subject to dengue virus or any member of the family or an equivalent thereof. The kit may be any convenient form of POC testing which allows for a binding partner in a biological sample to interact with an anti-dengue IgA captured dengue virus viral component. The result is an indication, by the presence of dengue virus specific binding partners such as
10 IgA in the biological sample, of prior exposure to dengue virus. Preferably the kit comprises a solid support such as described herein adapted to receive or comprise anti-dengue virus IgA captured components of dengue virus or an equivalent thereof. The kit may also comprise reagents, reporter molecules capable of providing detectable signals and optionally instructions for use.

15

[00119] The kit may be a modular kit comprising one or more members wherein at least one member is a solid support comprising loaded/sprayed dengue virus component of virus particle or equivalent or cell lysate comprising an immunogenic component derived from a dengue virus or equivalent thereof.

20

[00120] The present invention also provides individual components of the kit for use in the method of the present invention. The invention provides solid supports that include anti-dengue virus IgA reactive components of the dengue virus for use in the detection of exposure to the dengue virus. In one embodiment, a nitrocellulose membrane is used to
25 attach viral antigen, either for use as an immobilized anti-dengue virus IgA reactive dengue viral components or as a line blot. Preferably the membranes include components selected from the group of dengue viral antigens structural and non- structural proteins, flavivirus particles and fragments thereof, glycoproteins, lipids and carbohydrates derived from the flavivirus or any mixture thereof.

30

[00121] The solid support may also be a microtitre plate, glass slide or biological microchip wherein the components of the cell lysate are immobilised. These solid supports can then be subjected to the biological sample to detect flavivirus exposure. Preferably

nitrocellulose membrane is used to attach dengue virus antigen by spraying onto the membrane from dengue virus infected cells.

[00122] In yet another aspect, the present invention also provides a method of
5 assessing the relative risk of one or more subjects being exposed to dengue virus or an
equivalent thereof within a defined location (e.g. geographical area, housing estate, means
of transport or center for medical treatment or assessment), comprising; obtaining samples
from a representative population within a defined location; and assessing evidence of
10 exposure of individual members of a sample population to a dengue virus or equivalent
thereof by the method comprising the steps of - contacting a biological sample from the
subject with a mixture of dengue virus specific immunogenic components; and
determining the presence of a complex that forms between a binding partner in the
biological sample and a dengue virus specific immunogenic component and wherein the
15 presence of the complex is indicative of exposure of the subject to a dengue virus or
equivalent thereof; and assessing the relative risk of exposure within the defined location
by characterizing the binding partner in the complex.

[00123] Risk analysis may be conducted using software in a computer readable
form. Consequently, the present invention further relates to a computer readable program
20 and computer comprising suitable for analyzing exposure of subjects or group of subjects
or a risk of exposure of subject or group of subjects to a dengue virus or equivalent thereof.

[00124] The method or technique of the present invention allows for the
epidemiological study or sero-surveillance of outbreaks of infection caused by dengue
25 virus or any member of the family or equivalent thereof. Such studies provide valuable
information, which advance multiple facets of research in the area of dengue virus disease.
For example, epidemiological studies aid in the identification of the index of an infection.
Such information enables the identification of a defined location from which the source of
virus responsible for a viral outbreak originated.

30

[00125] Additionally the technique/method of the present invention permits for the
rapid identification or isolation of subjects who are infected with a dengue virus or
equivalent thereof without major laboratory equipment or even in field conditions. Such

information aids in identifying subjects, who require medical treatment as well as defining locations that require further investigation or disease control approaches such as identification of breeding places and its control. Further, the technique of the present invention allows for the monitoring of an infected patient to determine the presence of -
5 anti-dengue virus specific IgA. Alleviation of IgA titre or its presence in an early phase of infection may be the indication of secondary infection and thus, help the monitoring of the subsequent phases of dengue virus infection like DHF or DSS.

[00126] Further, the technique of the present invention provides a means for
10 identifying subjects who are infected with any specific member of the genus of dengue virus and serotypes involved, allowing for the rapid detection, risk of further infection, pointing to the location of an infection and disease control strategy.

[00127] The present invention is further directed to the use of IgA antibodies
15 specific for dengue virus or an immunological relative thereof or a dengue virus antigen or an immunogenic component thereof in the manufacture of a POCT to detect current or recent prior infection or exposure to dengue virus or an immunological relative thereof.

[00128] The present invention is now described with reference to the following non-
20 limiting Examples. It should be understood, however, that the following description is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

EXAMPLE 1

25 *Dengue type 2 antigen*

[00129] **a) Dengue antigen:** Dengue type 2 antigen (Biodesign, International, USA)
was used to develop POCT using reverse flow technology. Dengue virus was grown in the cell culture and concentrated from culture supernatants by precipitation and ultra-
30 centrifugation, which was then inactivated by formaldehyde. Later the formaldehyde was neutralized by sodium bisulphite and inactivation was confirmed by vero cell culture. The resultant inactivated dengue type 2 antigen was tested against antibodies against all four serotypes of dengue virus (1-4) and was found positive.

[00130] **b) Protein-L:** Protein L (Pierce Biotechnology, USA) is an immunoglobulins-binding recombinant protein that was originally derived from the bacteria *Peptostreptococcus magnus*. Protein L has the ability to bind with kappa light chain of all classes and subclasses of immunoglobulins without interfering antigen-antibody reactions and was used to bind IgA molecule from the biological samples used in this development.

[00131] **c) Anti-human IgA-Gold conjugate (40nm):** Goat anti-human IgA antibodies conjugated with gold colloid was prepared using our in-house protocol and compared with commercial product (Code-HIA10, Millenia Diagnostics, Inc, USA). The antihuman IgA gold conjugate was used in this test as secondary antibodies and was used at a dilution of 9 OD (520 nm).

15 [00132] **d) Serum samples:** A total 184 serum samples positive either by NS1 antigen or anti-dengue capture IgM ELISAs were used as dengue positive samples. An amount of 125 serum samples collected from healthy donors were used as negative control. All these samples were obtained from the library of serum bank of MP Biomedicals Asia Pacific Pt Ltd, Singapore. An additional 124 serum samples used for the field validation of the assay were obtained from Virology Department of Bangabandhu Sheikh Mujib Medical University, Bangladesh.

[00133] **e) Serological assays:** IgM capture ELISA, a commercial kit (Pan-bio, Australia) was used for the measurement of anti-dengue specific IgM antibodies in the samples used in this study. The test was performed according to the procedures described by the manufacturer.

[00134] **f) Dengue rapid diagnostics:** Three types of dengue rapid diagnostic kits available in the market either in the form of cassette or dipstrip were used as competitor kits to validate and evaluate Den-IgA POCT.

[00135] **g) Dengue NS1 antigen ELISA:** Dengue NS1 antigen commercial kit called Platelia Dengue NS1 Ag ELISA (Bio-Rad Corporate HQ, Hercules, USA) was used

for the detection of dengue specific NS1 protein from the serum samples used in this study. The test was carried out according to the procedures described by the manufacturer.

[00136] **h) Saliva samples:** Total 25 saliva samples collected from healthy donors were used as negative as well as spiked samples with dengue IgA positive sera.

[00137] **i) Whole blood:** Whole blood was collected from the health donor (5 ml) and used for the spiking of dengue IgA positive sera. These whole blood spiked samples were used to study the performance of Den-IgA POCT (Figure-6)

[00138] Optimization of Dengue Virus Antigen. The optimization of dengue virus antigen (Biodesign, International, USA) is carried out 1x PBS according to Table 1. A minimum volume of 500µl was made for each concentration before starting the optimisation.

Table 1
Optimization of dengue virus antigen

Name	E- Protein (mg/ml)
1	0.223
2	0.2
3	0 0.185
4	. 0175

[00139] Dengue E-protein antigen was sprayed at position set for Den-IgA Rapid Test striping on 4 pieces of membrane (30 cm each). Use Protein L as control line and crystal violet as indicator line. Following spraying, the membrane is dried, blocked using blocking buffers and dried again and then laminated, cut and assembly for test.

[00140] **j) Optimization of Protein L:** Protein L (Pierce Biotechnology, USA) was diluted in 1 x PBS according to Table 2 mentioned below. A minimum volume of 500µl was made for each concentration for the process of optimisation.

5

Table 2
Dilutions of Protein-L

Name	Protein-L mg/ml
Concentration-1	0.02
Concentration-2	0.025
Concentration-3	0.03
Concentration-4	0.04

10 [00141] The assembly cassette was used for the optimization of antigen and protein-L sprayed/loaded onto the membrane-using panel of strong, weak and negative anti-dengue IgA positive serum samples. For protein-L the C-line with 1.5 to 2.5 intensity with serum was considered as optimum, level of reaction while with saliva the intensity was 2.5 to 3. For the optimization of viral protein the T-line with strong positive serum 2.5, moderate
15 1.5 to 1.0, weak positive 0.5 to 0.25 while for negative no line were considered as optimum level of reactions.

[00142] Following optimization of antigen (Figure-1) and protein-L (Figures-2 & -4), 500 cassette were prepared for the study of sensitivity, specificity, positive predictive
20 value and negative predictive value.

[00143] Optimization of anti-human IgA gold-colloid: Anti-human IgA gold colloid was prepared using standard procedure and optimized using different OD level ranging from 06 to 10 and 09 OD was found optimum against dengue positive and negative
25 samples (Figure-3).

Assay procedure

Whole Blood:

- 5 [00144] **Step 1-** Added 20µl of whole blood sample to the samples square well followed by 1 drop of chase buffer to the same well.

- [00145] **Step 2 –** Sample absorbed by sample pad and then serum/plasma move over the membrane (not red blood cells) and once reached the blue control line (A), three (3)
10 drops Chase Buffer was added to the gold-pad.

- [00146] **Step 3 -**Pulled “plastic tab until resistance is felt and added another drop of chase buffer onto the sample pad square and started timing. Read result after 15 minutes.

15 **Serum / Plasma/saliva Samples**

- [00147] **Step 1-** Added 15µl of sample to the samples square well followed by 1 drop of chase buffer to the same well.

- 20 [00148] **Step 2 –** Sample absorbed by sample pad and then move over the membrane and once reached the blue control line (A), three (3) drops Chase Buffer was added to the conjugate pad.

- [00149] **Step 3 -**Pulled the “plastic tab (labeled with Den-IgA) until resistance is felt
25 and added another drop of chase buffer onto the sample pad square and started timing. Read the result after 15 minutes.

Interpretation of Den-IgA POCT

- 30 [00150] The result of POCT was interpreted using interpretation card which has a range of color intensities varied from 0.25 to 3.0. For test line (T-line) any intensity between 0.25 to 3.0 was considered as den-IgA positive sample while 0.0 is considered as

negative. For control line (C-Line) intensity between 1.5 to 3.0 was considered as validate test.

[00151] 1) Antigen captures anti-dengue IgA ELISA (ACA-ELISA): The 96 well plate (Max-absorb-NUNC) was coated with 100 µl per well of dengue antigen (3µg) using 1x PBS and incubated either over-night at 4⁰C or 1 hour at 37⁰C. After blocking the plate with blocking buffer for 1 hour at 37⁰C, plate was washed 4 washes with wash and used for ACA-ELISA. 100µl of test serum at 1:100 in diluent buffer was added to each well and 1 positive and 3 negative serum controls were kept in each plate. After 1 hour of incubation at RT, the plate was washed again 6 times using wash buffer and 100µl of rabbit anti- human IgA conjugated with HRP (1:4000) was added to each well and further incubated for 30 minutes at RT. Following incubation, the plate was washed again 6 times and 100µl of TMB (Sigma, USA) was added to each well and incubated for 10 minutes at RT. Further colour development was stopped using sulphuric acid (2.75%) and the plate was read in an ELISA reader at 450 nm. Results were calculated using a formula of OD value of the test sample divided by an average OD of negative samples and multiplying the value by 10.

f) Comparative analytical sensitivity of Den-IgA point of care test (POCT):

20

[00152] The analytical sensitivity of den-IgA POCT was studied by using strong, medium and weak dengue IgA positive serum samples. Serum samples were diluted at 1:5 to 1:160 in whole blood, serum or saliva from a healthy person who is negative to dengue antibodies (IgG, IgM and IgA). Fifteen-microlite (15µl) of each diluted sample was then added onto the cassette sample pad and test was performed as described above.

25

EXAMPLE 2

Comparative analysis of Den-IgA POCT against Den-capture IgM, ACA and NS1 antigen ELISAs

30

[00153] The level of sensitivity of Den- IgA POCT carried out using dengue positive sera obtained from R & D lab and compared with other ELISAs. Results showed

that Den-IgA POCT detected 77.52% of dengue cases compared to 59.55% NS1 protein, 60.67% den-capture IgM and 58.42% by anti-Den IgA ELISAs (Figure-5).

EXAMPLE 3

Den-IgA POCT

[00154] The performance of Den-IgA POCT was further evaluated using against commercially available POCT using 67 dengue positive sera (sera were positive either to NS1 antigen ELISA or den-capture IgM ELISA). The sensitivity of Den-IgA POCT was found 80.59% compared to 77.61%, 70.15% and 77.61% by Uni-check IgM/IgG Dipstick, Uni-cassette IgM/IgG and Pan-bio IgM/IgG cassette respectively (Figure-7).

EXAMPLE 4

Specificity and Sensitivity of Den-IgA POCT

[00155] Specificity of Den-IgA POCT using sera collected from healthy and non-dengue febrile illness.

Two by Two table showing sensitivity and specificity of Den-IgA POCT

Table-3

		Den NS1 and IgM ELISA		Total
		Positive	Negative	
Den-IgA POCT	Positive	106	11	117
	Negative	20	114	134
	Total	126	125	251
Sensitivity	84.13%		PPV	90.60%
Specificity	91.20%		NPV	85.07%
PPV= Positive predictive value				
NPV= Negative predictive value				

EXAMPLE 5

Den-IgA POCT

[00156] Performance of Den-IgA POCT against spiked saliva and whole blood with
5 positive and negative serum samples showed high level of consistency at 1:3 dilution
(Figure-6).

EXAMPLE-6

The kinetics of Den-IgA production by Den-IgA POCT

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[00157] Out of 67 dengue positive serum samples (Den-NS1 Ag and Den-Cap IgM
ELISAs) collected during acute phase of infection (1-8 days), 57.14% (12/21) between day
1-3, 90.48% (38/42) between day 4-6 and 100% (4/4) between day 7-8 showed the
presence of anti-dengue IgA (Figure-8).

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[00158] The newly developed Den-IgA POCT showed high level of performance
compared to other POCTs and early dengue ELISAs (capture IgM and NS1 antigen
ELISAs). This is because of eliminating the interference of non-dengue specific IgA
particularly in early phase of dengue illness. This performance is even better than ACA-
20 ELISA because of using high amount of serum (15µl compared to 1:100 in ACA-ELISA),
moreover Den-IgA POCT platform can be used against any type of samples collected from
patient like whole blood, serum/plasma and saliva.

[00159] Those skilled in the art will appreciate that the invention described herein is
25 susceptible to variations and modifications other than those specifically described. It is to
be understood that the invention includes all such variations and modifications. The
invention also includes all of the steps, features, compositions and compounds referred to
or indicated in this specification, individually or collectively, and any and all combinations
of any two or more of said steps or features.

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CLAIMS:

1. A method for detecting exposure of a subject to a dengue virus or an immunological relative thereof, said method comprising contacting a biological sample from the subject with a mixture of dengue virus specific immunogenic components; and determining the presence of a complex that forms between a binding partner in the biological sample and a dengue virus specific immunogenic component; characterizing the binding partner in the complex; and correlating the binding partner to exposure to dengue virus or its immunological relative.
2. A method of Claim 1 wherein the dengue specific immunogenic components are sprayed on solid surface from dengue virus infected cells or an equivalent thereof.
3. A method of Claim 1 wherein the dengue virus specific immunogenic components are sprayed on nitrocellulose membrane.
4. A method of Claim 1 wherein the dengue virus specific immunogenic component is selected from the list consisting of structural and non-structural proteins, dengue virus particles and fragments thereof, glycoproteins, lipids and carbohydrates derived from the dengue virus.
5. A method of Claim 4 wherein the structural protein is selected from the group including envelope proteins, Pr membrane proteins, and nucleocapsid proteins.
6. A method of Claim 5 wherein the structural protein is an envelope protein.
7. A method of Claim 1 wherein the dengue virus specific immunogenic component is selected from the group comprising dengue virus serotype immunogenic components selected from the group consisting of DEN1, DEN2, DEN3 and DEN4.
8. A method of Claim 1 wherein the method detects exposure to a dengue virus serotype selected from the group consisting of DEN1, DEN2, DEN3 and DEN4.

9. A method of Claim 1 wherein the immunogenic component is an antiidiotypic antibody to an antigen binding site of a dengue virus antibody generated in response to exposure to a component derived from dengue virus or an immunological relative thereof.
10. A method of Claim 1 wherein the binding partner is a dengue virus specific antibody or an immunological fragments thereof.
11. A method of Claim 10 wherein the binding partner is an antibody expressed in an early stage of a dengue virus infection, during acute or convalescence or derived from recent past infection.
12. A method of Claim 1 wherein the binding partner is an IgA antibody.
13. A method of Claim 1 wherein the dengue virus relative is selected from the group consisting of yellow fever virus, West-Nile and JE virus.
14. A method of Claim 12 wherein the binding partner antibody is an IgA antibody that is specific to all dengue serotypes (DEN1, DEN2, DEN3 or DEN4).
15. A method of Claim 1 wherein the biological sample is selected from the group consisting of saliva, blood, serum, plasma, cord fluid, B cells, urine and amniotic fluid.
16. A method of Claim 15 wherein the biological sample is saliva, whole blood, serum, plasma or urine.
17. A method of Claim 12 wherein the binding partner is characterized using an anti-IgA antibody.
18. A method of Claim 17 wherein the anti-IgA antibody is conjugated with gold colloid.
19. A method for detecting IgA in a subject that is specific for dengue virus thereof said method comprising: contacting a biological sample from the subject with a mixture of

dengue virus reactive immunogenic components; and determining the presence of a complex that forms between a binding partner in the biological sample and a dengue virus specific immunogenic component; and characterizing the binding partner in the complex with human anti-IgA antibody conjugated with gold colloid.

20. A method of Claim 19 wherein the dengue specific immunogenic components are sprayed on solid surface from dengue virus infected cells or an equivalent thereof.

21. A method of Claim 19 wherein the dengue virus specific immunogenic components are sprayed on nitrocellulose membrane.

22. A method of Claim 19 wherein the dengue virus specific immunogenic component is selected from the list consisting of structural and non-structural proteins, dengue virus particles and fragments thereof, glycoproteins, lipids and carbohydrates derived from the dengue virus.

23. A method of Claim 22 wherein the structural protein is selected from the group including envelope proteins, Pr membrane proteins, and nucleocapsid proteins.

24. A method of Claim 23 wherein the structural protein is an envelope protein.

25. A method of Claim 19 wherein the dengue virus specific immunogenic component is selected from the group comprising dengue virus serotype immunogenic components selected from the group consisting of DEN1, DEN2, DEN3 and DEN4.

26. A method of Claim 19 wherein the method detects exposure to a dengue virus serotype selected from the group consisting of DEN1, DEN2, DEN3 and DEN4.

27. A method of Claim 19 wherein the immunogenic component is an antiidiotypic antibody to an antigen binding site of a dengue virus antibody generated in response to exposure to a component derived from dengue virus or an immunological relative thereof.

28. A method of Claim 27 wherein the binding partner is an antibody expressed in an early stage of a dengue virus infection, during acute or convalescence or derived from recent past infection.
29. A method of Claim 19 wherein the dengue virus relative is selected from the group consisting of yellow fever virus, West-Nile and JE virus.
30. A method of Claim 29 wherein the binding partner antibody is an IgA antibody that is specific to all dengue serotypes (DEN1, DEN2, DEN3 or DEN4).
31. A method of Claim 19 wherein the biological sample is selected from the group consisting of saliva, blood, serum, plasma, cord fluid, B cells, urine and amniotic fluid.
32. A method of Claim 31 wherein the biological sample is saliva, whole blood, serum, plasma or urine.
33. A method of Claim 30 wherein the binding partner is characterized using an anti-IgA antibody.
34. A method of Claim 33 wherein the anti-IgA antibody is conjugated with gold colloid.
35. A solid support for use in a method of Claim 1 or 19, said support comprising dengue specific immunogenic components immobilized on the support.
36. A solid support of Claim 35 selected from the group consisting of a bead, a disc, a magnetic particle or a fiber optic sensor, a microtitre plate, glass slide or biological microchip or a membrane including nitrocellulose membranes, polytetrafluorethylene membrane filters, cellulose acetate membrane filters and cellulose nitrate membrane filters with filter paper carriers.
37. A kit for detecting IgA in a subject that is specific for a dengue virus or an immunological relative thereof or for detecting dengue virus exposure comprising a solid

support including a dengue virus specific immunogenic component or equivalent thereof; or a solid support including a dengue virus specific immunogenic component or equivalent thereof attached to a second support; at least one detection agent conjugated to a reporter group for detecting IgA in a biological sample that forms a complex with the dengue virus specific immunogenic component; and optionally instructions for using said kit to further identify the binding partner of the complex.

38. A kit of Claim 37 wherein the dengue virus specific immunogenic component is immobilized on nitrocellulose membrane.

39. A kit of Claim 38 wherein the dengue virus specific immunogenic component is selected from the group consisting of dengue virus structural and nonstructural proteins, virus particles and fragments thereof, glycoproteins, lipids and carbohydrates derived from the dengue virus.

40. A kit of Claim 39 wherein the structural protein is selected from the group including envelope proteins, Pr membrane proteins, and nucleocapsid proteins.

41. A kit of Claim 40 wherein the structural protein is an envelope protein.

42. A kit of Claim 41 wherein the dengue virus specific immunogenic component is selected from the group including dengue virus serotype immunogenic components DEN1, DEN2, DEN3 or DEN4.

43. A method of assessing the relative risk of one or more subjects being exposed to dengue virus or an immunological relative thereof within a defined location comprising; obtaining samples from a representative population within a defined location; and assessing evidence of exposure of individual members of a sample population to a dengue virus or immunological equivalent thereof by the method comprising the steps of contacting a biological sample from the subject with a mixture of dengue virus specific immunogenic components; and determining the presence of a complex that forms between a binding partner in the biological sample and a dengue virus specific immunogenic component and wherein the presence of the complex is indicative of exposure of the

subject to a dengue virus or equivalent thereof; and assessing the relative risk of exposure within the defined location by characterizing the binding partner in the complex.

44. The method of Claim 43 wherein the binding partner is IgA.

45. The method of Claim 43 or 44 wherein the defined location is a geographical area, housing estate, means of transport or center for medical treatment or assessment.

46. Use of IgA antibodies specific for dengue virus or an immunological relative thereof or a dengue virus antigen or an immunogenic component thereof in the manufacture of a POCT to detect current or recent prior infection or exposure to dengue virus or an immunological relative thereof.