

METHOD AND KIT FOR DETECTING A DENGUE VIRUS INFECTION

FIELD

[0001] The present invention relates to the field of diagnostic assays. More particularly, the present invention provides a multiplex-based Point Of Care Test (POCT) that can detect two contrast dengue infection related components (antigens and antibodies) in a single platform using a single drop of sample from a human subject to test for recent or prior and/or post exposure in the subject to a dengue virus or an immunologically related virus or to a dengue virus antigen. Point of care (POC) diagnostic and sero-evaluation of kits for the detection of dengue virus or viral components or 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 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.

[0004] Dengue is an acute systemic viral disease that has established itself globally in both endemic and epidemic transmission cycles. Dengue virus infection in humans is often inapparent^{1,2} but can lead to a wide range of clinical manifestations, from mild fever to potentially fatal dengue shock syndrome³. The lifelong immunity developed after infection with one of the four virus types is type-specific¹, and progression to more serious disease is frequently, but not exclusively, associated with secondary infection by heterologous types^{2,4}. No effective antiviral agents yet exist to treat dengue infection and treatment therefore remains supportive². Furthermore, no licensed vaccine against dengue infection is available, and the most advanced dengue vaccine candidate did not meet expectations in a recent large

trial^{5,6}. Current efforts to curb dengue transmission focus on the vector, using combinations of chemical and biological targeting of *Aedes* mosquitoes and management of breeding sites². These control efforts have failed to stem the increasing incidence of dengue fever epidemics and expansion of the Dengue is the most rapidly spreading vector borne disease^{7,8}. An estimated 390 million dengue infections occur annually and approximately 2.5 billion people live in dengue endemic countries¹⁰. An early and rapid dengue diagnosis is important due to the size of infection, complexity of differential diagnosis in order to institute lifesaving resuscitation and utility of public health surveillance and monitoring disease control¹¹. Current dengue diagnostics are based on either detection of viral agent (antigen/genome) or antibodies (IgM/IgG) which are produced in host body during infection. Although several commercial kits for dengue diagnosis are available, concerns have arisen with respect to the performance characteristics of these kits^{12,16}.

[0005] 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 non-structural (NS) protein genes.

[0006] 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 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 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 has been developed. At present, no effective dengue vaccine has achieved wide usage.

[0007] 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 viral isolation and characterization, detection of detection of viral components: nucleic acid by amplification technology assay and/or viral protein (NS1) or host component, virus-specific antibodies. After the onset of illness, the virus or viral components 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.

[0008] 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 exposed to dengue virus or other member of flaviviruses. A secondary antibody response is seen in individuals who have had a previous dengue or member of dengue family 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 patient management, epidemiological 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.*, 2008)¹³.

[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 IgM antibodies (6 months, as measured by E/M-specific capture IgM ELISA, or lifelong existence of IgG, 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.

[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 and/or diagnostic centres. 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)¹¹.

[0014] In dengue serological diagnostic, another dengue specific signature antibody molecule "IgA" is also playing an important role by detecting early dengue secondary cases (Yap et al., 2011)¹⁷. Currently one rapid test Assure Dengue IgA RT is available in the market which should high level of sensitivity and specificity (Ahmed et al., 2010 and Tan et al 2011)^{18,19}.

[0015] The combination of Dengue IgM/IgG rapid test with NS1 has complemented and enhanced the total detection infection of dengue infection up to 93% (Fry et al., 2011)²⁰. Two main findings were observed here: a) NS1 detection was overall only modestly sensitive for dengue diagnosis, with sensitivity highest in patients who presented early in their illness and b) a combined NS1 and IgM detection increased the overall sensitivity of dengue diagnostic was found to be 82% of confirmed dengue cases using samples collected in the first four days of fever (Guzman et al 2009)²¹.

[0016] The gap in current dengue diagnostic setting is to fail to detect an early and/or acute dengue secondary infections. This is because of interference of three biomarker based diagnostics: NS1 detection technique unable to detect dengue secondary infection due to the formation immuno-complex (NS1 and previous anti-dengue IgG)²²⁻²⁵, 30% dengue secondary infected patients unable to develop detectable label of IgM and/or the titre of anti-dengue IgG remain below the cut-off point of dengue secondary infection detection criteria²⁶. The detection dengue secondary infection at the early stage is important to address

patient management in order to avoid fatal outcomes like DHF, DSS and death. This important gap in dengue diagnostic is an urgent need to address, as many more dengue secondary infections are coming up in recent years due to the circulation of all four serotypes of dengue viruses all over dengue endemic countries.

[0017] The dramatic increase in the global burden of dengue has spurred increased public and private sector interest in developing improved diagnostics for dengue infections which drive the world community to find 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 with clear differentiation of dengue primary and secondary infections and to monitor possible dengue fatal cases and the efficacy of dengue virus immunity.

SUMMARY

[0009] In an aspect of the present invention, there is provided a method for detecting a flavivirus infection in a human subject, the method comprising:(a)providing at least one flavivirus specific immunogenic component;(b)contacting the at least one flavivirus specific immunogenic component with a biological sample obtained from the subject, the biological sample suspected of containing at least one binding partner capable of hybridising to the at least one flavivirus specific immunogenic component;(c)detecting the presence of a complex that forms between the at least one of the flavivirus specific immunogenic component and the at least one binding partner, which presence indicates a flavivirus infection in the subject.

[00010] By “flavivirus specific immunogenic component”, it is meant to include any component derived from cells or otherwise that has been infected with the dengue virus or an immunological relative thereof, including any molecule that is complementary to the component. These include any flavivirus antigen (which includes any protein, nucleotide and/or part of thereof) or antibody against such a flavivirus agent, or such anti-antibodies. By “complementary”, it is meant to refer to any molecule or component that has the affinity to bind or hybridise together to form a complex.

[00011] By “binding partner”, it is meant to include any molecule or component that can bind or hybridise to a flavivirus specific immunogenic component. Any such binding or

hybridisation method may be used that is known to a person skilled in molecular biology techniques.

[00012] Preferably, detecting the presence of the complex comprising contacting the complex with a molecule capable of hybridising to the at least one binding partner, the molecule further comprising at least one detectably labelled moiety. More preferably, the molecule may be any one selected from the group comprising: (a) a flavivirus antigen, (an anti-human antibody (for example, anti- IgG, IgM, or IgA); and (b) monoclonal and/or polyclonal antibodies against a flavivirus antigen. The flavivirus antigen may be selected from the group comprising of structural and non-structural of flavivirus particles and fragments thereof, glycoproteins, lipids and carbohydrates derived from the flavivirus. The molecule and its detectably labelled moiety may also be known collectively as a reporter group.

[00013] One set of mono/polyclonal antibodies is used for capturing and another set for detecting as conjugated with gold colloid.

[00014] Preferably, the at least one flavivirus specific immunogenic component is any one selected from the group comprising:(a)anti-human antibodies IgG, IgM and IgA against a flavivirus; and(b) monoclonal/poyclonal antibody against a flavivirus.

[00015] Preferably, the at least one binding partner is any one selected from the group comprising:(a)IgA, IgM and IgG antibodies against a dengue virus; and(b)any flavivirus antigen selected from the group comprising: NS1 and envelope protein.

[00016] Preferably, the detectably labelled moiety is a gold colloid.

[00017] Preferably, the flavivirus is selected from the group comprising dengue virus, yellow fever virus, West Nile virus and Japanese Encephalitis virus. More preferably, the flavivirus is a dengue virus serotype selected from the group comprising of DEN1, DEN2, DEN3 and DEN4.

[00018] Preferably, the biological sample is selected from the group comprising of saliva, whole blood, plasma, serum, urine and peripheral blood mononuclear cells.

[00019] The method may further comprise: (a) providing a solid support having a first end and a second end; (b) immobilising the flavivirus specific immunogenic components on the solid support, the flavivirus specific immunogenic components are immobilised separately and spaced apart from each other along the solid support; (c) immobilising the molecule on the second end of the solid support, the molecule capable of binding to the complex; (c) loading the sample on the first end of the solid support, the sample contacting the flavivirus specific immunogenic components as it travels along the solid support in a first direction along the solid support and towards the second end; and (d) loading a buffer on the second end of the solid support adjacent or on the molecule such that the buffer solution solubilise the molecule as it travels along the solid support in a second direction opposite the first direction, wherein the presence of the complex formed on the solid support is detected by the labelled moiety of the molecule. Preferably, the method further provides an absorbent pad adjacent the first end of the solid support, the absorbent pad and the solid support are separated by a separator, wherein removing the separator allows contact between the absorbent pad and the solid support to enhance the flow of the buffer along the solid support in the second direction.

[00020] In an aspect of the present invention, there is provided a method for detecting a flavivirus infection in a human subject, the method comprising:(a) providing at least one anti-human antibody;(b) contacting the at least one anti-human antibody with a sample suspected of containing human antibodies against a flavivirus obtained from the subject; and(c)detecting the presence of human antibodies bound to the at least one anti-human antibody by reacting any bound human antibodies with at least one flavivirus antigen, the flavivirus antigen further comprising at least one detectably labelled moiety,wherein detection of the detectably labelled moiety indicates a flavivirus infection in the human subject.

[00021] Preferably, the anti-human antibody is any one selected from the group comprising of IgA, IgM and IgG.

[00022] Preferably, the detectably labelled moiety is a gold colloid.

[00023] Preferably, the flavivirus antigen is any one selected from the group comprising: structural and non-structural of flavivirus particles and fragments thereof, glycoproteins, lipids and carbohydrates derived from the flavivirus.

[00024] Preferably, the flavivirus antigen is any one selected from the group comprising of NS1 and envelope protein. More preferably, the NS1 and envelope protein are dengue virus antigens although the flavivirus is selected from the group comprising dengue virus, yellow fever virus, West Nile virus and Japanese Encephalitis virus.

[00025] Preferably, the dengue virus serotype is any one selected from the group comprising of DEN1, DEN2, DEN3 and DEN4.

[00026] Preferably, the biological sample is any one selected from the group comprising of saliva, whole blood, plasma, serum, urine and peripheral blood mononuclear cells.

[00027] The method further comprising: (a) providing a solid support having a first end and a second end; (b) immobilising the anti-human antibodies on the solid support, the anti-human antibodies are immobilised separately and spaced apart from each other along the solid support; (c) immobilising the flavivirus antigen on the second end of the solid support, the flavivirus antigen capable of binding to the bound human antibodies; (c) loading the sample on the first end of the solid support, the sample contacting the anti-human antibodies as it travels along the solid support in a first direction along the solid support and towards the second end; and (d) loading a buffer on the second end of the solid support adjacent or on the flavivirus antigen such that the buffer solution solubilise the flavivirus antigen as it travels along the solid support in a second direction opposite the first direction, wherein the presence of the complex formed on the solid support is detected by the labelled moiety. Preferably, the method further provides an absorbent pad adjacent the first end of the solid support, the absorbent pad and the solid support are separated by a separator, wherein removing the separator allows contact between the absorbent pad and the solid support to enhance the flow of the buffer along the solid support in the second direction.

[00028] In an aspect of the present invention, there is provided a method for detecting a flavivirus infection in a human subject, the method comprising:(a)providing monoclonal antibodies against a flavivirus;(b)reacting the monoclonal antibodies against a flavivirus

with a sample suspected of containing a flavivirus antigen obtained from the subject; and(c)detecting the presence of a flavivirus antigen bound to the monoclonal antibodies reacting any bound flavivirus antigen with polyclonal antibodies against a flavivirus antigen, the polyclonal antibodies further comprising at least one detectably labelled moiety, wherein detection of the detectably labelled moiety indicates a flavivirus infection in the human subject.

[00029] Preferably, the monoclonal antibodies are against any flavivirus antigen selected from the group comprising of structural and non-structural of flavivirus particles and fragments thereof, glycoproteins, lipids and carbohydrates derived from the flavivirus. More preferably, the flavivirus antigen is any one selected from the group comprising of NS1 and envelope protein.

[00030] Preferably, the flavivirus is any one selected from the group comprising dengue virus, yellow fever virus, West Nile virus and Japanese Encephalitis virus. More preferably, the flavivirus is dengue virus and the dengue virus serotype is selected from the group comprising of DEN1, DEN2, DEN3 and DEN4.

[00031] Preferably, the detectably labelled moiety is a gold conjugate.

[00032] Preferably, the biological sample is any one selected from the group comprising of saliva, whole blood, plasma, serum, urine and peripheral blood mononuclear cells.

[00033] The method further comprising: (a) providing a solid support having a first end and a second end; (b) immobilising the monoclonal antibodies on the solid support, the monoclonal antibodies are immobilised separately and spaced apart from each other along the solid support; (c) immobilising the polyclonal antibodies on the second end of the solid support, the polyclonal antibodies capable of binding to the bound flavivirus antigen; (d) loading the sample on the first end of the solid support, the sample contacting the monoclonal antibodies as it travels along the solid support in a first direction along the solid support and towards the second end; and (e) loading a buffer on the second end of the solid support adjacent or on the polyclonal antibodies such that the buffer solution solubilise the polyclonal antibodies as it travels along the solid support in a second direction opposite the first direction, wherein the presence of the complex formed on the solid support is detected

by the labelled moiety. Preferably, the method further provides an absorbent pad adjacent the first end of the solid support, the absorbent pad and the solid support are separated by a separator, wherein removing the separator allows contact between the absorbent pad and the solid support to enhance the flow of the buffer along the solid support in the second direction.

[00034] In an aspect of the present invention, there is provided 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:(a)providing at least one flavivirus specific immunogenic component; (b)contacting the at least one flavivirus specific immunogenic component with a biological sample obtained from the subject, the biological sample suspected of containing at least one binding partner capable of hybridising to the at least one flavivirus specific immunogenic component; and(c)detecting the presence of a complex that forms between the at least one of the flavivirus specific immunogenic component and the at least one binding partner 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.

[00035] Preferably, detecting the presence of the complex comprising contacting the complex with a molecule capable of hybridising to the at least one binding partner, the molecule further comprising at least one detectably labelled moiety. The molecule and its detectably labelled moiety may also be known collectively as a reporter group.

[00036] Preferably, the molecule may be any one selected from the group comprising: (a) a flavivirus antigen; (b) an anti-human antibody; and (c) mono/polyclonal antibodies against a flavivirus antigen.

[00037] Preferably, the flavivirus antigen is any one selected from the group comprising of structural and non-structural of flavivirus particles and fragments thereof, glycoproteins, lipids and carbohydrates derived from the flavivirus.

[00038] Preferably, the at least one flavivirus specific immunogenic component is any one selected from the group comprising:(a) anti-human antibodies IgG, IgM and IgA against a flavivirus; (b) a flavivirus antigen, the flavivirus antigen is any one selected from the group comprising: NS1 and envelope protein and (c) monoclonal/polyclonal antibody against a flavivirus.

[00039] Preferably, the binding partners are selected from the group comprising:(a) IgA, IgM and IgG antibodies against dengue virus; and (b) a flavivirus antigen selected from the group comprising of NS1 and envelope protein.

[00040] Preferably, the defined location is a geographical area, housing estate, means of transport or center for medical treatment or assessment.

[00041] In aspect of the invention, there is provided a kit for detecting a flavivirus infection in a human subject, the kit comprising:(a)a solid support with at least one surface-bound flavivirus specific immunogenic component.

[00042] Preferably, the kit comprises a plurality of bound flavivirus specific immunogenic components, the components are immobilised on the solid support separately and spaced apart from each other.

[00043] Preferably, the kit further comprising a molecule capable of hybridising to a complex formed with at least one surface-bound flavivirus specific immunogenic component, the molecule further comprising at least one detectably labelled moiety. More preferably, the molecule comprises:(a) a flavivirus antigen, or(b) polyclonal antibodies against a flavivirus antigen.

[00044] Preferably, the kit provides for a sample that is obtained from the subject to be loaded on a first end of the solid support, and a buffer for solubilising the molecule to be loaded on a second end of the solid support opposite the first end.

[00045] Preferably, the polyclonal antibodies are against an NS1 or envelope protein of a dengue virus.

[00046] Preferably, the at least one flavivirus specific immunogenic component is any one selected from the group comprising: (a) anti-human antibodies IgG, IgM and IgA against a flavivirus; (b) a flavivirus antigen, the flavivirus antigen is any one selected from the group comprising: NS1 and envelope protein; and (c) monoclonal and/or polyclonal antibody against a flavivirus.

[00047] Preferably, the kit comprises: (a) a first reaction chamber, the first reaction chamber comprising a solid support with at least one surface-bound antibodies IgG, IgM and IgA against a human antibodies; and (b) a second reaction chamber, the second reaction chamber comprising a solid support with at least one surface-bound monoclonal/polyclonal antibody against a dengue virus, the dengue virus antigen selected from the group comprising of NS1 and envelope protein. Preferably, the reaction chambers have windows allowing one to view reaction or results in the chamber.

[00048] Preferably, the solid support is selected from the group comprising 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.

[00049] Preferably, the solid support is a nitrocellulose membrane.

[00050] Multiplexing of Dengue antibodies (IgA/IgM/IgG) and antigens (NS1 & Envelope protein (E-protein or Ep) could be an ideal rapid diagnostics to over-come the current early and accurate diagnostic challenge in dengue endemic world.

[00051] The present invention provides a MULTIPLEX based "point of care test" (POCT) for detection and differentiating of a subject into primary and secondary infections and/or acute or recent most infection who has had exposure to a dengue virus or immunological relative thereof or who is caring anti-dengue IgG against a dengue virus antigen due to previous exposure. The method is predicted in part on the simultaneous detection of two contrast binding partners: antibodies (generally IgA, IgM and IgG antibodies) which is specific for a dengue virus or an immunological relative thereof and dengue antigens (non-structural and structural proteins: NS1 and Ep) which produced by dengue infected cells during dengue infection cycle. The POCT comprises contacting a

biological sample from the subject with a mixture of dengue virus specific immunogenic and/or immunoglobulin components; and determining the presence of a complex that forms between a binding partner (e.g. IgA/IgM/IgG antibodies) and viral proteins (NS1& Ep) in the biological sample and a dengue virus specific immunogenic component; and characterizing the binding partner in the complex with an anti-binding partner specific antigen (e.g. NS1 and/or Ep antibodies and dengue antigen) or antibody (dengue NS1 or envelope proteins) conjugated with gold colloid using double chamber reverse flow technology (as disclosed in PCT/SG2014/000070, the contents of which are incorporated herein).

[00052] In an embodiment, the POCT relies on two separate components: i. identification of dengue antibodies (IgA or IgM or IgG or all antibodies), ii. detection of non-structural-1 and envelope proteins (NS1&Ep) specific for a dengue virus or its immunological relative in the biological sample.

[00053] 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 separately anti-human antibodies (IgA, IgM & IgG) and anti-den-NS1 and Ep antibodies (preferably monoclonal antibody), which subsequently captures binding partners identified as anti-dengue (IgA or IgM or IgG or all three) and dengue NS1 and Ep from a biological sample such as whole blood, serum, plasma, saliva, urine or tissue fluid of subjects to be tested for exposure to dengue virus and finally visualized by dengue antigen and anti-NS1 and Ep antibodies conjugated with gold particles.

[00054] Accordingly, the POCT of the present invention shows greater specificity and sensitivity using all three host-based biomarkers (IgA, IgG and IgM) and one virus-based biomarker (NS1 & Ep) compared to other conventional and most currently used dengue IgM/IgG and Den-NS1 rapid as well as dengue ELISAs (IgM, IgG and NS1) by providing a lateral flow double chamber rapid test platform. The Double window (reaction chamber) reverse flow platform (as disclosed in PCT/SG2014/000070, the contents of which are incorporated herein) that can specifically identify dengue antibodies (e.g. IgA, IgM and IgG) and antigens (NS1& Ep) produced during dengue infection. Alternatively, the present invention may only require a single window (reaction chamber) having a reverse flow platform) that can specifically identify dengue antibodies (e.g. IgA, IgM and IgG) and

antigens (NS1& Ep) produced during dengue infection. In a particular embodiment, the POCT identifies dengue infections and differentiated into acute and convalescence as well as dengue primary and secondary infections.

[00055] 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 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.

[00056] In an embodiment, in one of the windows the biological sample is applied to a nitrocellulose membrane previously loaded with anti-human immunoglobulins. Conveniently, the immunoglobulin components are derived from animals previously immunized with human immunoglobulin separately. The complex formed by an immunogenic component of the anti-human immunoglobulins with their binding partner then be detected using a detection agent that contains a reporter group, wherein the detection agent specifically binds to the component/binding partner complex. In the second window, dengue NS1 and EP antigens from patients samples binds with reactive monoclonal antibody previously coated on the nitrocellulose membrane. The complex formed either with dengue NS1 antigen and/or EP protein with their binding partner (monoclonal/polyclonal antibodies) then be detected using a detection agent second set of mono/polyclonal antibodies specific to NS1/Ep that contains a reporter group, wherein the detection agent specifically binds to the component/binding partner complex.

[00057] More particularly, in one of the windows, the binding partners are human IgA, IgM and IgG and the detection agent is dengue antigen conjugated with colloidal gold. In other diagnostic window, the binding partner are dengue NS1 protein and/or dengue envelope protein (Ep) and the detection agent is anti-NS1 and EP polyclonal/monoclonal antibodies conjugated with gold colloid.

[00058] Yet another aspect of the present invention provides a POCT kit in a cassette form 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; 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 (IgA, IgM & IgG) and dengue antigens (NS1 & Ep). In that aspect, the reporter group generally comprises dengue antigen and/or antibodies conjugated with gold particles and the platform is double chamber reverse flow technology (as disclosed in PCT/SG2014/000070, the contents of which are incorporated herein).

[00059] 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 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 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. In an embodiment, the binding partners are dengue antibodies (IgA, IgM & IgG) and dengue antigens (NS1 & Ep)

[00060] 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.

[00061] The present invention is further directed to the use of antibodies and antigens 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

[00062] In order that the present invention may be fully understood and readily put into practical effect, there shall now be described by way of non-limitative examples only preferred embodiments of the present invention, the description being with reference to the accompanying illustrative figures.

[00063] In the Figures:

[00064] Figures 1 to 5 are schematic diagrams showing test principle of each test line method according to an embodiment of the present invention;

[00065] Figure 6 is a schematic diagram of the device (also known as “Double Chamber Reverse Flow Device / Cassette” or “Den 4-1 RT”) according to an embodiment of the present invention indicating positions of test and control lines (G stand for IgG, M stands for IgM, A stands for IgA, C stands for control and NS1 stands for dengue non-structural protein-1);

[00066] Figure 7 is an external view of Double Chamber Reverse Flow Cassette;

[00067] Figure 8 shows the assembly of the Den 4-1 RT showing different components and their positions on the device using double chamber reverse flow technique;

[00068] Figure 9 is a schematic diagram of assay procedure of DEN 4-1 RT: sample addition, addition of buffer to release gold and removal of pull tab;

[00069] Figure 10 shows steps in assay procedure of DEN 4-1 RT using whole blood sample;

[00070] Figure 11 shows steps in assay procedure of DEN 4-1 RT using serum/plasma/saliva samples;

[00071] Figure 12 is a schematic diagrams of test interpretation of DEN 4-1 RT based on test and control lines appear following assay;

[00072] Figure 13 shows examples of some dengue suspected samples tested by DEN 4-1 RT;

[00073] Figure 14 shows a Rapid Test Reader for reading and interpretation of DEN 4-1 RT;

[00074] Figure 15 is a schematic drawing of a kit according to an embodiment of the present invention;

[00075] Figure 16 is a schematic drawing of using a kit according to embodiment of the present invention;

[00076] Figure 17 are charts showing the interpretation of the results; and

[00077] Figure 18 is a schematic drawing showing a cross section of the kit according to an embodiment of the present invention

DETAILED DESCRIPTION

[00078] All scientific citations, patents, patent applications and manufacturer's technical specifications referred to hereinafter are incorporated herein by reference in their entirety.

[00079] 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.

[00080] 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.

[00081] 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.

[00082] 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".

[00083] 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 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 partners such as anti-dengue antibodies (IgA, IgM & IgG) and dengue antigens (NS1 & Ep) in a subject that are specific for a dengue virus or immunological thereof and the test 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 partners in the biological sample and a dengue virus specific immunogenic component; and characterizing the binding partners in the complex with anti-dengue antibodies preferably monoclonal antibodies conjugated with gold colloid and/or dengue immunogen conjugated with gold colloid.

[00084] This method is specific for the identification of dengue virus-specific binding partner molecules. In a particular embodiment, binding partners is dengue antibodies (IgA, IgM & IgG) and dengue non-structural antigen 1 (NS1). Accordingly, the immunogenic components may be dengue-specific antibodies immunogenic components. The use of both antibodies and antigen specific to dengue viruses renders the POCT specific for dengue virus or its immunogenic components in the biological sample which are specific for dengue virus or its immunological relative. However, other binding partners may also be employed.

[00085] Referring to Figures 1 to 5, there is provided test principles of the assay and there is provided a method for detecting a flavivirus infection in a human subject. The method involves providing at least one human antibody specific component or dengue virus specific immunogenic component and indicated as 10. This component may be anti-antibodies IgG, IgM and IgA, and/or anti-antigen monoclonal antibody that is against a dengue antigen (examples include antibodies against NS1 and Envelope protein of dengue virus). As will be described later, the dengue virus specific immunogenic component 10 may be sprayed onto a solid support for use in a device / test kit. In an embodiment of the present invention, the various components mentioned in this paragraph may be sprayed onto the solid support at pre-determined locations or portions on the support. This is shown in the device indicated as 20 in the Figures 1 to 5. Various markings indicate where on the solid support (i.e. device) the components are sprayed on.

[00086] In carrying out the assay, a biological sample is obtained from a human subject who is suspected of having a dengue virus infection. The present invention may also be used to determine if any antibodies present in the subject's sample could be present due to a past exposure to the virus.

[00087] The biological sample is suspected of containing at least one binding partner indicated as 30 capable of hybridising to the at least one flavivirus specific immunogenic component. Such binding partners present in the sample may include any antibody against the dengue virus (for example, IgG, IgM and/or IgA) or dengue virus antigen (NS1 antigen or Envelope protein antigen). The assay involves allowing reaction or contact between the immobilised anti-human antibodies or flavivirus specific immunogenic component and the binding partners (if present) in the sample. If there is any binding or hybridisation, a complex is formed. Detecting the presence of the bound complex that forms between the flavivirus

specific immunogenic component and binding partner indicates a flavivirus infection in the test subject.

[00088] Detecting the presence of the bound complex (10 + 30) comprises contacting the bound complex with a molecule indicated as 40 that is capable of hybridising to the bound complex, or ie. the binding partner 30. The molecule 40 may be (a) a dengue virus antigen (in the case where the binding partner 30 is an antibody against a dengue antigen), or (b) polyclonal/monoclonal antibodies against a flavivirus antigen (in the case where the binding partner 30 is a dengue antigen). The molecule 40 further comprises at least one detectably labelled moiety, and may also be known collectively as a reporter group. The detectably labelled moiety may be a gold colloid.

[00089] Accordingly, one aspect of the present invention provides a method for detecting a binding partners in a subject that are 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 partners in the biological sample and a dengue specific immunogenic component; and characterizing the binding partners in the complex with antibody specific for the binding partner and/or antigen are conjugated with gold colloid.

[00090] In another embodiment, the present invention provides a method both antibodies and antigen in a subject that are 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 specific immunogenic component; and characterizing the binding partner in the complex with antibody and/or antigen conjugated with gold colloid.

[00091] In a further aspect, the present invention provides a POCT using double chamber 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.

[00092] In a particular embodiment, the binding partners are dengue antibodies (IgA, IgM & IgG) and antigens (NS1 & Ep).

[00093] Hence, in a particular aspect, the present invention provides a POCT double chamber reverse flow rapid test for dengue reactive antibodies (IgA, IgM & IgG) and antigens (NS1 & Ep) which in one particular embodiment, uses serum, whole blood and 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 simultaneous detection of dengue specific antibodies (IgA, IgM & IgG) and antigens (NS1 & Ep) compared to the detection of dengue-specific antibodies using ELISA techniques and/or other rapid test platforms. The POCT of the present invention is **ONE STOP** diagnostics at the primary health care level where molecular and ELISA based dengue diagnostic assays are not feasible.

[00094] 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 addition to this present technique will detect dengue at an early stage of infection (NS1and/or Ep) and differentiate into primary and secondary infections 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: both antibodies and antigen to dengue or an immunological relative thereof using double chamber reverse flow technology. The binding partners are generally subject-derived binding partners such as, but not limited to, immuno-interactive molecules. The most useful immuno-interactive molecules are antibodies (IgA, IgM & IgG) and antigens (NS1 & Ep). 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.

[00095] The present invention specifically utilizes double chamber reverse flow technique, where in one chamber anti-human antibodies (IgA, IgM & IgG) are sprayed separately or loaded onto the nitrocellulose for capturing of human antibodies (IgA, IgM & IgG) while mixture of anti-NS1 and anti-Ep monoclonal antibodies specific dengue three

serotypes (DEN-NS1-Serotype 1, DEN-NS1-Serotype 2 & DEN-NS1-Serotype 3) are sprayed in second chamber. Dengue reactive antibodies from the biological samples of the subject exposed to dengue infection captured along non-reactive antibodies in respective test line formed complex (IgA, IgM & IgG) is later detected by dengue antigen conjugated with gold particle. In the second chamber dengue specific antigens (NS1 & Ep) are captured by anti-dengue (NS1 and Ep) monoclonal/polyclonal antibodies and formed immunocomplex and detected by second set of anti-dengue NS1/Ep specific monoclonal/polyclonal antibodies conjugated with gold colloid. The multiplex based dengue combo (antibodies and antigen) technique will provide the presence or absence of all possible signature molecules so far established (antibodies: IgA, IgM & IgG) and antigens-NS1/Ep) in one platform and can be useful at any level of health care system which is called DEN 4-1 point of care (POC) testing.

[00096] Accordingly, the present invention shows greater sensitivity and specificity compared to other conventional and most currently used antibody and antigen combo rapid test where two antibodies (IgM & IgG) and Dengue antigens (NS1) are detected in two separate cassettes with two different volume of samples (10ul for antibodies and 100ul for NS1).

[00097] Therefore, the present invention provides a specific, rapid and economical MULTIPLEX based POCT with the detection of major dengue specific signature molecules with single drop of suspected samples (whole blood, serum/plasma and saliva) using dengue specific virus antigen and/or monoclonal antibodies. The test is conducted at room temperature (RT). It is a convenient technique for specific antibody detection. The POCT utilizes double chamber reverse flow platform in a cassette format coated with dengue specific monoclonal antibodies and/or anti-human antibodies and gold conjugated dengue specific antigen and/monoclonal/polyclonal antibodies. Furthermore, the POCT is used for testing a single sample for all five signature molecules (IgA, IgM, IgG, NS1 and Ep) with high sensitivity and specificity.

[00098] 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.

[00099] 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.

[000100] 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)³¹. 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.

[000101] 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 behaviour 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)²⁸⁻²⁹. 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)³⁰.

[000102] 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.

[000103] 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)²⁷.

[000104] 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.

[000105] Grade I: Fever with non-specific constitutional symptoms, and the only haemorrhagic manifestation is a positive tourniquet test and or easy bruising.

[000106] Grade II: In addition to the manifestation in grade I one spontaneous bleeding in the forms of skin or other haemorrhages.

[000107] 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.

[000108] Grade IV: Profound shock with undetectable blood pressure or pulse (WHO, 1997).

[000109] 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).

[000110] 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).

[000111] 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%.

[000112] 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 lifelong immunity to that serotype but only temporary protection against other serotypes.

[000113] The nucleoplasmid 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.

[000114] 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.

[000115] 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.

[000116] 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.

[000117] 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.

[000118] 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 antibodies (IgA, IgM & IgG) and antigens (NS1 & Ep) molecule.

[000119] 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.

[000120] 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.

[000121] 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.

[000122] 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 include, but are not limited to, Fab fragments, F(ab')₂ fragments, and Fc fragments.

[000123] In a particular embodiment, the binding partner is an antibody and/or antigen more particularly is a dengue virus-specific three antibodies (IgA, IgM & IgG) and antigens (NS1 & Ep) molecules specific to dengue antigen.

[000124] 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 partners. Such biological samples may be selected 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.

[000125] 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.

[000126] It should be noted that a biological sample might also be devoid of a binding partners 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.

[000127] 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.

[000128] 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, antibodies, particularly IgA, IgM & IgG 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 them and anti-human antibodies coated onto membrane. Simultaneously dengue virus specific non-structural antigens (NS1 & Ep) which is one of the early signature molecules will also detected using two sets of NS1 and Ep specific monoclonal/polyclonal antibodies (capturing and detecting).

[000129] Conveniently, the dengue virus antigen is either envelope recombinant protein and/or non-structural protein-1 (NS1). Monoclonal antibodies against NS1 and Ep are produced in mouse model against three major dengue circulating serotypes (serotype-1, serotype-2 and serotype-3) and reactive with all four serotypes.

[000130] However, the present invention is not to be limited solely to use E-protein and/or NS1 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 develop during the course of dengue virus exposure/infection may also be used.

[000131] Immuno-complexes form between antibodies, particularly IgA, IgM & IgG specific to dengue virus or immunological relative thereof and sets of NS1 and Ep capturing monoclonal/polyclonal antibodies and NS1 and Ep antigens from dengue suspected samples (except saliva).

[000132] The method of the present invention is to 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 infection/immunization.

[000133] The complex may comprise one or more binding partners bound to one or more components derived from dengue virus or an equivalent thereof and all possible signature molecules related with dengue virus infection like antibodies (IgA, IgM & IgG) and dengue antigens (NS1 & Ep) are captured during test assay procedure.

[000134] A second binding immunological molecule is also loaded on the nitrocellulose membrane in both chambers called goat anti-mouse IgG/rabbit anti-goat IgG and are acted

as a control line to indicate the validity of the assay.

[000135] 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 immuno-complexes.

[000136] The dengue virus specific immunogenic components and the biological sample are contacted so that a complexes may form between the components and a binding partners present within the biological sample. Conveniently, immunogens specific to human antibodies (IgA, IgM & IgG) in one chamber and dengue specific antigens (NS1 & Ep) against monoclonal/polyclonal antibodies, captured human antibodies against specific anti-human antibodies and NS1and Ep antigens by specific monoclonal antibodies and forms immuno-complexes.

[000137] Particularly, the specific binding partners are antibodies and/or antigen or fragments thereof present in the biological sample. These will only be present when the subject has been exposed/immunized to the dengue virus.

[000138] Particularly, the complex forms between antibodies (IgA, IgM & IgG), specific for the member of the dengue virus antigen or equivalent thereof are captured anti-human antibodies (IgA, IgM & IgG) loaded onto nitrocellulose membrane are indicative of dengue virus specific signature molecules in the sample and hence recent or prior exposure.

[000139] Another dengue early biomarkers (NS1 & Ep) presence in dengue suspected samples is captured by anti-NS1and/or Ep specific antibodies against major three dengue serotypes (serotype-1, serotype-2 & serotype-3) previously loaded onto nitrocellulose membrane are indicative of dengue virus specific signature molecules in the sample and hence indicate recent exposure.

[000140] In one embodiment, the present invention relies upon the detection of dengue virus specific binding partners: antibodies (IgA, IgM & IgG) and antigens: NS1and Ep present in the biological sample that are specific for a component of anti-human antibodies and anti-dengue NS1and Ep derived from goat IgG (anti-NS1 & Ep antibodies) and mouse IgG (anti-NS1/Ep monoclonal antibodies). The complex may comprise one or more binding partners bound to one or more components derived from dengue virus or an equivalent

thereof. However, it is the identification of all three anti-dengue antibodies and/or any one of them and dengue antigens (NS1 & Ep) to the complexes that is indicative of current or prior exposure to dengue infection in this present invention.

[000141] A non-immunological complex is formed with biological samples (whole blood, serum/plasma and saliva) along the control line loaded with goat anti-mouse IgG and/or rabbit anti-goat IgG does not indicate the exposure of dengue virus to the subject but does provide the validity of the assay.

[000142] 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 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 partners present in the biological sample and immunogenic components specific to dengue virus; and optionally characterizing the binding partner in the complex to correlate the binding partner to exposure to the dengue virus; the support comprising dengue virus specific immunogenic components immobilized on the support.

[000143] 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.

[000144] 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 and/or antibodies on the nitrocellulose membrane following spraying by BioDot machine. Immobilization by adsorption of antigen and/or antibodies is onto the membrane. 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. Four lines are sprayed in antibody detection chamber while three lines are sprayed onto membrane in antigen detection chamber.

Following spraying membrane is allowed dry in a dryer and finally is blocked by blocking buffer.

[000145] Anti-human antibodies (polyclonal and/or monoclonal) against IgA, IgM & IgG are 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.

[000146] A mixture of anti-dengue NS1 and Ep antibodies (monoclonal/polyclonal) against specific to dengue serotypes 1, 2 & 3 are loaded/sprayed separately onto the membrane and allowed to contact with biological samples which is passed over the membrane once added onto the sample pad, which allow the stable formation of immuno-complex.

[000147] Detecting the immuno-complexes 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.

[000148] 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.

[000149] 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 conjugated with enzyme, gold particle/colloid which permits detection of the complexes with the binding partners.

[000150] In an embodiment, the methods as herein described involve the use anti-

human antibodies (polyclonal and/or monoclonal) against IgA, IgM & IgG are 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. The complex formed by a component of anti-human specific antibodies and the binding partners bind against reporter group and specifically binds to the component/binding partner complex such as specific dengue antibody against anti-antibody (IgA with anti-IgA, IgM with anti-IgM and IgG with anti-IgG). Such detection agent may comprise, for example, an anti-antibody or other agent that specifically binds to the binding partner, such as an antiimmunoglobulin.

[000151] In an embodiment, the methods as herein described involve the use a mixture of anti-dengue NS1 antibodies (monoclonal/polyclonal) and Ep specific antibodies (monoclonal/polyclonal) against specific to dengue serotypes 1, 2 & 3 are loaded/sprayed onto the membrane and 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. The complex formed by a component of anti-dengue NS1 specific monoclonal antibodies and Ep mono/polyclonal antibodies and the binding partners (NS1&Ep) bind against reporter anti-dengue NS1 and Ep antibodies (serotype 1 with monoclonal antibody and so on). Such detection agent may comprise, for example, an antigen and antibody or other agent that specifically binds to the binding partner, such as an anti-immunoglobulin.

[000152] In an embodiment, the detection reagent is an antibody or an antigen-binding fragment thereof, capable of binding to the binding partners of the biological sample. Antibodies may be prepared by any of a variety of techniques known to those of ordinary skill in the art (See, for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988).

[000153] The second set of antibody and/or antigen 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 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.

[000154] Generally, the antibody either is NS1 monoclonal/polyclonal antibodies or Ep monoclonal/polyclonal recognizing different epitopes than those of capturing monoclonal antibodies sprayed are conjugated with gold colloid and therefore detects NS1 and/or Ep antigen captured by NS1 or Ep monoclonal/polyclonal antibodies.

[000155] Another component would be dengue antigens specific to dengue antibodies are conjugated with gold colloid and therefore detects antibodies (IgA, IgM & IgG) which ever presence in dengue suspected samples and captured by anti-human antibodies (IgA, IgM & IgG) .

[000156] 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 antibodies and/or antigen. 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.

[000157] 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 and/or captured 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.

[000158] The detection agent is either dengue antigen (for detecting antibodies) and dengue antibodies (monoclonal/polyclonal antibodies). Preferred reporter groups include those groups recited herein. The detection agent is then released from either side of gold-pad over the membrane where immunocomplex is formed with dengue virus antigen (NS1 and Ep in antigen detection window) and with dengue antibodies (IgA, IgM & IgG) in antibody detection window. An appropriate amount of time may 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 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 reporter group (commonly a radioactive or fluorescent group or an enzyme).

[000159] 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 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 10³ L/mol. The binding constant may be determined using methods well known in the art.

[000160] 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.

[000161] 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, a standardised sample comprising a detectable level of binding agent, and a secondary detection reagent, as herein described.

[000162] The method and test 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 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 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 dengue antigen and binding partners (antibodies) in the infected patient or patient derived it from his/her previous infection/infections.

[000163] In another aspect of the present invention there is provided a POCT kit for detecting both dengue antibodies (IgA, IgM & IgG) in a subject that is specific for a dengue virus or equivalent thereof or for detecting dengue virus antigens (NS1 & Ep) which grow in host cells comprising: a solid membrane support including a capturing of human

antibodies (IgA, IgM & IgG) specific to dengue virus specific immunogenic component or equivalent thereof; or a solid support including a dengue virus specific monoclonal antibodies or equivalent thereof attached to a second support; at least two 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.

[000164] Optionally, the test will also include additional parts such as plastic dropper, buffers, test device (cassette) and instructions as are necessary for conducting the method.

[000165] 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 of two different partners in a biological sample: one partner interact with anti-human antibodies (IgA, IgM & IgG) and second partner interact with dengue NS1 and Ep antigens specific monoclonal / polyclonal antibodies. The result is an indication, by the presence of dengue virus specific binding partners: antibodies (IgA, IgM & IgG) and antigens (NS1 & Ep) 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 dengue virus specific antibodies and/or antigens (NS1 & Ep) and finally detected by gold conjugated detecting partners: antigen (for antibodies) and monoclonal/polyclonal antibodies (for NS1 and Ep). The kit may also comprise reagents, reporter molecules capable of providing detectable signals and optionally instructions for use.

[000166] The kit may be a modular kit comprising one or more members wherein at least one member is a solid support comprising loaded/sprayed with capturing agents: anti-human antibodies (IgA, IgM & IgG), dengue envelope protein (Ep) and NS1 specific monoclonal/polyclonal antibodies.

[000167] 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 antibodies reactive components and dengue antigen for use in the detection of exposure to the dengue virus. In one embodiment, a nitrocellulose membrane is used to load human antibodies capturing agent (anti-human antibodies) and dengue viral specific

monoclonal/polyclonal antibodies (NS1 and Ep), either for use as an immobilized anti-dengue virus antibodies reactive anti-human antibodies and dengue antigens (NS1 & Ep) reactive to monoclonal antibodies specific to NS1 and Ep antigen as separate line blots in different reaction chamber. Preferably the membranes include components selected from anti-human antibodies against human IgA, IgM & IgG in one chamber (derived from either goat and/or mouse) and dengue specific NS1 and Ep monoclonal/polyclonal antibodies derived from hybridoma cells fused with mouse immune cells (B-cell) or other species of animals previously immunized against NS1 and Ep protein separately.

[000168] The solid support may also be a microtitre plate, glass slide or biological microchip wherein the components of capturing agents are immobilised. These solid supports can then be subjected to the biological sample to detect flavivirus exposure. Preferably nitrocellulose membrane is used to attach capturing agents by spraying onto the membrane.

[000169] In yet another aspect, the present invention also provides a method of 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 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 partners 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.

[000170] 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 equivalent thereof.

[000171] The method or technique of the present invention allows for the epidemiological study or sero-surveillance of outbreaks of infection caused by dengue 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.

[000172] 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 - anti-dengue virus specific antibodies (IgA, IgM & IgG) and dengue antigens (NS1 & Ep). Alleviation of one of the dengue specific antibodies titre or its presence in an early phase of infection particularly IgG in presence of dengue antigens NS1 and/Ep may be the indication of secondary infection and thus, help the monitoring of the subsequent phases of dengue virus infection like DHF or DSS.

[000173] Further, the technique of the present invention provides a means for 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.

[000174] The present invention is further directed to the use of 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.

[000175] The present invention is now described with reference to the following non-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

Test Platform

[000176] The double chamber reverse flow technique (described in PCT/SG2014/000070) is used for the development of the present test kit: the “DEN 4-1 Rapid Test”. The device and positions of test and control lines as follows:

[000177] Figure 6 shows a schematic diagram of a device that uses the double chamber reverse flow device. As can be seen, there are two reaction chambers having windows: an antibodies chamber and an antigen chamber. Markings on the device indicate positions of test and control lines (G stand for IgG, M stands for IgM, A stands for IgA, C stands for control and NS1 stands for dengue non-structural protein-1 and Ep stands for envelope protein).

Test reagents

[000178] **Anti-human antibodies:** Obtained from Immunology Consultants LLC, USA (1mg/ml) and used for the capturing of human antibodies (IgA, IgM & IgG)

[000179] **Pan anti-DEN-NS1 monoclonal antibodies:** Obtained from Immunology Consultants LLC, USA (1mg/ml) and used for the capturing of NS1 antigen from suspected dengue samples.

[000180] **Pan anti-DEN NS1Goat polyclonal antibodies:** Obtained from Immunology Consultants LLC, USA (1mg/ml) and used for the detection of captured NS1 antigen from suspected dengue samples.

[000181] **Pan DEN envelope protein goat polyclonal antibodies:** Obtained from Immunology Consultants LLC, USA (1mg/ml) and used for the detection of captured envelope antigen from suspected dengue samples.

[000182] **Pan DEN envelope protein monoclonal antibodies:** Obtained from Immunology Consultants LLC, USA (1mg/ml) and used for the detection of captured envelope antigen from suspected dengue samples.

[000183] **DEN serotype specific (1, 2&3) envelope protein monoclonal antibodies:** Obtained from Immunology Consultants LLC, USA (1mg/ml) and used for the capturing envelope antigen from suspected dengue samples.

[000184] **Rabbit anti-goat IgG:** Obtained from Immunology Consultants LLC, USA (1mg/ml) and used for the detection of control line in NS1 detection chamber of the cassette.

[000185] **Goat anti-mouse IgG:** Goat anti-mouse IgG is obtained from Bhat Bio-Teck India (P) Ltd and used for the detection of control line in dengue antibody chamber of the cassette.

[000186] **Dengue antigen:** Dengue NS1 antigens (Immunology Canada) and Dengue envelope proteins (Bhat Bio-Tech India (P) Ltd) are used for detection of dengue specific antibodies (IgA, IgM & IgG) from dengue suspected samples.

[000187] **Dengue Envelope protein Gold conjugate:** Developed using 40um gold colloid at Bhat Bio-Tech India Pte Ltd according to their in-house technology.

[000188] **Dengue NS1 polyclonal antibodies Gold conjugate:** Developed using 40um gold colloid at Bhat Bio-Tech India Pte Ltd using goat pan dengue NS1 polyclonal antibodies according to their in-house technology.

[000189] **Serum/Plasma samples:** A total 25 serum/whole blood samples positive and 11 dengue negative tested by NS1 antigen or anti-dengue capture IgM/IgG ELISAs were used as dengue positive and negative samples. These samples were obtained from Bhat Bio-Tech India Pte Ltd.

[000190] **Saliva samples:** Total 5 saliva samples collected from healthy donors were used as negative as well as spiked samples with dengue positive sera.

[000191] Whole blood: Five whole blood samples from healthy donors were used for validation of test platform.

[000192] Dengue rapid diagnostics: One type of dengue rapid diagnostic kits available in the market in the form of cassette was used as competitor kits to validate and evaluate DEN 4-1 RT.

Table-1. Optimization goat anti-human antibodies (serial dilution)

Name	Anti-human IgA (mg/ml)	Anti-human IgM (mg/ml)	Anti-human IgG (mg/ml)
	1.0	1.0	1.0
1	0.75	0.75	0.75
2	0.50	0.50	0.50
3	0.35	0.35	0.35
4	0.25	0.25	0.25

Table-2 Optimization anti-dengue NS1 monoclonal/polyclonal antibodies (serial dilution)

Name	NS1-serotype-1 (mg/ml)	NS1-serotype-2 (mg/ml)	NS1-serotype-3 (mg/ml)	NS1 polyclonal antibodies
1	0.75	0.75	0.75	0.50
2	0.50	0.50	0.50	0.35
3	0.35	0.35	0.35	0.25
4	0.25	0.25	0.25	0.15

[000193] One of the chambers of the device is sprayed with anti-human antibodies (IgA, IgM & IgG) were separately at positions indicated in Figure 6 for DEN 4-1 Rapid Test striping on 4 pieces of membrane (30 cm each) and goat anti-mouse IgG is used as control

line. Following spraying, the membrane is dried, blocked using blocking buffers and dried again and then laminated, cut and assembly for test.

[000194] The second chamber which will detect dengue NS1 and Ep antigens of the device is sprayed with a mixture of anti-dengue NS1/Ep monoclonal antibodies (serotype-1, serotype-2 & serotype-3) at positions indicated in Figure 6 for DEN 4-1 Rapid Test. Total four pieces of membrane (30 cm each) were sprayed along with goat anti-mouse IgG/Rabbit anti-goat IgG as control line. Following spraying, the membrane is dried, blocked using blocking buffers and dried again and then laminated, cut and assembly for test.

Table-3. Optimization anti-dengue envelope protein monoclonal/polyclonal antibodies

Name	Den serotype-1 (mg/ml)	Den serotype-2 (mg/ml)	Den serotype-3 (mg/ml)	Den-polyclonal antibodies
1	0.70	0.70	0.70	0.50
2	0.50	0.50	0.50	0.35
3	0.25	0.25	0.25	0.15

[000195] **Optimization of goat anti-mouse IgG:** Goat anti-mouse IgG (Bhat Bio-Tech India) was diluted in 1 x PBS according to Table 3 mentioned below. A minimum volume of 500µl was made for each concentration for the process of optimisation.

Table-4 Optimization goat anti-mouse IgG

Name	Goat anti-mouse IgG mg/ml
Concentration-1	1.0
Concentration-2	0.75
Concentration-3	0.50
Concentration-4	0.25

[000196] Figure 7 also shows the device/test kit of the present invention. It shows the first and second reaction chambers having a “test window” each. In addition, it also shows a “sample window” for allowing a sample from a human subject to be input into the device, and “gold windows” on either end of the device for allowing other reagents (e.g. the reporter group/molecule with gold conjugate) to be input into the device. The device/test kit or assembly cassette as shown in Figures 6 and 7 was used for the optimization of device against anti-human antibodies (IgA, IgM & IgG), dengue NS1 and/or E-protein monoclonal/polyclonal antibodies and goat anti-mouse/rabbit goat anti- IgG sprayed/loaded

onto the membrane-using panels of dengue positive (strong or weak) and negative serum samples. For the control lines with 2.5 to 3.0 intensity at both windows are considered as optimum level of reaction while with saliva the intensity was >3.0 . For the optimization of T-lines with strong positive serum 2.5, moderate 1.5 to 1.0, weak positive 0.5 to 0.25 while for negative should not have any visible line and considered as optimum level of reactions.

[000197] Figure 8 shows the internal view of the device or cassette according to an embodiment of the present invention showing different components of device using double chamber reverse flow technique.

[000198] Following optimization of anti-human antibodies, dengue NS1 and Ep monoclonal/polyclonal antibodies and goat anti-mouse IgG, 125 cassettes were prepared for the study of sensitivity, specificity, positive predictive value and negative predictive value.

Assay procedure

[000199] The assay procedure using the device/test kit/cassette of the present invention ("DEN 4-1 RT") for dengue suspected sample is shown in Figure 9 and explain details in different categories of samples (whole blood, serum/plasma and saliva). The steps 1, 2 and 3 are labelled correspondingly in the Figure 9.

Whole Blood Sample:

[000200] **Step 1**-Add two drops (60 μ l) of whole blood sample to the samples square well followed by 1 drop of chase buffer to the same well.

[000201] **Step 2** – Sample absorbed by sample pad and once sample released over the membrane (the nitrocellulose membrane solid support), three drops of buffer was added to the gold-pad at the both windows.

[000202] **Step 3** – Once gold conjugate released over the membrane from both windows pulled out the plastic tab and added another drop of buffer onto the sample pad square and started timing. Read result after 20 minutes. A plastic tab separates sample pad from absorbance pad so that sample will not be absorbed when added but once removed it allows to absorb reagents during reverse flows.

[000203] Figure 10 shows the steps involved in the assay procedure.

Serum / Plasma/ Saliva Sample:

[000204] **Step 1**-Added two drops of sample (60µl) to the samples window. If sample did not flow over membrane with 10-15 seconds, add 1 drop of buffer to the same well.

[000205] **Step 2** – Sample absorbed by sample pad and once sample released over the membrane, three drops of buffer was added to the gold-pad at the both windows

[000206] **Step 3** – Once gold conjugate released over the membrane from both windows pulled out the plastic tab and added another drop of buffer onto the sample pad square and started timing. Read result after 20 minutes.

[000207] Figure 11 shows the steps involved in the assay procedure.

Interpretation of results

[000208] The results of the assay are interpreted using both visual and Rapid test reader depending on the intensity of test and control lines and is summarized in Table-6 and Figure 14. Results can also be customized with Rapid Test Reader (Fig-14). Following the assay method, a lab tech will use the reader to read and interpreted the data- as show in table-7. Reader will read, analysis the data and will be view on reader screen as mentioned in table-7.

[000209] Figures 12 and 13 show schematic diagrams of the test interpretation / outcome of the assay.

Table-6: Appearance of test and control lines on device windows and result interpretation

Test lines	Control lines	Dengue infection	Primary	Secondary
Control lines	Only control lines appear	Test is valid and negative to dengue infection		
NS1and/Ep	appear	positive	positive	Negative
NS1and/or Ep+IgA	appear	positive	positive	Negative
NS1and/or Ep+IgM	appear	positive	positive	Negative
NS1and/or Ep+IgG	appear	positive	negative	positive

NS1 and/or +IgA+IgM+IgG	EP	appear	positive	Negative	positive
IgA		appear	positive	positive	Negative
IgA+IgM		appear	positive	positive	Negative
IgA+IgG		appear	positive	negative	positive
IgA+IgM+IgG		appear	positive	negative	positive
IgM		appear	positive	positive	Negative
IgM+IgG		appear	positive	negative	positive
IgG		appear	Could be passed infection, however retesting is suggested after 5 days of first blood collection		
All test lines		Donot appear	Test is invalid		

The test reader will read respective line, analysis the data and print out the interpretation as follows:

Table-7. Interpretations of DEN 4-1 RT by Test Reader

Sample	Disease status	Type of Infection	
		Primary	Secondary
Sample-1	Positive	Primary	
Sample-2	Positive		Secondary
Sample-3	Valid and Negative		
Sample-4	Past infection/ Retest		
Sample-5	Invalid		

[000210] The analytical sensitivity of the device was studied by using strong, medium and weak dengue specific antibodies and antigen with 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) and antigens (NS1 and EP).

EXAMPLE 2

[000211] Figure 15 shows another embodiment of the present invention – a single reverse flow chamber. In particular, Figure 15 shows a kit 50 for detecting a dengue virus infection. The kit 50 is similar in structure and workings as described above. The kit 50 includes a casing having an inlet Well A 55 for receiving a biological sample obtained from a subject, another inlet Well B 60 for receiving a chaser buffer (which will be described more later), and a reaction chamber 65. The reaction chamber 65 may be the portion of the kit 50 which includes a window allowing a user to see the results obtained when the assay method is carried out on the kit 50. The inlet 55 may also include a sample chamber for receiving the sample. The inlets 55, 60 and reaction chamber 65 are all in fluid communication with each other. Inlet 55 is opposite inlet 60. The kit 50 also includes any structure 70 having the flavivirus specific immunogenic component/ anti-human antibodies/ monoclonal antibodies against the flavivirus immobilised on it. The 70 may be made of any suitable material, for example a membrane that spans the inlets 55, 60 and includes the reaction chamber 65 where the flavivirus specific immunogenic component/ anti-human antibodies /monoclonal antibodies against the flavivirus are immobilised. The front of the casing of the kit 50 adjacent the window may include markings showing the locations of the various test lines. As such, the flavivirus specific immunogenic component/ anti-human antibodies/ monoclonal antibodies against the flavivirus are immobilised on the solid support in a “striped” and linear fashion as shown in Figure 15. They may be immobilised on the membrane using a Dispensing system, followed by a drying step. Next the “striped” membrane is subjected to blocking step with blocking solution containing Casein, Triton, PVP and 5X stabil coat for 1 min, followed by a drying step. The flavivirus specific immunogenic component/ anti-human antibodies/ monoclonal antibodies against the flavivirus may be immobilised by any suitable methods known to the skilled person.

[000212] The antibodies and antigens may be obtained commercially.

Antibody	Manufacturer
Mouse anti-human IgG	Fapon Biotech Inc.
Mouse anti-human IgM	Fapon Biotech Inc.
Goat anti-human IgA	General Bioscience
Anti-dengue NS1 monoclonal antibodies (subtype 1, 2, 3, and 4)	Fapon Biotech Inc.
Recombinant dengue antigens (Type I and II)	Fapon Biotech Inc.
Goat anti-biotin	Pierce Thermo Scientific

[000213] In an embodiment, and as shown in Figure 15, the flavivirus specific immunogenic component/ anti-human antibodies/ monoclonal antibodies against the flavivirus – IgG, IgM, IgA, and NS1 test lines – are immobilised on the structure 70. This is shown on the markings adjacent the window of the kit 50. These test lines are arranged separately and spaced apart in a linear fashion such that they line up along the length of the window of the kit 50. An indicator line (in an embodiment, the indicator line may be blue) is shown on the structure 70 to indicate when the sample has travelled across the immobilised flavivirus specific immunogenic component/ anti-human antibodies/ monoclonal antibodies against the flavivirus along the length of the structure 70. In addition to the indicator line, a control line may be included. Advantageously, having all four test lines allow determination of phase of infection.

[000214] In general, diagnostic of dengue is dependent on the phase of the infection, current dengue diagnostics are based on either detection of viral agent (antigen/genome) or antibodies (IgA/IgM/IgG) produced against it. Dengue nonstructural protein 1 (NS1), produced in both membrane-associated and secreted forms, has been utilized as an early marker due to its presence in the serum of patients during the viraemic phase of infection. NS1 circulates in the serum from 1 to 9 days after the onset of clinical signs, with a peak from 3 to 5 days. The host immune response to dengue infection consists of the production of antibodies that are primarily directed against the dengue virus envelope proteins. Understanding the features of host humoral immune response is important for the interpretation of dengue infection. A primary antibody response is observed in individuals who are not immune to dengue and a secondary immune response is observed in patients who have had a previous dengue infection. In primary infection, IgM antibodies develop by 3-10 days after the onset of infection and reach its peak level ~2 weeks later. IgM generally decline to undetectable levels over the next 2-3 months. After the end of first week of infection, IgG is detectable at low titre and slowly increases, and persists for life. By contrast, during a secondary infection, high level of IgG antibodies is detectable even in the acute phase and rapidly rises over the following 2 weeks. IgM antibodies may be detectable after 20 days of infection and are usually lower than in primary infections. IgA typically appears a day after IgM at a low level and decreases to undetectable level within 45 days following detection. As such, the present invention is intended as a rapid test, developed to detect antibodies specific to dengue IgA/IgM/ IgG and dengue NS1 antigens in human serum, plasma, finger pricked whole blood or whole blood with anti-coagulants. The test is a

reverse-flow immunochromatographic test and uses immobilized goat anti-human IgA, mouse anti-human IgM and IgG antibodies; and anti-dengue NS1 monoclonal antibodies for capturing IgA, IgM, IgG antibodies and NS1 dengue antigen in the human serum, plasma, finger pricked whole blood or whole blood with anticoagulants. The presence of IgA, IgM, IgG antibodies against dengue virus and NS1 dengue antigen are detected by the colloidal gold labeled dengue recombinant proteins and anti-dengue NS1 monoclonal antibody.

[000215] The kit 50 is a direct solid-phase immunochromatographic assay, based on MP Biomedicals' proprietary Reverse Flow technology (US Patent No.: 6,316,205) for simultaneous and differential detection of dengue antibodies and antigen in human serum, plasma, finger pricked whole blood or whole blood with anti-coagulants. The structure 70 has Goat anti-human IgA, mouse anti-human IgM and IgG; and anti-dengue NS1 monoclonal antibodies are striped on the membrane as 4 separate test lines 75. The dengue antibodies and/ or antigen in the test sample (serum, plasma, finger pricked whole blood or whole blood with anticoagulants) form antibody-antigen complexes with anti-human antibodies and dengue NS1 monoclonal antibodies immobilized on nitrocellulose membrane in the test viewing window as the test sample migrates upward from the sample well. The bound antibody-antigen complexes are subsequently detected by dengue antigens and dengue NS1 monoclonal antibody conjugates carried by chase buffer that flows downward giving a pink-purplish color. In addition, Biotinylated-BSA is immobilized on structure 70 which can be recognized by colloidal gold-labeled goat anti-biotin is used as an internal control for proper function of the reagents.

[000216] The kit may further include any molecule that is capable of binding to a complex that may be formed with the flavivirus specific immunogenic component/anti-human antibodies/monoclonal antibodies against the flavivirus immobilised on the membrane.

[000217] Figure 18 shows the kit of the present invention in greater detail, in particular it shows a cross section of it. For ease of description, the components shown are separated from each other. In the actual kit, these components are in contact with each other.

[000218] In an embodiment, the solid support 85 is a strip which includes a sample pad 80, a membrane 70, and a gold conjugate pad 90. The sample pad 50 receives the sample from the inlet 55. The solid support 85, particularly the nitrocellulose membrane 70, includes

the test markers 75. The structure 70 may be made from a material that is different to that of the sample pad 80 and gold conjugate pad 90. For example, the structure 70 is made of a nitrocellulose membrane. This is the part of the strip that is shown in the window 65. The gold conjugate pad 90 has the molecule that is capable of binding to the antibody-antigen complex. This molecule has a gold conjugate for detection purposes. The gold conjugate pad 90 receives the buffer from the inlet 60. As such, the sample pad 80 is at a first end of the strip while the gold conjugate pad 90 is at a second end of the strip. First and second ends are opposite each other.

[000219] At the first end of the strip, i.e. the sample pad 80 is an absorbent pad 95. The absorbent pad 95 is placed beneath the sample pad 80. The absorbent pad 95 and sample pad 80 are separated by a separator 100, i.e. the “pull tab” as shown in the Figures.

[000220] In operation, a sample is received in inlet 55 and gets absorbed by the sample pad 80. The sample then travels along the strip in the direction A to the membrane 70 by capillary action where it comes into contact with the flavivirus specific immunogenic component/anti-human antibodies/monoclonal antibodies against the flavivirus that are immobilised on the membrane 70. Depending on the infection in the sample, complexes will be formed on membrane 70 at the relevant marker positions as indicated in 75. A buffer is then received in inlet 60 and re-solubilise the molecule on the gold conjugate pad 90. When the separator 100 is removed by pulling the tab, the sample pad 80 and absorbent pad 95 comes into contact. This helps to draw the resolubilized gold conjugate and buffer from the gold conjugate pad 90 to travel the strip in a direction that is opposite that of the sample, i.e. Direction B. The binding between molecule and the antigen-antibody complex is detected by the labelled moiety. Other types of detection systems such as biotin/avidin may be used.

[000221] The gold conjugate is re-solubilised when 3 drops added to the oval well (Well B). The gold conjugate is sprayed and dry on the gold conjugate pad 60. Once chase buffers added, the buffer will dissolve the gold conjugate and the gold conjugate in liquid format will flow downwards (direction B).

[000222] After pull-tab pulled, 1 drop of chase buffer was used to make contact between sample pad and absorbent pad to facilitate the flow downwards. The drop of chase buffer connects the sample pad portion of the solid support to the absorbent pad.

[000223] The kit 50 is allowed to warm to room temperature ($25^{\circ}\text{C} \pm 3^{\circ}\text{C}$) before running the assay for optimal binding. For best results, the assay is conducted at room temperature ($25^{\circ}\text{C} \pm 3^{\circ}\text{C}$).

[000224] The assay method is diagrammatic represented in Figure 16. Whole blood can be collected in anti-coagulant containing tubes and used as outlined in the assay procedure immediately or stored at 2°C to 8°C for not more than 72 hours before use. The presence of 6 anti-coagulants, Acid Citrate Dextrose (ACD), Citrate-phosphate-dextrose (CPD), Ethylenediaminetetraacetic acid (EDTA), Potassium Oxalate (K-Oxalate), Lithium Heparin (LiHep) and Sodium Citrate (Na Citrate) were found to have no effects on the performance of MULTISURE Dengue Ab/Ag Rapid Test. Serum / plasma samples should be stored at 2°C to 8°C if the test is to be run within 7 days of collection or frozen at -20°C or colder if the test is to be delayed for more than 7 days. Clear, nonhemolyzed samples are preferred. Lipemic, icteric or contaminated (particulate or bacterial) samples should be filtered ($0.45\mu\text{m}$) or centrifuged before testing. Sample can be inactivated but this is not a requirement for optimal test performance.

[000225] Inactivate as follows:

1. Loosen cap of sample container.
2. Heat-inactivate sample at 56°C for 30 minutes in a water bath.
3. Allow sample to cool down before retightening cap.
4. Sample can be stored frozen until analysis.
5. Repeated freeze-thawing of the sample is not recommended. Do not use specimens (serum & plasma) with more than five (5) freeze-thaw cycles.

Strict adherence to the assay procedure will ensure optimal performance. Deviations from the procedure may lead to aberrant results.

Note:

☐ Allow the kit to warm to room temperature ($25^{\circ}\text{C} \pm 3^{\circ}\text{C}$)

before running the assay.

☐ For best results, conduct the assay at room temperature ($25^{\circ}\text{C} \pm 3^{\circ}\text{C}$).

☐ Chase buffer with crystallization may affect test results.

☐ In order to prevent crystallization in chase buffer, it is highly recommended that the chase buffer to be stored at 18°C -

28°C.

□ Check the chase buffer carefully. If crystals are present in the chase buffer, make sure that they are fully dissolved before use. Warming at 37°C helps to expedite the dissolving process.

1. Open the pouch by tearing at the tear notch on the right side of the pouch. Conduct the test immediately after removing the test device from the pouch.
2. Label the test device with the sample name.
3. Proceed with the assay procedures as shown in the diagrams below.

With reference to Figure 17, the interpretation of the results is as follows:

1. Positive for dengue

A test is dengue positive if Control Line (C) appears with any visible test lines (IgG, IgM, IgA, NS1).

- Presence of IgG indicates secondary or past infection.
- Presence of IgM and/or IgA indicates current infection.
- Presence of NS1 indicates acute dengue infection.

2. Negative for dengue

A test is negative if Control line (C) appears with no visible test line(s). Retest in 3-5 days if dengue infection is suspected.

2. Invalid

A test is invalid if Control Line (C) is absent. The assay should be repeated using a new device.

[000226] The following tables show the total diagnostic performance.

Total Diagnostic performance

Diagnostic Parameter	Performance of MULTISURE Dengue Ab/Ag Rapid Test	95% Confidence Interval
Sensitivity (n=291)	94.16%	90.81% to 96.56%
Specificity (n=425)	93.65%	90.89% to 95.77%
Positive Predictive Value	91.03%	87.21% to 94.01%
Negative Predictive Value	95.90%	93.52% to 97.60%

Overall Sensitivity

Method used	Reference assay: commercial ELISAs (n=291)		
	Test Line	Positive	Sensitivity
MULTISURE Dengue Ab/Ag Rapid Test	IgG	160	54.98%
	IgM	223	76.63%
	IgA	127	43.64%
	NS1	85	29.21%
	Total	274	94.16%

Sensitivity performance of individual test lines

Method used	Primary Reference: Commercial ELISAs Secondary Reference: Commercial Rapid Tests			
	Test Line	Positive	Total	Sensitivity
MULTISURE Dengue Ab/Ag Rapid Test	IgG*	136	156	87.18%
	IgM*	206	224	91.96%
	IgA [#]	127	291	43.64%
	NS1*	84	100	84.00%

*Discordant samples with primary reference are verified by secondary reference. Disagreed samples between primary and secondary reference are excluded for calculation.

[#]IgA reactivity rate in dengue positive samples based on Primary Reference (IgG, IgM and/or NS1).

Primary infection defined by commercial ELISAs

Method used	Reference assay: commercial ELISAs (n=127)		
	Test Line	Positive	Sensitivity
MULTISURE Dengue Ab/Ag Rapid Test	IgG	20	15.75%
	IgM	89	70.08%
	IgA	33	25.98%
	NS1	74	58.27%
	Total	120	94.49%

Secondary infection defined by commercial ELISAs

Method used	Reference assay: commercial ELISAs (n=164)		
	Test Line	Positive	Sensitivity
MULTISURE Dengue Ab/Ag Rapid Test	IgG	140	85.37%
	IgM	134	81.71%
	IgA	94	57.32%
	NS1	11	6.71%

	Total	154	93.90%
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Specificity performance of individual test lines

Method used	Reference assay: commercial ELISAs (n=425)		
	Test Line	Negative	Specificity
MULTISURE Dengue Ab/Ag Rapid Test	IgG	413	97.18%
	IgM	410	96.47%
	IgA	423	99.53%
	NS1	425	100.00%
	Total	398	93.65%

Specificity performance in various populations

Study	Category	Total Sample Size (n)	Diagnostic Specificity of MULTISURE Dengue Ab/Ag Rapid Test
In-house study	Blood Donors /Healthy Donors	156	96.80% (151/156)
In-house study	Hospitalized / Clinical	98	91.84% (90/98)
In-house study	Cross-reactive	66	95.46% (63/66)
In-house study	Interference	55	98.18% (54/55)
In-house study	Pregnancy	50	80.00%(40/50)
Total		425	93.65% (398/425)

Potential cross-reacting

Sample Profile	Total Sample Size	Performance of MULTISURE Dengue Ab/Ag Rapid Test
Rheumatoid Factor (RF)	10	100.00% (10/10)
Hepatitis A	10	100.00% (10/10)
HBsAg	5	100.00% (05/05)
Hepatitis C	7	100.00% (07/07)
Measles IgG	9	100.00% (09/09)
Human T-lymphotropic Virus Type I (HTLV-I)	10	90.00% (09/10)
HIV 1/2	9	88.89% (08/09)
Hepatitis E	6	83.33% (05/06)
Total	66	95.45% (63/66)

Potential Interferences

Sample Profile	Total Sample Size	Performance of MULTISURE Dengue Ab/Ag Rapid Test
Icteric	8	100.00% (08/08)
Hemolyzed	8	100.00% (08/08)
Triglyceride	8	100.00% (08/08)
Lipemic	8	100.00% (08/08)

Sample Profile	Total Sample Size	Performance of MULTISURE Dengue Ab/Ag Rapid Test
Total Protein	6	100.00% (06/06)
Total bilirubin	8	100.00% (08/08)
Antinuclear Antibody (ANA)	9	88.89% (08/09)
Total	55	98.18% (54/55)

With the titration of one dengue antibody and antigen positive specimen, the reactivity end point for MPD MULTISURE Dengue Ab/Ag Rapid Test were found to be at 1:1024 and 1:32 dilutions respectively. The inter-assay (between-run) and intra-assay (within-run, withinday and day-to-day) reproducibility of MPD MULTISURE Dengue Ab/Ag Rapid Test have been evaluated using a set of control panel members. All results obtained were consistently fall within the acceptance criteria, indicating the MPD MULTISURE Dengue Ab/Ag Rapid Test is robust, reproducible and consistent across three lots studied.

EXAMPLE 3

Comparative analysis of DEN 4-1 against DEN IgM, IgG and NS1 and/or Ep antigen Rapid tests

[000227] The level of sensitivity of DEN 4-1 RT carried out using 25 dengue positive and 11 negative sera/whole blood samples obtained from R & D lab and compared with other ELISAs. Results showed that DEN 4-1 RT detected overall 92% of dengue cases with 90% specificity.

EXAMPLE 4

DEN 4-1 RT

[000228] The performance of DEN 4-1 RT was further evaluated using against commercially available POCT using 25 dengue positive and 11 dengue negative samples (sera were positive either to NS1 antigen ELISA or den-capture IgM ELISA). The sensitivity of DEN 4-1 RT was found 92% compared to 87.61%, and Bhat Bio-Tech India (P) Ltd gM/IgG and NS1 cassette respectively.

EXAMPLE 5

Specificity and Sensitivity of DEN 4-1 RT

[000229] Specificity of DEN 4-1 RT using sera collected from healthy and non-dengue febrile illness.

Two by Two table showing sensitivity and specificity of DEN 4-1 RT

		Den NS1 and IgM/IgG ELISAs		Total
		Positive	Negative	
DEN 4-1 RT	Positive	23	10	33
	Negative	2	1	3
	Total	25	11	36
Sensitivity (%)	92% (23/25)			
Specificity (%)	90.90% (10/11)			
Diagnostic accuracy (%)	91.67% (33/36)			

EXAMPLE 6

DEN 4-1 RT

[000230] Performance of DEN 4-1 RT against 3 dengue patients saliva and 5 whole blood samples showed high 100% sensitivity (although sample size were too small to make statistically significance).

[000231] The newly developed DEN 4-1 RT showed high level of performance compared to other POCTs and early dengue ELISAs (capture IgM and NS1 antigen ELISAs). This is because of combination of three antibodies and antigen in the same platform particularly the presence of dengue specific IgA antibodies and dengue antigens (NS1 and Ep) which appears earlier among dengue secondary infected patients.

[000232] Those skilled in the art will appreciate that the invention described herein is 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 a flavivirus infection in a human subject, the method comprising:
 - (a) providing at least one flavivirus specific immunogenic component;
 - (b) contacting the at least one flavivirus specific immunogenic component with a biological sample obtained from the subject, the biological sample suspected of containing at least one binding partner capable of hybridising to the at least one flavivirus specific immunogenic component; and
 - (c) detecting the presence of a complex that forms between the at least one of the flavivirus specific immunogenic component and the at least one binding partner, which presence indicate as flavivirus infection in the subject.
2. The method according to claim 1, wherein detecting the presence of the complex comprising contacting the complex with a molecule capable of hybridising to the at least one binding partner, the molecule further comprising at least one detectably labelled moiety.
3. The method according to claim 2, wherein the molecule any one selected from the group comprising:
 - (a) a flavivirus antigen;
 - (b) an anti-human antibody; and
 - (c) monoclonal/polyclonal antibodies against a flavivirus antigen.
4. The method according to claim 3, wherein the flavivirus antigen is any one selected from the group comprising: structural and non-structural of flavivirus particles and fragments thereof, glycoproteins, lipids and carbohydrates derived from the flavivirus.
5. The method according to any one of the preceding claims, wherein the at least one flavivirus specific immunogenic component is selected from the group comprising:
 - (a) antibodies IgG, IgM and IgA against human;
 - (b) a flavivirus antigen, the flavivirus antigen is any one selected from the group comprising of: NS1 and envelope protein; and

- (c) monoclonal/polyclonal antibody against a flavivirus.
6. The method according to any one of the preceding claims, wherein the at least one binding partner is any one selected from the group comprising:
- (a) IgA, IgM and IgG antibodies against dengue; and
 - (b) a flavivirus antigen selected from the group comprising: NS1 and envelope protein.
7. The method according to claim 2, wherein the detectably labelled moiety is a gold colloid.
8. The method according to any one of the preceding claims, wherein the flavivirus is any one selected from the group comprising: dengue virus, yellow fever virus, West Nile virus and Japanese Encephalitis virus.
9. The method according to claim 8, wherein the dengue virus serotype is any one selected from the group comprising: DEN1, DEN2, DEN3 and DEN4.
10. The method according to any one of the preceding claims, wherein the biological sample is any one selected from the group comprising: saliva, whole blood, plasma, serum, urine and peripheral blood mononuclear cells.
11. The method according to any one of claims 2 to 10, the method further comprising:
- (a) providing a solid support having a first end and a second end;
 - (b) immobilising the flavivirus specific immunogenic components on the solid support, the flavivirus specific immunogenic components are immobilised separately and spaced apart from each other along the solid support;
 - (c) immobilising the molecule on the second end of the solid support, the molecule capable of binding to the complex;
 - (c) loading the sample on the first end of the solid support, the sample contacting the flavivirus specific immunogenic components as it travels along the solid support in a first direction along the solid support and towards the second end; and

(d) loading a buffer on the second end of the solid support adjacent or on the molecule such that the buffer solution solubilise the molecule as it travels along the solid support in a second direction opposite the first direction, wherein the presence of the complex formed on the solid support is detected by the labelled moiety of the molecule.

12. The method according to claim 11, further providing an absorbent pad adjacent the first end of the solid support, the absorbent pad and the solid support are separated by a separator, wherein removing the separator allows contact between the absorbent pad and the solid support to enhance the flow of the buffer along the solid support in the second direction.

13. A method for detecting a flavivirus infection in a human subject, the method comprising:

- (a) providing at least one anti-human antibody;
- (b) contacting the at least one anti-human antibody with a sample suspected of containing human antibodies against a flavivirus obtained from the subject; and
- (c) detecting the presence of human antibodies bound to the at least one anti-human antibody by reacting any bound human antibodies with at least one flavivirus antigen, the flavivirus antigen further comprising at least one detectably labelled moiety,

wherein detection of the detectably labelled moiety indicates a flavivirus infection in the human subject.

14. The method according to claim 13, wherein the anti-human antibody is any one selected from the group comprising: IgA, IgM and IgG.

15. The method according to any one of claims 13 or 14, wherein the detectably labelled moiety is a gold colloid.

16. The method according to any one of claims 13 to 15, wherein the flavivirus antigen is any one selected from the group comprising: structural and non-structural of flavivirus

particles and fragments thereof, glycoproteins, lipids and carbohydrates derived from the flavivirus.

17. The method according to claim 16, wherein the flavivirus antigen is any one selected from the group comprising: NS1 and envelope protein.

18. The method according to any one of claims 13 to 17, wherein the flavivirus is any one selected from the group comprising: dengue virus, yellow fever virus, West Nile virus and Japanese Encephalitis virus.

19. The method according to claim 18, wherein the dengue virus serotype is any one selected from the group comprising: DEN1, DEN2, DEN3 and DEN4.

20. The method according to any one of claims 13 to 19, wherein the biological sample is any one selected from the group comprising: saliva, whole blood, plasma, serum, urine and peripheral blood mononuclear cells.

21. The method according to any one of claims 13 to 20, the method further comprising:

- (a) providing a solid support having a first end and a second end;
- (b) immobilising the anti-human antibodies on the solid support, the anti-human antibodies are immobilised separately and spaced apart from each other along the solid support;
- (c) immobilising the flavivirus antigen on the second end of the solid support, the flavivirus antigen capable of binding to the bound human antibodies;
- (c) loading the sample on the first end of the solid support, the sample contacting the anti-human antibodies as it travels along the solid support in a first direction along the solid support and towards the second end; and
- (d) loading a buffer on the second end of the solid support adjacent or on the flavivirus antigen such that the buffer solution solubilise the flavivirus antigen as it travels along the solid support in a second direction opposite the first direction,

wherein the presence of the complex formed on the solid support is detected by the labelled moiety.

22. The method according to claim 21, further providing an absorbent pad adjacent the first end of the solid support, the absorbent pad and the solid support are separated by a separator, wherein removing the separator allows contact between the absorbent pad and the solid support to enhance the flow of the buffer along the solid support in the second direction.

23. A method for detecting a flavivirus infection in a human subject, the method comprising:

- (a) providing monoclonal antibodies against a flavivirus;
- (b) reacting the monoclonal antibodies against a flavivirus with a sample suspected of containing a flavivirus antigen obtained from the subject; and
- (c) detecting the presence of a flavivirus antigen bound to the monoclonal antibodies reacting any bound flavivirus antigen with polyclonal antibodies against a flavivirus antigen, the polyclonal antibodies further comprising at least one detectably labelled moiety,

wherein detection of the detectably labelled moiety indicates a flavivirus infection in the human subject.

24. The method according to claim 23, wherein the monoclonal antibodies are against any flavivirus antigen selected from the group comprising: structural and non-structural of flavivirus particles and fragments thereof, glycoproteins, lipids and carbohydrates derived from the flavivirus.

25. The method according to claim 24, wherein the flavivirus antigen is any one selected from the group comprising: NS1 and envelope protein.

26. The method according to any one of claims 23 to 25, wherein the flavivirus is any one selected from the group comprising: dengue virus, yellow fever virus, West Nile virus and Japanese Encephalitis virus.

27. The method according to claim 26, wherein the dengue virus serotype is any one selected from the group comprising: DEN1, DEN2, DEN3 and DEN4.

28. The method according to any one of claims 23 to 27, wherein the detectably labelled moiety is a gold conjugate.
29. The method according to any one of claims 23 to 28, wherein the biological sample is any one selected from the group comprising: saliva, whole blood, plasma, serum, urine and peripheral blood mononuclear cells.
30. The method according to any one of claims 13 to 20, the method further comprising:
- (a) providing a solid support having a first end and a second end;
 - (b) immobilising the monoclonal antibodies on the solid support, the monoclonal antibodies are immobilised separately and spaced apart from each other along the solid support;
 - (c) immobilising the polyclonal antibodies on the second end of the solid support, the polyclonal antibodies capable of binding to the bound flavivirus antigen;
 - (c) loading the sample on the first end of the solid support, the sample contacting the monoclonal antibodies as it travels along the solid support in a first direction along the solid support and towards the second end; and
 - (d) loading a buffer on the second end of the solid support adjacent or on the polyclonal antibodies such that the buffer solution solubilise the polyclonal antibodies as it travels along the solid support in a second direction opposite the first direction,
- wherein the presence of the complex formed on the solid support is detected by the labelled moiety.
31. The method according to claim 30, further providing an absorbent pad adjacent the first end of the solid support, the absorbent pad and the solid support are separated by a separator, wherein removing the separator allows contact between the absorbent pad and the solid support to enhance the flow of the buffer along the solid support in the second direction.
32. 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:

- (a) providing at least one flavivirus specific immunogenic component;
- (b) contacting the at least one flavivirus specific immunogenic component with a biological sample obtained from the subject, the biological sample suspected of containing at least one binding partner capable of hybridising to the at least one flavivirus specific immunogenic component; and
- (c) detecting the presence of a complex that forms between the at least one of the flavivirus specific immunogenic component and the at least one binding partner 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.

33. The method according to claim 32, wherein detecting the presence of the complex comprising contacting the complex with a molecule capable of hybridising to the at least one binding partner, the molecule further comprising at least one detectably labelled moiety.

34. The method according to claim 33, wherein the molecule is any one selected from the group comprising:

- (a) a flavivirus antigen;
- (b) an anti-human antibody; and
- (c) mono/polyclonal antibodies against a flavivirus antigen.

35. The method according to claim 34, wherein the flavivirus antigen is any one selected from the group comprising: structural and non-structural of flavivirus particles and fragments thereof, glycoproteins, lipids and carbohydrates derived from the flavivirus.

36. The method according to any one of claims 32 to 35, wherein the at least one flavivirus specific immunogenic component is any one selected from the group comprising:

- (a) anti-human antibodies IgG, IgM and IgA against a flavivirus;
- (b) a flavivirus antigen, the flavivirus antigen is any one selected from the group comprising: NS1 and envelope protein; and

- (b) monoclonal and polyclonal antibody against a flavivirus.

37. The method according to any one of claims 32 to 36, wherein the binding partners are any one selected from the group comprising:

- (a) IgA, IgM and IgG antibodies against a dengue virus; and
- (b) any flavivirus antigen selected from the group comprising: NS1 and envelope protein.

38. The method according to any one of claims 32 to 37, wherein the defined location is a geographical area, housing estate, means of transport or center for medical treatment or assessment.

39. A kit for detecting a flavivirus infection in a human subject, the kit comprising:

- (a) a solid support with at least one surface-bound flavivirus specific immunogenic component.

40. The kit according to claim 39, further comprising a plurality of bound flavivirus specific immunogenic components, the components are immobilised on the solid support separately and spaced apart from each other.

41. The kit according to claim 33, further comprising a molecule capable of hybridising to a complex formed with the at least one at least one surface-bound flavivirus specific immunogenic component, the molecule further comprising at least one detectably labelled moiety.

42. The kit according to claim 41, wherein a sample obtained from the subject is loaded on a first end of the solid support, and a buffer for solubilising the molecule is loaded on a second end of the solid support opposite the first end.

43. The kit according to any one of claims 41 or 42, wherein the molecule is any one selected from the group comprising:

- (a) a flavivirus antigen;
- (b) an anti-human antibody; and
- (b) monoclonal or polyclonal antibodies against a flavivirus antigen.

44. The kit according to claim 43, wherein the polyclonal antibodies are against an NS1 or envelope protein of a dengue virus.
45. The kit according to any one of claims 39 to 44, wherein the at least one flavivirus specific immunogenic component is any one selected from the group comprising:
- (a) anti-human antibodies IgG, IgM and IgA against a flavivirus; and
 - (b) Poly/monoclonal antibody against a flavivirus.
46. The kit according to 39, wherein the kit comprises:
- (a) a first reaction chamber, the first reaction chamber comprising a solid support with at least one surface-bound anti-human antibodies IgG, IgM and IgA against a dengue virus; and
 - (b) a second reaction chamber, the second reaction chamber comprising a solid support with at least one surface-bound monoclonal/polyclonal antibody against a dengue virus, the dengue virus antigen selected from the group comprising of NS1 and envelope protein.
47. The kit according to any one of claims 39 to 46, wherein the solid support is any one selected from the group comprising 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.
48. The kit according to any one of claims 39 to 47, wherein the solid support is a nitrocellulose membrane.