DIAGNOSTIC TEST

FIELD OF THE INVENTION

The present invention relates generally to a technique of detecting exposure of human and or animal to a dengue virus or equivalent thereof. More particularly, the present invention relates to a rapid and easy analysis of a biological sample taken from a subject (animal or human) in order to determine if the subject had been exposed to a dengue virus or equivalent thereof. The present invention further provides medical diagnostic kits for the detection of dengue virus infection or equivalent thereof.

BACKGROUND

The dengue is the most common and widespread arthropod-borne viral infection in the world and more than 50 million dengue infection occur annually and the geographical spread, incidence and severity of dengue fever are increasing in the tropics (WHO, May 2002). There are four distinct virus serotypes, each capable of producing a wide spectrum of signs and symptoms and a primary infection with one of the four serotypes confers lasting immunity to that type. The chance of secondary infection with different serotypes still remains.

Although dengue is endemic in most of the tropical and subtropical countries, it occurs mainly in developing countries that lack the healthcare infrastructure or financial capabilities to readily diagnose dengue effectively. In Singapore, an uneven distribution of the two vectors resulted in the uneven transmission of dengue within the population. By doing a serological survey, it was determined that a change in the location where dengue was acquired could have occurred. This finding may aid in the modification of vector control programs to target new areas of transmission. However, only four sero-epidermal surveys have been completed so far. The high cost of laboratory tests or the lack of reagents needed contributes to so few surveys being conducted. Traditionally, hemagglutination inhibition (HAI) was used to detect antibodies against dengue.

However this method requires mouse brain-derived antigens that are not readily available due to the difficulties in growing such cells. Another method commonly used is complement fixation but this requires antibodies that are short-lived. More and more laboratories are resorting to the commercial enzyme-linked immunosorbent assay (ELISA) kits. These tests require the use of an expensive material (polystyrene) on which the immunogen would adhere to. As such, a cost-effective yet reliable alternative to the currently available IgG diagnostic kit is required.

The sensitivities of the performance of six commercially available immunoassay systems for the detection of dengue virus specific immunoglobulin M (IgM) and IgG antibodies in serum (Goren *et al.* 2000) have recently been evaluated. The sensitivities of the dengue virus-specific assays evaluated varied between 71 and 100% for IgM and between 52 and 100% for IgG, with specification of 86 to 96% and 81 to 100% respectively. The total test time of these tests varied from 7 to 480 minutes. In addition to this, all tests require laboratory equipment support except Pan-bio RIT and its sensitivity and specificity for dengue IgG assay were very low (75% and 52% respectively).

The immune response to dengue virus varies in primary versus secondary infections and from person to person. Therefore, several techniques are often used in combination to confirm a case of dengue. Not all dengue infections result in detectable immunoglobulin M (IgM), even several days after the infection has cleared. The kinetics of immunoglobulin A (IgA) response to dengue infection has been studied and found that IgA appears almost at the same time of IgM and can be used as one of the diagnostic tools of dengue infection (Balmaseda *et al.*, 2003)

Alternate diagnostic assays for dengue diagnosis are therefore needed which can provide accuracy specifically and speed and convenience. Serological detection procedures have the potential to play a role in clinical diagnosis to confirm dengue infection from other infectious diseases similar to dengue as well as its sero-surveillance studies.

SUMMARY OF THE INVENTION

In a first aspect of the present invention, there is provided a method for detecting exposure of a subject to a dengue virus or equivalent thereof, said method comprising:

contacting a biological sample from the subject with a mixture of immunogenic components derived from a lysate of cells infected with dengue virus or an equivalent; and

determining the presence of a complex that forms between a binding partner in the biological sample and a immunogenic component of the lysate.

The present invention results from a need to develop a rapid, low cost and straightforward assay to determine present or prior exposure to dengue virus. The invention specifically utilizes a cell lysate, which includes a mixture of dengue virus immunogenic components including dengue virus particles amongst other antigens indicative of dengue virus infection. These may include structural and non-structural viral proteins. The lysate as used in the present invention provides for a full spectrum of dengue immunogens that may be used for detecting anti-dengue antibodies (IgG, IgM and/or IgA) or prior exposure to the dengue virus or equivalents thereof. Most preferably, the lysate contains structural and non-structural proteins including but not limited to NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 along with other dengue virus immunogens.

Accordingly, the present invention shows greater sensitivity compared to other conventional and most currently used dengue IgG ELISA by providing a platform that can identify antibody produced against dengue virus or immunological relatives thereof at any stage of the infection.

In yet another aspect, the biological sample may be applied to a membrane such as, but not limited to, a nitrocellulose membrane and the component derived from dengue virus or an equivalent thereof added. The complex formed by a component of the dengue cell lysate and the binding partner may then be

detected using a detection agent that contains a reporter group and specifically binds to the component/binding partner complex.

In another aspect of the present invention there is provided a kit for detecting dengue virus exposure comprising;

a) a solid support including a component derived from a lysate of cells infected with dengue virus or equivalent thereof;
or
b) a solid support including a component derived from a lysate of cells infected with dengue virus or equivalent thereof attached to a second support;
c) an incubation container;
d) blocking buffer;
e) washing buffer;
f) at least one detection agent conjugated to a label for detecting a complex that forms in a biological sample; and optionally
g) instructions for using said kit.

The present invention also provides individual components of the kit for use in the method of the present invention.

The present invention also provides a method of assessing the relative risk of one or more subjects being exposed to dengue virus or its equivalent thereof within a defined location (e.g. geographical area, housing estate, means of transport or center for medical treatment or assessment), comprising;

a) obtaining samples from a representative population within a defined location; and
b) 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 -
   i) contacting a biological sample from subject with a component derived from the dengue virus or equivalent thereof to form a

complex between the components and a subject-derived binding partner of the component contained within the biological sample;

ii) determining the presence of the complex, wherein the presence of the complex is indicative of exposure of the subject to a dengue virus or equivalent thereof; and

iii) assessing the relative risk of exposure within the defined location.

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 analysing 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.

FIGURES

Figure-1 shows the analysis of both dengue IgG positive and negative sera using DB-EIA protocol.

Figure-2 shows the results of 20 dengue IgG positive and dengue IgG negative serum samples using dot-blot EIA.

Figure-3 shows the analysis of both dengue positive and negative sera using the DB-dipstick protocol.

Figure-4 shows the analysis of both dengue IgA and IgA negative sera using DB-EIA protocol.

Figure-5 shows results of both dengue IgA positive and IgA negative sera using DB-EIA.

Figure-6 shows the results of IgA assays using 5 pairs of dengue serum samples.

Figure-7 shows the results of dengue IgA dipstick technique.

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect of the present invention, there is provided a method for detecting exposure of a subject to a dengue virus or equivalent thereof, said method comprising:

contacting a biological sample from the subject with a mixture of immunogenic components derived from a lysate of cells infected with dengue virus or an equivalent; and

determining the presence of a complex that forms between a binding partner in the biological sample and a immunogenic component of the lysate.

The present invention results from a need to develop a rapid, low cost and straightforward assay to determine present or prior exposure to dengue virus. In accordance with the present invention, the subjects including animals such as mammals and in particular humans are screened for the presence of binding partners preferably IgG, IgM and/or IgA to dengue virus or its immunological components. The preferred binding partners are subject-derived binding partners such as, but not limited to immunointeractive molecules. The most immunointeractive molecules are antibodies particularly immunoglobulin G (IgG), Immunoglobulin M (IgM) and Immunoglobulin A (IgA). The identification of such binding partners is then used as evidence of present or prior exposure of the subject to dengue virus or an equivalent thereof.

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.

The invention specifically utilizes a cell lysate, which includes a mixture of dengue virus immunogenic components including dengue virus particles amongst other antigens indicative of dengue virus infection. These may include structural and non-structural viral proteins. The use of the cell lysate may include viral particles as well as the fragments of the viral particles which may be expressed at any stage of the dengue virus multiplication in cells. In the

present invention the cell lysate preferably comprises a mixture of dengue virus immunogens, which includes virus particles and both the structural and non-structural viral proteins. Preferably the dengue virus immunogens are immunogenic components of the lysate that are capable of eliciting an immunological reaction to a binding partner in the biological sample. The advantage of the use of non-structural proteins along with a viral particle particularly the non-structural protein NS1 is to preferably trap anti-dengue IgG, IgM and/or IgA that developed at the early stage of the dengue infection in the host body. These may be expressed in higher concentrations at this early stage.

In particular, the presence of anti-dengue IgA in a patient's body appears at an acute phase of dengue infection and remains for a short period of time (30-45 days following infection) (Balmaseda et. al., 2003).

The presence of dengue IgA in host serum or saliva indicates the recent history of dengue infection. In addition to this, IgA is the only one anti-dengue antibody that appears both in the primary and secondary dengue infection. Hence the presence of this antibody may provide a vital diagnostic tool for the diagnosis of dengue infection and perhaps can provide an indication of secondary infection while the patient is positive to anti-dengue IgG but negative to IgM. Hence the present invention preferably identifies dengue IgG alone or in combination with IgA.

Accordingly, the present invention shows greater sensitivity compared to other conventional and most currently used dengue IgG ELISA by providing a platform that can identify antibody produced against dengue virus or immunological relatives thereof at any stage of the infection.

Therefore, the present invention provides a new economical detection method using lysate of cells infected with dengue virus comprising a mixture of dengue virus components, preferably immunogenic components of the lysate described above, which permits the specific detection of dengue binding partners, preferably IgG, IgM and/or IgA that may be present in the test serum. The test

is rapid, preferably providing results within 30-60 minutes at room temperature. Apart from it being a simple and convenient technique for antibody diagnosis, one of the major advantages of the present invention is the usage of an inexpensive and degradable membrane as the antigen-adhering platform. Furthermore, the present invention makes the testing of small numbers of samples easier.

Throughout the description and claims of this specification, use of the word "comprise" and variations of the word, such as "comprising" and "comprises", is not intended to exclude other additives, components, integers or steps.

The term "dengue virus" is used herein the claims and description refers to all dengue serotypes (Den-1, Den-2, Den-3 and Den-4) associated with a 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 preferred in accordance with the present invention. However, the invention includes any subject that can respond to an infection or immunization by the dengue virus or an equivalent thereof.

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. 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.

Dengue virus is transmitted to humans through the bites of infected female Aedes mosquitoes, principally the *A. 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 cases 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 (Newton *et al.*, 1992). 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*, 1997). Such behaviour has been used to explain the epidermological 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.*, 2001).

Following the bite of an infective female mosquito, the virus undergoes an intrinsic incubation period of 3 to 14 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 source for the virus (Putnam *et al.*, 1995; Gubler *et al.*, 1976; WHO, Fact sheets, 2002).

Dengue virus infection causes a spectrum of illness in humans depending on the infecting virus, the host 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)

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 dengue shock syndrome (DSS).

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

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

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.

Grade IV: Profound shock with undetectable blood pressure or pulse.

Classical dengue fever is more common in older children, adolescents and adults, and they are less likely to be asymptomatic (Sharp *et al.*, 1995). The fever is abrupt in its onset with high fever, headache, incapacitating myalgias and arthralgias, nausea vomiting and macular or maculopapular rash (Waterman, 1989). The fever usually lasts for 5-7 days and sometimes can follow a biphasic course (Saddle back appearance).

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 DJ., 1998)

DSS is the terminal stage of DHF and is manifested by hypovolaemic shock due to plasma leakage. 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% (Rigau-Perez JG, *et al.*, 1998).

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, haemmagglutination and neutralization. Infection in humans by one of the serotypes provides life long immunity to that serotype but only temporary protection against other serotypes.

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.

The term "equivalent" 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).

The present invention is applicable to detecting exposure to the dengue virus or an equivalent thereof. Exposure may be present or prior exposure to the dengue virus or an equivalent thereof. Preferably, 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 equivalent thereof. Once the subject is exposed, the method of the present invention may be applied at any stage of exposure as described above. Preferably, the method is used to detect exposure where there are no signs and symptoms that are obvious of a dengue infection. Preferably, the method detects exposure of the subject at an early convalescence stage of exposure to dengue virus or equivalent thereof. The

exposure may not always manifest in a dengue infection or notable signs or symptoms but it will cause a response so as to induce a binding partner. Preferably, the response is an immunological response.

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.

Accordingly, the binding partner is any molecule or cell that is produced against the foreign dengue virus or equivalent thereof. Preferably, the binding partner is an antibody or immunologically active fragment thereof, or a cytotoxic cell. The binding partner includes an immunointeractive molecule that can interact with a dengue antigen or equivalent.

As indicated herein, the preferred binding partner is an immunointeractive molecule, which preferably refers to any molecule comprising an antigen binding portion or a derivative thereof. Preferably, the immunointeractive molecule is an antibody against any portion of dengue viral proteins produced during a humoral response in the subject of a dengue virus infection or exposure.

As indicated herein the preferred 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.

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.

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 Fv fragments.

Preferably the binding partner is an antibody. More preferably, it is an anti-dengue IgG, IgM and/or IgA antibody molecule. In a further embodiment, the binding partner is a reference to any combination of anti-dengue IgG, IgM and IgA antibody molecules.

Cytotoxic cells contained within the biological sample may also serve as binding partners. The cell may interact directly with the dengue virus or any component of a cell lysate from cells infected with dengue virus or an equivalent thereof.

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

It is also preferred that the biological sample is obtained from subjects suspected of exposure to a dengue virus. A biological sample may also be modified prior to use, such as by dilution, purification of various fractions, centrifugation 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.

It should be noted that a biological sample may also be devoid of a binding partner that can interact with dengue virus or an equivalent thereof. This occurs when the subject has not been exposed to dengue virus or an equivalent thereof. Hence "determining the presence of a complex that forms between a binding partner in the biological sample and a component of the lysate" may yield a zero result as a complex cannot form in the absence of binding partners.

It should also be noted that a biological sample may comprise a plurality of binding partners that can interact with dengue virus or an equivalent thereof. Hence, as noted earlier, "determining the presence of a complex that forms between a binding partner in the biological sample and a component of the lysate" encompasses a determination of the presence of a plurality of complexes that form between a plurality of different binding partners in the biological sample and components of the lysate. For example, in a preferred embodiment, an anti-dengue IgA antibody molecule can be detected in combination with an anti-dengue IgM and/or IgG antibody molecule. It is considered that the detection of a plurality of different binding partners will increase the diagnostic sensitivity of the present invention.

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

The biological sample is contacted with a mixture of viral immunogenic components derived from the lysate of cells infected with dengue virus or equivalent thereof. The lysate 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 infection, the development of IgG, IgM and/or IgA antibody molecules is one of the indications of dengue infection and this may be detected by the formation of a complex between it and a component, preferably an immunological component of the lysate.

The lysate as used in the present invention provides for a full spectrum of dengue immunogens that may be used for detecting anti-dengue antibodies (IgG, IgM and/or IgA) or prior exposure to the dengue virus or equivalents thereof. Preferably, the lysate contains dengue virus immunogens. Most preferably, the lysate contains structural and non-structural proteins including but not limited to NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 along with other dengue virus immunogens. Importantly, the lysate is a mixture of components derived from a cell that has been infected by the dengue virus or equivalent thereof.

When a subject is exposed to dengue virus, the body reacts to initially remove the virus. This causes a chain of events generally manifesting in an immunological response to the plethora of antigens presented by the dengue virus or an equivalent thereof.

The lysate of the present invention may be obtained from any source of cells that have been infected with the dengue virus or equivalent thereof. Preferably, the cells are cells infected in an *in vivo* culture with dengue virus or equivalent thereof.

Any type of cell may be infected. Preferably, the cell type is capable of infection and culture of dengue virus. However, it is preferred that cells capable of producing high titres of dengue virus are infected in accordance with the methods of the present invention, including, but not limited to, continuous cell lines commonly available (e.g. Vero cells (Vero-PM strain), CV-1 cells, LLC-MK2, C6/36 and AP-61 cells), primary cell lines such as fetal Rhesus lung (FRhL-2) cells, BSC-1 cells, and MRC-5 cells, or human diploid fibroblasts. A combination of cell types is also envisaged by the present invention. C6/36 or

AP-61 cells are infected with dengue virus or equivalent. Most preferably the cell type is C6/36.

Cells may be cultured for any period, preferably for a period which allows the dengue virus to establish and infect the cell. More preferably, the cells are cultured until a cytopathic effect is apparent in the cell culture thereby indicating active infection of the virus in the cells.

At this point, the cells may be lysed by any method available to the skilled addressee. Generally, the use of a hypotonic buffer including a detergent such as Triton X may be used providing the lysing buffer does not affect the imunogens of the dengue virus or equivalent thereof but inactivates the live virus particles.

It should be appreciated that the lysate will contain a mixture of viral immunogenic components including structural and non-structural dengue virus antigens as well as whole dengue virus particles selected from the group including DEN1, 2, 3 or 4. The present invention seeks to identify a mixture of antigens by a mixture of binding partners that are generated in the biological sample in response to exposure to the dengue virus of an equivalent.

The lysate may be processed in any manner. Preferably, the lysate is clarified to remove nuclei and cellular debris and whole dengue virus particles. The lysate may be aliquoted and stored at -80°C for future use.

The lysate and biological sample is contacted so that a complex may form between the viral immunogenic components of the lysate and the binding partner contained within the biological sample. Preferably, immunogens of the dengue virus particles, including but not limited to those of the structural and non-structural proteins, will form complexes with binding partners. Preferably, the binding partners are antibodies or fragments thereof derived from the biological sample. These will only be present when the subject has been exposed/immunized to the dengue virus.

Preferably, the complex will form between an antibody, preferably an IgG, IgM or an IgA antibody for the dengue virus or equivalent thereof and a dengue immunogens.

The binding partner may also be a cytotoxic cell such as a cytotoxic T-cell from the biological sample that reacts to the dengue immunogens.

Preferably the dengue immunogen is derived from a structural or non-structural protein of the dengue virus. More preferably, the structural protein is selected from the group including C-Capsid, M-Membrane and E-Envelope proteins. More preferably, the non-structural proteins are selected from the group including NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5. Most preferably the non-structural protein is NS-1.

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

Previous methods use specific dengue antigens DEN 1, 2, 3 and 4 (present in the supernatant of dengue virus infected cells) to detect antibodies indicative of dengue virus infection. However, the present invention does not solely use these antigens but a mixture of dengue virus molecules/ immunogens (present in the dengue virus infected cells, which may contain virus particles and other immunological components, preferably structural and non-structural proteins) against which antibodies develop in the course of a dengue exposure.

The preferred method of the present invention relies upon the detection of the complex formed between a binding partner contained within the biological sample and a component of the lysate derived from a cell infected with the dengue virus or an equivalent thereof. The complex may comprise one or more binding partners bound to one or more components derived from dengue virus or an equivalent thereof.

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

In yet another aspect, the biological sample may be applied to a membrane such as, but not limited to, a nitrocellulose membrane and the component derived from dengue virus or an equivalent thereof added. The component derived from a dengue virus or equivalent thereof is then left in contact with the biological sample for a period of time sufficient and under conditions, which allow the stable formation of a complex. Once a complex is formed, a detection system is then added to facilitate the detection of the complex.

Detecting the complex between the components derived from dengue virus or immunological relative 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.

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, or variations or combinations of these or other techniques such as are known in the art.
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 a preferred embodiment, the method of detection employs a further detection agent, which permits detection of said complexes.

In a preferred embodiment, the methods as herein described involve the use of a cell lysate derived from a cell infected with a dengue virus, or the purified components therefrom (herein referred to interchangeably as the "components" or the "components of the cell lysate"), immobilized on a solid support such as a nitrocellulose membrane to which a binding partner of a biological sample may

bind. The complex formed by a component of the cell lysate and the binding partner may then be detected using a detection agent that contains a reporter group and specifically binds to the component/binding partner complex. Such detection agent may comprise, for example, an antibody or other agent that specifically binds to the binding partner, such as an anti-immunoglobulin (i.e. antibody), protein G, protein A or a lectin. Alternatively, a competitive assay may be utilized, in which a detection agent capable of binding an antigen derived from a dengue virus, is labelled with a reporter group and allowed to bind to the immobilized component of the cell lysate in combination with the binding partner of the biological sample. The extent to which the binding partner of the biological sample inhibits the binding of the labelled dengue virus detection agent to the immobilized component is indicative of the reactivity of the binding partner of the biological sample with the immobilized component.

In a preferred embodiment, the detection reagent is an antibody or secondary antibody or an antigen-binding fragment thereof, capable of binding to the binding partner of the biological sample. Antibodies may be prepared by any of a variety of techniques known to those of ordinary skill in the art (See, for example, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988). In general, antibodies can be produced by cell culture techniques, including the generation of monoclonal antibodies, or via transfection of antibody genes into suitable bacterial or mammalian cell hosts, in order to allow for the production of recombinant antibodies.

The secondary antibody, which may be conjugated to a label, can be added to the complex to facilitate detection. A range of labels providing a detectable signal may be employed. The label may be selected from a group including chromogen, an enzyme, a catalyst, a fluorophore and a direct visual label. In the case of a direct visual label, use may be made of a colloidal metallic or non-metalic 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. 4366241, 4843000 and 4849338. Suitable enzyme labels in the present invention include alkaline phosphatase, 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 color change, preferably achieves the detection of the complex.

The solid support may be any material known to those of ordinary skill in the art to which a binding partner or component of the cell lysate 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. The support may also be a magnetic particle or a fiber optic sensor, such as those disclosed, for example, in U.S. Pat. No. 5,359,681.

The binding partner or the component of the cell lysate 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 to both non-covalent association, such as adsorption, and covalent attachment (which may be a direct linkage between the antigen or nucleotide and functional groups on the support, or may be a linkage by way of a cross-linking agent). Immobilization by adsorption to a well in a microtitre plate or to a membrane is preferred. In such cases, adsorption may be achieved by contacting the binding partner or a component of the cell lysate, in a suitable buffer, with the solid support for a suitable amount of time. The contact time varies with temperature, but is typically between about 1 hour and about 1 day.

Covalent attachment of a binding partner or a component of the cell lysate to a solid support may also generally be achieved by first reacting the support with a bi-functional reagent that will react with both the support and a functional group, such as a hydroxyl or amino group, of a binding partner or a component of the cell lysate. For example, the binding partner or component may be covalently attached to supports having an appropriate polymer coating using benzoquinone or by condensation of an aldehyde group on the support with an

amine and an active hydrogen of the component (see, for example, *Pierce Immunotechnology Catalog and Handbook*, 1991, at A12-A13).

In certain embodiments, the assay is a two-step sandwich assay. This assay may be performed by first contacting a binding partner of a biological sample that has been immobilized on a solid support, commonly the well of a microtitre plate, with the component of a cell lysate as herein described, such that a component is allowed to bind to the immobilized binding partner. Unbound sample is then removed from the immobilized complex and a detection reagent (preferably a second antibody capable of binding to the binding partner or the component, containing a reporter group) is added. The amount of detection reagent that remains bound to the solid support is then determined using a method appropriate for the specific reporter group.

More specifically, once the binding partner or a component of the cell lysate 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 Tween 20 ™ (Sigma Chemical Co., St. Louis, Mo.) is used. The immobilized binding partner or component is then incubated with a component of a cell lysate or binding partner, respectively, such that a complex between the binding partner and the component is allowed to form. The component or binding partner may also be diluted with a suitable diluent, such as phosphate-buffered saline (PBS) prior to incubation. In general, an appropriate contact time (i.e., incubation time) is a period of time that is sufficient to allow a component of the cell lysate to bind to the immobilized binding partner, or vice versa. Preferably, the contact time is sufficient to achieve a level of binding that is at least about 95% of that achieved at equilibrium between the bound and unbound binding partner or component of the cell lysate. 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. At room temperature, an incubation time of about 30-60 minutes is generally sufficient.

Unbound components may then be removed by washing the solid support with an appropriate buffer, such as PBS containing 0.1% Tween 20™. A detection agent which is capable of binding to the component of the cell lysate or the binding partner, and which contains a reporter group, may then be added to the solid support. Preferred reporter groups include those groups recited below. The detection agent is then incubated with the immobilized binding partner-component complex for an amount of time sufficient to detect the bound component or binding partner. 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), rhodamine, Texas Red and phycoerythrin. Biotin may be detected using avidin, coupled to a different reporter group (commonly a radioactive or fluorescent group or an enzyme). Enzyme reporter groups may generally be detected by the addition of substrate (generally for a specific period of time), followed by spectroscopic or other analysis of the reaction products. The substrate can be selected from a group of agents consisting of 4-chloro-l-naphtol (4CN), diaminobenzidine (DAB), aminoethyl carbazole (AEC), 2,2'azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ophenylenediamine (OPD) and tetramethyl benzidine (TMB).

It may also be desirable to couple more than one reporter group to a detection agent. In one embodiment, multiple reporter groups are coupled to one detection agent molecule. In another embodiment, more than one type of reporter group may be coupled to one detection agent. Regardless of the particular embodiment, detection agents with more than one reporter group may be prepared in a variety of ways. For example, more than one reporter group

may be coupled directly to a detection agent, or linkers that provide multiple sites for attachment can be used.

In a related embodiment, the method as herein described may be performed in a flow-through or strip test format, wherein the binding partner of a biological sample or a component of the cell lysate is immobilized on a membrane, such as nitrocellulose. In the flow-through test, for example, a component of the cell lysate is capable of binding to the immobilized binding partner as the sample passes through the membrane. Alternatively, a binding partner in a biological sample is capable of binding to the immobilized component of the cell lysate as the sample passes through the membrane. A second, labelled detection agent then binds to the binding partner-component complex as a solution containing the detection agent flows through the membrane. The detection of bound detection agent may then be performed as described above.

In the strip test format, one end of the membrane to which a component of the cell lysate is bound is immersed in a solution containing the biological sample. The binding partner in the biological sample migrates along the membrane through a region containing a detection agent and to the area of the immobilized component. The concentration of detection agent at the area of immobilized binding partner-component complex indicates the presence of binding agent in a biological sample. Typically, the concentration of the detection reagent at that site generates a pattern, such as a line, that can be read visually. The absence of such a pattern indicates a negative result. In general, the amount of binding partner immobilized on the membrane is selected to generate a visually discernible pattern when the biological sample contains a level of a binding agent that would be sufficient to generate a positive signal in the sandwich assay, in the format discussed above. Such tests can typically be performed with a very small amount of biological sample.

In a simpler version of the strip test or dipstick test, components of the cell lysate can be immobilised onto a membrane, such as a nitrocellulose membrane. Strips of membrane may then be subjected to biological samples to form complexes between the components and a binding partner in the biological

sample. The complex is then detectable by any means described above using a detection agent, such as an antibody. The dipstick test can provide a quick indication of previous exposure without using large biological samples.

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 10^3 L/mol. The binding constant may be determined using methods well known in the art.

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 dengue virus 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.

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 immunoblotting 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 components of the cell lysate is immobilized, an appropriate buffer, a standardised sample comprising a detectable level of binding agent, and a secondary detection reagent, as herein described.

The method and kits of the present invention can detect exposure of human or animals to a dengue virus 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. The "convalescent phase" refers to the stage of dengue 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 a binding partner in the infected patient.

In another aspect of the present invention there is provided a kit for detecting dengue virus exposure comprising;

g) a solid support including a component derived from a lysate of cells infected with dengue virus or equivalent thereof;

or

h) a solid support including a component derived from a lysate of cells infected with dengue virus or equivalent thereof attached to a second support;

i) an incubation container;

j) blocking buffer;

k) washing buffer;

l) at least one detection agent conjugated to a label for detecting a complex that forms in a biological sample; and optionally

g) instructions for using said kit.

The present invention further provides a kit for detecting exposure of a subject to dengue virus or an equivalent thereof. The kit may be any convenient form but generally comprises a solid support such as described herein adapted to receive or comprise isolated immunogenic components or lysate of a cell infected with dengue virus or an equivalent thereof. The kit may also comprise reagents, reporter molecules capable of providing detectable signals and optionally instructions for use. The kit may be in modular form wherein individual components may be separately purchased.

The kit may be a modular kit comprising one or more members wherein at least one member is a solid support comprising a component of a lysate of a cell infected with dengue virus or equivalent or cell lysate comprising an immunogenic component derived from a dengue virus or equivalent thereof.

In an alternative embodiment the solid support comprises an array of binding partners for one or more components of one or more dengue virus or equivalent thereof from one or more subjects.

The present invention also provides individual components of the kit for use in the method of the present invention. The invention provides solid supports which include components of the dengue virus for use in the detection of exposure to the dengue virus. In one embodiment, the invention provides a nitrocellulose membrane, either for use as a dot blot or use as a dip stick, which includes components of the dengue virus or equivalent thereof. Preferably, the membranes include components selected from the group including dengue virus structural and non-structural proteins, dengue virus particles and fragments thereof, glycoproteins, lipids and carbohydrates derived from the dengue virus or any mixture thereof.

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

The second support may be a holder which holds the solid support to improve manipulation of the solid support which has immobilised components of the dengue virus. For instance, the nitrocellulose membrane may be supported on a stick which enables the membrane to be dipped into a biological sample such as serum. This is useful as a component of a kit since small amounts of biological sample can be tested simultaneously.

The present invention also provides a method of assessing the relative risk of one or more subjects being exposed to dengue virus or its equivalent thereof

within a defined location (e.g. geographical area, housing estate, means of transport or center for medical treatment or assessment), comprising;

a) obtaining samples from a representative population within a defined location; and

b) 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 -

   i) contacting a biological sample from subject with a component derived from the dengue virus or equivalent thereof to form a complex between the components and a subject-derived binding partner of the component contained within the biological sample;

   ii) determining the presence of the complex, wherein the presence of the complex is indicative of exposure of the subject to a dengue virus or equivalent thereof; and

   iii) assessing the relative risk of exposure within the defined location.

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 analysing 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.

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

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 which 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 IgA or the alternations of antibody level (IgG). Alleviation of IgG or IgA titre or their presence in an early phase of infection may be monitored to determine the subsequent phases of dengue infection like dengue hemorrhagic fever or dengue shock syndrome.

Further, the technique of the present invention provide a means for identifying subjects who are infected with dengue virus, allowing for the rapid detection, pointing the location of infection and disease control strategy.

Examples of the procedures used in the present invention will now be more fully described. 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.

EXAMPLES

Example 1: The Dot-Blot EIA (DB-EIA)

a) Preparation of dengue virus lysate antigens

C6/36 cells were grown in 75mm^3 tissue culture flasks (Nunc, Denmark) in Leibovitz 15 medium containing 5% heat inactivated foetal bovine serum. Virus stocks were inoculated onto confluent monolayers after removal of growth medium and allowed to adsorb to the cells by rocking the flasks at room temperature for one hour, after which the flasks were replenished with 20 ml of maintenance medium (Leibovitz 15 medium with 2% heat inactivated bovine serum and antibiotics), then incubated stationary in an incubator set at 30°C. Maintenance medium was changed after 2 to 4 days (depending on the virus) and the culture supernatants and infected cells were harvested when cytopathic effect was apparent throughout the monolayer. The culture supernatants were clarified by centrifugation for 10 minutes at 1000 rpm at 4°C in a Universal 32R, Hettich with a 1452 rotor, and stored in aliquots at -80° until use. The infected monolyers were washed one time with phosphate buffered saline (PBS) and

lysed in 2 ml of a hypotonic buffer (10mM Hepes-0.25 ml 10mM, KCL-0.1ml, 1.5mM, Cl237.5ul) containing 1% TX-100. Intact nuclei were removed by brief centrifugation at 14,000 rpm for one minute in a microcentrifuge (18 Microcentrifuges, BECKMAN COULTER) and the lysate supernatants (referred to as "lysates") were aliquoted and stored at -80°C until use.

(b) Dengue positive and negative sera

Serum samples from fifty-eight positive cases were used in this study. Five dengue positive sera were used for the development of the test protocol. One hundred and twenty two dengue negative serum samples were used as negative control. The negative control included serum collected from 16 laboratory staff and 104 from foreign workers who were negative to dengue infection for pan-bio IgG assays.

(c) DB-EIA protocol

The dot-blot was performed onto nitrocellulose membrane (Schleincher & Schuell BA-S 83, 0.45µm, Keene, NH) in a 48 well format. For the assay, 3ul of dengue virus lysate (1, 2, 3 & 4, as prepared above) were dotted into the labelled nitrocellulose (NC) membrane in duplicate and then dried at room temperature. Each dot on the NC membrane was cut into 7mm x 7mm pieces and placed into each well of a 48 well plastic plate (NUNC, USA) in such a way that the labelled side was facing up and then the NC dots were blocked at room temperature for 15 minutes using blocking buffer containing 5% skim milk (Merck, Germany) and 0.1% triton x 100 (Duchefa, The Netherlands). After incubation, the blocking buffer was aspirated off and the wells were washed 3 times with washing buffer (1xPBS with 0.05% Tween 20) allowing one minute in each wash with gentle agitation and then used for an anti-dengue-IgG assay. The antigen dotted membranes were incubated at room temperature for 10 minutes with 100-200µl serum sample diluted 1:100 in diluent buffer (5% skim milk with 0.1% Triton x 100) in duplicate in 48 well plates. Positive and negative serum controls and membrane controls were kept in the first column of the plate. After incubation, strips were washed four times with washing buffer as described above. After a final wash 200µl of rabbit anti-human IgG-HRP (1:1000, Code No. P0214, Lot 012, Edition 11.03.02, DAKO, Denmark) was

added into each well and incubated again at room temperature for 10 minutes and the strips were washed again for 5 times using washer buffer as described above. After a final wash, 75-100µl of substrate solution (DAB, Code no-K3466, DakoCytomation, Denmark) was added to each well. The presence of anti-dengue IgG was indicated with the development of a well-defined circle of a brownish stain within 3 minutes on the NC membrane. The strips were then washed 2 times with deionised water, dried at room temperature and the results were interpreted visually. The intensity of the stain/colour developed in the dot is roughly proportional to the amount of anti-dengue IgG in the serum samples (Figures 1 and 2). Development of an unclear dot is considered as non-conclusive and needs the testing of a second sample at least five days interval of the first sampling.

For the titration of the level of anti-dengue IgG, a range of dilutions of serum were made starting from 1:100 to 1:6400 and were performed in duplicate in a 48 well plate format.

The DB-EIA was developed using dengue virus and confirmed 5 positive and 16 dengue negative sera collected from laboratory staff. Virus neutralization tests against all serotypes of dengue viruses was used to confirm reactivity of the serum samples.

Sera were tested at ranges of 1:12.5 to 1:800 dilutions. Results showed that all dengue confirmed 5 convalescents showed high levels of reaction against dengue lysate antigen (>1:800) while 16 negative sera did not show any reaction even at 1:50 dilutions except two serum samples which showed mild reaction up to a level of 1:50 dilutions (one of them had a history of vaccination against Japanese encephalitis virus while the other one had a vaccination against Japanese encephalitis, Yellow fever and Tick-borne encephalitis virus and have a neutralizing positive antibody against yellow fever vaccine virus). Hence the cut-off point of serum dilution for the DB-EIA was set at 1:100 and used throughout the study.

Dengue confirmed 4 convalescent positive sera were further titrated in ranges of dilutions starting from 1:100 to 1:6400 that showed positive reaction to the dilutions of 1:6400. All results of DB-EIA were also evaluated in terms of intra- and inter assays and showed high level of consistency.

Example 2: Comparison of DB-EIA to a Dengue Indirect IgG ELISA and a competitive slide-enzyme immunoassay (C-EIA).

(a) Dengue Indirect IgG ELISA

The PanBio Dengue Indirect IgG ELISA Test (PanBio, Brisbane, Australia) was used to detect antibodies according to the method described by the manufacturer. The commercially available kit was used as the standard technique against dot-blot EIA. Dengue antigen coated polystyrene microtite plates supplied were used for the test. Test sera were diluted in 1:100 with the serum diluent provided, 100µl/well of the positive, negative; cut-off and patients' sera were added to the plate containing anti-human IgG. Human IgG will be attached if they are present in the patient's sera. The plate was then incubated for 30 minutes at 37°C. Following incubation, the plate was washed 6 times with the wash buffer provided and 100µl/well of horseradish peroxidase (HRP) conjugated anti-human IgG was added. These complexes were then captured by dengue specific IgG during incubation of 30 minutes at 37°C. Once again, the plate was washed six times with wash buffer and bound complexes were visualised through the addition of a 100µl tetramethylbenzidine substrate. After 15 minutes, the reaction was stopped by the addition of 100µl/well 1M phosphoric acid. The color intensity in the plate was then read at 450nm using an ELISA plate reader.

The optical density values obtained from the reader were converted to PanBio Units to determine whether the sample sera were positive or negative using the formula below:

PanBio Units = 10 x Absorbance of Sample/Mean Absorbance of cut-off

Pan Bio values below 9 were regarded as negative, values between 9 and 11 as equivocal and values above 11 as positive.

(b) Competitive slide-enzyme immunoassay (C-EIA)

Competitive slide-immunoassay (C-EIA) was carried out on a twelve-welled rectangular glass slide (Pathology, China), approximately 5cm x 8cm with three columns and four rows. The glass slide was used as a coating plate for antigen. Each well was coated with 4µl dengue virus antigen, air-dried and then fixed in ice-cold 80% acetone for 15 minutes. Following fixation, the slide was again air-dried and 20µl of blocking-buffer (PBS supplemented with 0.1% [v/v] Tween-20 and 0.3% [v/v] flavivirus negative human serum) was added to each well and 5µl of test serum was added in two wells as duplicated sample (1:5). Finally 25µl of Mab (either flavivirus specific or dengue virus specific), derived from the supernatant of the hybridoma culture was added to each well except the well which was kept as a conjugate control, or slide control (100% competition). The well with no test serum was the Mab control (0% competition). The serum controls included wells with either strong positive or a negative serum to ensure that similar levels of inhibition were obtained in each test. Following 45 minutes of incubation at 37°C, the plate was washed three times with a washing solution (1:5 in PBS) and 50µl goat anti-mouse serum conjugated with HRP (1:3000 in blocking buffer, Dako-cytomation Code Nr P 0447) was added to each well and incubated for 45 minutes at 37°C. The slide was again washed three times in washing solution and 50µl/well of hydrogen peroxide/orthophenylene diamine (OPD) (Sigma, USA) was added and allowed to develop color for 15 minutes. Further development of color was stopped by the addition of 50µl/well of 6.8% sulphuric acid. The optical densities obtained were read by ELISA plate reader at an absorbance of 492 nm and converted to percentage of inhibition (PI) by using the following formula:

$$PI = 100 - (OP \text{ in the test well} / OD \text{ of Mab}) \times 100$$

PI values greater than 50% were considered as positive to the dengue antigens.

(c) DB-EIA

This was performed according to Example 1.

(d) Comparison of the tests

The same set of serum samples were examined in dengue IgG indirect ELISA, competitive EIA and the DB-EIA.

The sensitivity and specificity of DB-EIA were analysed using 58 confirmed convalescent and 122 negative sera. All these 58 Dengue confirmed convalescent sera showed very clear positive reaction with DB-EIA thus the sensitivity of DB-EIA was 100%. All those 122 serum samples collected and tested negative to dengue indirect IgG ELISA and C-EIA were negative in DB-EIA and hence showed 100% specificity. A Chi-squared analysis with Yates continuity correction showed that this is highly significant (χ^2 = 166.70; $p<0.0005$). The odds ratio approached infinity to positive and negative serum samples.

Table 1: Evaluation of Dot-Blot with Pan-bio dengue ELISA using 2 by 2 table

Dot Blot –EIA

		Positive	Negative	Total
Pan-bio ELISA	Positive	58	0	58
	Negative	0	122	122
	Total	58	122	180

Sensitivity = 58/58 = 1x100=100%, Specificity = 122/122 = 1x100=100%

Table 2: Evaluation of Dot-blot EIA with Competitive EIA using 2 by 2 Table

Dot-Blot EIA

		Positive	Negative	Total
C-EIA	Positive	58	0	58
	Negative	0	122	122
	Total	58	122	180

Sensitivity = 58/58 = 1x100=100%, Specificity = 122/122 = 1x100=100%

Results of the comparison clearly indicated that the DB-EIA might be a suitable test for the detection of anti-dengue IgG antibodies. Among the three antibody assays, DB-EIA is able to detect dengue antibody positive from sera at the highest dilution of 1:6400 within 30 minutes carried out at room temperature while ELISA and C-EIA are able to detect 1:180 and 1:80 dilution respectively and both the tests need 3 to 4 hours test time along with laboratory equipment support. This indicates that the DB-EIA would be able to detect low levels of antibodies to dengue compared to indirect ELISA and C-EIA.

The DB-EIA is also simple and rapid and can be interpreted visually and can also be preserved as a record. The entire procedure requires only 30 minutes. Unlike indirect ELISA and C-EIA, an experienced technician is not required for this assay. The level of antibody can be judged by the intensity of the color range although the microtitre plate ELISA would be able to provide a more useful quantitative assessment with the appropriate absorbance reading.

Example 3: A dipstick Assay for Dengue

For the dipstick assay, 3µl of cell lysate dengue antigen (1, 2, 3 & 4) was dotted onto the labelled nitrocellulose membrane (Schleincher & Schuell BA-S 83, 0.45µm, Keene, NH) in duplicates and dried at room temperature. The lysate was prepared as in Example 1. The membrane was then cut into pieces (7mm x 2cm) and placed in a disposable trough in such as way that the labelled side was facing up. The membrane was blocked using blocking buffer for a period of 15 minutes. After incubation, the blocking buffer was aspirated off and the wells were washed 3 times with washing buffer (1x PBS with 0.05% Tween 20) allowing one minute in each wash with gentle agitation and then dried in air. The non-dotted part of the membrane was then fixed onto the transparent plastic strip with double sided adhesive tape in such a way that the labelled side facing up was not touched with the dotted portion of the membrane. The antigen dotted stick membranes were placed in tubes with 300µl serum sample (1:100 in diluent buffer) and incubated at room temperature for 10 minutes. Positive and negative serum controls as in Example 1 were kept separately for each batch of assay. After incubation, sticks were washed four times with

washing buffer allowing one minute for each washing. After a final wash 300µl of rabbit anti-human IgG-HRP (1:1000, Code No. P0214, Lot 012, Edition 11.03.02, DAKO, Denmark) was added into each tube and incubated again at room temperature for 10 minutes and sticks were then washed again for 5 times using washing buffer as described above. After final wash, 300µl of substrate solution ((DAB, Code no-K3466, DakoCytomation, Denmark) were added into each tube and allowed to react for 3 minutes. The presence of anti-dengue IgG was indicated with the development of two well-defined circles of brownish stain on NC membrane. The sticks were then washed 2 times with deionised water, dried at room temperature and results were interpreted visually. The intensity of the stain/color developed in the dot is roughly proportional to the amount of anti-dengue IgG in the serum samples (Figure 3). The dipstick technique was developed using the same test protocol as in Example 1 for the DB-EIA. It was found to be as good as the DB-EIA and can be used for the handling of small numbers of samples for the detection of anti-dengue IgG. Furthermore, the dipstick technique can be more suitable at the field and patient bedside screening.

In conclusion, both the dot blot EIA and dipstick are a useful method for detecting IgG antibodies to dengue and has showed the best performance compared to indirect ELISA and C-EIA in detecting low levels of antibodies to dengue virus as well as to distinguish between dengue virus antibodies positive from the negative sera.

Example 4: Development of a Dot-blot for the detection of anti-dengue IgA.

(a) Preparation of dengue cell lysate

Dengue cell lysate was prepared using the same protocol described above in Example 1.

(b) Dengue IgA positive and negative samples.

Sixty four serum samples from clinically positive dengue patient which confirmed either by PCR or anti-dengue IgM assays were used in this assays as dengue positive samples. Five pairs of dengue positive serum samples (collected 1-5 days interval of first collection) were also used in this study in

order to find the alleviation of the titres of IgA and IgG in the samples. Twenty two dengue negative sera (which were negative to IgM and IgG using Pan-Bio dengue kit) were used as dengue IgA negative samples in this assay.

(c) DB-EIA protocol:

The dot-blot EIA was performed onto nitrocellulose membrane (Schleincher & Schuell BA-S 83, 0.45μm, Keene, NH) in a 48 well format. For the assay, 3ul of dengue cell lysate (1, 2, 3 & 4) was dotted onto the labeled nitrocellulose (NC) membrane in duplicates and then dried at room temperature. Each dot on the NC membranes was cut into piece (7mm x 7mm) and placed into each well of 48 wells plastic plate (NUNC, USA) in such way that the labeled side facing up and then the NC dots were blocked at room temperature for 15 minutes using blocking buffer containing 5% skim milk (Merck, Germany) and 0.1% Triton X 100 (Duchefa, The Netherlands). After incubation, the blocking buffer was aspirated off and the wells were washed 4 times with washing buffer (1x PBS with 0.05% Tween 20) allowing one minutes in each wash with gentle agitation and then used for anti-dengue- IgA. The antigen dotted membranes was incubated at room temperature for 30-45 minutes with 100-200μl serum sample diluted 1:50 in diluent buffer (5% skim milk with 0.1% Triton X100) in duplicates in 48 well plates. A positive and negative serum controls were kept in the first column of the plate. After incubation, strips were washed four-five times with washing buffer described above. After a final wash 200μl of rabbit anti-human IgA-HRP (1:1000) was added into each well and incubated again at room temperature for 30-45 minutes and strips were washed again for 5 times using washing buffer as described above. After final wash, 75-100 μl of substrate solution ((DAB, Code no-K3466, DakoCytomation, Denmark) added to each well. The presence of anti-dengue IgA was indicated with the development of brownish dot within 3 minutes on the NC membrane. The strips were then washed 2 times with deionized water, dried at room temperature and results were interpreted visually. The intensity of the stain/color developed in the dot is roughly proportional to the amount of anti-dengue IgA in the serum samples (Figures 4 & 5).

For the titration of level of anti-dengue IgA, a range of dilutions of serum were made starting from 1:50 to 1: 6400 and were performed in duplicates in 48 well

plate format. Ten pairs (1-5 days interval) of serum samples from clinically confirmed dengue patients were analysed using anti-dengue IgA assays by serial dilutions (Figure 6).

Example 5: Comparison of dengue IgA dot-blot assay with dengue IgM assays using Focus Technologies and Pan-bio test kits

(a) Focus Technologies IgM assays

The test was conducted by the hospital using the procedures described by the manufacturer. In short, the technique involves the capturing of IgM from test serum using anti-IgM antibodies coated on the wells of a microtitre plate which is followed by attachment of known dengue virus antigen. The antigen-antibody complex was detected by a second set of antibody conjugated with enzymes and finally indicated the reaction by the addition of substrate which produced colour. The colour was stabilized by stopping buffer and the results read by an ELISA reader (Technical summary, Focus Technologies).

(b) Pan-Bio dengue IgM Capture ELISA

Pan-bio IgM capture ELISA was carried out using the instructions manual of the manufacturer (Dengue IgM capture ELISA, Cat No. E-DENO1M, PanBio). Anti-human IgM coated on the microplate test strips bases the principle of the test on the capturing of human IgM present in the test serum followed by the addition of pre-treated dengue antigen with monoclonal tracer. The positive reaction is indicated by the addition of indicator system Tetramethylbenzidine/hydrogen peroxidase (TMB/HO) which produced blue colour in the positive samples. After adding stopping buffer, the TMB become yellow and then measured the optical density by ELISA reader.

The sensitivity and specificity of anti-dengue IgA DB-EIA were analysed using 64 confirmed IgM positive and 24 negative sera. Of 64 Dengue confirmed IgM sera, 62 serum samples showed very clear positive reaction with DB-EIA thus the sensitivity of DB-EIA was 96.87%. All those 22 serum samples collected and tested negative to dengue IgM capture ELISAs (Pan Bio and Focus Technologies) were negative in DB-EIA and hence showed 100% specificity

Table 3: Evaluation of Dot-Blot with dengue IgM capture ELISA using 2 by 2 table

Anti-dengue IgA Dot Blot –EIA

		Positive	Negative	Total
IgM Capture ELISA	Positive	62	2	64
	Negative	0	22	22
	Total	62	24	86

Sensitivity= 62x100/64 = 96.87%

Specificity= 22x100/22 = 100%

(c) Dipstick Assay:

For dipstick assay, 3µl of dengue cell lysate (1, 2, 3 & 4) was dotted onto the labeled nitrocellulose membrane (Schleincher & Schuell BA-S 83, 0.45µm, Keene, NH) in duplicates and dried at room temperature. The membrane was then cut into pieces (7mmx2 cm) and placed in disposable trough in such way that the labeled side facing up and then block the membrane using blocking buffer for a period of 15 minutes. After incubation, the blocking buffer was aspirated off and the wells were washed 3 times with washing buffer (1x PBS with 0.05% Tween 20) allowing one minutes in each wash with gentle agitation and then dried in air. The non-dotted part of the membrane was then fixed onto the transparent plastic strip with double sided adhesive tap in such way that the labeled side facing up and not touched dotted portion of the membrane. The antigen dotted stick membranes were put in tubes with 300µl serum sample (1:50 in diluent buffer) and incubated at room temperature for 20-30 minutes. A positive and negative serum controls were kept separately for each batch of assay. After incubation, sticks were washed four times with washing buffer allowing 3 minutes for each washing. After a final wash 300µl of rabbit anti-human IgA-HRP (1:1000) was added into each tube and incubated again at room temperature for 30-45 minutes and sticks were then washed again for 5 times using washing buffer as described above. After final wash, 200 µl of substrate solution ((DAB, Code no-K3466, DakoCytomation, Denmark) were added into each tube and allowed to react for 3 minutes. The presence of anti-dengue IgA was indicated with the development of well-defined circle of

brownish stain on NC membrane. The sticks were then washed 2 times with deionized water, dried at room temperature and results were interpreted visually. The intensity of the stain/color developed in the dot is roughly proportional to the amount of anti-dengue IgA in the serum samples (Figure 7).

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Finally it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

CLAIMS

1. A method for detecting exposure of a subject to a dengue virus or an equivalent thereof, said method comprising:

contacting a biological sample from the subject with a mixture of immunogenic components derived from a lysate of cells infected with dengue virus; and

determining the presence of a complex that forms between a binding partner in the biological sample and an immunogenic component of the lysate.

2. A method according to claim 1 wherein the mixture of immunogenic components includes dengue virus structural and non-structural proteins, dengue virus particles and fragments thereof, glycoproteins, lipids and carbohydrates derived from the dengue virus.

3. A method according to claim 2 wherein structural proteins are selected from the group including envelope proteins, Pr membrane proteins, and nucleocapsid proteins.

4. A method according to claim 2 or 3 wherein the non-structural protein is selected from a group including NS-1, NS-2a, NS-2b, NS-3, NS-4a, NS-4b and NS-5.

5. A method according to claim 4 wherein the non-structural protein is NS-1.

6. A method according to claim 2 wherein the component is an anti-idiotypic antibody to an antigen binding site of a dengue antibody generated in response to exposure to a component derived from dengue virus or equivalents thereof.

7. A method according to any one of claims 1 to 6 further including determining the presence of a complex formed between a binding partner and any one of DEN 1, 2, 3 or 4 immunogens or equivalent thereof.

8. A method according to any one of claims 1 to 7 wherein the dengue virus is any one of dengue virus serotypes including DEN-1, DEN-2, DEN-3 or DEN-4.

9. A method according to any one of claims 1 to 8 wherein the binding partner is an antibody or an immunological fragment thereof.

10. A method according to claim 9 wherein the binding partner is an antibody expressed in an early stage of dengue virus infection or convalescence.

11. A method according to claim 10 wherein the antibody is an IgG, IgM and/or an IgA antibody.

12. A method according to any one of claims 9 to 11 wherein the immune fragment of the antibody is any one of a Fab fragment, $F(ab')_2$ fragment or an Fv fragment.

13. A method according to any one of claims 1 to 12 wherein the biological sample is selected from the group including blood, saliva, cord fluid, B cells, T cells, plasma, serum, urine and amniotic fluid.

14. A method according to claim 13 wherein the biological sample is serum and or saliva.

15. A dengue virus infected cell lysate composition when used for the detection of exposure of an animal or human to a dengue virus or immunological relative thereof, said lysate obtained by lysing a cell infected with the dengue virus.

16. A lysate composition according to claim 15 wherein the dengue virus is selected from the group including the dengue virus serotypes including DEN-1, DEN-2, DEN-3 or DEN-4.

17. A lysate composition according to claim 15 or 16 comprising a mixture of immunogenic components including dengue virus structural and non-structural proteins, dengue virus particles and fragments thereof, glycoproteins, lipids and carbohydrates derived from the dengue virus.

18. A lysate according to claim 17 wherein structural proteins are selected from the group including envelope proteins, Pr membrane proteins, and nucleocapsid proteins.

19. A lysate according to claim 17 or 18 wherein the non-structural protein is selected from a group including NS-1, NS-2a, NS-2b, NS-3, NS-4a, NS-4b and NS-5.

20. A lysate according to claim 19 wherein the non-structural protein is NS-1.

21. A kit for detecting a dengue virus exposure comprising;

a) a solid support including an immunogenic component derived from a lysate of a cell infected with dengue virus or equivalent thereof; or

b) a solid support including an immunogenic component derived from a lysate of a cell infected with dengue virus or equivalent thereof attached to a second support;

c) an incubation container;

d) blocking buffer;

e) washing buffer;

f) at least one detection agent conjugated to a label for detecting a complex that forms in a biological sample; and optionally

g) instructions for using said kit.

22. The kit according to claim 21, where the solid support in (a) is used alone or in combination with (b).

23. The kit according to claim 21 or 22, wherein said incubation container is a mictrotitre plate.

24. The kit according to any one of claims 21 to 23, wherein said label is selected from a group including fluorochrome, a chromogen, enzyme, or radioactive molecule.

25. The kit according to any one of claims 21 to 24 wherein said solid support is a nitrocellulose membrane.

26. The kit according to claims 21 to 25 where the solid support is a dot-blot membrane or a dipstick.

27. A dot-blot membrane for use in the detection of dengue virus exposure, said membrane comprising dengue virus structural and non-structural proteins, dengue virus particles and fragments thereof, glycoproteins, lipids and carbohydrates derived from the dengue virus.

28. A dipstick for use in the detection of dengue virus exposure, said dipstick membrane comprising dengue virus structural and non-structural proteins, dengue virus particles and fragments thereof, glycoproteins, lipids and carbohydrates derived from the dengue virus.

29. A method for assessing the relative risk of one or more subjects being exposed to a dengue virus or immunological relative thereof within a defined location, comprising

a) obtaining biological sample from a representative population within the defined location ; and
b) assessing the evidence of exposure of individual members of a sample population to dengue virus or its immunological relative thereof by the steps of;

I. contacting a biological sample from the subject with a component derived from the dengue virus or immunological relative thereof for a time or under conditions sufficient to facilitate the formation of a complex between the component and a subject derived binding partner of the component contained within the biological samples; and

II. determining the presence of the complex, wherein the presence of complex is indicative of exposure of the subject to a dengue virus or immunological relative thereof and thereby assessing the risk of exposure within the defined location;