An immunoassay for the diagnosis of viral infections

Aspects of the present inventions relate to a polypeptide comprising a sequence selected from the group comprising SEQ ID NO1, SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9 or a variant thereof, preferably SEQ ID NO1 or a variant thereof, preferably a dimer and/or hexamer thereof, more preferably a dimer thereof, for the diagnosis of a disease, a diagnostically useful carrier comprising a means for specifically capturing an antibody to SEQ ID NO1 in a sample from a subject, a kit comprising the diagnostically useful carrier, and a method, preferably for diagnosing a disease, comprising the step detecting in a sample from a subject the presence or absence of an antibody to SEQ ID NO1.

Zika virus (ZIKV) is an emerging mosquito-transmitted flavivirus currently causing large epidemics in South and Central America as well as in the Carribean. It is closely related to other human pathogenic members of the flavivirus family like dengue (DENV), West Nile (WNV), Powassan virus (PWV), Japanese encephalitis (JEV), Usutsu and Yellow Fever (YFV) virus. Besides their structural resemblance, most of these viruses share a partially overlapping geographical distribution, tropical and sub-tropical regions representing the favorable environment of the main vector, mosquitos of the genus *Aedes*.

Clinically, Zika fever resembles dengue fever, but is generally less severe. As over 80% of infections are asymptomatic, most cases remain unnoticed. The symptoms comprise fever, rash, arthralgia and conjunctivitis and infections are normally self-limiting. In contrast, in 5% of DENV infections, severe complications lead to the dengue shock syndrome or dengue haemorrhagic fever with high mortality rates. The current ZIKV epidemic, in particular in Brazil, has hardened the suspicion about two potential severe complications in ZIKV infections initially suspected during the 2007 outbreak in Micronesia. Firstly, a significant raise in cases of the rare Guillain-Barré syndrome (GBS), an autoimmune disease resulting in damage of the peripheral nerve myelin, was triggered by infections. Secondly, a 20-fold increase in microcephaly cases in newborns from the highly endemic regions in Brazil, followed by the first reports of ZIKV genome detection in the amniotic fluid of two pregnant women, carrying fetuses with microcephaly, and in the brain of a fetus aborted after the

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intrauterine diagnosis of microcephaly, provided a strong causative link between fetal abnormalities and ZIKV infection during early pregnancy.

Besides the two representatives from the flavivirus family, chikungunya virus (CHIKV), a member of the Alphavirus family, should also be considered in the differential diagnosis. CHIKV is transmitted by the same mosquito vector and is endemic in the same regions. The common distribution and similar clinical presentation in combination with high varieties in disease outcome and the necessity of differentiated treatment of ZIKV, DENV and CHIKV infected patients substantiate the need for specific and reliable diagnostic possibilities.

At present, diagnosis of ZIKV infections is challenging, because the only specific tool is direct proof of viraemia using nucleic acid-based testing, but the viraemic phase usually lasts only up to seven days after symptom onset. Thus, methods such as RT-PCR may already give negative results by the time a patient consults their doctor. Plaque-reduction neutralization tests (PRNT) can measure virus-specific neutralizing antibodies and discriminate between cross-reacting antibodies. This is highly relevant in regions where two or more flaviviruses co-occur. However, PRNT is time-consuming, difficult to perform and not amenable to testing large numbers of sera. In contrast, ELISA-based measurement of virus-specific antibody response is a rapid, scalable and technically mature approach. As reported, IgM antibodies are produced starting four to seven days after symptom onset and IgG antibodies appear a few days later.

A major limitation of conventional serological assays for diagnosing flaviviral infections, for example those based on glycoprotein E (gpE), is their extensive cross-reactivity within the flavivirus genus.

Another limitation is the fact that a range of patients, particular with a background of past flavivirus infections, appear to be deficient in IgM, which is an antibody class that may emerge at the early stage of flavirus infection, prior to detectable levels of IgG class antibodies. In such patients, the results of IgM-based diagnostic tests, as frequently used for the diagnosis of flavivirus infections, give a false-negative result, with severe implications for the health of the patients and, if they are pregnant, their babies.

Another limitation, which concerns research into the field of flavivirus such as Zika virus infections, for example the diagnosis or therapy of flavivirus infections or any basic research, is that sera from patients with confirmed Zika infections are in short supply. Quite often not

only one sample is required, but several samples taken from a range of time points following infection or the onset of symptoms, for example if the kinetics of the disease is investigated or control samples are required for studies related to a therapeutic invention at an early stage of the infection.

Therefore, the problem underlying the present invention is to provide a diagnostic assay overcoming any shortcomings associated with state of the art assays for the diagnosis of flaviviruses such as the Zika virus, in particular those based on the detection of antibodies to flaviviral antigens.

Another problem underlying the present invention is to provide an assay that allows for the specific diagnosis of an infection, preferably a flaviviral infection, more preferably a Zika virus infection, more specifically a distinction between infections with the Zika virus and related flaviviruses such as those selected from the group comprising dengue virus, Yellow fever virus, Tick-borne encephalitis virus, Usutu virus, West Nile virus and Japanese encephalitis virus, preferably dengue virus.

Another problem underlying the present invention is to provide an assay and reagents for the detection of a flavivirus infection, wherein the sensitivity and/or specificity is improved compared to state of the art assays, in particular regarding the early phase of an infection.

Another problem underlying the present invention is to provide a vaccine against a flavivirus, preferably Zika virus infection.

Another problem underlying the present invention is to provide a resource-efficient, yet diagnostically reliable test for distinguishing a flavivirus infection from another disease.

Another problem underlying the present invention is to provide a test providing a longer time window for the diagnosis.

Another problem underlying the present invention is to provide a test that requires a lower amount of patient sample.

Another problem underlying the present invention is to provide an assay for distinguishing an acute infection by Flaviruses, in particular Zika virus, from a vaccination or a previous infection by the same or another Flavivirus, preferably dengue virus.

Another problem underlying the present invention is to provide a vaccine against a flavivirus, preferably Zika virus infection.

Another problem underlying the present invention is to provide a resource-efficient, yet diagnostically reliable test for distinguishing a flavivirus infection from another disease.

Another problem underlying the present invention is to provide a test that may be used during a longer time window between the initial exposure or the onset of symptoms and the day the sample for the diagnosis is obtained.

Another problem underlying the present invention is to provide a test for distinguishing between a primary Flavivirus infection, preferably with a Flavivirus other than the Zika virus, and a secondary Flavivirus infection, preferably Zika infection, which method is diagnostically more reliable than state of the art methods, in particular with regard to avoiding false positive or negative results, and may ideally be applied to samples from patients having an IgM deficiency.

The problem underlying the present invention is solved by the subject-matter of the attached independent and dependent claims.

In a first aspect, the problem underlying the present invention is solved by a polypeptide comprising a sequence selected from the group comprising SEQ ID NO1, SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9 or a variant thereof, preferably SEQ ID NO1 or a variant thereof, preferably a dimer and/or hexamer, more preferably a dimer, for the diagnosis of a disease.

In a second aspect, the problem is solved by a diagnostically useful carrier comprising a means for specifically capturing an antibody to SEQ ID NO1 in a sample from a subject.

In a preferred embodiment, the carrier further comprises one or more than one means, which means is for specifically capturing an antibody to an antigen from the group comprising SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9.

In a preferred embodiment, the carrier is selected from the group comprising a bead, preferably a paramagnetic particle, a test strip, a microtiter plate, a blot, preferably from the group comprising western blot, line blot and dot blot, lateral flow test, a glass surface, a slide, a biochip and a membrane, and is preferably a bead a line blot or microtiter plate, more preferably a microtiter plate.

In a third aspect, the problem is solved by a kit comprising the diagnostically useful carrier according to the present invention, optionally as well as a means for specifically detecting a captured antibody.

In a preferred embodiment, the kit comprises the diagnostically useful carrier which further comprises one or more means, which means is for specifically capturing an antibody to one or more further antigens from the group comprising SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9,

wherein the means for specifically capturing an antibody to SEQ ID NO1 and the means for specifically capturing an antibody to one or more further antigens are coated on, preferably covalently linked to separate carriers.

In a preferred embodiment, the kit comprises the diagnostically useful carrier which further comprises one or more means, which means is for specifically capturing an antibody to one or more further antigens from the group SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9,

wherein the means for specifically capturing an antibody to SEQ ID NO1 and the means for specifically capturing an antibody to one or more further antigens are coated on one, preferably covalently linked to one carrier.

In a 4th aspect, the problem is solved by a method, preferably for diagnosing a disease, comprising the step detecting in a sample from a subject the presence or absence of an antibody to SEQ ID NO1.

In a preferred embodiment, the method further comprises the step detecting in a sample, preferably blood or CSF sample, from a subject the presence or absence of an antibody to one or more further antigens from the group comprising SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9.

In a preferred embodiment, the presence or absence of an antibody to SEQ ID NO1 and the presence or absence of an antibody to one or more further antigens from the group comprising SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9 is detected simultaneously.

In a preferred embodiment, the presence or absence of an antibody to SEQ ID NO1 and the presence or absence of an antibody to one or more further antigens is detected in spatially separate binding reactions.

In a preferred embodiment, the presence or absence of an antibody to SEQ ID NO1 and the presence or absence of an antibody to one or more further antigens is detected in a one-pot reaction.

In a preferred embodiment, the method comprises the step contacting the diagnostically useful carrier according to the present invention with a sample from the subject.

In a preferred embodiment, the subject suffers from or is suspected of suffering from an infectious disease, preferably a viral infection, more preferably an infection by a flavivirus, preferably from the group comprising Zika, dengue, Yellow fever, TBEV, Usutu, Powassan, West Nile and JEV, preferably Zika.

In a preferred embodiment, the antibody is a mammalian, preferably human antibody, more preferably a human IgA, IgM or IgG class antibody, preferably IgG.

In a 5th aspect, the problem underlying the present invention is solved by diagnostically useful carrier configured for capturing an IgA class antibody to NS1 from a flavivirus, preferably the Zika virus, in a sample from a subject,

preferably for diagnosing a Flavivirus infection, more preferably for distinguishing a primary from a secondary Flavivirus, preferably Zika virus infection.

In a preferred embodiment, the carrier is in addition configured for capturing and specifically detecting an IgM and/or IgG class antibody, preferably an IgM class antibody, to NS1 from said Flavivirus

and/or is in addition configured for capturing an antibody to an envelope glycoprotein of a Flavivirus.

In another preferred embodiment the diagnostically useful carrier is in complex with an IgA class antibody to NS1 from a Flavivirus.

In another preferred embodiment said complex further comprises the NS1 from a Flavivirus or a variant thereof, and preferably further comprises a detection label which is more preferably associated with an antibody to be detected or the NS1 from a Flavivirus or variant thereof.

In another preferred embodiment, the diagnostically useful carrier comprises a dimer or hexamer of the NS1 of said Flavivirus.

In a 6th aspect, the problem is solved by a kit comprising the diagnostically useful carrier according to the present invention.

In a 7th aspect, the problem is solved by a method for diagnosing a Flavivirus infection, preferably for distinguishing a primary from a secondary Flavivirus infection, comprising the step

a) detecting in a first sample from a subject an IgA class antibody to NS1 of said Flavivirus.

In another preferred embodiment, the diagnostically useful carrier according to the present invention is used.

In another preferred embodiment, the method further comprises the step

b) detecting in a second sample from said subject an IgA class antibody to NS1 of said Flavivirus,
wherein the second sample was obtained from said subject at least three days later than the first sample.

In another preferred embodiment, in addition an IgM class antibody to NS1 of said Flavivirus is detected as part of step a) and/or step b), preferably step a).

In another preferred embodiment, an IgG class antibody to NS1 of said Flavivirus is detected in addition in step a) and/or step b), preferably step a).

In another preferred embodiment, in addition in step a) and/or step b), preferably step a), at least one class of antibody to an envelope glycoprotein of said Flavivirus is detected,

wherein preferably the at least one class of antibody to an envelope glycoprotein of said Flavivirus is selected from the group comprising IgG, IgM and IgA, preferably IgA and IgM, or IgA and IgG, or IgM and IgG, more preferably IgA.

In another preferred embodiment, each antibody is detected in spatially separate binding reactions, separated according to antigen and antibody class to be detected.

In another preferred embodiment, the Flavivirus is selected from the group comprising Zika virus, dengue virus, Yellow fever virus, Tick-borne encephalitis virus, Usutu virus, West Nile virus and Japanese encephalitis virus, preferably Zika virus.

In an 8th aspect, the problem is solved by a use of an IgA class antibody to NS1 from a Flavivirus, preferably the Zika virus, or a diagnostically useful carrier for immobilizing and optionally detecting said antibody, for distinguishing a primary from a secondary Flavivirus infection, preferably Zika virus infection.

In a 9th aspect, the problem is solved by a use of an IgA class antibody to NS1 from a Flavivirus, preferably the Zika virus, or a diagnostically useful carrier for immobilizinig and optionally detecting said antibody, for diagnosing a flaviviral infection, preferably a Zika virus infection, in an IgM-deficient subject.

In a 10th aspect, the problem is solved by a use of an IgA class antibody to NS1 from a Flavivirus, preferably the Zika virus, or a diagnostically useful carrier for immobilizinig and optionally detecting said antibody, for increasing the diagnostic reliability, preferably sensitivity, of a diagnostic assay for diagnosing a flaviviral infection, preferably a Zika infection, more preferably at the early stages of an infection.

The present invention sets forth the detection of an antibody to NS1 from Zika virus (SEQ ID NO1) as part of a diagnostic method practiced on a sample from a patient suspected of suffering from an infection, preferably a flaviviral infection, more preferably a Zika virus infection.

The present inventors have surprisingly found that a Zika virus infection may be diagnosed and distinguished from other flaviviral infections by detecting antibodies in a sample from a patient, with a surprisingly high degree of diagnostic reliability, in particular relative to the NS1 antigens derived from other flaviviruses, with an unexpected low degree of cross reactivity.

The inventors have also surprisingly found that some patients, despite having been exposed to a Flavivirus, do not have titers of IgM that allow for the monitoring of the course of the infection, but have surprisingly dynamic titers of IgA to NS1 of said Flavivirus that may be used. More surprisingly, these IgA antibodies do not show a degree of cross reactivity, as would have to be expected, that would make the distinction between an acute Zika virus infection and a previous Flavivirus infection, preferably with a Flavivirus other than the Zika virus, insufficiently reliable.

The inventors have also surprisingly found that Zika virus NS1 antigen exists in oligomeric forms and complexes that have surprising properties relevant for application in diagnostic assays, among them monomers, dimers and hexamers, and complexation with mammalian

apolipoproteins, which, when in complex with Zika virus NS1, enhance the diagnostic reliability and stability of the antigen.

The invention relates to a diagnostically useful carrier, which is preferably a solid carrier made from an artificial material such as glass or plastic for contacting a means, which means is associated with said carrier, which means is for specifically capturing an antibody with a bodily fluid sample from a subject, preferably a mammalian subject, more preferably a human subject. In a preferred embodiment, the term "captured" or "specifically captured", as used herein, means that the binding between the means for specifically capturing and the antibody to be captured is stronger than a binding reaction characterized by a dissociation constant of 1×10^{-5} M, more preferably 1×10^{-7} M, more preferably 1×10^{-8} M, more preferably 1×10^{-9} M, more preferably 1×10^{-10} M, more preferably 1×10^{-11} M, more preferably 1×10^{-12} M, as determined by surface plasmon resonance using Biacore equipment at 25 °C in PBS buffer at pH 7. The diagnostically useful carrier may comprise one or more controls, preferably selected from a control confirming that sample has been added and/or a control confirming that a secondary antibody has been added.

In a preferred embodiment, the antibody captured specifically may be an antibody from a certain antibody class, preferably selected from IgG, IgM and IgA, more preferably IgA. In another preferred embodiment, the antibody captured specifically may be an antibody to a Flavivirus antigen, preferably selected from the group comprising NS1 and Envelope glycoprotein, preferably NS1. In a more preferred embodiment, the antibody captured specifically is an IgM or IgA, preferably IgA class antibody to SEQ ID NO1, preferably to an epitope from SEQ ID NO1 sufficiently long to be recognized by an antibody, which epitope comprises, with reference to SEQ ID NO1, one or more amino acids from the group comprising Arg62, Ile66, Arg 69, Glu72, Glycine73, the latter of which may be substituted with serine or alanine, preferably the peptide comprising the sequence Arg 62 to Glycine 73; one or more amino acids from the group comprising Gln102, Pro105, the latter of which could be substituted with a neutral amino acid and a short side chain such as Ser or Ala, and Glu110; preferably the peptide comprising the sequence Gln102 to Glu110; the peptide comprising residues Ser121 to Thr129, the peptide comprising residues Asp138 to Lys141, the peptide comprising residues Asp174 to Glu178 and the peptide comprising Ser322 to Lys326. In a preferred embodiment, the solid carrier is a diagnostic device, more preferably selected from the group comprising a bead, preferably a paramagnetic particle, a test strip, a microtiter plate, blot, a glass surface, a biochip and a membrane, more preferably from the group comprising a bead, a blot, a test strip and a microtiterplate.

The diagnostically useful carrier may be a microtiter plate comprising a range of wells configured for an immunoassays such as an ELISA assay. In a preferred embodiment, the term "microtiter plate" is a diagnostic device, preferably made from glass or plastic, more preferably plastic, comprising one or more, preferably more than one, more preferably at least 8 wells, in which reactions in liquid buffer may be run separately without cross-contamination. At least one of the well is coated with a polypeptide, preferably an antigenic polypeptide that may be used to specifically capture a diagnostically useful antibody. If more than one means for specifically detecting an antigen is used, then preferably each means is in a well separate from other means. The microtiter plate may be used for running several samples in parallel, preferably in an automated fashion. The wells are preferably compatible with at least one routine detection techniques such colourimetry, immunofluorescence, detection of enzymatic activity, chemiluminscence, radioactivity or the like. Suitable microtiter plates are commercially available. If the diagnostically useful carrier is a microtiter plate, it is preferred that at least 50%, 60%, 70%, 80% or 90%, preferably 50% of any Flavivirus NS1, preferably Zika virus NS1, is a hexamer or dimer, preferably dimer.

The diagnostically useful carrier may be a bead configured for an immunoassay comprising a polypeptide comprising SEQ ID NO1 or a variant thereof. In a more preferred embodiment, the bead is a paramagnetic microparticle which may be removed from a solution and concentrated, preferably at the surface of a vessel, by applying a magnetic field. The bead comprises an antigen linked to the bead by a covalent or non-covalent bond. If more than one bead is used, a preparation of beads may be made by covalently attaching to the beads a universal ligand which binds strongly to a tag which may be fused to one or more polypeptides according to the present invention by way of genetic engineering. The preparation of beads may then be divided into more than one batches, and each batch is contacted with a different polypeptide fused to a tag to the effect that a range of carriers, each with a different immobilized polypeptide, is produced. A mixture of beads, for example one of which carrying a polypeptide comprising SEQ ID NO1 or a variant thereof, and at least one more carrying a polypeptide comprising a sequence selected from the group comprising SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9 may be made. If the diagnostically useful carrier is a bead, it is preferred that at least 50%, 60%, 70%, 80% or 90%, preferably 50% of any Flavivirus NS1, preferably Zika virus NS1, is a monomer, hexamer or dimer, preferably monomer.

The diagnostically useful carrier may be a line blot (Raoult, D., and Dasch, G. A. (1989), The line blot: an immunoassay for monoclonal and other antibodies. Its application to the serotyping of gram-negative bacteria. J. Immunol. Methods, 125 (1-2), 57-65; WO2013041540). In a preferred embodiment, the term "line blot", as used herein, refers to a test strip, more preferably membrane-based, that has been coated with one or more means for capturing an antibody, preferably a polypeptide each. If two or more means are used, they are preferably spatially separated on the carrier. Preferably, the width of the bands is at least 30, more preferably 40, 50, 60, 70 or 80 % of the width of the test strip. The test strip may comprise one or more control bands for confirming that it has been contacted with sample sufficiently long and under adequate conditions, in particular in the presence of human serum, antibody conjugate, or both. A multitude of line blots are commercially available, for example from EUROIMMUN AG, Lübeck, Germany. If the diagnostically useful carrier is a line blot, it is preferred that at least 50%, 60%, 70%, 80% or 90%, preferably 50% of any Flavivirus NS1, preferably Zika virus NS1, is a hexamer or dimer, preferably a hexamer.

The diagnostically useful carrier may be a glass slide coated with a cell, preferably a eukaryotic cell, more preferably an insect or mammalian cell, preferably a human cell expressing a Flavivirus NS1, preferably a polypeptide comprising SEQ ID NO1. The cell may be a live cell, but is preferably a fixed cell. Immunofluorescence may then be used to detect the antibody. Preferably the cell is a recombinant cell overexpressing the Flavivirus NS1. The Flavivirus NS1 may be localized at a cell membrane, preferably at the surface of the cell. If the diagnostically useful carrier is a glass slide, it is preferred that at least 50%, 60%, 70%, 80% or 90%, preferably 50% of any Flavivirus NS1, preferably Zika virus NS1, is a hexamer or dimer, preferably a dimer.

According to the present invention, the diagnostically useful carrier is configured for capturing an antibody to a Flavivirus antigen. The carrier comprises one or more means for specifically capturing an antibody, preferably one or more, more preferably two or more, more preferably three or more, more preferably four or more such means, each of them capable of specifically capturing a different antibody. Alternatively, one or more carriers, preferably two or more, three or more or four or more, each comprising a means for specifically capturing an antibody, may be used. The means for specifically capturing an antibody may be an antibody binding to all antibodies of a certain Ig glass, preferably selected from the group comprising

IgG, IgM and IgA, more preferably IgA class antibodies. Said means is preferably immobilized on said carrier. In a preferred embodiment, the means for specifically capturing an antibody is a polypeptide, preferably a dimer and/or hexamer, more preferably a dimer, comprising or consisting of an antigen from the group comprising SEQ ID NO1, SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9 or a variant thereof, preferably SEQ ID NO1, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8 and SEQ ID NO9 or a variant thereof, and at least one, most preferably one of SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5 or a variant thereof. In another preferred embodiment, a polypeptide comprising SEQ ID NO1, a polypeptide comprising SEQ ID NO6, a polypeptide comprising SEQ ID NO7, a polypeptide comprising SEQ ID NO8, and at least one polypeptide, preferably all polypeptides, from the group comprising a polypeptide comprising SEQ ID NO 2, a polypeptide comprising SEQ ID NO 3, a polypeptide comprising SEQ ID NO 4 and a polypeptide comprising SEQ ID NO 5 or a variant thereof is used. In another preferred embodiment, a polypeptide comprising SEQ ID NO1, a polypeptide comprising SEQ ID NO2, a polypeptide comprising SEQ ID NO3, a polypeptide comprising SEQ ID NO4, a polypeptide comprising SEQ ID NO5 and a polypeptide comprising SEQ ID NO6 or a variant thereof is used. Preferably at least 0.001, 0.01, 0.05, 0.1, 0.2, 0.5, 1, 5, 10 or 100 µg of polypeptide are used for each carrier as a means for specifically capturing an antibody.

In a preferred embodiment, the diagnostically useful carrier comprises one or more means for specifically capturing an antibody to a Flavivirus envelope glycoprotein, preferably a antigenic polypeptide comprising a sequence selected from the group comprising SEQ ID NO11, SEQ ID NO12, SEQ ID NO13, SEQ ID NO14, SEQ ID NO15, SEQ ID NO16, SEQ ID NO17, SEQ ID NO18 and SEQ ID NO27 and a variant thereof.

The diagnostically useful carrier may comprise one or more means, each for capturing an antibody to a Flavivirus antigen from the group comprising NS1 of a Flavivirus and Envelope glycoprotein of a Flavivirus, preferably comprising two means for capturing two antibodies, one to NS1 and one to envelope glycoprotein antigens from the same Flavivirus, more preferably one to NS1 from Zika virus and one to the envelope glycoprotein from Zika virus (SEQ ID NO1 and SEQ ID NO11, respectively).

Said antigen, together with the insoluble carrier to which it is attached, may be separated from a sample from a subject in a straightforward manner, for example by filtration, centrifugation, magnetism or decanting. Said antigen may be immobilized in a reversible or

irreversible manner. For example, the immobilization is reversible if the molecule interacts with the carrier via ionic interactions which may be masked by addition of a high concentration of salt or if the molecule is bound via a cleavable covalent bond or a non-covalent bond. By contrast, the immobilization is irreversible if the molecule is tethered to the carrier via a covalent bond that cannot be cleaved in aqueous solution. The polypeptide may be indirectly immobilized, for example by immobilizing an antibody or other entity having affinity to the polypeptide, followed by addition of the polypeptide and formation of a polypeptide-antibody complex. A non-covalent bond may be made by chemically attaching a ligand to the carrier, preferably via a covalent bond, and fusing to the polypeptide according to the present invention a polypeptide having affinity to the ligand. In a preferred embodiment, the ligand is selected from the group comprising biotin, in which case the polypeptide having affinity may be streptavidin or a variant thereof binding to biotin, glutathione (polypeptide having affinity: glutathione-S-transferase), Nickel (polypeptide having affinity: His tag), Flag tag (polypeptide having affinity: anti-flag antibody), carbohydrate such as maltose or cellulose (polypeptide having affinity: maltose or cellulose binding protein), and is preferably biotin.

The polypeptide according to the present invention comprising SEQ ID NO1 or a variant thereof or, in addition a polypeptide comprising a sequence selected from the group comprising SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9 and a variant thereof, may be immobilized via the diagnostically relevant antibody to be detected, which is immobilized on the carrier via another antibody directly attached to the carrier. The other antibody may be an Ig class-specific antibody, preferably from the group comprising IgM, IgG and IgA-class specific antibody, more preferably an IgA class specific antibody. The binding site of such a class-specific antibody, which is commercially available, may be the constant region of a human antibody.

The teachings of the present invention may not only be carried out using polypeptides, for example SEQ ID NO1, optionally in combination with one or more further antigens such as SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9, having the exact sequences referred to in this application explicitly, for example by function, name, sequence or accession number, or implicitly, but also using variants of such polypeptides.

In a preferred embodiment, the term “variant”, as used herein, may refer to at least one fragment of the full length sequence referred to, more specifically one or more amino acid or nucleic acid sequence which is, relative to the full-length sequence, truncated at one or both termini by one or more amino acids. Such a fragment comprises or encodes for a peptide having at least 10, 15, 25, 50, 75, 100, 150, 200, 250 or 300 successive amino acids of the original sequence or a variant thereof. The total length of the variant may be at 25, 30, 40, 50, 60, 70, 80, 90, 100 or more amino acids.

In another preferred embodiment, the term "variant" relates not only to at least one fragment, but also a polypeptide or a fragment thereof comprising amino acid sequences, preferably a fragment comprising at least 25, more preferably 50, more preferably 200, more preferably 300 successive amino acids, that are at least 40, 50, 60, 70, 75, 80, 85, 90, 92, 94, 95, 96, 97, 98 99, 99.4, 99.5, 99.6, 99.7, 99.8 or 99.9 % identical to the reference amino acid sequence referred to or the fragment thereof, wherein amino acids other than those essential for the biological activity, for example the ability to bind specifically to an antibody of interest, or the fold or structure of the polypeptide are deleted or substituted and/or one or more such essential amino acids are replaced in a conservative manner and/or amino acids are added or deleted such that the biological activity of the polypeptide is at least partially preserved. Known methods comprise various methods that may be used to align two given nucleic acid or amino acid sequences and to calculate the degree of identity, see for example Arthur Lesk (2008), Introduction to bioinformatics, Oxford University Press, 2008, 3rd edition. In a preferred embodiment, the ClustalW software (Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., Higgins, D. G. (2007): Clustal W and Clustal X version 2.0. Bioinformatics, 23, 2947-2948) is used applying default settings.

In a preferred embodiment, variants may, in addition, comprise chemical modifications, for example isotopic labels or covalent modifications such as glycosylation, phosphorylation, acetylation, decarboxylation, citrullination, hydroxylation and the like. The person skilled in the art is familiar with methods for the modification of polypeptides. Moreover, variants may also be generated by way of fusion with other known polypeptides or variants thereof.

The variant of the polypeptide has biological activity. In a preferred embodiment, such biological activity is the ability to bind to, preferably capture specifically the respective antibody if the variant is a variant of a sequence from the group comprising SEQ ID NO1, SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID

NO8, SEQ ID NO9, SEQ ID NO11, SEQ ID NO12, SEQ ID NO13, SEQ ID NO14, SEQ ID NO15, SEQ ID NO16, SEQ ID NO 17, SEQ ID NO18, SEQ ID NO19 and SEQ ID NO27, preferably SEQ ID NO1. For example, a variant of SEQ ID NO1 has the ability to bind specifically to an antibody to SEQ ID NO1 in a sample obtained from a subject suspected of suffering from a viral infection. Such variants have at least one epitope recognized by the antibody to be captured, for example one epitope in SEQ ID NO1 if an antibody to SEQ ID NO1 is captured. The person skilled in the art is capable of designing variants by starting from the original SEQ ID NO1 sequence, introducing modifications such as point mutations, truncations and the like and subsequently confirming that the variant still has biological activity by testing whether said variant binds to an antibody to SEQ ID NO1 in a sample obtained from a subject suffering from the disease to be diagnosed, preferably an infection, more preferably a viral infection, more preferably an infection with a Flavivirus, most preferably an infection with a Zika virus. The 3D protein structure of the Zika Virus NS1 and related Flaviviruses have been published and may be used for guidance in the design of variants and choice of the sequences that may be varied without compromising the biological activity and to distinguish them from important epitopes (for example Xu *et al.*, Contribution of intertwined loop to membrane association revealed by Zika virus full-length NS1 structure (EMBO J., published on August 30, 2016, open access; Akey *et al.*, Flavivirus NS1 structures reveal surfaces for associations with membranes and the immune system, Science 21;343(6173):881-5. doi: 10.1126/science; WO2015/095735). For example, with reference to SEQ ID NO1, regions that are unique to Zika NS1 and should not be substituted, in particular not in a non-conservative manner, include residues 62 to73, preferably those comprising Arg62, Ile66, Arg 69, Glu72, Glycine73 (which could be substituted with a neutral amino acid); 102 to 110, preferably Gln102, Pro105 (which could be substituted with a neutral amino acid and a short side chain such as Ser or Ala) and Glu110; residues 121 to129, residues 138 to 141, 174 to 178 and 322 to 326. The biological activity of mammalian, preferably bovine apolipoprotein provided and used according to the present invention is the ability to bind to and form a complex with a polypeptide comprising Flavivirus NS1, preferably SEQ ID NO1. Variants may be identified by identifying naturally occurring fragments of such apolipoproteins derived from the full-length protein or a precursor thereof, for example by purifying them using NS1 as an affinity ligand followed by N-terminal Edman sequencing and/or tryptic digest in combination with mass spectrometry, and using them to practice the invention. Conservative amino acid substitutions may be used for all variants.

If a polypeptide is used as the means for specifically capturing an antibody, said polypeptide, preferably comprising one or more sequences selected from the group comprising SEQ ID

NO1, SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID , SEQ ID NO8, , SEQ ID NO19, SEQ ID NO9, SEQ ID NO11, SEQ ID NO12, SEQ ID NO13, SEQ ID NO14, SEQ ID NO15, SEQ ID NO16, SEQ ID NO 17, SEQ ID NO18, SEQ ID NO19 and SEQ ID NO27, preferably SEQ ID NO1, when used to carry out the teachings of the present invention, may be provided in any form and at any degree of purification, from tissues or cells comprising said polypeptide in an endogenous form, more preferably cells overexpressing the polypeptide, crude or enriched lysates of such cells, to purified and/or isolated polypeptide which may be essentially pure. In a preferred embodiment, the term "overexpressing", as used herein, means that the cell, preferably a eukaryotic, more preferably a mammalian or insect, more preferably a mammalian, more preferably a human cell, most preferably a HEK293 or HEK293T cell, has been genetically engineered such that it expresses more of the protein of interest than a non-engineered wild type cell would. In a preferred embodiment, the polypeptide is a native polypeptide, wherein the term "native polypeptide", as used herein, refers to a folded polypeptide comprising at least 15, 30, 50, 100 150, 200, 300 or 350 amino acids, preferably more than 30 amino acids, more preferably to a folded polypeptide purified from tissues or cells, more preferably from mammalian cells or tissues, optionally from non-recombinant tissues or cell. In another preferred embodiment, the polypeptide is a linear peptide having at least 7, more preferably at least 10 amino acid residues. If a native polypeptide is used, it is preferably enriched compared to its natural state. A recombinant polypeptide may comprise a C-terminal or N-terminal tag for affinity purification, immobilization or detection such as a His tag, as exemplified by SEQ ID NO10, or a streptavidin tag, preferably a streptavidin, which tag may preferably be removed by cleavage using a protease recognizing a protease cleavage site in a polypeptide linker between the tag and the N terminus or C-terminus, respectively, as part of the purification or method. The cleaved polypeptide may subsequently be attached to a diagnostically useful carrier to yield the diagnostically useful carrier according to the present invention. In another preferred embodiment, the means for specifically capturing an antibody is a Zika virus-infected eukaryotic, preferably human cell. Such a cell may be evaluated by fluorescence microscopy. The cells may be transiently or stably transfected, preferably transiently transfected.

According to the present invention, a nucleic acid encoding the polypeptide according to the present invention such as a polypeptide comprising SEQ ID NO1 or a variant thereof, optionally with an inducible promotor, which polypeptide is preferably for use for the diagnosis of a disease or the manufacture of a kit or reagent for such use, is provided. Said nucleic acid may be a vector, preferably for expressing said nucleic acid. A eukaryotic or

prokaryotic, preferably eukaryotic cell comprising this vector and preferably expressing the polypeptide encoding by the vector, is also provided. The nucleic acid, the vector and the cell may be used for the manufacture of a kit for use according to the present invention such as use of an antibody to NS1, preferably IgA class antibody to NS1 from a Flavivirus, preferably the Zika virus, or a diagnostically useful carrier for immobilizing and optionally detecting said antibody, for distinguishing a primary from a secondary, preferably the Zika virus infection use of an IgA class antibody to NS1 from a Flavivirus, preferably the Zika virus, or a diagnostically useful carrier for immobilizinig and optionally detecting said antibody, for diagnosing a flaviviral infection, preferably a Zika virus infection, in an IgM-deficient subject, such as use of an IgA class antibody to NS1 from a Flavivirus, preferably the Zika virus, or a diagnostically useful carrier for immobilizinig and optionally detecting said antibody, for increasing the diagnostic reliability, preferably sensitivity, of a diagnostic assay for diagnosing a flaviviral infection, preferably a Zika infection, more preferably at the early stages of an infection or such as for distinguishing a primary from a secondary, preferably the Zika virus infection or such as for increasing the diagnostic reliability, preferably sensitivity, of a diagnostic assay for diagnosing a flaviviral infection, preferably a Zika infection, more preferably at the early stages of an infection. The nucleic acid may be expressed, the polypeptide encoded purified and used, preferably coated on a diagnostically useful carrier, in order to make the diagnostically useful carrier according to the present invention. In a preferred embodiment, the term “early stage” refers to the time period before the first 60, preferably first 40 days after symptom onset, wherein more preferably no increase in IgG class antibodies can be observed.

A polypeptide provided or used in a method or as part of a carrier or used in any other way according to the present invention may be glycosylated or non-glycosylated, preferably glycosylated. A glycosylated polypeptide comprising SEQ ID NO1, SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO8, SEQ ID NO19 or SEQ ID NO9 or a variant thereof may be obtained by purifying the polypeptide from a eukaryotic cell, preferably a HEK293 or HEK293T cell. A homogenously glycosylated polypeptide may be obtained by purifying the polypeptide from the cytosolic fraction of a eukaryotic cell, a heterogeneously glycosylated polypeptide may be obtained by purifying the polypeptide from the cell culture supernatant medium following culture of a eukaryotic cells expressing the polypeptide. A non-glycosylated polypeptide may be obtained by enzymatic deglycosylation

of a polypeptide purified from a eukaryotic cell or by purification of a polypeptide expressed in a prokaryotic cell.

In a preferred embodiment, a polypeptide comprising a sequence from the group comprising SEQ ID NO1, SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9 or a variant thereof, preferably SEQ ID NO1 or a variant thereof, may be provided or used, as part of a diagnostically useful carrier, method or use according to the present invention in various oligomeric forms that comprise one or more than one monomer, for example 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 monomers, and may be used, for example, as a means for capturing an antibody to Flavivirus N1, preferably comprising SEQ ID NO1 or a variant thereof, optionally an IgA class antibody to a Flavivirus NS1, in the form of a monomer, dimer or a hexamer, preferably a dimer. In a preferred embodiment said monomer, dimer or hexamer, preferably dimer, has been purified from a eukaryotic cell, preferably recombinant eukaryotic cell, such as a HEK293T or HEK293 cell, preferably from the cytoplasmic fraction, which contains the dimeric form, or the cell medium in which the cell was grown, which contains the hexameric form secreted into the medium, preferably from the cytosolic fraction. In another preferred embodiment, a mixture of oligomeric forms, preferably purified from a eukaryotic cell, is used, wherein the molar ratio of dimer to hexamer is at least 0.1:1, preferably, 0.5:1, 1:1, 1.5:1, 2:1, 5:1 or 10:1. In another preferred embodiment, a mixture of oligomeric forms, preferably purified from a eukaryotic cell, is used, wherein the molar ratio of hexamer to dimer is at least 0.1:1, preferably, 0.5:1, 1:1, 1.5:1, 2:1, 5:1 or 10:1. In a preferred embodiment, the hexamer is used as a means to capture an IgG class. In another preferred embodiment, the dimer is used to capture an IgM class antibody.

Alternatively, a prokaryotic cell or chemical synthesis may be used to express or obtain and to purify the polypeptide oligomer, preferably dimer or hexamer, optionally by chemical crosslinking and isolating the oligomer, preferably dimer or hexamer. The person skilled in the art is familiar with techniques for isolating or enriching certain oligomeric forms, for example using size-exclusion chromatography. The interface of the monomers in an oligomeric form, which causes the monomers to associate to the oligomer, is preferably made of SEQ ID NO1 or a variant thereof that is part of sequence of the monomers in the oligomeric form.

An oligomer comprising more than one monomer may be stabilized by a non-covalent or covalent bond, preferably covalent bond between the two or more monomers that form such

oligomer. In a preferred embodiment, the oligomer is stabilized by one or more covalent bond via one or more Cystein side chains between the monomers. The covalent bond may be a disulfide bond or comprise a linker comprising two functional groups that are reactive with thiol groups, which linker links two side chain residues following reaction of the two functional groups with two thiol groups. In a more preferred embodiment, this covalent bond is between two cysteine residues side chain that would not normally form a disulfide bond in the native, as mentioned in (Xu *et al.*, (Contribution of intertwined loop to membrane association revealed by Zika virus full-length NS1 structure (EMBO J., published on August 30, 2016, open access), natural state of the protein. In another preferred embodiment, the oligomer is stabilized by non-covalent bonds between monomers, and at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 11 or 12 cystein residues are oxidized, forming intermonomeric disulfide bonds.

In a preferred embodiment, the Flavivirus NS1, preferably SEQ ID NO1 or a variant thereof, is in complex with a lipid, which lipid is preferably derived from a cell membrane, more preferably from a Eukaryotic cell membrane such as a HEK293 cell membrane.

Oligomeric forms such as dimers or hexamers may be used, when practicing the present invention, in the form of homogenous or heterogeneous oligomers, wherein homogeneous oligomers comprise two or more different monomers, optionally derived from different flavivirus sequences from the group comprising SEQ ID NO1, SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9 or a variant thereof, preferably of SEQ ID NO1 or a variant thereof. For example, a heterogeneous dimer may comprise a monomer comprising SEQ ID NO1 and a monomer comprising SEQ ID NO2. By contrast, heterogeneous oligomers comprise two monomers which are both derived from the same sequence from the group comprising SEQ ID NO1, SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9 or a variant thereof, preferably of SEQ ID NO1 or a variant thereof, and are optionally identical.

In a preferred embodiment, the antibody to be detected may be an antibody to a monomer, dimer and/or hexamer, preferably to a dimer, of a polypeptide from the group comprising SEQ ID NO1, SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8 and SEQ ID NO9 or a variant thereof, preferably SEQ ID1 or a variant thereof. Said antibody or an antibody binding to a polypeptide from the group comprising SEQ ID NO1, SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9 or a variant thereof, preferably SEQ ID1

or a variant thereof regardless of the oligomeric state, may be provided as an isolated and/or recombinant antibody or antibody fragment.

In another preferred embodiment, the polypeptide comprising a sequence from the group comprising SEQ ID NO1, SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9 and a variant thereof, preferably SEQ ID1 or a variant thereof, preferably a dimer and/or hexamer, preferably the hexamer, is used in a mixture with a mammalian, preferably non-human polypeptide such as a bovine polypeptide such as mammalian Apolipoprotein A-I, more preferably human (NCBI Reference Sequence: NP_000030.1) or bovine Apolipoprotein A-I (GenBank: AAI02942.1; all data base codes cited in this document refer to the entry in the respective data base at the date of priority), or human (NCBI: NP_000375.2) or bovine Apolipoprotein B-100 isoform X1 (NCBI: XP_015329038.1 or a variant thereof) most preferably bovine Apolipoprotein A-I or a variant thereof. The mixture may comprise a complex of the polypeptide comprising a sequence from the group comprising SEQ ID NO1, SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9 and a variant thereof, preferably SEQ ID NO1 or a variant thereof, preferably a dimer and/or hexamer, preferably the hexamer, and, as a second component of the complex, a mammalian Apolipoprotein A-I, more preferably human (NCBI Reference Sequence: NP_000030.1) or bovine Apolipoprotein A-I (GenBank: AAI02942.1), most preferably bovine Apolipoprotein A-I or a variant thereof, which complex may be used for practicing the invention, for example for the diagnosis of a disease, more specifically as a means for capturing an antibody. In the mixture, preferably the complex, the molar ratio between polypeptide monomers and the mammalian Apolipoprotein A-1 may be at least 1:1, 2:1, 5:1, 6:1, 10:1, 50:1 or 100:1.

According to the present invention, the polypeptide may be a recombinant protein, wherein the term "recombinant", as used herein, refers to a polypeptide produced using genetic engineering approaches at any stage of the production process, for example by fusing a nucleic acid encoding the polypeptide to a strong promoter for overexpression in cells or tissues or by engineering the sequence of the polypeptide itself. The person skilled in the art is familiar with methods for engineering nucleic acids and polypeptides encoded (for example, described in Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989), Molecular Cloning, CSH or in Brown T. A. (1986), Gene Cloning – an introduction, Chapman & Hall) and for producing and purifying native or recombinant polypeptides (for example Handbooks „Strategies for Protein Purification", „Antibody Purification", published by GE Healthcare Life

Sciences, and in Burgess, R. R., Deutscher, M. P. (2009): Guide to Protein Purification). In another preferred embodiment, the polypeptide according to the present invention and used for the various embodiments of the present invention is an isolated polypeptide, wherein the term "isolated", as used herein, means that the polypeptide has been enriched compared to its state upon production using a biotechnological or synthetic approach and is preferably pure, *i.e.* at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 95 or 99 percent of the polypeptide in the respective sample consists of said polypeptide as judged by SDS polyacrylamide gel electrophoresis followed by Coomassie blue staining and visual inspection.

The subject according to the present invention is an organism producing antibodies, preferably IgA, IgM and/or IgG class antibodies, more preferably from a mammal, most preferably a human. According to the present invention, IgM and IgG class antibodies to SEQ DI NO1 may be detected in separate assay reactions, for example to determine the time when the subject was infected for the first time.

Within the scope of the present invention is a diagnostically useful carrier comprising a means for specifically capturing an antibody to an antigen such as SEQ ID NO1. In a preferred embodiment, the term "specifically capturing an antibody", as used herein, refers to the ability to bind specifically to the antibody of interest, preferably an IgA, IgM or IgG class antibody, to the effect that it is bound and removed from the sample, whereas other antibodies, preferably from the same class and/or to another antigen, are essentially not bound and remain in the sample. The antibody is preferably an antibody that binds to the antigen of interest only such as the one represented by SEQ ID NO1, but not to other homologous antigens from other viruses such as those represented by SEQ ID NO1, SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8 and SEQ ID NO9.

The diagnostically useful carrier according to the invention serves as a scaffold for the one or more means for specifically capturing an antibody, preferably a diagnostically relevant antibody to a Flavivirus antigen such as the one represented by SEQ ID NO1. Said carrier is suitable for carrying out a diagnostic method. By using a carrier rather than free, soluble means for specifically capturing an antibody, it is more straightforward to isolate and separate from the sample a complex comprising the means and the antibody and to wash said complex, for example for the purpose of removing any molecules binding non-specifically to the means, complex or carrier. In a preferred embodiment, the diagnostically

useful carrier is a diagnostic device, preferably selected from the group comprising a bead, preferably a paramagnetic particle, a test strip, a microtiter plate, a blot and a membrane, and is preferably a line blot or microtiter plate, more preferably a microtiter plate.

In a preferred embodiment, the diagnostically useful device is a microtiter plate comprising a well coated with a means for specifically capturing an antibody to SEQ ID NO1, which means is preferably a polypeptide comprising SEQ ID NO1 or a variant thereof. In addition, said well comprises a means for detecting an antibody to at least one of SEQ ID NO2, SEQ ID NO3, SEQ ID NO4 and SEQ ID NO5, preferably all of them, preferably a polypeptide comprising at least one of SEQ ID NO2, SEQ ID NO3, SEQ ID NO4 and SEQ ID NO5 or a variant thereof. In addition, said well comprises means for specifically capturing an antibody to each of SEQ ID NO6, SEQ ID NO 7, SEQ ID NO8 and SEQ ID NO9, preferably a polypeptide comprising SEQ ID NO6 or a variant thereof, SEQ ID NO 7 or a variant thereof, SEQ ID NO 8 or a variant thereof and SEQ ID NO9 or a variant thereof. In addition, a separate well may include one or more antigens for detecting a chikungunya virus infection.

In a preferred embodiment, the term "specifically detecting a captured antibody", as used herein, means that the antibody binding specifically to the means for specifically capturing the antibody, preferably a polypeptide comprising SEQ ID NO1 or a variant thereof, following capture, is detected rather than any other antibody present in the sample. In a preferred embodiment, the term "binding specifically", as used herein, means that the binding is stronger than a binding reaction characterized by a dissociation constant of 1×10^{-5} M, more preferably 1×10^{-7} M, more preferably 1×10^{-8} M, more preferably 1×10^{-9} M, more preferably 1×10^{-10} M, more preferably 1×10^{-11} M, more preferably 1×10^{-12} M, as determined by surface plasmon resonance using Biacore equipment at 25 °C in PBS buffer at pH 7.

In a preferred embodiment, the means for specifically capturing an antibody to SEQ ID NO 1 and the means for specifically capturing an antibody to one or more further antigens, preferably selected from the group comprising SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9, are on separate carriers. This means that the means are not attached to a single carrier, but one or more carriers that are separate and/or separable without damaging them. For example, the means for specifically capturing an antibody to SEQ ID NO 1 may be attached

to a first test strip, and the means for specifically capturing an antibody to SEQ ID NO 2 is attached to another test strip which is separate from the first test strip.

In a preferred embodiment, the means for specifically capturing an antibody to SEQ ID NO 1 and the means for specifically capturing an antibody to one or more further antigens, preferably selected from the group comprising SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9, are on one, preferably covalently linked to one carrier. This means that the means are attached to one carrier which may not be disassembled, without damaging the carrier, such that the means are on separate carriers. For example, the means may be all coated on one test strip, particular in the form of a line blot.

According to the present invention, a means for specifically detecting a captured antibody is provided, optionally as part of a kit.

The inventive teachings provide a kit, preferably for diagnosing an infection, more preferably for diagnosing a flavivirus infection, most preferably a Zika virus infection. Such a kit is a container that comprises specific reagents required to practice the inventive method, in particular the diagnostically useful carrier according to the present invention, optionally in addition to one or more solutions required to practice the inventive method, preferably selected from or all from the group comprising sample dilution buffer, washing buffer and buffer comprising a means for detecting any specifically captured antibody, such as a secondary antibody and optionally a means for detecting the specifically captured antibody, which may optionally be attached to the secondary antibody, for example a fluorescent, enzymatically active, radioactive, chemiluminescent, preferably electrochemiluminscent label or a spin label. The kit may comprise a chemical solution for carrying out a detection reaction such as 3,3',5,5'-tetramethylbenzidine, *p*-Nitrophenyl Phosphate, 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid or *o*-phenylenediamine dihydrochloride for a colorimetric reaction tripropylamin for an electrochemiluminescence reaction. Furthermore, it may comprise instructions detailing how to use the kit and the inventive diagnostically useful carrier for contacting the inventive polypeptide with a bodily fluid sample from a subject, preferably a human subject, for example a line blot, wherein the inventive means for specifically capturing SEQ ID NO1, preferably a polypeptide comprising SEQ ID NO1 or a variant thereof, is immobilized on the line blot. Furthermore, the kit may comprise a positive control, for example a recombinant antibody known to bind to SEQ ID NO1, and a negative control, for example a protein having no detectable affinity to SEQ ID NO1. Finally, the kit

may comprise a standard solution comprising a SEQ ID NO1-binding antibody for preparing a calibration curve. In a preferred embodiment, the kit comprises a device, preferably a blot-based device such as a line blot coated with a means for specifically capturing an antibody to SEQ ID NO1 and, optionally, an antibody to one or more further antigens such as SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and/or SEQ ID NO9. The kit may comprise one or more further control selected from a control confirming that sample has been added and/or a control confirming that a secondary antibody has been added.

According to the invention, a means for detecting the one or more captured antibodies may be used. The person skilled in the art is aware of many methods that may be used, which are also described in the state of the art, for example in Zane, H. D. (2001), Immunology – Theoretical & Practical Concepts in Laboratory Medicine, W. B. Saunders Company, in particular in Chapter 14. In a preferred embodiment, a secondary antibody binding to the constant region of the one or more captured antibodies, which is the corresponding primary antibody, is used, which secondary antibody may be associated with a label that is straightforward to detect. Alternatively, an antigenic polypeptide, preferably from the group of polypeptides comprising a sequence from the group comprising SEQ ID NO1, SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9 and a variant thereof, preferably SEQ ID NO1, or a variant thereof may be used to detect the diagnostically relevant antibody, preferably following its immobilization, wherein said polypeptide preferably comprises a label that is straightforward to detect. Such antigenic polypeptide may bind to any immobilized diagnostically useful antibody to allow the specific detection. The label that is straightforward to detect may be selected from the group comprising label that is straightforward to detect, for example a fluorescent, chemiluminescent such as electrochemiluminescent, radioactive label, spin label or enzymatically active label, the latter of which may catalyze a chemiluminescent reaction, or it may bring about the generation of a molecule detectable or a signal such as a photon using colorimetry, fluorescence detection such as fluorescence microscopy, photomultiplication or spectroscopy or another analytical method.

In a preferred embodiment, the term „diagnosis“, as used herein, refers to any kind of procedure aiming to obtain information instrumental in the assessment whether a patient suffers or is likely or more likely than the average or a comparative subject, the latter preferably having similar symptoms, to suffer from a certain disease or disorder in the past,

at the time of the diagnosis or in the future, to find out how the disease is progressing or is likely to progress in the future or to evaluate the responsiveness of a patient with regard to a certain treatment, for example the administration of suitable drugs such as drugs for the desensitization of allergic patients. In other words, the term "diagnosis" comprises not only diagnosing, but also prognosticating and/or monitoring the course of a disease or disorder.

Therefore, the term "diagnosis" does preferably not imply that the diagnostic methods or agents according to the present invention will be definitive and sufficient to finalize the diagnosis on the basis of a single test, let alone parameter, but may refer to a contribution to what is referred to as a "differential diagnosis", *i.e.* a systematic diagnostic procedure considering the likelihood of a range of possible conditions on the basis of a range of diagnostic parameters. The term "diagnosis" may also refer to a method or agent used to choose the most promising treatment regime for a patient. In other words, the method or agent may relate to selecting a treatment regimen for a subject.

The present invention relates to a method comprising the step detecting in a sample from a subject the presence or absence of an antibody to an antigenic polypeptide such as a polypeptide comprising a SEQ ID NO1 or a variant thereof. This method preferably comprises immobilizing said antibody followed by specific detection of said antibody, for example by way of the steps a) providing a sample from a subject, b) contacting the sample with the diagnostically useful carrier according to the present invention under conditions compatible with the formation of a complex comprising the diagnostically useful carrier and the antibody, more specifically the means for specifically capturing the antibody and the antibody, c) isolating any said complex, for example by removing the sample, d) optionally washing said complex, and e) optionally detecting said complex. The method is preferably an *in vitro* method. The detection of the complex for the prognosis, diagnosis, methods or test kit according to the present invention comprises the use of a method selected from the group comprising immunodiffusion techniques, immunoelectrophoretic techniques, light scattering immunoassays, agglutination techniques, labeled immunoassays such as those from the group comprising radiolabeled immunoassay, enzyme immunoassays such as colourimetric assays, chemiluminscence, preferably electrochemiluminescence, immunoassays and immunofluorescence techniques. The person skilled in the art is familiar with these methods, which are also described in the state of the art, for example in Zane, H. D. (2001): Immunology – Theoretical & Practical Concepts in Laboratory Medicine, W. B. Saunders Company, in particular in Chapter 14. The method may further involve testing the avidity of antibodies to SEQ ID NO1 in the sample, preferably of antibodies to SEQ ID NO1.

A product obtained when practicing the inventive method is a diagnostically useful carrier comprising a means for specifically detecting an antibody to SEQ ID NO1 in complex with the antibody to SEQ ID NO1 and optionally a means for specifically detecting the antibody to SEQ ID NO1 such as a secondary antibody. If the antibody to SEQ ID NO1 is an IgM class antibody, the secondary antibody is a labeled antibody binding to a constant region of IgM class antibodies. If the antibody to SEQ ID NO1 is an IgG class antibody, the secondary antibody is a labeled antibody binding to a constant region of IgG class antibodies. If the antibody to SEQ ID NO1 is an IgA class antibody, the secondary antibody is a labeled antibody binding to a constant region of IgA class antibodies. The diagnostically useful carrier may be a microtiter plate with one or more than one wells, one well comprising a means for specifically capturing an antibody to SEQ ID NO1, and at least one or more, two or more, three or more, for or more wells each comprising a means for specifically capturing an antibody to a sequence from the group comprising SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and/or SEQ ID NO9.

In many cases, detecting the absence or presence of an antibody, optionally meaning determining whether the concentration of the antibody is beyond a certain threshold, often suggested by the detection limit, in the sample, is sufficient for the diagnosis. If the antibody can be detected, this could be information used for clinician's diagnosis and indicates an increased likelihood that the patient suffers from a disease. In a preferred embodiment, the relative concentration of the antibody in serum, compared to the level that may be found in an average healthy subject, may be determined. In a preferred embodiment, the term "detecting the presence or absence", as used herein, means that it is sufficient to check whether a signal sufficiently beyond any background level may be detected using a suitable complex detection method that indicates that the antibody of interest is present or more antibody of interest is present than would be in a healthy subject. In a more preferred embodiment, this may involve determining whether the concentration is at least 0.1, preferably 0.2, 0.5, 1, 2, 5, 10, 20, 25, 50, 100, 200, 500, 1000, 10000 or 100000 times higher than the concentration of the antibody of interest found in the average healthy subject.

The disease to be diagnosed is an infection, preferably a viral, more preferably Flavivirus, most preferably Zika virus infection. Preferably, a Zika virus infection may be distinguished from another Flavivirus infection, more preferably from an infection with a Flavivirus selected from the group comprising dengue virus, Yellow fever virus, Tick-borne encephalitis virus,

Usutu virus, West Nile virus and Japanese encephalitis virus or all of them, preferably from the dengue virus.

The invention may be used to provide a prognosis whether a pregnant woman's newborn child is likely to suffer from a deformity if the sample from the pregnant woman is tested. Preferably, the pregnant woman may have symptoms suggesting that she may suffer from a flaviviral infection or may very actually suffer from an infection.

The invention may be used to diagnose whether deformities in a child, such as microcephaly, are a result of a previous Zika virus infection or not.

The invention may be used to diagnose whether a subject is suffering or likely to suffer, following onset of the infection, from an autoimmune disease such as the Guillan Barré syndrome. More specifically, if antibodies to SEQ ID NO1 are detected in a sample from a subject, said subject is more likely to suffer from an autoimmune disease than a subject having no antibodies to SEQ ID NO1.

The invention may be used to test samples comprising autoimmune antibodies such as ANA autoantibodies which may obscure results obtained using conventional assays and so may be used in combination with a method comprising the step detecting in a sample from a subject ANA autoantibodies. Commercially available kits may be used for detecting ANAs for example EUROPLUS ANA Mosaic 20A, ANA screen 11, ANA Profile 3 or Anti-ENA ProfilePlus. This may allow distinguishing an autoimmune disease from a viral infection or an antibody relating to an autoimmune disease and those relating to a viral infection.

The invention may be used to distinguish Flavivirus infections from other viral infections, preferably alphavirus infections, more preferably a chikungunya virus infection.

The invention may be used to screen blood given by blood donors for previous infections.

In a preferred embodiment, the absence or presence of one or more antibodies, such as an antibody to SEQ ID NO1, is detected simultaneously, *i.e.* at the same time. This is convenient in terms of efficient diagnostic procedures, as a maximum of diagnostic information is obtained in a given period of time. Of course, a prerequisite is that sufficient capacity is available for running all reactions.

In a preferred embodiment, the absence or presence of at least two antibodies, such as an antibody to SEQ ID NO1 and one and more antibodies to an antigen from the group comprising SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19, SEQ ID NO9, SEQ ID NO10, SEQ ID NO11, SEQ ID NO12, SEQ ID NO13, SEQ ID NO14, SEQ ID NO15, SEQ IDNO16, SEQ ID NO17, SEQ ID NO18 and SEQ ID NO27, is detected in spatially separate reactions. This means that these reactions run in different reaction mixtures in separate vessels, for example separate wells of a microtiter plate or separate compartments each comprising a different bead or the same compartment used subsequently with more than one bead.

If more than one antibody is to be detected, the method may, in another preferred embodiment, be carried out in a one-pot reaction. Preferably, the term "one-pot reaction", as used herein, means that two or more, preferably all reactions carried out for the purpose of detecting the presence or absence of an antibody are carried out in the same reaction mixture in one reaction vessel, without physical barriers between the reactions, by contrast to experimental settings contemplating that at least two reactions are carried out in separate solutions and reaction vessels.

The invention provides a pharmaceutical composition or a vaccine, which composition or immunogenic composition such as a vaccine comprises a polypeptide comprising SEQ ID NO1 or a variant thereof, optionally in combination with one or more further antigens such as one or more selected from the group comprising SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and/or SEQ ID NO9, preferably in addition to previously used antigens such as an Zika virus Envelope glycoprotein, preferably comprising the sequence AHL16749.1 (UNIPROT data base as online on priority date) or a variant thereof or SEQ ID NO 11, preferably SEQ ID NO 11 or a variant thereof, and/or a Zika virus. Moreover, the composition or immunogenic composition may comprise an antigen comprising a sequence selected from the group comprising SEQ ID NO12, SEQ ID NO13, SEQ ID NO14, SEQ ID NO15, SEQ ID NO16, SEQ ID NO17, SEQ ID NO18, SEQ ID NO19 and SEQ ID NO27 and a variant thereof or a variant thereof. An immunogenic composition or vaccine may comprise components to inactivate a virus or bacteria and stabilize the vaccine, helping to preserve the vaccine and prevent it from losing its potency over time. Adjuvants are added to vaccines to simulate the production of antibodies against the vaccine to make it more effective. An adjuvant could be organic or inorganic. The most common inorganic adjuvants for human vaccines include aluminum

phosphate and aluminum hydroxide. Organic adjuvants could be based on the organic compound squalene and an oil [squalene] in water adjuvant can be used. An immunogenic composition may comprise stabilizers that help the vaccine to maintain its effectiveness during storage, e.g., $MgCl_2$, $MgSO_4$, lactose-sorbitol, or sorbitol-gelatin, and preservatives to prevent bacterial and fungal growth, e.g., thiomersal, formaldehyde, or phenol derivatives, antibiotics. The composition is preferably suitable for administration to a subject, preferably a mammalian subject, more preferably to a human. Such a pharmaceutical composition may comprise a pharmaceutically acceptable carrier. The pharmaceutical composition may, for example, be administered orally, parenterally, by inhalation spray, topically, by eyedrops, rectally, nasally, buccally, vaginally or via an implanted reservoir, wherein the term "parentally", as used herein, comprises subcutaneous, intracutaneous, intravenous, intramuscular, intra-articular, intrasynovial, instrasternal, intrathecal, intralesional and intracranial injection or infusion techniques. The pharmaceutical composition may be provided in suitable dosage forms, for example capsules, tablets and aqueous suspensions and solutions, preferably in sterile form. It may be used in a method of treatment of a disease, which method comprises administering an effective amount of the inventive polypeptide to a subject.

The inventive method for diagnosing a Flavivirus infection, preferably for distinguishing a primary from a secondary Flavivirus infection, may comprise the step detecting in a first sample from a subject an IgA class antibody to NS1 of a Flavivirus, optionally further comprising detecting in said first sample an IgM and/or IgG class, preferably IgM class antibody to NS1 of a Flavivirus, more preferably SEQ ID NO1. In a preferred embodiment, the method further comprises the step detecting in a second sample obtained from said subject an IgA class antibody to NS1 of said Flavivirus, optionally further comprising detecting in said second sample an IgM and/or IgG class, preferably IgM class antibody to NS1 of a Flavivirus such as SEQ ID NO1, optionally further comprising detecting in said first sample an IgM and/or IgG class, preferably IgM class antibody to NS1 of a Flavivirus, more preferably SEQ ID NO1.

A dynamic titer of IgA or IgM, preferably IgA class antibodies to NS1 of a Flavivirus, preferably to SEQ ID NO1, increasing and decreasing significantly relatively to the background before emergence of IgG class antibodies (i.e. seroconversion), may indicate an acute Zika infection, which is a primary Flavivirus infection. By contrast, a parallel increase of IgA and IgG (albeit the latter at higher levels) may indicate an acute Zika infection, which is a

secondary Flavivirus infection. In a preferred embodiment, the first sample is obtained at least 3, 4, 5, 6 days, 1, 2 3 or 4 weeks following the subject's exposure or suspected exposure to a Flavivirus. In a preferred embodiment, the first sample is taken in the two weeks after onset of symptoms. The presence or absence of antibodies may be determined as well as their relative levels over time. The second sample may be obtained at least 3, 4, 5, 6 days, 1, 2, 4, 6, 8, 12, 16, 20, 24, 28 or 32 weeks later than the first sample, preferably at least 3 days, more preferably at least 7 days. A total number of at least 2, 3, 4, 5 or six samples may be taken, preferably at least 2 samples, optionally each sample at least 1 day, 3 days, one week, preferably one week after the previous sample. The total concentrations of IgG, IgM and/or IgA, preferably IgM, may be determined in addition, for example to rule out insufficiencies. This way, the titers of the respective antibodies may be monitored over time.

In a preferred embodiment, the titer of IgM, IgG and/or IgA, preferably IgA and IgG, more preferably IgA to NS1, preferably to SEQ ID NO1, is monitored by detecting the presence or absence or, preferably relative level over time for a period of at least 3, 4, 5, 6, 10, 14, 21, 28, 35 or 42 days, preferably at least 6 days, with the first sample being taken at least five days, preferably at least 7 days following onset of the symptoms. Seroconversion may be detected by monitoring the presence or absence or relative level over time of IgG class antibodies to NS1, preferably SEQ ID NO1. This may help identify the time window in which the increase and decrease of IgM and/or IgA class antibodies would be expected or concluding that this time window has passed.

The inventive method, kit and carriers may be used to distinguish between a primary and a secondary Flavivirus infection. In a preferred embodiment, the term "primary infection", as used herein, refers to an infection of a person who has never had an infection with said Flavivirus or another Flavivirus, preferably said Flavivirus, more preferably the Zika virus, by contrast to a secondary infection in a patient who has been exposed to a virus or immunogenic compositions derived thereof before. In a preferred embodiment, this may involve distinguishing a primary Zika virus infection from a secondary infection with another Flavivirus, preferably selected from the group comprising dengue virus, Yellow fever virus, Tick-borne encephalitis virus, Usutu virus, West Nile virus and Japanese encephalitis virus, preferably dengue virus.

The present invention is further illustrated by the following examples, sequences and figures from which further features, embodiments, aspects and advantages of the present invention may be taken. All methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, with suitable methods and materials being described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety, as are priority applications EP16000422.2, EP16000442.0, EP16000454.5 and EP16000454.5. Further, the materials, methods, and examples are illustrative only and are not intended to be limiting, unless otherwise specified.

Fig. 1 shows SDS-PAGE and Coomassie staining of 1 µg purified recombinant sNS1 and mNS1, the latter pure and in complex with bovine Apolipoprotein A1. For each lane, 1 µg protein was separated on a 4-12 % denaturing NuPage Bis-Tris gel, documenting high protein purity. Molecular weight markers are indicated on the left.

Fig. 2 shows Receiver operating characteristic (ROC) analysis of ELISA for the detection of anti-ZIKV IgM and IgG. Panel A shows the diagnostic performance for ZIKV infections (n=29) against infections or vaccinations with other flaviviruses (DENV, n=38; YFV, n=12; WNV, n=34; JEV, n=25). Panel B shows the diagnostic performance for ZIKV infections (n=29) against healthy controls (pregnant women, n=100; Argentinian blood donors, n=99; US-American blood donors, n=100; German blood donors; n=500). AUC, area under the curve.

Fig. 3 shows anti-ZIKV reactivity in different cohorts as determined by ELISA. Sera from patients infected with ZIKV, DENV, WNV, JEV or CHIKV or vaccinated against YFV, as well as samples from pregnant women (PREG), Argentinian blood donors (BD1), US-American blood donors (BD2) and German blood donors (BD3) were analyzed for anti-ZIKV IgM (Panel A) and anti-ZIKV IgG (Panel B) by ELISA based on NS1 antigen. Plotted data points represent ratios (extinction of patient sample / extinction of calibrator). Cut-off values for borderline results (≥0.8) and positive results (≥1.1) are indicated by horizontal dotted lines. Positive and total cases are indicated in parentheses. Triangles indicate patients with confirmed ZIKV infection that had a ratio for anti-ZIKV IgM or IgG below the cut-off (<1.1), but a corresponding positive result in IgG or IgM testing, respectively. Panel C shows a comparison between anti-ZIKV IgM and IgG detection in the cohort of ZIKV infected patients. Panel D depicts the time course of anti-ZIKV IgM and IgG antibody levels in the serum of a representative ZIKV-infected patient.

Fig. 4 shows the results of gel filtration with the aim to isolate Zika NS1 oligomers as carried out in Example 2.

Fig. 5 shows the generation of Zika NS1 monomers and dimers (SDS resistant) under various conditions.

Fig. 6 shows the reaction of two patients' sera with monomeric and dimeric mNS1 and sNS1. Dimeric NS1 is shown to be more sensitive.

Fig. 7 shows the results of stability studies, more specifically exposure to harsh buffer exchange conditions. A complex comprising sNS1 and bovine apolipoprotein AI is more stable than NS1 that is not part of such a complex. mNS1 and sNS1 alone can be partially pelleted after incubation on ice or at room temperature, indicating that 30-50% of total protein amount forms aggregates.

Fig. 8 shows the results of an ELISA to compare the reactivity of various NS1 preparations. A higher reactivity of mNS1 and sNS1+ApoAI compared to sNS1 could be shown.

Fig. 9. Anti-ZIKV reactivity in patients with RT-PCR-confirmed (n = 27) and suspected (n = 85) ZIKV infection as determined by ELISA for (A) IgM and (B) IgG[a]; time course analysis of anti-ZIKV antibody levels in follow-up samples from (C) a German patient returning from Colombia (probable primary ZIKV infection)[b] and (D) a Colombian patient with RT-PCR-confirmed ZIKV infection (probable secondary flavivirus infection)[c]

RT-PCR: reverse transcription-PCR; US: United States; WHO: World Health Organization; ZIKV: Zika virus.

[a] Per patient, one sample was examined for anti-ZIKV IgM and IgG antibodies. Plotted data points represent ratio values ($extinction_{sample}/extinction_{calibrator}$). Cut-off values for borderline results (≥ 0.8 to < 1.1) and positive results (≥ 1.1) are indicated by horizontal dashed lines. Positive and total cases are indicated in parentheses. Triangles indicate samples with a ratio for anti-ZIKV IgM or IgG below the cut-off (< 1.1), but a corresponding positive result in IgG or IgM testing, respectively.

[b] Samples were provided by the WHO Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research, Hamburg, Germany. Cut-off ratio: ≥ 1.1.

[c] Samples were provided by Biomex US LLC, Coconut Creek, Florida, US. Cut-off ratio: ≥ 1.1.

Fig. 10. Anti-ZIKV reactivity in potentially cross-reactive samples (n = 252) and healthy controls (n = 1,015) as determined by ELISA for (A) IgM and (B) IgG[d,e], study evaluating a novel NS1-based ELISA, Germany 2016

ARG: Argentina; CHIKV: chikungunya virus; CHIL: children; DENV: dengue virus; GER: Germany; JEV: Japanese encephalitis virus; NS: non-structural protein; PLAS: *Plasmodium*; PREG: pregnant women; US: United States; WNV: West Nile virus; YFV: Yellow fever virus; ZIKV: Zika virus; ZIM: Zimbabwe.

[d] Plotted data points represent ratio values ($extinction_{sample}/extinction_{calibrator}$); one data point per patient. Cut-off values for borderline results (≥ 0.8 to < 1.1) and positive results (≥ 1.1) are indicated by horizontal dashed lines. Positive and total cases are indicated in parentheses.

[e] To provide high levels of potentially cross-reactive anti-DENV IgM and IgG antibodies, the DENV-infected patients were divided into two groups: DENVa, high median ratio (3.9) anti-DENV IgM, anti-DENV IgM ratio ≥ 3.0 in 79% of cases (inset Panel A); DENVb, high median ratio (3.9) anti-DENV IgG, anti-DENV IgG ratio ≥ 3.0 in 80% of cases (inset Panel B). Cut-off ratio (anti-DENV ELISA, EUROIMMUN): ≥ 1.1.

Figs. 11A and **11B** show measurements of IgG, IgA and IgM antibodies against ZIKV-NS1 antigen in the sequential samples of the two Colombian patients.

Fig. 12 shows the results of Zika IgM Capture ELISA and NS1-based Anti-Zika virus ELISA IgM and IgG.

SEQ ID NO1: Zika virus NS1 antigen
SEQ ID NO2: dengue virus 1 NS1 antigen
SEQ ID NO3: dengue virus 2 NS1 antigen
SEQ ID NO4: dengue virus 3 NS1 antigen
SEQ ID NO5: dengue virus 4 NS1 antigen
SEQ ID NO6: West Nile virus NS1 antigen
SEQ ID NO7: Tick-borne encephalitis virus NS1 antigen
SEQ ID NO8: Japanese encephalitis virus NS1 antigen
SEQ ID NO9: Yellow fever virus NS1 antigen
SEQ ID NO10: Zika virus NS1 antigen with C-terminal His tag
SEQ ID NO 11: Zika virus envelope glycoprotein
SEQ ID NO12: dengue virus 1 envelope glycoprotein

SEQ ID NO13: dengue virus 2 envelope glycoprotein
SEQ ID NO14: dengue virus 3 envelope glycoprotein
SEQ ID NO15: dengue virus 4 envelope glycoprotein
SEQ ID NO16: West Nile virus envelope glycoprotein
SEQ ID NO17: Tick-borne encephalitis virus envelope glycoprotein
SEQ ID NO18: Japanese encephalitis virus envelope glycoprotein
SEQ ID NO19: Powassan virus NS1 antigen
SEQ ID NO20: Zika virus NS1 antigen with C-terminal His tag and additional fused peptide
SEQ ID NO21: Zika virus NS1 epitope
SEQ ID NO22: Zika virus NS1 epitope
SEQ ID NO23: Zika virus NS1 epitope
SEQ ID NO24: Zika virus NS1 epitope
SEQ ID NO25: Zika virus NS1 epitope
SEQ ID NO26: Zika virus NS1 epitope
SEQ ID NO27: Yellow fever virus envelope glycoprotein

Example 1: Studying the diagnostic performance of Zika NS1-based ELISA

METHODS

Human serum samples

Serum samples from patients with ZIKV infection (n=29) and patients with other flaviviral or non-flaviviral infections as well as yellow fever vaccinations (n=128) were examined in this study. Sera from healthy pregnant women (n=100) and blood donors living in flavivirus endemic and non-endemic areas (n=699) served as negative controls. Follow-up samples from a German patient with clinically and serologically confirmed ZIKV infection contracted during a stay in Colombia were tested by the WHO Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research (Hamburg, Germany) and used for time-course analysis of anti-ZIKV antibody levels. All sera were stored at -20 °C until assayed. The samples were used anonymously to maintain confidentiality and the study protocol conformed to the recommendations of the Central Ethical Committee of Germany.

Protein expression and purification

Recombinant NS1[ZIKV] was expressed in HEK293T cells using standard cloning an expression methods based on the pTriEx-1 plasmid with an artificial signal sequence and a

C-terminal His tag (SEQ ID NO20). Transfected cells were cultured at 37 °C and 8.5 % CO_2 in Dulbecco's modified eagle's medium with 10 % fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin for three to five days. Cells were harvested, resuspended in 20 mM Tris-HCl pH 7.4, 10 % (w/v) sucrose, 5 mM EDTA, 1 mM PMSF and stored at -80 °C until further use.

Cells were resuspended in 20 mmol/l tris chloride pH 8.0, 600 mmol/l sodium chloride, 20 mmol/l magnesium chloride, 20 mmol/l imidazole, 1 mmol/l PMSF, 0.5 mmol/l dithiotreitol, 0.1 % Triton X-100 and lysed by homogenization. Cell debris was removed by centrifugation for 60 minutes at 100,000xg, 4 °C. The soluble protein fraction was applied to Nickel Rapid Run (Agarose Bead Technologies, Miami, FL, USA) equilibrated with 5 mmol/l tris chloride pH 8.0, 150 mmol/l sodium chloride, 0.015 % (w/v) Triton X-100, 0.5 mmol/l dithiotreitol, 20 mmol/l imidazole and eluted by increasing the imidazole concentration to 150 mmol/l. The eluates were pooled, diluted with two volumes 20 mmol/l tris chloride pH 8.5, 5 mmol/l EDTA, 1 mmol/l PMSF, 0.015 % (w/v) Triton X-100, 1 mmol/l dithiotreitol and cleared by centrifugation at 100,000xg and 4 °C for 60 minutes. The supernatant was loaded onto a HiTrap Q FF column (GE Lifesciences, Freiburg, Germany) equilibrated with 20 mmol/l tris chloride pH 8.5, 2.5 mmol/l EDTA, 1 mmol/l PMSF, 0.015 % (w/v) Triton X-100, 1 mmol/l dithiotreitol, 50 mmol/l sodium chloride, washed and eluted with 20 mmol/l tris chloride pH 8.5, 2.5 mmol/l EDTA, 1 mmol/l PMSF, 0.015 % (w/v) Triton X-100, 1 mmol/l dithiotreitol with stepwise increase of sodium chloride from 50 to 1000 mmol/l. All fractions containing NS1[ZIKV] were pooled and concentrated by ultrafiltration (VivaSpin, Sartorius, Göttingen, Germany). The final preparations were stored at -80°C until further use.

Enzyme-linked immunosorbent assay

NS1-coated microtiter-plates (Nunc, Roskilde, Denmark) and standardized reagents from the commercially available Anti-Zika Virus IgG and IgM ELISA (EUROIMMUN, Lübeck, Germany) were used as recommended by the manufacturer. In brief, sera diluted 1:101 in PBS plus 0.1 % (w/v) casein were added to the wells and allowed to react for 60 minutes at 37 °C. In case of IgM detection, sera were pre-incubated with rheumatoid factor absorbent for 10 minutes. Bound antibodies were detected applying rabbit anti-human IgG peroxidase conjugate or goat anti-human IgM peroxidase conjugate for 30 min, followed by staining with tetramethylbenzidine for 15 min. The enzymatic reaction was stopped by addition of one volume of 0.5 mol/l sulphuric acid. Optical density was determined photometrically at 450 nm (reference 620 nm). Unless indicated otherwise, all assay procedures were carried out at room temperature.

The cut-off for positivity was validated and optimized by receiver-operating characteristics (ROC). A highly positive index patient serum was diluted to generate a cut-off calibrator that was incubated in every experiment. A ratio of the extinction value of the patient sample over the extinction value of calibrator was calculated.

Statistics

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA) and SigmaPlot 13.0 analysis software (SSI, San Jose, CA, USA). Confidence intervals (CI 95%) were calculated according to the modified Wald method.

RESULTS

Eukaryotic expression and purification of ZIKV-specific NS1

ZIKV-specific NS1 was expressed in the human cell line HEK293T and purified from the cell lysate (mNS1) or culture supernatant (sNS1). When separated by SDS-PAGE, mNS1 and sNS1 migrated essentially according to their predicted molecular mass (43.9 kDa; **Fig. 1**).

Development of NS1-based anti-ZIKV ELISA

Purified recombinant mNS1 and sNS1 were used as solid phase antigens in ELISA for the detection of anti-ZIKV IgM and IgG, respectively. ROC analyses were performed based on 29 sera from patients with ZIKV infection and 908 controls, including 109 patients with flavivirus infections or vaccinations, 100 pregnant women and 699 blood donors. Areas under the curve (IgM, ≥ 0.979; IgG, ≥0.956) indicated excellent diagnostic performance (**Fig. 2**). The cut-off ratio [$OD_{patient\ sample}$ / $OD_{calibrator}$] for assay positivity was set at ≥1.1 for either Ig class. This threshold exceeds the cut-off level with maximum sum of sensitivity and specificity to ensure high assay specificity. Ratios in the range of ≥0.8 to <1.1 were classified as borderline.

Diagnostic performance of the anti-ZIKV ELISA

Sensitivity: Out of 29 serum samples from patients with clinically and serologically confirmed ZIKV infection, 24 (82.8 %) were positive for anti-ZIKV IgM, 20 (69.0 %) for IgG, and 28 (96.6 %) for IgM and/or IgG (**Fig. 3A and 3B**). 16 sera showed positive reactivity for both IgM and IgG, while 12 sera were positive for either IgM or IgG. Accordingly, highest diagnostic sensitivity is achieved by parallel testing of both Ig classes (**Fig. 3C**). This approach also allows the categorization of patients by disease state (acute or past infection). For example, analysis of follow-up samples from a patient who showed clinical symptoms after returning

from a stay in Colombia revealed a decrease in anti-ZIKV IgM and a significant increase in IgG levels over a period of 16 weeks, confirming acute infection (**Fig. 3D**).

Specificity: Among 799 healthy controls, only 1/99 (1.0 %) Argentinian and 1/500 (0.2 %) German blood donors were found anti-ZIKV IgM positive, while 100 healthy US-American blood donors and 100 healthy pregnant women were negative. Anti-ZIKV IgG was present in 1/100 (1.0%) US-American and 1/500 (0.2%) German blood donors, but absent in the cohorts of healthy Argentinian blood donors and pregnant women. Thus, overall specificity amounted to 99.7% for either Ig class (**Fig. 3A and 3B**).

Cross-reactivity: Serum panels from 128 clinically and serologically well characterized patients or vaccinees with high titers of antibodies of class IgM and/or IgG against flaviviruses (DENV, YFV, WNV or JEV) and CHIKV were analyzed. Anti-ZIKV IgM reactivity was detectable in 1/34 (2.9 %) patients infected with WNV and anti-ZIKV IgG in 1/25 (4.0 %) patients infected with JEV (**Fig. 3A and 3B**). In both cases, double infections cannot be excluded, so that it remains unclear if ELISA positivity was due to cross-reactions with antibodies against other flaviviruses (false-positive) or due to coinfection with ZIKV (true positive). Considering an overall positivity rate of 1/128 (0.8 %) for either Ig class, cross-reactivity can almost entirely be excluded when using the NS1-based ELISA.

Example 2: Preparation of Zika NS1 antigen

Recombinant NS1[ZIKV] was expressed in HEK293T cells using standard cloning and expression methods based on the pTriEx-1 plasmid with an artificial signal sequence and a C-terminal His tag (SEQ ID NO20).Transfected cells were cultured at 37°C and 8.5% CO2 in Dulbecco's modified eagle's medium with 10% fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin for three to five days. Cell culture supernatant was decanted and stored until further use. Cells were harvested, resuspended in 20 mM Tris-HCl pH 7.4, 10% (w/v) sucrose, 5 mM EDTA, 1 mM PMSF and stored at -80°C until further use.

To prepare mNS1, cells were resuspended in 20 mmol/l tris chloride pH 8.0, 600 mmol/l sodium chloride, 20 mmol/l magnesium chloride, 20 mmol/l imidazole, 1 mmol/l PMSF, 0.5 mmol/l dithiotreitol, 0.1% Triton X-100 and lysed by homogenization. Cell debris was removed by centrifugation for 60 minutes at 100,000xg, 4°C. The soluble protein fraction was

applied to Nickel Rapid Run (Agarose Bead Technologies, Miami, FL, USA) equilibrated with 5 mmol/l tris chloride pH 8.0, 150 mmol/l sodium chloride, 0.015% (w/v) Triton X-100, 0.5 mmol/l dithiotreitol, 20 mmol/l imidazole and eluted by increasing the imidazole concentration to 150 mmol/l. The eluates were pooled, diluted with two volumes 20 mmol/l tris chloride pH 8.5, 5 mmol/l EDTA, 1 mmol/l PMSF, 0.015% (w/v) Triton X-100, 1 mmol/l dithiotreitol and cleared by centrifugation at 100,000xg and 4°C for 60 minutes. The supernatant was loaded onto a HiTrap Q FF column (GE Lifesciences, Freiburg, Germany) equilibrated with 20 mmol/l tris chloride pH 8.5, 2.5 mmol/l EDTA, 1 mmol/l PMSF, 0.015% (w/v) Triton X-100, 1 mmol/l dithiotreitol, 50 mmol/l sodium chloride, washed and eluted with 20 mmol/l tris chloride pH 8.5, 2.5 mmol/l EDTA, 1 mmol/l PMSF, 0.015% (w/v) Triton X-100, 1 mmol/l dithiotreitol with stepwise increase of sodium chloride from 50 to 1000 mmol/l. All fractions containing NS1[ZIKV] were pooled and concentrated by ultrafiltration (VivaSpin, Sartorius, Göttingen, Germany). The final preparations were stored at -80°C until further use.

To prepare sNS1, cell culture supernatant was adjusted to 5 mmol/l tris chloride pH 8.0, 164 mmol/l sodium chloride, 50 mmol/l magnesium chloride, 20 mmol/l imidazole, 0,1% Triton X-100, cleared by centrifugation for 30 minutes at 17,600xg, 4°C, applied to Nickel Rapid Run (Agarose Bead Technologies, Miami, FL, USA) equilibrated with 5 mmol/l tris chloride pH 8.0, 300 mmol/l sodium chloride, 20 mmol/l imidazole and eluted by increasing the imidazole concentration to 150 mmol/l. All fractions containing NS1[ZIKV] were pooled and concentrated by ultrafiltration (VivaSpin, Sartorius, Göttingen, Germany). The final preparations were stored at -80°C until further use.

To prepare sNS1 in complex with bovine apolipoprotein AI, cell culture supernatant was adjusted to 5 mmol/l tris chloride pH 8.0, 164 mmol/l sodium chloride, 50 mmol/l magnesium chloride, 20 mmol/l imidazole, cleared by centrifugation for 30 minutes at 17,600xg, 4°C, applied to Nickel Rapid Run (Agarose Bead Technologies, Miami, FL, USA) equilibrated with 5 mmol/l tris chloride pH 8.0, 300 mmol/l sodium chloride, 20 mmol/l imidazole and eluted by increasing the imidazole concentration to 150 mmol/l. All fractions containing NS1[ZIKV]/Apo AI[Bos taurus]-complexes were pooled and concentrated by ultrafiltration (VivaSpin, Sartorius, Göttingen, Germany). The final preparations were stored at -80°C until further use.

When separated by SDS-PAGE, NS1 migrated essentially according to its predicted molecular mass (43.9 kDa). Protein identity was verified by mass spectrometry.

Example 3: Preparation of NS1 oligomers

Protein preparations of mNS1 and sNS1 prepared as in Example 2 were analyzed by analytical gel filtration using a Superdex 200 pg column (GE Healthcare, Munich, Germany). in 20 mmol/l tris chloride pH 8.5, 2.5 mmol/l EDTA, 1 mmol/l PMSF, 0.015% (w/v) Triton X-100, 1 mmol/l dithiotreitol, 300 mmol/l sodium chloride or 5 mmol/l tris chloride pH 8.0, 300 mmol/l sodium chloride, 150 mmol/l imidazole. Protein mixtures with known molecular weights were run separately and used as a calibrator. Retention times of individual peaks were used to calculate the molecular weight of the observed NS1 populations and eluate fractions were analyzed using denaturing/non-denaturing gel electrophoresis under reducing conditions followed by silver staining.

Fig. 4 shows the results of a representative gel filtration run. The retention times of the major mNS1 and sNS1 peaks reveal a molecular weight of 212 kDa (mNS1) and 227 kDa (sNS1) which is in good agreement with hexameric populations ($M_W[NS1_{monomeric}]$ = 43,9 kDa). mNS1 shows an additional peak at 44 kDa, which most probably resembles its monomeric form. This interpretation is underpinned by the fraction's electrophoretic mobility: both a heat-denatured and a non-denatured aliquot migrates at the same position below 50 kDa, whereas dimeric NS1 would migrate at 70 kDa in the non-heat-denatured aliquot if present in this population.

Protein preparations of mNS1 and sNS1 were treated with or without 16 mmol/l dithiotreitol and incubated at 70°C or at room temperature for 10 minuntes, followed by SDS gel electrophoresis and Coomassie staining. **Fig. 5** shows the generation of monomers and dimers (SDS resistant) under various conditions.

Dimeric mNS1 or sNS1 populations could also be generated by detergent treatment of the hexameric population, e.g. 0,1% Triton X-100 or 0,2% sodium dodecylsulfate. Analytical gel filtration clearly showed that mNS1 and sNS1 exist mainly as hexamers *in vitro* with a molecular mass of slightly above 200 kDa, however, only the SDS-resistant dimer, migrating with an apparent molecular mass of 70 kDa, could be visualized in an SDS-containing gel. The dimer was further converted into the monomer ($MW[NS1_{monomeric}]$ = 43,9 kDa) by heat denaturation. This process is independent of disulfide bonds.

Example 4: NS1 monomers and dimers may be used to detect antibodies in patient serum by way of Western blotting

Protein preparations of mNS1 and sNS1 prepared as in Example 2 were treated with or without 16 mmol/l dithiotreitol and were incubated for 10 min at 70°C or at room temperature to achieve monomeric or oligomeric NS1. Both populations were mixed and separated using SDS electrophoresis followed by transfer to a nitrocellulose membrane. Proteins were either stained unspecifically by Ponceau S staining to demonstrate NS1 in monomeric and dimeric form or were incubated with anti-His antibody as a positive control, buffer devoid of serum as negative control and four human sera (dilution 1:51), two of them from healthy blood donors and two from patients suffering from Zika virus infection.

Fig. 6 shows that both patients' sera react with monomeric and dimeric mNS1 and sNS1, but dimeric NS1 is more sensitive. Reduction of monomeric NS1 with dithiotreitol leads to denaturation and further reduces sensitivity. As shown in a subsequent experiment, the analytical sensitivity of reduced NS1 monomers is at least 10 times less than that of non-reduced monomers in a Western blot.

Example 5: NS1 Antigen stability is increased in the presence of bovine apolipoprotein AI

The following experiment shows that a complex of a polypeptide comprising SEQ ID NO1 and mammalian apolipoprotein is more stable in a solution than the polypeptide by itself. Therefore, a mammalian apolipoprotein may be used to stablilize the polypeptide and devices and kits comprising it.

Protein preparations of mNS1, sNS1 and a complex consisting of sNS1 and bovine apolipoprotein AI, the latter made by preparing protein as in Example 1 followed by addition of chromatography fractions comprising the apolipoprotein, were transferred into 50 mmol/l sodium phosphate pH 7.4, 150 mmol/l sodium chloride using desalting spin columns (Zeba Spin, ThermoFisher Scientific, Waltham, USA). An aliquot of each preparation was kept on ice or at room temperature over night to allow for precipitation of non-PBS-soluble proteins. Aliquots were centrifuged for 30 min at 4°C and 100,000xg, and supernatants and pellets (resuspended in an equivalent volume of 50 mmol/l sodium phosphate pH 7.4, 150 mmol/l sodium chloride, 8 mol/l urea) were analyzed using denaturing gel electrophoresis under reducing conditions followed by Coomassie staining.

Harsh buffer exchange conditions were chosen to provoke aggregation of potentially unstable proteins.

Fig. 7 shows that mNS1 and sNS1 alone can be partially pelleted after incubation on ice or at room temperature, indicating that 30-50% of total protein amount forms aggregates.

On the other hand, the entirety of sNS1 in complex with bovine apolipoprotein AI remains in the supernatant after centrifugation, indicating a stabilizing effect on sNS1.

Example 6: Comparing reactivity of various NS1 preparations

The following experiment was performed to evaluate the reactivity of different preparations of Zika Virus NS1 antigen in an indirect ELISA for the detection of anti-Zika virus antibodies in human sera. It shows that complexation with a mammalian apolipoprotein increases the reactivity of NS1 and thus the sensitivity of the assay.

Preparation of coated microtiter plates

Three different Zika NS1 preparations were used: sNS1 (soluble NS1 purified from cell culture supernatant), mNS1 (membranous NS1 purified from cells), sNS1+ApoAI (soluble NS1 purified from cell culture supernatant, in complex with bovine ApoAI).

For use in microtiter ELISA these three NS1 preparations were diluted in PBS to final concentrations of 0.5, 1.0 and 2.0 µg/ml, respectively. Microtiter plates were coated with 100 µl antigen dilution per well.

Incubation of samples:

A set of anti-Zika-NS1 IgM positive or negative human sera was used for evaluation of reactivity of the different antigen preparations. All reagents used during incubation are included in every EUROIMMUN IgM ELISA Test-Kit for infectious diagnostics (e.g. EI 2668-9601 M). Sera were diluted 1:101 in IgM sample buffer containing IgG/RF absorbent and incubated at room temperature for 10 min to absorb rheumatoid factors and IgG. Samples were applied to microtiter plates and incubated as described for commercial EUROIMMUN anti-Zika virus ELISA (IgM) (EI 2668-9601 M). In brief: 60 min at 37 °C; 3 washing steps using EUROIMMUN wash buffer; addition of 100 µl of peroxidase-labelled anti-human IgM conjugate (goat) per well; incubation for 30 min at room temperature; 3 washing steps using EUROIMMUN wash buffer; addition of 100 µl of chromogen/substrate solution (TMB/H2O2) per well; incubation for 15 min at room temperature; addition of 100 µl stop-solution (0.5 M sulfuric acid); measurement of optical density at 450 nm.

Interpretation of results:

Fig. 8 shows the results, more specifically a higher reactivity of mNS1 and sNS1+ApoAI compared to sNS1. mNS1 and sNS1+ApoAI show comparable reactivity concerning positive sera. With regard to negative sera sNS1+ApoAI shows a lower reactivity compared to mNS1 (even in highest applied coating concentrations) suggesting a higher specificity of the complexed antigen. NS1 could also be shown to form a complex bovine Apolipoprotein B-100 isoform X1, which may also be used for stabilizing the antigen.

Example 7: A multicohort study of NS1-based ELISA assay performance shows lack of cross-reactivity with dengue virus antibodies

The diagnostic performance of the assay according to the present invention was examined

using sera from returning travellers and patients from ZIKV-endemic areas with laboratory-confirmed ZIKV infection, potentially cross-reactive samples from patients with flaviviral and other infections, as well as control panels from blood donors of different ages and geographical origin.

Methods

Human sera

The study included serum samples from 27 patients who had tested positive for ZIKV RNA by reverse transcription PCR (RT-PCR); Group 1: travellers returning from endemic areas (n = 8); Group 2: residents in ZIKV-endemic areas (n = 19). On the basis of direct detection of the pathogen's genome, these cases were referred to as having RT-PCR-confirmed ZIKV infection. Samples from a further 85 patients had been pre-characterised by anti-ZIKV indirect immunofluorescence assay (IIFA; EUROIMMUN, Lübeck, Germany) based on whole virus antigen, showing reactivity for anti-ZIKV IgM and/or IgG; Group 3: travellers returning from endemic areas (n = 26); Group 4: residents in ZIKV-endemic areas (n = 59). Since false-positive results due to cross-reactivity of this IIFA cannot be excluded, these cases were referred to as having suspected ZIKV infection (**Table 1**).

Classification into three stages of ZIKV infection was according to the Pan American Health Organization (PAHO) /World Health Organization (WHO) recommendations on ZIKV surveillance in the Americas: ≤ 5 days post symptom onset, initial stage; 6–20 days post symptom onset, active stage; > 20 days post symptom onset, late stage. Samples from travellers returning from endemic areas were provided by the diagnostic institutes (listed in **Table 1**) to which they had been sent for routine diagnostic testing. Samples from patients residing in Latin America (i.e. Dominican Republic and Colombia) were purchased from Boca Biolistics (Coconut Creek, Florida, United States (United States (US)), Allied Research Society (Miami Lakes, Florida, US) and Biomex GmbH (Heidelberg, Germany). As confirmed by these institutes and companies, written informed consent had been obtained from all patients, and there were no legal or ethical restrictions to using the samples.

To evaluate cross-reactivity, samples were used from 252 patients with either a post-YFV vaccination status (n = 12), or with other flaviviral (DENV = 93; WNV = 34, JEV = 25), non-flaviviral (CHIKV = 19) and *Plasmodium* spp. (PLAS: n = 69) infections. In samples from DENV-infected patients, the confirmation of DENV as the infectious agent was based on NS1

antigen detection. Sera from 1,015 healthy individuals (pregnant women, blood donors and children) living in flavivirus-endemic and non-endemic areas served as negative controls. Pre-characterisation data for all control cohorts are reported in **Table 2**. To the best of the authors' knowledge, none of these samples were analysed in previous studies.

TABLE 1. Characteristics of patients with RT-PCR-confirmed (n = 27) and suspected (n = 85) Zika virus infection, study evaluating a novel NS1-based ELISA, Germany 2016

Case ID	Age groups in years	Sex	Country of infection	Current/former residence	Sampling Dpso	Phase of infection[a]	Clinical symptoms[b]	Diagnostic centre/ provider of samples	ZIKV-RT-PCR assay/ performed at	ZIKV-RT-PCR result[c]	Virus neutralisation assay titre	IIFA IgM titre[d]	IIFA IgG titre[d]
Group 1: RT-PCR-confirmed ZIKV infection, travellers returning from ZIKV-endemic areas (n = 8)													
1	20–29	M	NA	Non-endemic	7	Active	Yes	WHOCC, Hamburg, Germany	RealStar Zika Virus RT-PCR (Altona Diagnostics, Hamburg, Germany)/ WHOCC	Pos	NA	1:3,200	1:3,200
2	30–39	F	Haiti	Non-endemic	≥ 4	Active	Yes			Pos	NA	1:320	1:32,000
3	50–59	M	NA	Non-endemic	3	Initial	No			Pos	NA	NA	NA
4	50–59	F	NA	Non-endemic	< 4	Initial	NA			Pos	NA	1:100	1:1,000
5	20–29	F	NA	Non-endemic	17	Active	NA	ITM, Antwerp, Belgium	RealStar Zika Virus RT-PCR (Altona Diagnostics, Hamburg, Germany)/ ITM	Pos	> 1:640	NA	NA
6	40–49	M	NA	Non-endemic	11	Active	NA			Pos	1:243	NA	NA
7	0–9	M	NA	Non-endemic	3	Initial	NA			Pos	NA	NA	NA
8	20–29	F	NA	Non-endemic	11	Active	NA			Pos	1:788	NA	NA
Group 2: RT-PCR-confirmed ZIKV infection, residents in ZIKV-endemic areas (n = 19)													
1	60–69	F	Suriname	The Netherlands/Suriname[e]	3	Initial	NA	AMC, Amsterdam,	In-house Zika RT-PCR/AMC	Pos	NA	NA	NA

2	50–59	M	Suriname	The Netherlands/Suriname[e]	5	Initial	NA	the Netherlands			Pos	NA	NA	NA	
3	40–49	F	Suriname	The Netherlands/Suriname[e]	11	Active	NA				Pos	NA	NA	NA	
4	40–49	M	Suriname	The Netherlands/Suriname[e]	9	Active	NA				Pos	NA	NA	NA	
5	50–59	F	Suriname	The Netherlands/Suriname[e]	6	Active	NA				Pos	NA	NA	NA	
6	50–59	M	Suriname	The Netherlands/Suriname[e]	6	Active	NA				Pos	NA	NA	NA	
7	50–59	F	Suriname	The Netherlands/Suriname[e]	53	Late	NA				Pos	NA	NA	NA	
8	50–59	F	Suriname	The Netherlands/Suriname[e]	17	Active	NA				Pos	NA	NA	NA	
9	60–69	F	Suriname	The Netherlands/Suriname[e]	24	Late	NA				Pos	NA	NA	NA	
10	70–79	M	Suriname	The Netherlands/Suriname[e]	6	Active	NA				Pos	NA	NA	NA	
11	0–9	M	Dominican Republic	The Netherlands	1	Initial	NA				Pos	NA	NA	NA	
12	50–59	F	Dominican Republic	Dominican Republic	20	Active	Yes	Boca Biolistics, Coconut Creek, Florida, US	Trioplex real-time RT-PCR (CDC, Atlanta, Georgia, US)/CDC		Pos	NA	0	**1:32,000**	
13	50–59	F	Dominican Republic	Dominican Republic	31	Late	Yes				Pos	NA	**1:100**	**1:32,000**	
14	20–29	M	Colombia	Colombia	3	Initial	Yes	Allied Research	Trioplex real-time RT-PCR		Pos	NA	0	**1:1,000**	
15	40–49	F	Colombia	Colombia	5	Initial	Yes				Pos	NA	0	**1:1,000**	

16	50–59	F	Colombia	Colombia	4	Initial	Yes	Society, Miami Lakes, Florida, US	(CDC, Atlanta, Georgia, US)/CDC	Pos	NA	1:10	1:3,200
17	10–19	M	Colombia	Colombia	3	Initial	Yes			Pos	NA	0	1:3,200
18	20–29	F	Colombia	Colombia	6	Active	Yes	Biomex GmbH, Heidelberg, Germany	RealStar Zika Virus RT-PCR (Altona Diagnostics, Hamburg, Germany)/ Altona Diagnostics	Pos	NA	1:3,200	1:32,000
19	10–19	M	Colombia	Colombia/US	15	Active	Yes		Trioplex real-time RT-PCR (CDC, Atlanta, Georgia, US)/CDC	Pos	NA	1:10	1:32,000
Group 3: Suspected ZIKV infection, travellers returning from ZIKV-endemic areas (n=26)													
1	NA	NA	NA	Non-endemic	NA	NA	NA	WHOCC, Hamburg, Germany	NA	NA	NA	1:3,200	1:10,000
2	NA	NA	NA	Non-endemic	NA	NA	NA			NA	NA	1:1,000	1:10,000
3	NA	NA	NA	Non-endemic	NA	NA	NA			NA	NA	1:3,200	1:10,000
4	NA	NA	Brazil	Non-endemic	NA	NA	NA			NA	NA	1:1,000	1:32,000
5	NA	NA	Brazil	Non-endemic	NA	NA	NA			NA	NA	1:1,000	1:3,200
6	NA	NA	Brazil	Non-endemic	NA	NA	NA			NA	NA	1:3,200	1:10,000
7	NA	NA	Brazil	Non-endemic	NA	NA	NA			NA	NA	1:100	< 1:100
8	NA	NA	NA	Non-endemic	NA	NA	NA			NA	NA	1:1,000	1:100

9	NA	NA	NA	Non-endemic	NA	NA	NA					NA	NA	1:320	1:10,000
10	NA	NA	NA	Non-endemic	NA	NA	NA					NA	NA	1:320	1:32,000
11	NA	NA	Brazil	Non-endemic	19	Active	Yes					NA	NA	1:320	1:10,000
12	NA	NA	Brazil	Non-endemic	NA	NA	NA					NA	NA	1:100	1:100,000
13	NA	NA	Brazil	Non-endemic	NA	NA	NA					NA	NA	1:1,000	1:320
14	NA	NA	Brazil	Non-endemic	NA	NA	NA					NA	NA	1:320	1:3,200
15	NA	NA	Brazil	Non-endemic	NA	NA	NA					NA	NA	1:320	1:1,000
16	NA	NA	Brazil	Non-endemic	NA	NA	NA					NA	NA	1:1,000	1:10,000
17	NA	NA	Brazil	Non-endemic	NA	NA	NA					NA	NA	1:320	1:10,000
18	NA	NA	NA	Non-endemic	32	Late	NA					NA	NA	1:100	1:32,000
19	NA	NA	Colombia	Non-endemic	45	Late	NA					NA	NA	1:100	1:3,200
20	NA	NA	NA	Non-endemic	NA	NA	NA					NA	NA	1:1,000	1:10,000
21	NA	NA	Denmark	Non-endemic	NA	NA	NA					NA	NA	1:100	1:32,000
22	NA	NA	NA	Non-endemic	NA	NA	NA					NA	NA	1:3,200	1:32,000
23	NA	NA	Colombia	Non-endemic	NA	NA	NA					NA	NA	1:100	1:10,000
24	NA	NA	Brazil	Non-endemic	NA	NA	NA					NA	NA	1:320	1:32,000
25	NA	NA	Brazil	Non-endemic	NA	NA	NA					NA	NA	1:320	1:32,000
26	NA	NA	Colombia	Non-endemic	15	Active	NA					NA	NA	1:3,200	1:10,000
Group 4: Suspected ZIKV infection, residents in ZIKV-endemic areas (n=59)															
1	30–39	F	Colombia	Colombia	6	Active	Yes	Allied Research Society, Miami Lakes, Florida, US	NA			NA	NA	1:1,000	1:320,000
2	20–29	M	Colombia	Colombia	8	Active	Yes					NA	NA	1:100	1:1,000
3	30–39	F	Colombia	Colombia	11	Active	Yes					NA	NA	0	1:1,000
4	40–49	M	Colombia	Colombia	14	Active	Yes					NA	NA	1:3,200	1:320,000
5	30–39	F	Colombia	Colombia	17	Active	Yes					NA	NA	1:3,200	1:320,000
6	80–89	M	Colombia	Colombia	20	Active	Yes					NA	NA	1:320	1:320,000
7	50–59	F	Colombia	Colombia	23	Late	Yes					NA	NA	1:320	1:10,000

8	30–39	M	Colombia	Colombia		30	Late	Yes					NA	NA	1:3,200	1:320,000		
9	40–49	F	Colombia	Colombia		49	Late	Yes					NA	NA	1:100	1:10,000		
10	10–19	F	Colombia	Colombia		54	Late	Yes					NA	NA	1:10	1:1,000		
11	50–59	F	Colombia	Colombia		6	Active	Yes					NA	NA	0	1:3,200		
12	40–49	F	Colombia	Colombia		4	Initial	Yes					NA	NA	0	1:1,000		
13	10–19	M	Colombia	Colombia		66	Late	Yes					NA	NA	0	1:32,000		
14	40–49	F	Colombia	Colombia		68	Late	Yes					NA	NA	1:10	1:32,000		
15	50–59	F	NA	Colombia		70	Late	Yes					NA	NA	0	1:32,000		
16	40–49	F	NA	Colombia		2	Initial	Yes					NA	NA	0	1:10,000		
17	20–29	F	Colombia	Colombia		7	Active	Yes					NA	NA	1:100	1:320,000		
18	50–59	F	NA	Colombia		4	Initial	Yes					NA	NA	1:100	1:100,000		
19	40–49	M	Colombia	Colombia		3	Initial	Yes					NA	NA	1:10,000	1:32,000		
20	40–49	F	Colombia	Colombia		4	Initial	Yes					NA	NA	1:32	1:32,000		
21	30–39	M	Colombia	Colombia		4	Initial	Yes					NA	NA	1:32	1:32,000		
22	40–49	F	Colombia	Colombia		4	Initial	Yes					NA	NA	0	1:100,000		
23	30–39	M	Colombia	Colombia		4	Initial	Yes					NA	NA	0	1:32,000		
24	20–29	F	Colombia	Colombia		5	Initial	Yes					NA	NA	1:10	1:10,000		
25	40–49	F	Colombia	Colombia		5	Initial	Yes					NA	NA	1:1,000	1:100,000		
26	30–39	F	Colombia	Colombia		3	Initial	Yes					NA	NA	0	1:3,200		
27	40–49	F	Colombia	Colombia		4	Initial	Yes					NA	NA	0	1:32,000		
28	20–29	F	Colombia	Colombia		3	Initial	Yes					NA	NA	0	1:320		
29	50–59	F	Colombia	Colombia		4	Initial	Yes					NA	NA	0	1:10,000		
30	20–29	F	Colombia	Colombia		3	Initial	Yes					NA	NA	1:32	1:10,000		
31	30–39	F	Colombia	Colombia		3	Initial	Yes	Biomex GmbH, Heidelberg, Germany	NA			NA	NA	0	1:32,000		
32	20–29	F	Colombia	Colombia		4	Initial	Yes					NA	NA	1:100	1:32,000		
33	10–19	F	Colombia	Colombia		9	Active	Yes					NA	NA	1:100	1:32,000		

34	20–29	F	Colombia	Colombia	12	Active	Yes			NA	NA	0	1:32,000
35	10–19	F	Colombia	Colombia	20	Active	Yes			NA	NA	1:100	1:10,000
36	20–29	F	Colombia	Colombia	27	Late	Yes			NA	NA	1:320	1:10,000
37	30–39	F	Colombia	Colombia	36	Late	Yes			NA	NA	1:10	1:32,000
38	10–19	F	Colombia	Colombia	56	Late	Yes			NA	NA	1:100	1:10,000
39	30–39	F	Colombia	Colombia	67	Late	Yes			NA	NA	1:10	1:32,000
40	10–19	F	Colombia	Colombia	2	Initial	Yes	Allied Research Society, Miami Lakes, Florida, US	NA	NA	NA	0	1:10,000
41	30–39	F	Colombia	Colombia	5	Initial	Yes			NA	NA	1:320	1:10,000
42	20–29	F	Colombia	Colombia	6	Active	Yes			NA	NA	1:100	1:10,000
43	20–29	F	Colombia	Colombia	8	Active	Yes			NA	NA	1:100	1:32,000
44	30–39	F	Colombia	Colombia	15	Active	Yes			NA	NA	0	1:10,000
45	20–29	F	Colombia	Colombia	21	Late	Yes			NA	NA	1:10	1:100,000
46	20–29	F	Colombia	Colombia	29	Late	Yes			NA	NA	1:320	1:32,000
47	20–29	F	Colombia	Colombia	38	Late	Yes			NA	NA	1:1,000	1:320,000
48	10–19	F	Colombia	Colombia	50	Late	Yes			NA	NA	1:10	1:10,000
49	20–29	F	Colombia	Colombia	88	Late	Yes			NA	NA	0	1:1,000
50	40–49	F	Colombia	Colombia	2	Initial	Yes			NA	NA	0	1:3,200
51	20–29	M	Colombia	Colombia	5	Initial	Yes			NA	NA	1:1,000	1:32,000
52	30–39	F	Colombia	Colombia	6	Active	Yes			NA	NA	0	1:1,000
53	20–29	M	Colombia	Colombia	8	Active	Yes			NA	NA	0	1:10,000
54	30–39	F	Colombia	Colombia	15	Active	Yes			NA	NA	1:320	1:320,000
55	30–39	M	Colombia	Colombia	21	Late	Yes			NA	NA	1:100	1:32,000
56	40–49	M	Colombia	Colombia	29	Late	Yes			NA	NA	1:32,000	1:32,000
57	40–49	F	Colombia	Colombia	38	Late	Yes			NA	NA	0	1:320
58	50–59	F	Colombia	Colombia	50	Late	Yes			NA	NA	0	1:100,000
59	50–59	M	Colombia	Colombia	85	Late	Yes			NA	NA	0	1:32,000

AMC: Academic Medical Center; CDC: Centers for Disease Control and Prevention; dpso: days post symptom onset; F: female; IIFA: indirect immunofluorescence assay; ITM: Institute of Tropical Medicine, M: male; NA: not available; NS: non-structural protein; Pos: positive; US: United States; WHOCC: World Health Organization Collaborating Centre (for Arbovirus and Haemorrhagic Fever Reference and Research); ZIKV: Zika virus.

[a] Phase of infection at the time of sample collection: initial phase: ≤ 5 dpso; active phase: 6 to 20 dpso; late phase: > 20 dpso.

[b] Fever, skin rash, joint pain, myalgia, headache, conjunctivitis, eye pain, diarrhoea and malaise.

[c] ZIKV-RT-PCR results can also refer to serum or urine samples taken at an earlier date than the samples used for anti-ZIKV serological testing.

[d] IIFA was performed at EUROIMMUN, Lübeck, Germany, using the Anti-Zika Virus IIFA test kit (EUROIMMUN). Cut-off IgM: ≥ 1:10; IgG: ≥ 1:100.

[e] Sera from Dutch residents who were born and raised in Suriname and/or had visited their country of origin occasionally.

TABLE 2. Characteristics of control cohorts, study evaluating a novel NS1-based ELISA, Germany 2016

Cohort	n	Origin of sample donors	Type	Diagnostic centre (provider of samples)	Sample receipt	Pre-characterisation
Flavivirus infection or vaccination						
DENVa (high IgM)	47	Germany, Italy	Returning travellers from endemic areas with DENV infection (contracted e.g. in Brazil, Bali, Thailand, Laos, Philippines, India, Cambodia, Taiwan)	MVZ Diamedes GmbH Bielefeld, Germany; University of Bologna, Bologna, Italy; WHOCC, Hamburg, Germany	2011–2014	• Panbio or BIO-RAD DENV-NS1 ELISA[a,b]: 47/47 (100%) DENV-NS1 positive • DENV-RT-PCR (only 8/47 tested)[b]: n = 4 subtype DENV-1, n = 2 subtype DENV-2, n = 2 subtype DENV-3 • EUROIMMUN Anti-DENV ELISA (IgM, IgG)[c]: 40/47 (85%) anti-DENV IgM positive, 30/47 (64%) anti-DENV IgG positive, 37/47 (79%) anti-DENV IgM ratio ≥ 3.0, 10/47 (21%) anti-DENV IgM ratio < 3.0, anti-DENV IgM median ratio = 3.9
DENVb (high IgG)	46	Germany, Italy	Returning travellers from endemic areas with DENV infection (contracted e.g. in Brazil, Bali, Thailand, Laos, Philippines, India, Cambodia, Taiwan)	MVZ Diamedes GmbH Bielefeld, Germany; University of Bologna, Bologna, Italy	2011–2014	• DENV-NS1 ELISA[a,b]: 46/46 (100%) DENV-NS1 positive • DENV-RT-PCR (only 1/46 tested)[b]: n = 1 subtype DENV-4 • EUROIMMUN Anti-DENV ELISA (IgM, IgG)[c]: 35/46 (76%) anti-DENV IgM positive, 40/46 (87%) anti-DENV IgG positive, 37/46 (80%) anti-DENV IgG ratio ≥ 3.0, 9/46 (20%) anti-DENV IgG ratio < 3.0, • anti-DENV IgG median ratio = 3.9
YFV	12	France	Individuals vaccinated against YFV	Cerba Specimen Services, Saint-Ouen l'Aumône, France	2015	• YFV seroneutralisation test[d]: 12/12 (100%) anti-YFV positive • EUROIMMUN Anti-WNV ELISA (IgM, IgG)[c]: 0/12 (0%) anti-WNV IgM positive, 0/12 (0%) anti-WNV IgG positive • EUROIMMUN Anti-CHIKV ELISA (IgM, IgG)[c]: 0/12 (0%) anti-CHIKV IgM positive, 1/12 (8%) anti-CHIKV IgG positive

WNV	34	US	Patients from endemic areas with WNV infection	MAYO Clinic, Scottsdale, Arizona, US	2014	• WNV PRNT[e]: 34/34 (100%) anti-WNV positive • EUROIMMUN Anti-WNV ELISA (IgM, IgG)[c]: 23/34 (68%) anti-WNV IgM positive, 26/34 (76%) anti-WNV IgG positive
JEV	25	Vietnam	Patients from endemic areas with JEV infection	National Hospital of Tropical Disease, Hanoi, Vietnam	2016	• DRG JE IgM capture ELISA[f]: 25/25 (100%) anti-JEV IgM positive • EUROIMMUN Anti-JEV ELISA (IgM, IgG)[c]: 25/25 (100%) anti-JEV IgM positive, 19/25 (76%) anti-JEV IgG positive
Non-flavivirus infection						
CHIKV	19	Réunion	Patients from endemic areas with CHIKV infection	Cerba Specimen Services, Saint-Ouen l'Aumône, France	2015	• CHIKV VRP neutralisation test[g]: 19/19 (100%) anti-CHIKV positive • EUROIMMUN Anti-CHIKV ELISA (IgM, IgG)[c]: 0/19 (0%) anti-CHIKV IgM positive, 19/19 (100%) anti-CHIKV IgG positive
Parasite infection						
PLAS	69	France (including overseas department and region Mayotte), French Guiana, Tunisia, Madagascar, Switzerland	Blood donors living in and travellers returning from *Plasmodium*-endemic areas, acute or past *Plasmodium* infection	TheBindingSite, Schwetzingen, Germany Cerba Specimen Services, Saint-Ouen l'Aumône, France Swiss Red Cross, Bern, Switzerland	2016	• BioMérieux Plasmodium IFA (IgM, IgG)[d,h]: 1/15 (7%) anti-*Plasmodium* IgM positive, 15/15 (100%) anti-*Plasmodium* IgG positive • BIO-RAD Malaria ELISA (IgG)[i]: 54/54 (100%) anti-*Plasmodium* positive
Healthy controls: pregnant women, blood donors and children						

PREG	100	Germany	Pregnant women from non-flavivirus endemic areas without clinical symptoms	Laboratory Schottdorf, Augsburg, Germany	2007	• EUROIMMUN Anti-DENV ELISA (IgM, IgG)[c]: 2/100 (2%) anti-DENV IgM positive, 7/100 (7%) anti-DENV IgG positive • EUROIMMUN Anti-WNV ELISA (IgM, IgG)[c] 3/100 (3%) anti-WNV IgM positive, 4/100 (4%) anti-WNV IgG positive • EUROIMMUN Anti-JEV ELISA (IgM, IgG)[c]: 2/100 (2%) anti-JEV IgM positive, 14/100 (14%) anti-JEV IgG positive • EUROIMMUN Anti-CHIKV ELISA (IgM, IgG)[c]: 0/100 (0%) anti-CHIKV IgM positive, 0/100 (0%) anti-CHIKV IgG positive
ZIM	128	Zimbabwe	Blood donors from flavivirus and parasite endemic areas without clinical symptoms	National Blood Transfusion Service, Zimbabwe, Africa	2003	• EUROIMMUN Anti-DENV ELISA (IgG)[c]: 4/128 (3%) anti-DENV IgG positive • EUROIMMUN Anti-CHIKV ELISA (IgG)[c]: 3/128 (2%) anti-CHIKV IgG positive • EUROIMMUN Anti-Plasmodium ELISA (IgG)[c]: 36/128 (28%) anti-*Plasmodium* IgG positive
ARG	99	Argentina	Blood donors from flavivirus endemic areas without signs of viral infection (routine samples for parasitology)	IACA Laboratory, Buenos Aires, Argentina	2014	• EUROIMMUN Anti-DENV ELISA (IgM, IgG)[c]: 2/99 (2%) anti-DENV IgM positive, 4/99 (4%) anti-DENV IgG positive • EUROIMMUN Anti-WNV ELISA (IgM, IgG)[c]: 2/99 (2%) anti-WNV IgM positive, 3/99 (3%) anti-WNV IgG positive • EUROIMMUN Anti-CHIKV ELISA (IgM, IgG)[c]: 3/99 (3%) anti-CHIKV IgM positive, 1/99 (1%) anti-CHIKV IgG positive • EUROIMMUN Anti-Trypanosoma ELISA (IgM, IgG)[c]: 2/99 (2%) anti-Trypanosoma IgM positive, 1/99 (1%) anti-Trypanosoma IgG positive
US	100	US	Blood donors without clinical symptoms (n): Hispanic (25), African American (30), Caucasian (43), Asian (1), Colombian (1)	Serologix, New Hope, Pasadena, US	2014	• EUROIMMUN Anti-DENV ELISA (IgM, IgG)[c]: 1/100 (1%) anti-DENV IgM positive, 6/100 (6%) anti-DENV IgG positive • EUROIMMUN Anti-WNV ELISA (IgM, IgG)[c]: 0/100 (0%) anti-WNV IgM positive, 4/100 (4%) anti-WNV IgG positive • EUROIMMUN Anti-CHIKV ELISA (IgM, IgG)[c]: 0/100 (0%) anti-CHIKV IgM positive, 4/100 (4%) anti-CHIKV IgG positive
GER	500	Germany	Blood donors from non-	University Medical Center	2012	NA

CHIL	88	Germany	flavivirus endemic areas without clinical symptoms	Children (≤ 10 years) form non-flavivirus endemic areas without clinical symptoms	Schleswig-Holstein, Campus Lübeck, Lübeck, Germany	Praxis Dr Fischer-Wassels, Dortmund, Germany	2007–2008	• EUROIMMUN Anti-DENV ELISA (IgM, IgG)[c]: 0/100 (0%) anti-DENV IgM positive, 0/100 (0%) anti-DENV IgG positive • EUROIMMUN Anti-WNV ELISA (IgM, IgG)[c]: 1/100 (1%) anti-WNV IgM positive, 0/100 (0%) anti-WNV IgG positive • EUROIMMUN Anti-JEV ELISA (IgM, IgG)[c]: 0/100 (0%) anti-JEV IgM positive, 0/100 (0%) anti-JEV IgG positive • EUROIMMUN Anti-CHIKV ELISA (IgM, IgG)[c]: 0/100 (0%) anti-CHIKV IgM positive, 0/100 (0%) anti-CHIKV IgG positive

ARG: Argentina; CHIKV: chikungunya virus; CHIL: children; DENV: dengue virus; IFA: immunofluorescence assay; GER: Germany; JEV: Japanese encephalitis virus; IIFA: indirect immunofluorescence assay; NA: not available; PLAS: *Plasmodium*; PREG: pregnant women; PRNT: plaque reduction neutralisation test; RT-PCR: reverse transcription-PCR; US: United States; WHOCC: World Health Organization Collaborating Centre (for Arbovirus and Haemorrhagic Fever Reference and Research); WNV: West Nile virus; YFV: yellow fever virus; ZIKV: Zika virus; ZIM: Zimbabwe.

[a] Performed at MVZ Diamedis GmbH, Bielefeld, Germany.

[b] Performed at the University of Bologna, Italy.

[c] Performed at EUROIMMUN, Lübeck, Germany.

[d] Performed at Cerba Specimen Services, Saint-Ouen l'Aumône, France.

[e] Performed at the University of Leipzig, Germany.

[f] Performed at the National Hospital of Tropical Disease, Hanoi, Vietnam.

[g] Performed at the University of Bonn, Germany.

[h] Performed at TheBindingSite, Schwetzingen, Germany.

[i] Performed at the Swiss Red Cross, Bern, Switzerland.

anonymised to the Institute for Experimental Immunology (affiliated to EUROIMMUN). All sera were stored at -20 °C until assayed. The study was performed according to the recommendations of the Central Ethical Committee of Germany [29].

Enzyme-linked immunosorbent assays

Anti-Zika Virus IgM and IgG ELISA (EUROIMMUN) were used as recommended by the manufacturer. These kit assays are based on standardised reagents and microtitre plates coated with recombinant ZIKV-NS1. Briefly, sera diluted 1:101 in sample buffer were added to the wells and allowed to react for 60 min at 37 °C. Before IgM detection, sera were pre-incubated with sample buffer containing IgG/rheumatoid factor (RF) absorbent (EUROIMMUN) to remove class IgG antibodies and class IgM RF from the sample. This step prevents specific IgG from displacing IgM from the antigen (leading to false IgM-negative results) and RF-IgM from reacting with specifically bound IgG (leading to false IgM-positive results). Bound antibodies were detected by applying goat anti-human IgM peroxidase conjugate or rabbit anti-human IgG peroxidase conjugate for 30 min at room temperature, followed by staining with tetramethylbenzidine for 15 min. The enzymatic reaction was stopped by addition of one volume 0.5 mol/L sulphuric acid. A calibrator (chicken–human chimeric ZIKV antibody with a concentration adjusted to give an extinction value defining the upper limit of the reference range of non-infected persons) as well as positive and negative controls were provided with the test kit and assayed with each test run. Colour intensity of the enzymatic reactions was determined photometrically at 450 nm (reference 620 nm), resulting in extinction values. A signal-to-cut-off ratio ($extinction_{sample}/extinction_{calibrator}$) was calculated for each sample.

Receiver-operating characteristics (ROC) analysis based on the initial validation dataset of positive and negative samples was done by the manufacturer to evaluate assay performance at each possible cut-off, demonstrating optimal sensitivity and specificity at ratio values of 0.8 (IgM) and 0.6 (IgG). To ensure high specificity, the borderline range (≥ 0.8 to < 1.1) was established between the highest negative and the lowest positive validation sample, resulting in a positivity cut-off of ≥ 1.1.

Anti-dengue Virus IgM and IgG ELISA (EUROIMMUN) were used.

Statistics

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, California, US) and SigmaPlot 13.0 (SSI, San Jose, California, US). Sensitivity was calculated as the proportion of ZIKV patients (referring to groups 1 to 4 as indicated) identified as positive by the assay. Specificity was calculated as the proportion of negative test results obtained among healthy controls. We calculated 95% confidence intervals (CIs) according to the modified Wald method. The study was performed in compliance with the Standards for Reporting of Diagnostic accuracy (STARD) statement.

Results

Sensitivity of the enzyme-linked immunosorbent assay

The sensitivity of the novel NS1-based anti-ZIKV ELISA was evaluated in sera from 27 patients with RT-PCR-confirmed ZIKV infection that had been sub-grouped into travellers returning from ZIKV-endemic areas and endemic-area residents. Among eight infected travellers returning from ZIKV-endemic areas (group 1), positive anti-ZIKV IgM and IgG reactivity was found in seven (87.5%) and three (37.5%) cases, respectively. Of 19 infected residents in endemic-areas (group 2), six (31.6%) were positive for anti-ZIKV IgM and 15 (79.0%) for IgG. In addition, sera from 85 patients with suspected ZIKV infection were examined. Here, of 26 infected travellers returning from ZIKV-endemic areas (group 3) 21 (80.8%) were positive for anti-ZIKV IgM and 18 (69.2%) for IgG, while among 59 infected residents in endemic-areas (group 4), six (10.2%) showed positive reactivity for anti-ZIKV IgM and 53 (89.9%) for IgG. For the total of RT-PCR-confirmed and suspected cases, the combined ELISA sensitivity (IgM and/or IgG) amounted to 23/27 (85.2%) and 78/85 (91.8%), respectively.

Confining the time point of serological evaluation to the active and late phase of ZIKV infection, i.e. ≥ 6 days after symptom onset, anti-ZIKV IgM reactivity was observed in 10/17 (58.8%) patients with positive ZIKV-RT-PCR and 3/38 (7.9%) patients with suspected ZIKV infection, while anti-ZIKV IgG was detectable in 15/17 (88.2%) and 34/38 (89.5%) cases, respectively. Thus, the combined sensitivity (IgM and/or IgG) reached 17/17 (100%) among RT-PCR-confirmed cases and 34/38 (89.5%) among suspected cases (**Table 3**).

TABLE 3. Anti-ZIKV reactivity in patients with RT-PCR-confirmed (n = 27) and suspected (n = 85) ZIKV infection as determined by ELISA for IgM and IgG, study evaluating a novel NS1-based ELISA, Germany 2016

Group	Characteristics		Anti-ZIKV ELISA reactivity (≥ 1 day post symptom onset)[c]				Anti-ZIKV ELISA reactivity (≥ 6 days post symptom onset)[d,e]			
			n	IgM	IgG	IgM / IgG	n	IgM	IgG	IgM / IgG
1	RT-PCR-confirmed ZIKV infection, travellers returning from ZIKV-endemic areas	Positive	8	7	3	7	5	5	3	5
		Sensitivity %[b] (95% CI)	–	87.5 (50.8–99.9)	37.5 (13.5–69.6)	87.5 (50.8–99.9)	–	100 (51.1–100)	60.0 (22.9–88.4)	100 (51.1–100)
2	RT-PCR-confirmed ZIKV infection, residents in ZIKV-endemic areas[a]	Positive	19	6	15	16	12	5	12	12
		Sensitivity %[b] (95% CI)	–	31.6 (15.2–54.2)	78.9 (56.1–92.1)	84.2 (61.6–95.3)	–	41.7 (19.3–68.1)	100 (71.8–100)	100 (71.8–100)
Total 1 + 2	RT-PCR-confirmed ZIKV infection	Positive	27	13	18	23	17	10	15	17
		Sensitivity %[b] (95% CI)	–	48.1 (30.7–66.0)	66.7 (47.7–81.5)	85.2 (66.9–94.7)	–	58.8 (36.0–78.4)	88.2 (64.4–98.0)	100 (78.4–100)
3	Suspected ZIKV infection, travellers returning from ZIKV-endemic areas	Positive	26	21	18	25	NA[e]			
		Sensitivity %[b] (95% CI)	–	80.8 (61.7–92.0)	69.2 (49.9–83.7)	96.2 (79.6–100)				
4	Suspected ZIKV infection, residents ZIKV-endemic areas	Positive	59	6	53	53	38	3	34	34
		Sensitivity %[b] (95% CI)	–	10.2 (4.4–20.8)	89.9 (79.2–95.6)	89.9 (79.2–95.6)	–	7.9 (2.0–21.5)	89.5 (75.3–96.4)	89.5 (75.3–96.4)

Total 3 + 4	Suspected ZIKV infection	Positive	85	27	71	78	38[e]	3	34	34
		Sensitivity %[b] (95% CI)	–	31.8 (22.8–42.3)	83.5 (74.1–90.1)	91.8 (83.7–96.2)	–	7.9 (2.0–21.5)	89.5 (75.3–96.4)	89.5 (75.3–96.4)

CI: confidence interval; NA: not available or not applicable; NS: non-structural protein; RT-PCR: reverse transcription-PCR; ZIKV: Zika virus.

[a] This group contains 10 sera from residents of the Netherlands who were born and raised in Suriname and/or had visited their country of origin occasionally.

[b] Referring to the total number of samples in the respective patient group during the indicated sampling period.

[c] Referring to the whole study population of ZIKV-infected patients, i.e. samples (one per patient) taken between day 1 and day 88 post symptom onset, representing the initial (day 1–5 post symptom onset), active (day 6–20) and late phase (> 20 days) of infection.

[d] Samples (one per patient) taken between day 6 and day 88 post symptom onset, representing the active (day 6 to 20 post symptom onset) and late phase (> 20 days post symptom onset) of infection.

[e] Group 3 is not represented in the sampling period ≥ 6 days post symptom onset, because the sampling date was available for only four out of a total of 26 samples in this group.

TABLE 4. Anti-ZIKV reactivity in potentially cross-reactive specimens (n = 252) and healthy controls (n = 1,015) as determined by ELISA for IgM and IgG, study evaluating a novel NS1-based ELISA, Germany 2016

Cohort	Characteristics		Prevalence % (CI 95%)[c]		Specificity (CI 95%)[c]	
			IgM	IgG	IgM	IgG
DENVa	Dengue virus infection (high median anti-DENV IgM)[a]	47	0 (0–9.0)	0 (0–9.0)	100 (91.0–100)	100 (91.0–100)
DENVb	Dengue virus infection (high median anti-DENV IgG[b]	46	0 (0–9.2)	0 (0–9.2)	100 (90.8–100)	100 (90.8–100)
YFV	Yellow fever virus vaccination	12	0 (0–28.2)	0 (0–28.2)	100 (71.8–100)	100 (71.8–100)
WNV	West Nile virus infection	34	2.9 (0–16.2)	0 (0–12.1)	97.1 (83.8–100)	100 (87.9–100)
JEV	Japanese encephalitis virus infection	25	0 (0–15.8)	4.0 (0–21.1)	100 (84.2–100)	96.0 (78.9–100)
CHIKV	Chikungunya virus infection	19	0 (0–19.8)	0 (0–19.8)	100 (80.2–100)	100 (80.2–100)
PLAS	*Plasmodium* spp. Infection	69	1.4 (0–8.5)	0 (0–6.3)	98.6 (91.5–100)	100 (93.7–100)
Total	Potentially cross-reactive samples	252	0.8 (0–3.0)	0.4 (0–2.4)	99.2 (97.0–100)	99.6 (97.6–100)
PREG	German pregnant women	100	0 (0–4.4)	0 (0–4.4)	100 (95.6–100)	100 (95.6–100)
ZIM	Zimbabwean blood donors	128	0 (0–3.5)	0 (0–3.5)	100 (96.5–100)	100 (96.5–100)
ARG	Argentinian blood donors	99	1.0 (0–6.1)	0 (0–4.5)	99.0 (94.0–100)	100 (95.5–100)
US	US-American blood donors	100	0 (0–4.4)	1.0 (0–6.0)	100 (95.6–100)	99.0 (94.0–100)
GER	German blood donors	500	0.2 (0–1.2)	0.2 (0–1.2)	99.8 (98.8–100)	99.8 (98.8–100)
CHIL	German children	88	0 (0–5.0)	0 (0–5.0)	100 (95.0–100)	100 (95.0–100)
Total	Healthy control samples	1,015	0.2 (0–0.8)	0.2 (0–0.8)	99.8 (99.2–100)	99.8 (99.2–100)

ARG: Argentina; CHIKV: chikungunya virus; CHIL: children; DENV: dengue virus; GER: Germany; JEV: Japanese encephalitis virus; PLAS: *Plasmodium*; PREG: pregnant women; US: United States; WNV: West Nile virus; YFV: yellow fever virus; ZIKV: Zika virus; ZIM: Zimbabwe.

[a] Median anti-DENV IgM ratio 3.9 (79% of samples with anti-DENV IgM ratio ≥ 3.0), as indicated in the inset of **Fig. 9A**.

[b] Median anti-DENV IgG ratio 3.9 (80% of samples with anti-DENV IgG ratio ≥ 3.0), as indicated in the inset of **Fig. 9B**.

[c] Referring to the total number of samples in the individual cohorts.

Comparing ZIKV-infected travellers returning from endemic areas (groups 1 and 3) with infected residents in these areas (groups 2 and 4), a tendency of distinct ZIKV antibody kinetics could be observed: in most returning travellers, high IgM ratio values (median 5.6; interquartile range (IQR): 4.6–6.9,) and moderate IgG ratios (median 2.2; IQR 0.9–2.8,) were detectable in the active phase of infection (cut-off ratio: 1.1). By contrast, the majority of endemic-area residents had infections with very high IgG ratios (median 4.8; IQR 3.3–5.9) during the active phase, while IgM ratios were variable, but predominantly negative or low (median 0.5; IQR 0.2–1.3) (**Fig. 9A** and **9B**).

Time course analysis of a German patient who showed clinical symptoms after returning from a stay in Colombia revealed very high anti-ZIKV IgM ratios on first testing (day 10 after symptom onset), while IgG ratios increased to moderate levels during the acute phase of infection and thereafter (**Fig. 9C**). On the other hand, follow-up samples taken from a Colombian resident with RT-PCR-confirmed ZIKV infection indicated a significant rise in the ZIKV-specific IgG response between days 3 and 15 after symptom onset, followed by a slow decrease, while anti-ZIKV IgM was negative 3 days after symptom onset and remained below detection threshold for 14 weeks (**Fig. 9D**).

Cross-reactivity of the enzyme-linked immunosorbent assay

Cross-reactivity was analysed first in sera from 93 DENV-infected patients whose diagnosis had been secured by positive DENV-NS1 detection. This cohort was divided into one group (DENVa) with high anti-DENV IgM (median ratio 3.9) and another group (DENVb) with high anti-DENV IgG (median ratio 3.9), ensuring the presence of high levels of potentially cross-reactive antibodies. In both groups, anti-ZIKV reactivity was below the threshold, indicating absence of cross-reactivity in these specimens. Further testing, on a supplementary basis, included 159 sera from patients positive for IgM and/or IgG against YFV, WNV, JEV, CHIKV or PLAS. Anti-ZIKV IgM was positive in 1/34 (2.9%) patients infected with WNV and 1/69 (1.4%) patients infected with PLAS. Anti-ZIKV IgG was found in 1/25 (4.0%) patients infected with JEV (Figure G). For the total of 252 potentially cross-reactive samples, the overall positivity rate amounted to 2/252 (0.8%) for IgM and 1/252 (0.4%) for IgG (**Table 4**).

Specificity of the enzyme-linked immunosorbent assay

Assay specificity was assessed by testing 1,015 sera from healthy controls. Only 1/99 (1.0%) Argentinian and 1/500 (0.2%) German blood donors were found anti-ZIKV IgM positive, while

all 128 Zimbabwean and 100 US American blood donors as well as 100 German pregnant women and 88 children in Germany were negative. Anti-ZIKV IgG was present in 1/100 (1.0%) US American and 1/500 (0.2%) German blood donors, but absent in the cohorts of Zimbabwean and Argentinian blood donors, pregnant women and children. Thus, overall specificity amounted to 99.8% for either Ig class (**Table 4, Figs. 10A** and **10B**).

Discussion

The serological diagnosis of ZIKV infections has been challenging due to cross-reactions with other flaviviruses, secondary infections and previous vaccinations, which complicate interpretation, sometimes leading to unreliable or false-positive results. Here, we evaluated a newly-developed ELISA with recombinant ZIKV-NS1 protein as solid-phase antigen. Huzly et al. (2016 Apr 21;21(16). doi: 10.2807/1560-7917) recently provided evidence that this assay is highly specific, as demonstrated on a limited number of European patients with DENV, YFV, tick-borne encephalitis virus (TBEV) or hepatitis C virus infection. In the present study, testing on specimens collected ≥ 6 days after onset of symptoms (i.e. after the viraemic phase) revealed a combined sensitivity (IgM/IgG) of 100% for RT-PCR-confirmed cases of ZIKV infection at 99.8% specificity. Among suspected ZIKV cases, the combined sensitivity amounted to 89.5%. Notably, we included only one serum sample for each of the studied patients in our analysis, except for the time course analysis. For the serological diagnosis of patients, however, the evaluation of follow-up samples is important and recommended to demonstrate seroconversion or a 4-fold increase in antibody titre. In four of 27 RT-PCR-confirmed ZIKV cases, samples were negative for both IgM and IgG against ZIKV-NS1, presumably because all of them were taken only ≤ 4 days after symptom onset, i.e. when antibodies had not yet reached detectable levels. Among 85 suspected ZIKV patients, too early sampling may account for two cases with negative IgM and IgG, while the remaining five double-negative cases could be due to the absence of ZIKV infection (deficits in pre-characterisation) or to false-negative results.

Cross-reactivity with high-level DENV antibodies was not detectable and, according to preliminary analysis with a limited amount of samples, there was no indication for DENV serotype-dependent differences in cross-reactivity (data not shown). To better judge assay performance in endemic areas, samples from endemic residents who experienced multiple DENV (and other flavivirus) infections should be included in further assessments, as these samples have a potential for increased cross-reactivity. Analysis of all potentially cross-reactive specimens resulted in positive rates of 0.8% (IgM) and 0.4% (IgG) caused by one

case each with WNV and PLAS with low-level anti-ZIKV IgM and one JEV case with low-level anti-ZIKV IgG. In these cases, however, double infections cannot be excluded, so it remains unclear if ELISA positivity resulted from the presence of ZIKV antibodies due to co-infection with ZIKV (true-positive) or from cross-reactivity (false-positive). In case of PLAS infection, PLAS-induced polyclonal B-cell activation may cause the production of potentially cross-reactive antibodies. Among patients with current PLAS infection, up to 30% false-positive or borderline reactions were reported using the presented NS1-based ELISA, which is in contrast to only 1.4% in the present study and probably explained by the fact that our cohort was comprised mainly of individuals with past PLAS infection status. Possible interferences should thus be considered when applying the assay.

In sera from travellers returning from ZIKV-endemic areas, we observed a tendency of ZIKV-specific IgM to appear at high ratios during the active phase of infection, paralleled by a moderate rise in IgG. In contrast, most residents in endemic areas had high anti-ZIKV IgG and low/negative IgM ratio values, irrespective of whether their samples were taken during the initial, active or late phase of infection. IgM responses in travellers returning from ZIKV-endemic areas tended to be higher compared with residents in such areas, whereas the IgG-positivity rate was higher in the latter subgroup. Such differences in ZIKV antibody kinetics were also illustrated by time course analysis of antibody levels in two representative patients, possibly reflecting that travellers returning from ZIKV-endemic countries predominantly had a primary flavivirus/ZIKV infection, while most residents probably contracted ZIKV as a secondary flavivirus infection. Similar kinetics have been described for primary and secondary infections in the Micronesian ZIKV epidemic and for DENV-infected patients, suggesting that the detection of both specific IgM and IgG is diagnostically important and relevant for differentiating primary from secondary infections. Regarding our comparison of patients residing in endemic countries vs travellers, however, systematic differences in the background of these populations (e.g. genetic, ethnic) cannot be excluded.

Another limitation of our study is that it does not comprise side-by-side testing with additional assays, such as the Zika MAC-ELISA (Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, US) or PRNT, to provide comparative data on these current tests. In addition, the non-deliberate absence of a uniform serological reference standard for the pre-characterisation of all ZIKV samples resulted in a high number of suspected cases of ZIKV infection.

Although ZIKV usually causes rather mild infections, there has been convincing evidence of a causal link to neuronal impairment, such as newborn microcephaly and GBS [37].

Furthermore, there have been studies showing that DENV NS1 antibodies have the potential of inducing autoantibodies in secondary infections, probably mediated by cross-reactive binding of antigens on platelets and endothelial cells, followed by cellular damage and inflammatory activation. Basic research is needed to fully elucidate the causal relations between neuronal disorders and ZIKV infection. Epidemiologic assessment of pregnant women and their babies, and of travellers returning from endemic areas, the surveillance of donated blood and the investigation of ZIKV prevalence in endemic and non-endemic areas may provide crucial information. These studies need reliable, fast, and easy-to-handle diagnostic tests that have low cross-reactivity and allow a definite diagnosis.

In conclusion, our study revealed that the NS1-based anti-ZIKV ELISA is a sensitive and highly specific tool for the serodiagnosis of ZIKV infections, eliminating cross-reactions with antibodies to DENV and other flaviviurses. The assay format is suitable for use in routine laboratories worldwide enabling high-throughput testing in epidemic settings. Serological identification of ZIKV infections is maximised by parallel testing for IgM and IgG. Further studies will be necessary to determine the accuracy of this and other current assays in a larger set of well-defined samples, and to clarify how ZIKV infection triggers GBS, newborn microcephaly and other neurological manifestations.

Example 8: Anti-Zika virus IgA may indicate an acute infection in anti-Zika virus IgM-negative patients

This example shows that IgA to SEQ ID NO1 and related reagents and methods may be used for distinguishing an acute infection from a past and thus a primary from a secondary infection.

Methods

Serum samples were taken at several time points from two Columbians with a background of past flavivirus infections and from two German travellers, all presenting with confirmed ZIKV infections. Titers of anti-ZIKV IgM and IgG were measured using a commercial NS1-based Anti-Zika virus ELISA (Euroimmun AG, Germany). An indirect immunofluorescence test (Arbovirus Fever Mosaic 2, IgM, cut-off ≥ 1:10, Euroimmun AG, Germany) based on cells infected with ZIKV was used additionally for IgM measurement. For determination of anti-ZIKV IgA, a corresponding ELISA was adapted, applying an anti-human IgA conjugated with peroxidase. In all assays, the cut-off was set to a ratio of 1.1.

Results

In the German travellers, anti-ZIKV IgM was detected at day 9 and day 16, respectively, irrespective of the method. Active infections were subsequently confirmed by anti-ZIKV IgG seroconversion. IgA measurements were above 1.1 in all samples except for one, showing an initial increase and a subsequent decrease (**Table 5**)

Patient	Country of origin	Country of infection	Days after symptoms	Anti-ZIKV IgA ratio; pos: >1.1	Anti-ZIKV IgM ratio; pos: >1.1	Anti-ZIKV IgG ratio; pos: >1.1
1	Colombia	Colombia	-16	0.1	0.0	0.6
			6	0.6	0.0	**2.0**
			24	**3.4**	0.0	**4.9**
			66	0.6	0.0	**3.3**
2	Colombia	Colombia	3	0.2	0.1	**2.0**
			15	**2.9**	0.5	**5.9**
			38	0.7	0.2	**5.2**
			52	0.6	0.1	**5.0**
			66	0.5	0.1	**4.9**
			76	0.6	0.1	**5.1**
			95	0.5	0.1	**4.7**
3	Germany	Martinique	11	**4.1**	1.0	0.1
			16	**9.0**	**2.7**	**1.4**
			36	**1.5**	**1.2**	**2.5**
4	Germany	Nicaragua	4	0.3	0.2	0.2
			9	**7.6**	**2.4**	1.0
			30	**2.4**	0.9	**3.0**

In the sequential samples of the two Colombian patients (results shown in **Figs. 11A** and **11B**), measurements of IgM antibodies against ZIKV-NS1 antigen were persistently below the cut-off. In accordance, testing for IgM against full Zika virus was negative in all but one, weak positive sample (1:10). Anti-ZIKV IgG was positive already within the first week in both patients. IgA, however, showed a titer increase, peaking above the cut-off in week three and four before dropping below the threshold again.

Conclusion

When specific IgM is not detectable neither with NS1- nor full virus-based assays as observed in the Colombian patients, measurement of anti-ZIKV IgA may allow discrimination of acute from past infections.

Example 9: Absence of specific IgM in week six post symptom onset in a patient with confirmed Zika virus infection

This example shows that detecting the presence or absence of both IgG and IgM to SEQ ID NO1 and related reagents and methods may be used for increasing the diagnostic reliability of an assay for diagnosing a Zika virus infection compared to assays based on the detection of Ig from one class only.

Introduction

Subsequently to the severe outbreak of Zika virus (ZIKV) infections in the Americas, CDC recommends nucleic acid testing in samples collected within two weeks after symptom onset. Serum samples collected after day 14 and up to day 84 should be tested for anti-ZIKV IgM antibodies assuming that these are present any time from near day four post symptom onset to up to twelve weeks.

However, absence of specific IgM has been frequently reported in patients with secondary Dengue virus (DENV) infections, since DENV and ZIKV are related flaviviruses immunological response may be comparable in ZIKV infections.

Methods

A Colombian woman of 42 years with ZIKV infection as confirmed by RT_PCR five days after appearance of the first symptoms was additionally tested for specific IgM and IgG antibodies. Serum samples taken at day five and day 41 post symptom onset were analysed using ZIKV IgM Capture ELISA, based on the full virus antigen (cut-off ratio 1.8; InBios, USA) and NS1-based Anti-Zika virus ELISA IgM as well as IgG (cut-off ratio 1.1; Euroimmun AG, Germany) according to instructions of the manufacturer.

Results

Results are shown in **Fig. 12** and **Table 6**. The two serum samples revealed negative results in both IgM assays. Ratios in the IgM Capture ELISA ranged from 0.81 (day 5) to 0.12 (day 41), and measurements with the Anti-Zika virus ELISA IgM revealed ratios of 0.1 (day 5) and 0.4 (day 41). In contrast, testing for IgG yielded positive results at day 5 (ratio 1.9) as well as day 41 (ratio 5.6).

Conclusion

Both available serum samples of this patient dated from the suggested anti-ZIKV IgM-positive time frame (12 weeks) but were tested IgM-negative independently of the antigenic substrate used – full virus or NS1.

Instead, the two samples revealed an increasing IgG titer suggesting that parallel testing for anti-ZIKV IgM and IgG in two consecutive serum samples should be performed to detect either seroconversion or a significant IgG titer increase in order to avoid missing patients tested anti-ZIKV IgM-negative.

Table 6:

Days after onset of symptoms	Ratio		
	IgM Capture ELISA Cut-off: 1.8	NS1-based ELISA IgM Cut-off: 1.1	NS1-based ELISA IgG Cut-off.1.1
5	0.81	0.1	1.9
41	0.12	0.4	5.6

Claims

1. A recombinant polypeptide comprising SEQ ID NO1 or a variant thereof, which polypeptide is a monomer, dimer or hexamer.

2. The polypeptide according to claim 1, wherein the polypeptide is expressed in and preferably isolated from a eukaryotic cell, preferably a mammalian or insect cell, more preferably a human cell.

3. A composition comprising the polypeptide according to any of claims 1 to 2 and a mammalian apolipoprotein or a variant thereof

and/or

a Zika virus envelope glycoprotein, preferably a polypeptide comprising SEQ ID NO11 or a variant thereof.

4. A diagnostically useful carrier comprising a means for specifically capturing an antibody to SEQ ID NO1 in a sample from a subject, wherein the diagnostic device is preferably selected from the group comprising bead, preferably a paramagnetic particle; a test strip, a microtiter plate, a blot, preferably from the group comprising western blot, line blot and dot blot; lateral flow test, a glass surface, a slide, a biochip and a membrane.

5. The diagnostically useful carrier according to claim 4, wherein the means for specifically capturing an antibody to SEQ ID NO1 is the polypeptide according to any of claims 1 to 2 or the composition according to claim 3.

6. The diagnostically useful carrier according to any of claims 4 to 5, further comprising one or more than one means, which means is for specifically capturing an antibody to an antigen from the group comprising SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9, preferably SEQ ID NO2,

and/or

a means for specifically capturing an antibody to an antigen from the group comprising SEQ ID NO 11, SEQ ID NO 12, SEQ ID NO13, SEQ NO 14, SEQ ID NO15, SEQ ID NO16, SEQ ID NO17, SEQ ID NO18, SEQ ID NO27, preferably, SEQ ID NO18.

7. A kit comprising the polypeptide, composition or diagnostically useful carrier according to any of claims 1 to 6, preferably further comprising a means for specifically detecting a captured antibody.

8. A method comprising the step detecting in a sample from a subject, preferably blood or CSF sample, the presence or absence of an antibody to SEQ ID NO1.

9. The method according to claim 8, comprising the step contacting the diagnostically useful carrier according to any of claims 4 to 6 with a sample from the subject.

10. The method according to step 9, further comprising the step detecting in a sample from a subject the presence or absence of an antibody to one or more antigens from the group comprising SEQ ID NO2, SEQ ID NO3, SEQ ID NO4, SEQ ID NO5, SEQ ID NO6, SEQ ID NO7, SEQ ID NO8, SEQ ID NO19 and SEQ ID NO9, SEQ ID NO11, SEQ ID NO12, SEQ ID NO13, SEQ ID NO14, SEQ ID NO15, SEQ ID NO16, SEQ ID NO17, SEQ ID NO18, SEQ ID NO27, preferably SEQ ID NO2.

11. The method according to any of steps 9 or 10, wherein the presence or absence of an antibody to SEQ ID NO1 and the presence or absence of an antibody to one or more further antigens is detected in spatially separate binding reactions.

12. The method according to any of claims 9 to 11, comprising the step monitoring an IgA, IgM and/or IgG, preferably IgA and IgG, more preferably IgA class antibody to SEQ ID NO1 for a time period of at least three days, preferably at least 6 days.

13. The method according to claim 14, wherein the total concentration of IgM to SEQ ID NO1 is detected in addition.

14. The diagnostically useful carrier, kit or method according to any of claims 4 to 13, wherein the antibody is a mammalian, preferably human antibody, more preferably a human IgA, IgM or IgG class antibody.

15. A use of the polypeptide, composition, diagnostically useful carrier or kit according to any of claims 1 to 7 and 14 or an IgA class antibody to SEQ ID NO1 for the diagnosis of a Flavivirus infection, more preferably a Zika virus infection, wherein preferably a Zika infection is distinguished from an infection with a Flavivirus other than Zika virus.

16. A use of the polypeptide, composition, diagnostically useful carrier or kit according to any of claims 1 to 7 and 14 or an IgA class antibody to SEQ ID NO1 for distinguishing a primary from a secondary Flavivirus infection, preferably Zika virus infection.

17. A use of the polypeptide, composition, diagnostically useful carrier or kit according to any of claims 1 to 7 and 14 or an IgA class antibody to SEQ ID NO1 for diagnosing a Zika virus infection, preferably for increasing the reliability of the diagnosis, more preferably at an early stage of the infection.

18. A use of the polypeptide, composition or diagnostically useful carrier according to any of claims 1 to 6 and 14 or a nucleic acid encoding the polypeptide for the manufacture of the kit according to claim 7.

19. The composition according to claim 3, wherein the composition is a pharmaceutical composition, optionally comprising a pharmaceutically acceptable composition, or an immunogenic composition, optionally comprising an adjuvant.