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(54) **Title:** METHOD FOR THE DIAGNOSIS OR THE SCREENING OF AN ARBOVIRUS INFECTION, REAGENTS USEFUL IN SAID METHOD AND THEIR APPLICATIONS

(57) **Abstract:** Method for the diagnosis or the screening of an arbovirus infection and preferably a flaviviridae infection and more preferably a flavivirus infection, reagents useful in said method and their applications. Said method comprises: (i) contacting a sample from the subject or animal with a solid support sensitized with an Ig binding protein which is directed against a specific class of Ig molecules of the subject or animal species under consideration and (ii) incubating the immunocomplex formed in (i) with a detector molecule consisting of a hybrid protein comprising at least an arboviral ED3 domain and an alkaline phosphatase (PhoA), the detection of said immunocomplex being the sign of the presence of an arbovirus in said sample.

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METHOD FOR THE DIAGNOSIS OR THE SCREENING OF AN ARBOVIRUS INFECTION, REAGENTS USEFUL IN SAID METHOD AND THEIR APPLICATIONS

The present invention relates to a method for the diagnosis or the screening of an arbovirus infection and preferably a *flaviviridae* infection and more preferably a flavivirus infection, reagents useful in said method and their applications.

Arboviruses (*ar*thropod-*bo*rne *viruses*) are viruses maintained in nature in cycles involving haematophagous arthropod vectors and susceptible vertebrate hosts. All arboviruses comprising an envelope protein are included in the present invention, even though the description is focused mainly on the flavivirus genus.

Many arboviruses and particularly many flaviviruses are responsible for serious human or animal diseases, in particular the yellow fever virus (YFV), dengue virus (DENV), West Nile virus (WNV), etc.

Flaviviral infections are currently detected by several methods, including virus isolation, viral-RNA detection by RT-PCR and immunochemical assays, targeted either at viral proteins or anti-viral immunoglobulin molecules. The kinetics of the appearance and the disappearance for the viral RNAs, viral proteins, virions and different classes of antibodies (IgM, IgA and IgG) are well documented for a number of flaviviruses, during primary or subsequent infections.

The detection of antibodies that are directed against a virus and present in the serum of patients, by an immunosorbent assay constitutes a well established and recommended method for the diagnosis of infections by flaviviruses (Kuno, 2003; WHO, 1997). The purposes of these diagnoses are at least two-fold: case confirmation to differentiate flaviviral diseases from other diseases with similar clinical presentations; and surveillance of the transmission.

The diagnosis of flaviviral infections is complicated by several factors. Most serological tests currently in use to measure antibodies against one flavivirus, cross-react with other members of this family (Kuno, 2003). These cross-reactions may be a problem in areas where several flaviviruses co-circulate. For example, many antibodies that are directed against WNV, cross react with JEV (Japanese Encephalitis Virus), SLEV (St Louis Encephalitis Virus) and even DENV

(Granwehr et al., 2004); many antibodies, that are directed against DENV, cross-react with YFV and JEV (Vorndam and Kuno, 1997).

The four serotypes of DENV pose a special problem. The pathogenesis of the severe forms of dengue, the dengue hemorrhagic fever (DHF) and shock syndrome (DSS), remains controversial. Two main theories have been proposed. The commonly accepted hypothesis is the secondary infection or immune enhancement theory (Halstead, 2003; Mongkolsapaya et al., 2003). The other hypothesis emphasizes the involvement of viral factors (McBride and Bielefeldt-Ohmann, 2000). The differentiation between primary and secondary infections is therefore a key issue for understanding the pathogenesis of DHF. The viral mRNA and antigens are present in both primary and subsequent infections (Alcon et al., 2002). Therefore, the detection of DENV antibodies provides the only method for differentiating the different modes of infection.

Current diagnostic assays utilize either ELISA or dipstick formats for the identification of flavivirus infections.

The immunosorbent assays (ISA) for the detection of viral antibodies in the serum of patients belong to two main types: the indirect ISA and the antibody-specific capture ISA.

. In an indirect ISA, a solid support is sensitized with the viral antigen (virAg). The immobilized antigen is reacted with the human or animal serum under analysis. Finally, the bound antibodies are revealed with a reporter system, which generally consists of a conjugate between an immunoglobulin binding protein (@Ig) and an enzyme (Enz), typically horseradish peroxidase (HRP) or alkaline phosphatase (PhoA). This being an enzyme-linked ISA (ELISA). Other types of probes can be used, e. g. a fluorophore or colloidal gold. The general scheme for an indirect ISA is the following:

Support-virAg :: Serum :: @Ig-Reporter (1)

where "-" stands for a covalent bond or immobilization; and "::", for non-covalent interactions. The Ig binding protein may be specific for a particular class of Ig (@IgX, where X = M, A or G). In that case, one speaks of an IgX-specific indirect ISA. Several variations of the indirect ISA have been described, in particular

the antigen capture ISA, the epitope blocking ISA, and the avidity ISA (Blitvich et al., 2003; Johnson et al., 2000; Matheus et al., 2005).

. In the IgM, -A or -G specific capture ISA, a solid support is sensitized with an Ig binding protein (@IgX, with X = M, A or G), which is directed against a specific class of Ig molecules of the animal species under consideration and most generally consists of heterologous antibodies. The immobilized Ig binding protein is reacted successively with the serum under analysis, the viral antigen and then a reporter system, which generally consists of a conjugate between an antigen binding molecule (@virAg) and an enzyme (Enz). A generic IgX specific capture ISA (XAC-ISA) can be schematized as follows:

Support-@IgX :: Serum :: virAg :: @virAg-Reporter (2)

Depending on the Ig binding protein (@IgX), one can speak of IgM, IgG or IgA antibody capture immunosorbent assays (MAC-ELISA, GAC-ELISA or AAC-ELISA).

The immunosorbent assays for IgM antibodies are among the most useful serologic procedures for determining recent infections by flaviviruses, since these IgM molecules appear early in infection, rise rapidly in the course of the disease, and are usually less cross-reactive with other viruses than IgG antibodies (Kuno, 2003). IgM molecules can be detected as soon as the 5th day after infection but their affinity for a monomeric antigen is generally lower than that of other immunoglobulin molecule types.

The MAC-ELISA is preferred over the IgM-specific indirect ELISA because the IgG antibodies from previous infections by related viruses can have a suppressive effect on the sensitivity of the latter assay (Vorndam and Kuno, 1997). It is recommended by WHO for the serological diagnosis of several flaviviral infections and in particular dengue (WHO, 1997).

The MAC-ELISA has the following advantages: If paired serum samples are available, a rising, stable or falling titer in IgM can indicate the time of infection. The ratio of IgM to IgG antibody in parallel MAC- and GAC-ELISA on a single sample can be used to differentiate primary from secondary infections since the IgM/IgG ratio is higher than one in the former case and lower in the latter (Innis et al., 1989). It can detect anti-flaviviral IgM in the cerebrospinal fluid and saliva (Kao et al.,

2005; Teles et al., 2005). IgA specific ELISAs have also been developed. The IgA response develops after the IgM response but before the IgG one. The IgA/IgM ratio in parallel MAC- and AAC-ELISAs can indicate whether the infection is recent or dates from a few months, for DENV and WNV (Prince and Lape-Nixon, 2005; Talarmin et al., 1998).

The specificity of the immunosorbent assays comes mainly from the interaction between the serum under analysis and the antigen and thus depends on the nature of the antigen preparation. However, it may also come from the nature of the reporter molecule.

Until recently, the antigens in use for ISA were mainly extracts of suckling mouse brains (SMB) or cell cultures, infected by the virus under consideration. These are being progressively replaced with recombinant prM/gpE virus like particles (VLP), where prM and gpE are the precursor of the membrane protein and envelope glycoprotein of the virus or with a recombinant extracellular domain (sE) of gpE. The non-structural protein NS1 has also been used as an antigen in both IgG-specific indirect ELISA and MAC-ELISA. NS1 can differentiate between primary and secondary infections and correctly identify the serotype of the infecting DENV in the sera of patients with primary infection (Shu et al., 2004; Shu et al., 2003; Shu et al., 2002).

Many MAC-ELISAs use antiviral polyclonal antibodies as detector molecules. These polyclonal antibodies vary in potency from batch-to-batch and can be virus cross-reactive, which limits the specificity of the tests (Martin et al., 2000). Therefore, monoclonal antibodies (mAbs) are more advantageous than polyclonal antibodies (pAbs) and reduce the variations in specificity. Broadly cross-reactive mAbs, such as mAb4G2 and mAb6B6C-1, have been conjugated with enzymes and widely used as detector molecules (Kuno, 2003). Neutralization escape variants at positions S169P and G257R have mapped the epitope of mAb4G2 at the interface between domains 1 and 2 of gpE (Serafin and Aaskov, 2001).

Other types of ISA exist such as sandwich ELISA (R. J. Kerschbaumer et al., 1996) whose format is the following:

Support-@GST :: GST-(3D6 epitope) :: scFv3D6-PhoA,

where @GST represents antibodies directed against the Glutathione-S-Transferase (GST); GST-(3D6 epitope), a hybrid protein between GST and an epitope of antibody 3D6; and scFv3D6-PhoA, a hybrid protein between a single-chain variable fragment (scFv) of antibody 3D6 and alkaline phosphatase.

Sandwich ELISA assays are used to detect the presence of an antigen in the serum of patients, but not antibodies directed against an infectious agent as in the current invention. Moreover such methods require the isolation and characterization of at least two non-competing antibodies to be used in the assay.

Another type of ISA is a reverse ELISA (D. Ludolfs et al., 2007) whose format is the following:

Support-RF :: serum :: rED3-HRP

where the Rhumatoid Factor (RF) is an autoimmune antibody that recognizes the Fc fragment of the IgG immunoglobulins; and rED3-HRP is a chemical conjugate between Horseradish Peroxydase (HRP) and a recombinant domain 3 (rED3) of the envelope protein E from the West-Nile virus. HRP is a monomeric protein, whereas alkaline phosphatase is dimeric, and the rED3-HRP hybrid protein was obtained by chemical coupling of the two partners, rED3 and HRP.

The authors in this work explicitly mention that their "reverse ELISA" does not detect any specific IgM antibodies (page 472, left column, line 31 and following). They conclude that their reverse ELISA could improve knowledge about the prevalence of West-Nile virus infections in the world. Such a methodology is therefore most suitable for long term epidemiological studies into the prevalence of viral infections.

Another type of ISA is an indirect IgG ELISA (D. W. C. Beasley et al., 2004) whose format is the following:

Support-rED3 :: serum :: @IgG-HRP

where @IgG-HRP is a chemical conjugate between Horseradish Peroxydase and an antibody directed against human IgGs.

The authors mention explicitly that the recombinant domain rED3 is poorly bound by antibodies coated in the wells of microtiter plates (page 2764, lines 34-39), and therefore is not suitable for antibody capture ELISA.

Antigens for use in capture ELISA

* SMB- or cell culture-derived viral antigen

The specificity of the GAC- or MAC-ELISA does not differ significantly when using either SMB- or cell culture-derived viral antigen (Cardosa et al., 1992). MAC-ELISAs that are performed with such preparations of antigen are generally specific of a viral sero-complex but can hardly differentiate the infecting virus within a sero-complex. For example, they can differentiate between infections by DENV and either JEV or WNV (Innis et al., 1989; Martin et al., 2002). However, they have difficulty in differentiating between infections by the four DENV serotypes, even though the signal is the highest for the infecting serotype in most cases (Nawa et al., 2000). They can differentiate between WNV infections and either SLEV or JEV infections, if testing for these flaviviruses of the JEV serocomplex is done simultaneously and with a precise and specific diagnostic algorithm (Martin et al., 2002; 2004). However, such a specific diagnosis is only possible in primary infections because cross-reactivities are more important in patients experiencing secondary and further infections (Kao et al., 2005; Teles et al., 2005).

* Recombinant prM/gpE-VLPs and sE as antigens

The prM/gpE VLPs from several flaviviruses perform as well as or better than SMB-derived antigen in MAC-ELISA, according to a number of criteria measuring sensitivity, specificity, accuracy and other statistical tests (Holmes et al., 2005; Martin et al., 2002; Martin et al., 2000; Muerhoff et al., 2002). For DENV, VLPs can successfully detect the infecting serotype in primary infections (Shu et al., 2002; Shu and Huang, 2004). For SLEV, VLPs do not cross-react with IgM antibodies that are directed against WNV or the Powassan virus, contrary to the SMB-derived antigen (Purdy et al., 2004). For TBEV (Tick-Borne Encephalitis Virus), VLPs do no cross-react with IgM antibodies that are directed against JEV, contrary to the commercial antigen (Yoshii et al., 2003). For WNV however, VLPs do cross-react with a high proportion of sera from patients that are either infected with or vaccinated against other flaviviruses (JEV, SLEV, DENV, YFV) (Hogrefe et al., 2004). The extracellular domain sE of gpE, expressed as a recombinant protein in drosophila cells, is used in a chromatographic format of the MAC- and GAC-ELISAs. This immunochromatographic assay, using recombinant sE domains from the four

serotypes of DENV, has specificities and sensivities that are comparable to those of conventional MAC- and GAC- ELISAs, performed with SMB extracts as antigens (Cuzzubbo et al., 2001).

* rED3 as an antigen

Several factors are relevant to the use of the ED3 domain as an antigen in immunoassays: it is highly antigenic and immunogenic; the most strongly neutralizing antibodies are directed against this domain (Crill and Roehrig, 2001; Sanchez et al., 2005); the sequences of the ED3 domains are more distant than those of the other domains of gpE (Gritsun et al., 1995); the antibodies that cross-react with different flaviviruses are directed towards domains ED1 and ED2 of gpE more than towards ED3 (Crill and Chang, 2004; Kanai et al., 2006; Modis et al., 2005; Roehrig, 2003; Sanchez et al., 2005). For DENV, hybrids TrpE-ED3 between the TrpE protein from *E. coli* and the four serotypes of the ED3 domain have been compared with cell culture-derived viral antigens. The two kinds of antigens are equally sensitive for detecting IgM or IgG antibodies, directed against DENV, in convalescent sera. However, the TrpE-ED3 antigens are more specific than the cell culture-derived antigens for discrimination between DENV infections and YFV or JEV vaccinations (Simmons et al., 1998). For DENV, recombinant isolated ED3 domains (rED3) can successfully detect the infecting serotype in an immunoblot strip assay (Ludolfs et al., 2002). For WNV, rED3 gives a more sensitive and specific response than an SMB-derived antigen in an IgG-specific indirect ELISA, on panels of monkey, human and horse sera. It can clearly discriminate an IgG response against WNV from those against other related flaviviruses (JEV, SLEV, MVEV) (Beasley et al., 2004). For TBEV, rED3 also gives a more sensitive and specific response than an SMB-derived antigen in an IgG-specific indirect ELISA. It can distinguish between tick-borne (TBEV) and mosquito-borne (YFV, DENV) flaviviruses but cannot distinguish between members of the TBEV serocomplex of flaviviruses (Holbrook et al., 2004).

However, isolated ED3 domains have been used only in IgG- or IgM-specific indirect ISA for reasons explained below.

When using capture ELISA method with recombinant antigens, problems of valence and folding may arise and problems of detection may also arise.

- Valence and folding

Indeed, preliminary experiments have suggested that an rED3 domain from WNV is poorly bound by antibodies coated in the wells of microtiter plates or may be captured but not bound by detecting antibodies due to steric hindrance. It was therefore concluded that rED3 may not be immediately suitable for a capture assay format (Beasley et al., 2004). However, other explanations are equally plausible. The flaviviruses display 180 monomers (90 dimers) of gpE at their surface and therefore gpE and its ED3 domain are present in multiple and adjacent copies (Kuhn et al., 2002; Mukhopadhyay et al., 2003). The same molecule of IgG, IgA or IgM can bind simultaneously two to five copies of its epitope and this multivalent mode of binding results in a strong apparent affinity (avidity). The valence of the antigen is also high in prM/gpE VLPs (Ferlenghi et al., 2001); it is two for a recombinant antigen like the soluble gpE (sE), which is a dimer (Kanai et al., 2006; Modis et al., 2003; Modis et al., 2005; Rey et al., 1995), but only one for an isolated ED3 domain. Therefore, the affinity between a monomeric rED3 domain and one binding site of an IgM may be insufficient for a MAC-ELISA. Similar problems may be encountered with IgG- or IgA-capture ELISAs, especially for a primary infection. To overcome this limitation of the monomeric rED3 domains, it may be necessary to engineer their oligomerization.

The ED3 domain contains two cysteine residues. They form a disulfide bond which is necessary for the proper folding and antigenic integrity of the domain (Roehrig et al., 2004). rED3 can be produced in the periplasmic space of *Escherichia coli,* where the essential disulfide bond can form, in a properly folded state. The production in the periplasmic space has the added advantage that the protein can be extracted from the producing bacteria by a simple osmotic shock, in a concentrated and partially purified form.

- Detection

To compare the response of a serum towards several different antigens quantitatively (e. g. towards different viral serotypes) and thus deduce its specificity, the detection system of the assay must be the same for all the tested antigens. This may not be the case when one uses polyclonal antibodies. The use of a monoclonal antibody, directed against a common epitope of different viruses or viral

serotypes may lead to the following problems. (i) The binding of the antigen to the human serum may mask the epitope of the tracer monoclonal antibody. (ii) The affinities between the tracer antibody and different antigens may depend on the structural context of the epitope. As a result, the relation between the output signal of the assay and the amount of captured antigen may vary for different antigens.

Therefore there is a need for reagents better adapted to ELISA tests and more preferably to XAC-ELISA tests than the reagents of the prior Art.

Therefore, the present invention relates to a method for the diagnosis or the screening of an arbovirus in a subject or animal host, characterized in that it comprises:

(i) contacting a sample from the subject or animal with a solid support sensitized with an Ig binding protein which is directed against a specific class of Ig molecules of the subject or animal species under consideration and most generally consists of heterologous antibodies (anti-IgX antibodies) and

(ii) incubating the immunocomplex formed in (i) with a detector molecule consisting of a hybrid protein comprising at least an arboviral ED3 domain and preferably a flaviviral ED3 domain and an alkaline phosphatase (PhoA), the detection of said immunocomplex being the sign of the presence of an arbovirus in said sample.

According to an advantageous mode of carrying out said method, the Ig binding protein is selected in the group consisting of anti-IgM, anti-IgG and anti-IgA (@IgX, with X = M, A or G).

According to another advantageous mode of carrying out said method, said arbovirus is preferably a flavivirus.

According to another advantageous mode of carrying out said method, said alkaline phosphatase is selected from the group consisting of rat, mouse, chicken, bovine, yeast and bacterial alkaline phosphatases, preferably alkaline phosphatase of *E. coli.*

According to a further advantageous mode of carrying out said method, said hybrid protein further comprises a polypeptide tag, useful, for instance for purifying said hybrid protein from a periplasmic extract. Examples of such

polypeptide tags may be HIS (hexahistidine), c-MYC, HA, VSV-G, HSV, V5 and FLAG (Sigma products).

Thus, according to a further advantageous mode of carrying out said method, said hybrid protein comprises preferably a hexahistidine, an appropriate flaviviral ED3 domain and the alkaline phosphatase of *E. coli* and comprises SEQ ID NO:25.

Preferably the alkaline phosphatase consists of SEQ ID NO: 25.

According to said mode of carrying out the method, said alkaline phosphatase of *E. coli* is modified. More preferably said alkaline phosphatase of *E. coli* includes two mutations in its active site: D153G and D330N and comprises SEQ ID NO: 24 (with the numbering of Le Du et al., 2002). Such a modified PhoA have been described in European Patent Application n° 0 752 475.

Preferably the alkaline phosphatase consists of SEQ ID NO: 24.

Other modifications to the alkaline phosphatase are possible and are encompassed by the present invention.

Unexpectedly, by using the detector molecule as specified here above, i.e. comprising at least the flaviviral domain ED3 and an alkaline phosphatase of *E. coli*, and preferably hybrid proteins between a hexahistidine, a flaviviral domain ED3 and an alkaline phosphatase of *E. coli*, preferably a modified alkaline phosphatase, the IgX antibody capture immunosorbent assay for the detection of flaviviruses is significantly improved at several levels:

(i) replacement of the crude preparations of reagents (antigen or detection system) that are used in some assays, by defined and homogeneous molecular species

(ii) decrease of the number of reagents and steps that are necessary for the assays

(iii) replacement of all the elements of the assays that involve the manipulation of infectious viruses, animals, or cell culture in safety laboratories for their preparation, by recombinant elements that can be produced in bacteria and purified easily.

Thus, the detector molecule is preferably a $(H6\text{-}ED3\text{-}PhoA)_2$ hybrid; therefore, the binding of the $(H6\text{-}ED3\text{-}PhoA)_2$ hybrids was revealed through the

enzymatic activity of their PhoA portion. The numerous substrates of PhoA, that produce colorimetric or chemo-luminescent reactions, may be used for this revelation. Preferably, microtiter plates to immobilize the anti-IgG or anti-IgM antibodies were used. Other types and formats of supports could be used for these immobilizations, in particular optical fibers.

Thus, an assay with the (H6-ED3-PhoA)$_2$ hybrids may be performed for the detection of other immunoglobulin types, IgA and IgE, directed against the ED3 domain, to the detection of immunoglobulins from man and different animals, for instance mouse, bovine and horse, and to their detection in other body fluids than serum. This assay may also be performed with the ED3 domains from other arboviruses and other flaviviruses than those cited since the E glycoproteins from the viruses of this taxonomic group have highly homologous structures. It may be performed with hybrids between other antigenic proteins or protein fragments, whether they come from pathogenic agents or not, and whether they are present in a monomeric or multimeric state in these agents. It may be extended to bifunctional hybrid proteins that would include another tracer than PhoA. Finally, it could be extended to cases where the oligomerization of the antigen is obtained by genetic fusion or chemical coupling with a specific protein module, distinct from the tracer. The construction of the hybrids is greatly facilitated by the possibility of chemically synthesizing the DNA segment coding for the ED3 domain on the basis of its nucleotide or amino-acid sequence.

Bifunctional dimeric hybrids like (H6-ED3-PhoA)$_2$ or more generally (Ag-PhoA)$_2$ have numerous applications. They can be used (i) to detect antibodies directed against the antigen (Ag) that is fused with PhoA; (ii) to detect antibodies directed against the pathogen which the antigen comes from or mimics; (iii) to diagnose infections by a pathogen or validate a vaccination by a pathogen or an immunogen; (iv) to study the epidemiology of a pathogen; (v) to study the interaction between the protein or protein fragment that is fused with PhoA, and molecules, proteins or cells; (vi) to screen and identify, in a chemical library, molecules that modify the interaction between the fused protein or protein fragment and a target molecule, protein of cell.

The use of rED3 to successfully detect IgM in the serum of infected individuals has not been described previously.

The current invention relies on the possibility of simultaneously dimerising a recombinant antigen and fusing it with an enzymatic tracer, by constructing a hybrid between its gene sequence and the alkaline phosphatase gene. In this way, a reagent is obtained that can detect low affinity antibodies; for example IgM immunoglobulins, which are pentameric and appear early in infections by arboviruses. This early detection can be used as a tool in the management of epidemics. Therefore, it clearly differs from assays that are based on the detection of IgGs and are only used in retrospective studies, such as variously sandwich, reverse and indirect ELISA.

In particular the advantages of the methods and reagents according to the current invention over the prior art include: (i) the production of diagnosis reagents in low safety laboratories; (ii) the production of a single reagent per virus, in a single step and without any chemical reaction step; (iii) the ability to detect IgMs, which appear early in infection and have low affinities, with artificial dimeric antigens; (iv) the specificity of detection towards the types of viruses and infections; (iv) the simplification and speeding up of the diagnosis assay by fusion between the antigen and an enzymatic tracer.

Therefore preferably, said hybrids $(H6\text{-}ED3\text{-}PhoA)_2$ were constructed, at the genetic level between sequences encoding a hexahistidine, the viral domain ED3, and the alkaline phosphatase of *E. coli.* The hexahistidine tag enabled the purification of the hybrids on a column of nickel ions. PhoA is a dimeric periplasmic protein. The fusion of a passenger protein with PhoA at the genetic level results in the dimerisation of the hybrid protein, its export into the periplasmic space, and the preservation of the folds and functions of the two partners (Boulain and Ducancel, 2004). Moreover, the symmetrical points of insertion for the passenger protein in the crystal structure of the PhoA dimer are located on the same side of the molecule, close to one another (17.6 Å) and far from the catalytic sites (> 32.5 Å) (Le Du et al., 2002).

Therefore, the construction of hybrids $(ED3\text{-}PhoA\text{-}H6)_2$ solves the problem of the antigenic valence. The hybrids include their own enzymatic tracer and

the enzymatic portion of the hybrid does not depend on the nature of its antigenic portion. With this new reagent, a MAC- or AAC- or MAC-ELISA involves only three participating molecules, according to the scheme:

Support-@IgX :: serum :: $(H6\text{-}ED3\text{-}PhoA)_2$ (3)

where X = M, A or G.

According to another mode of carrying out the method of the invention, the envelope protein domain 3 polypeptide is selected in the group consisting of a yellow fever virus envelope protein domain 3 polypeptide, a West Nile virus envelope protein domain 3 polypeptide, a Dengue virus envelope protein domain 3 polypeptide, a St Louis encephalitis virus envelope protein domain 3 polypeptide, a Murray Valley encephalitis virus envelope protein domain 3 polypeptide and a Japanese encephalitis virus envelope protein domain 3 polypeptide.

More preferably, the ED3 domain is in particular from WNV (noted ED3.WN), from Yellow fever virus (ED3-YF) or from Dengue virus (serotypes 1, 2, 3 or 4) and preferably from serotype 1 of DENV (noted ED3.DEN1).

ED3 polypeptides of Flavivirus are described for instance in International PCT Application WO 2004/016586.

These new reagents unexpectedly simplify the MAC-, AAC- and GAC-ELISAs, contribute to make them more reproducible and quantitative, and therefore specific. They need only low levels of biological security and technical means for their preparation.

The instant invention also relates to a hybrid protein, characterized in that it comprises an appropriate polypeptide tag, an arbovirus ED3 domain and an alkaline phosphatase.

In particular the present invention relates to a hybrid protein which can be used in a method according to the current invention.

According to an advantageous embodiment of said hybrid protein, it comprises hexahistidine, an appropriate flaviviral ED3 domain and the alkaline phosphatase of *E. coli*.

Said hybrid protein is preferably in a multimeric form and more preferably in a dimeric form, such that for instance $(H6\text{-}ED3\text{-}PhoA)_2$.

According to another advantageous embodiment of said hybrid protein:

- when the ED3 domain is from DEN1 virus, said hybrid protein (H6-ED3.DEN1-PhoA) presents the sequence (SEQ ID NO:2).

- when the ED3 domain is from DEN2 virus, said hybrid protein (H6-ED3.DEN2-PhoA) presents the (SEQ ID NO:4).

- when the ED3 domain is from DEN3 virus, said hybrid protein (H6-ED3.DEN3-PhoA) presents the sequence (SEQ ID NO:6).

- when the ED3 domain is from DEN4 virus, said hybrid protein (H6-ED3.DEN4-PhoA) presents the sequence (SEQ ID NO:8).

- when the ED3 domain is from West Nile virus, said hybrid protein (H6-ED3.WN-PhoA) presents the sequence (SEQ ID NO:10) and

- when the ED3 domain is from yellow fever virus, said hybrid protein (H6-ED3.YF-PhoA) presents the sequence (SEQ ID NO:12).

The invention also relates to the nucleic acids encoding the hybrid proteins according to the invention.

Preferably, said nucleic acid is selected in the group consisting of SEQ ID NO:1 encoding H6-ED3.DEN1-PhoA hybrid protein, SEQ ID NO:3 encoding H6-ED3.DEN2-PhoA hybrid protein, SEQ ID NO:5 encoding H6-ED3.DEN3-PhoA hybrid protein, SEQ ID NO:7 encoding H6-ED3.DEN4-PhoA hybrid protein, SEQ ID NO:9 encoding H6-ED3.WN-PhoA hybrid protein and SEQ ID NO:11 encoding H6-ED3.YF-PhoA hybrid protein.

Said hybrid proteins may be obtained according to a method similar as the ones described in EP 0 407 259 and in EP 0 752 475.

Preferably they are obtained by inserting the correct ED3 in the expression vector pEBL1 (SEQ ID NO:13), containing a modified alkaline phosphatase (SEQ ID NO: 24) comprising two mutations (D153G and D330N), with the numbering of Le Du et al., 2002.

Said expression vector has been deposited at the CNCM (Collection Nationale de Culture de Microorganismes, 28 rue du Docteur Roux, 75015 PARIS) on April 23, 2007 under the accession number I-3747.

The invention also relates to a method of preparing a hybrid protein according to the invention, said method being characterized in that it comprises:

(a) obtaining an expression vector containing the sequence encoding an hybrid protein as defined here above by inserting the sequence coding for the appropriate arboviral ED3 polypeptide and preferably flaviviral ED3 polypeptide in the vector pEBL1 (SEQ ID NO:13),

(b) transforming an appropriate *E. coli* strain, preferably the XL1-blue strain (described by Bullock et al., 1997) with the expression vector obtained in (a),

(c) culturing said modified strains in an appropriate medium and

(d) purifying the tag-ED3-PhoA hybrid protein from the periplasmic extract.

When the tag is an hexahistidine, step (d) of purifying is performed by affinity chromatography on a column of NiNTA resin.

The different expression vectors thus obtained comprise the sequence expressing the appropriate hybrid proteins:

Vector	Hybrid protein expression
pEBL11	H6-ED3-DEN1-PhoA
pEBL12	H6-ED3-DEN2-PhoA
pEBL13	H6-ED3-DEN3-PhoA
pEBL14	H6-ED3-DEN4-PhoA
pEBL15	H6-ED3-WN-PhoA
pEBL17	H6-ED3-YF-PhoA

According to a mode of carrying said method, the expression vector of step (a) is selected in the group consisting of an expression vector of a hybrid protein as defined here above and more preferably the hybrid protein H6-ED3.DEN1-PhoA (pEBL11, deposited at the CNCM (Collection Nationale de Culture de Microorganismes, 28 rue du Docteur Roux, 75015 PARIS) on April 23, 2007 under the accession number I-3748) and the hybrid protein H6-ED3.WN-PhoA (pEBL15, deposited at the CNCM (Collection Nationale de Culture de Microorganismes, 28 rue du Docteur Roux, 75015 PARIS) on April 23, 2007 under the accession number I-3749).

The present invention also relates to a method for screening for arbovirus antibodies and preferably flavivirus antibodies in a subject or an animal, said method comprising:

(i) contacting a sample from said subject or animal with a solid support sensitized with an Ig binding protein which is directed against a specific class of Ig molecules of subject or the animal species under consideration,

(ii) incubating the immunocomplex formed in (i) with a detector molecule consisting of a hybrid protein comprising at least a arboviral ED3 domain and an alkaline phosphatase and

(iii) detecting the presence of said arbovirus antibodies.

Said detection is preferably performed by adding pNPP and measuring the formation of paranitrophenol.

In all the mentioned methods and kits the Ig binding protein, the ED3 domain, the alkaline phosphatase and the polypeptide tag are as defined above.

The invention also relates to a kit for diagnosing and/or screening for arbovirus antibodies and preferably flavivirus antibodies in a subject comprising:

- a solid support sensitized with an Ig binding protein which is directed against a specific class of Ig molecules of the animal species under consideration and most generally consists of heterologous antibodies (anti-IgX antibodies) and

- at least a hybrid protein comprising at least an arbovirus ED3 domain and an alkaline phosphatase,

- at least one positive control, preferably a reference serum from an infected individual and

- at least one negative control, preferably a reference serum from a non-infected individual.

Preferably, the Ig binding protein is selected in the group consisting of anti-IgM, anti-IgG and anti-IgA (@IgX, with X = M, A or G), and said hybrid protein comprises a hexahistidine, a viral ED3 domain of an appropriate flavivirus and the alkaline phosphatase of *E. coli*.

According to an advantageous embodiment of said kit, the alkaline phosphatase is a modified alkaline phosphatase including two mutations in its active site: D153G and D330N (with the numbering of Le Du et al.).

Preferably the alkaline phosphatase comprises SEQ ID NO: 24.

The invention also relates to the use of a hybrid protein comprising an appropriate antigen of a pathogen and an alkaline phosphatase, for an *in vitro* diagnostic of infections by said pathogen or for studying the epidemiology of said pathogen.

The invention also relates to the use of a hybrid protein comprising an appropriate antigen of a pathogen and an alkaline phosphatase, for an *in vitro* validation of a vaccination against said pathogen or an immunogen thereof.

The invention also relates to the use of a hybrid protein comprising a protein or a fragment thereof and alkaline phosphatase to study the interaction between said protein or fragment thereof fused with PhoA and molecules, proteins or cells.

The invention also relates to a method for the diagnosis of an infection by a pathogen, for validating a vaccination by a pathogen or an immunogen thereof or for studying the epidemiology of said pathogen, characterized in that it comprises:

(i) contacting a sample from a subject or an animal with a solid support sensitized with an Ig binding protein which is directed against a specific class of Ig molecules of the animal species under consideration,

(ii) incubating the immunocomplex formed in (i) with a detector molecule consisting of a hybrid protein comprising an appropriate antigen of a pathogen and alkaline phosphatase, the presence of said immunocomplex being the sign of said infection.

The invention also relates to a method for studying the interaction between a protein or a fragment thereof fused to PhoA and molecules, proteins or cells, characterized in that it comprises:

(i) contacting said molecule, protein or cell with a hybrid protein comprising the protein or a fragment thereof fused to PhoA and

(ii) detecting the complex eventually formed between the protein or a fragment thereof fused to PhoA and said molecule, said protein or said cell.

The invention also relates to a method for screening for anti-arbovirus compounds, said method comprising:

(i) contacting an anti-arbovirus antibody or a receptor of a surface molecule of an arbovirus, eventually bound to a solid support with a hybrid protein comprising an epitope of an arbovirus fused to PhoA

(ii) detecting the complex formed between said anti-arbovirus antibody or said receptor and said epitope by measuring an appropriate signal, for instance the formation of paranitrophenol

(iii) adding a compound to be tested and

(iv) detecting if the amount of complex formed between said anti-arbovirus antibody or said receptor and said epitope has decreased in relation to the amount of complex detected in step (ii), by measuring an appropriate signal and comparing the signal obtained with the signal obtained in (ii).

In all the methods the formation of the immunocomplex is directly detected by adding 4-nitrophenylphosphate (pNPP) and measuring the formation of paranitrophenol.

Besides the above provisions, the invention also comprises other provisions which would emerge from the following description, which refers to examples of implementation of the invention and also to the attached drawings, in which:

Figure 1. Structures of plasmids pLB11, pVP5, pLIP5GN-H6 and pEBL1. The *bla* and *aph* genes code for resistances to ampicillin and kanamycin respectively. *Ss* for signal sequence and H6 for hexahistidine. Bottom part, details of the sequence between the 5'-end of the *phoA* signal sequence and the main part of the *phoA* gene in pLIP5GN-H6 and pEBL1. The vertical arrow indicates the cleavage site of the signal peptide. The residues that do not belong to the *phoA* gene or its product are italicized.

Figure 2. Simplified GAC-ELISA of murine serums, performed with the H6-ED3.DEN1-PhoA hybrid. Closed symbols, serum from a mouse infected with DENV1; open symbols, control serum of a non-infected mouse. Squares, revela-

tion for 2.5 h at 25°C; Circles, revelation overnight at 4°C; diamond, average value of the blanks. The signals of the control serum after 2.5 h and overnight superimpose.

Figure 3. Simplified MAC-ELISA of murine serums, performed with the H6-ED3.WN-PhoA hybrid. Closed symbols, serum from a mouse immunized with gpE.WN; open symbols, control serum of a non-immunized mouse. Squares, revelation for 3 h at 25°C; circles, revelation overnight at 4°C; diamond, average value of the blanks.

Figure 4. Specificity of a simplified GAC-ELISA towards the antigen. The assay was performed with the H6-ED3.DEN1-PhoA and H6-ED3.WN-PhoA hybrids in parallel. Closed symbols, serum from a mouse infected with DENV1; open symbols, control serum of a non-infected mouse. Circles, cognate H6-ED3.DEN1-PhoA antigen; squares, non-cognate H6-ED3.WN-PhoA antigen; diamond, average value of the blanks. The revelation was conducted overnight at 4°C.

Figure 5. Specificity of a simplified MAC-ELISA towards the antigen. The assay was performed with the H6-ED3.DEN1-PhoA and H6-ED3.WN-PhoA hybrids in parallel. Closed symbols, serum from a mouse infected with WNV; open symbols, control serum of a non-infected mouse. Circles, cognate H6-ED3.WN-PhoA antigen; squares, non-cognate H6-ED3.DEN1-PhoA antigen; diamond, average value of the blanks. The revelation was conducted overnight at 4°C.

Figure 6. Concentration dependence of the signal in a simplified MAC-ELISA of human serums, performed with the H6-ED3.DEN1-PhoA hybrid. Closed symbols, serums from patients who had experienced a primary infection with DENV1; open symbols, secondary infections with DENV1. The revelation was conducted for 3 h at 25°C.

Figure 7. Simplified MAC- and GAC-ELISA of serums from patients who had experienced infections with the four serotypes of DENV, performed with the H6-ED3.DEN1-PhoA hybrid. The serums were diluted 400-fold and the revelation of the assays was conducted for 3 h at 25°C. (A) Simplified MAC-ELISA. (B) Simplified GAC-ELISA. (C) Ratio *r* of the signals in the MAC- and GAC-ELISA. Samples 1.1, serums of primary infections by DENV1; 1.2, serums of secondary infections with DENV1; 2, 3 and 4, serums of infections by DENV2, -3 and -4; C,

serums of healthy individuals; N and B, signals in assays where the serum or the anti-human Ig was omitted respectively.

The following examples illustrate the invention but in no way limit it.

EXAMPLE 1: Materials and methods

- Media, buffers and kits

The culture media LB (Sambrook and Russell, 2001) and SB (Plückthun, 1996) have been described. Ampicillin was used at 200 μg/mL and kanamycin at 50 μg/mL. LB medium with ampicillin was used for all the genetic constructions. The preparations of plasmid DNA were performed with the Qiaprep Spin Miniprep Kit, the extraction of DNA from agarose gels with the Gel Extraction Kit (both from Qiagen), the ligations of DNA with the Quick Ligation Kit (Roche), and the polyacrylamide gel electrophoreses with the NuPAGE Novex System (Invitrogen). The enzyme linked immunosorbent assays (ELISA) were performed in 96 wells microtitration plates (Maxisorb, Nunc). The PBS buffer (phosphate buffer saline) was purchased from Invitrogen or Sigma-Aldrich; bovine serum albumin (BSA) from Roche; low-fat dry milk from Regilait; Tween 20, 4-nitrophenyl phosphate (pNPP) and 5-bromo-4-chloro-3-indolyl phosphate (Xp) from Sigma-Aldrich. Buffer A contained 50 mM Tris-HCl, pH 8.0, 500 mM NaCl; buffer B, 0.05% Tween in PBS; buffer C, 0.1% Tween in PBS; buffer D, 10% ethanolamine, pH 9.8, 0.01 M $MgSO_4$; and buffer E, 20 μM $ZnCl_2$ in buffer D.

- Bacterial, plasmid and viral strains

The XL1-Blue strain of *E. coli* (Bullock et al., 1987) and plasmids pET20b+ (www.novagen.com), pUC-4K (Genbank accession N° X06404) (Vieira and Messing, 1982), pCR-Blunt (Bernard et al., 1994), pQUANTAbody (Boulain and Ducancel, 2004), pLB11 (Lisova et al., 2007) and pVP5 (Lisova et al., 2007) have been described. Hypercompetent cells of XL1-Blue (Stratagene), pCR-Blunt (Invitrogen), pET20b+ (Novagen) and pUC-4K (Amersham Biosciences) were purchased from commercial suppliers. Plasmid pLIP5GN-H6 is a derivative of pQUANTAbody (figure 1). The FGA/89 strain of serotype 1 of the dengue virus (DENV1; Genbank accession number AF226687) (Duarte dos Santos et al., 2000), the IS-98-ST1 strain of the West Nile virus (WNV; Genbank AF481864; (Malkinson et

al., 2002)), the recombinant form MV_{Schw} of the Schwarz strain of the measles virus, and its derivative MV_{Schw}-sE_{WNV} (Despres et al., 2005) have been described. pUC-4K carries the *aph* gene, which confers resistance to kanamycin, in the form of a DNA cassette that is easily mobilisable. pQUANTAbody carries a mutant allele of the *phoA* gene from *E. coli,* under control of promoter *ptac.* This allele codes for an alkaline phosphatase (PhoA) with two mutations in its active site, D153G and D330N, and improved catalytic properties (Boulain and Ducancel, 2004; Le Du et al., 2002; Muller et al., 2001). pLIP5GN-H6 differs from pQUANTAbody by the presence of six codons of histidine (H6) and the multiple cloning site region, which are both located between codons 27 and 28 of *phoA,* downstream of the signal sequence (Figure 1). pLB11 and pVP5 carry the gene segments that code for ED3.DEN1 and ED3.WN respectively between the NcoI and XhoI restriction sites of pET20b+ (figure 1). MVSchw-sEWNV expresses the soluble form of gpE from WNV.

- Antibodies and antiserums

The goat anti-human IgM and IgG (Sigma-Aldrich) were purchased from commercial suppliers. Human serums were from the collection of the National Center of Reference for Arboviruses, Institut Pasteur of French Guiana. They were collected from patients who displayed the basic clinical symptoms of dengue (fever, headache, myalgia, arthralgia), associated or not with rash and minor hemorrhagic manifestations. The serums were characterized with standard diagnosis methods, in particular GAC- and MAC-ELISAs using mouse-brain extracts as antigens.

The goat anti-mouse IgM (Pierce) and IgG (Sigma-Aldrich) were purchased from commercial suppliers. The mouse monoclonal antibody mAb4E11 has been described (Bedouelle et al., 2006). Its epitope at the surface of the ED3.DEN1 domain has been mapped; it is discontinuous and conformational (Lisova et al., 2007). A murine serum, directed against DENV1, was obtained by infection of BALB/c mice with the virus on day J0, challenge with the same virus on day J28, and bleeding on day J53. A control serum was obtained from non-infected mice of the same species. The titer in IgG of the positive serum, defined as below and measured by an indirect ELISA against domain ED3.DEN1, was equal to 30 000 (Despres et al., 2005). A serum, directed against sE from WNV, was obtained by infection of CD46-IFNAR mice with the recombinant virus MV_{Schw}-sE_{WNV} on day J0, and bleeding on day J8. A

control serum was obtained by infection of mice with the "empty" virus MV_{Schw}. The titers in IgM of the positive and control serums were equal to 1000 and 100 respectively.

EXAMPLE 2: Construction of the intermediate vector pEBL1, deposited at the CNCM (Collection Nationale de Culture de Microorganismes, 28 rue du Docteur Roux, 75015 PARIS) on April 23, 2007 under the accession number I-3747

The restriction sites that are located in the cloning region of plasmid pLIP5GN-H6, are very close and double restriction cuts in this region are difficult to monitor. Therefore a cassette of resistance to kanamycin was inserted in the SalI site of this region. Plasmid pUC-4K was digested with the SalI enzyme and the DNA fragment that contained the *aph* gene, was purified by agarose gel electrophoresis. pLIP5GN-H6 was also digested with SalI. The purified fragment and the linear vector were recombined by ligation. The recombinant plasmid, pEBL1 (SEQ ID NO:13), was recovered by transformation of the ligation mixture into competent cells of XL1-Blue and selection of the transformed cells on LB medium containing both ampicillin and kanamycin.

More precisely:

*** XL1-Blue(pEBL1)**

XL1-Blue(pEBL1) is an *Escherichia coli* strain containing the pEBL1 plasmid. pEBL1 was engineered to simplify the construction of fusion proteins between an hexahistidine, a desired passenger protein (ED3 of flavivirus) and an alkaline phosphatase from *E.coli* with improved catalytic properties. In pEBL1, a DNA cassette which confers resistance to kanamycin is inserted at the position of the passenger gene. Thus the insertion of the passenger gene is easier to perform and to monitor, according to a cloning strategy previously described by Hermann et al.,1990.

*** Bacterial strains and plasmids used for the construction of XL1-Blue(pEBL1)**

See Example 1.

*** Activities to be checked confirming the viability of the micro-organism**

The organism is resistant to ampicillin and kanamycin: this phenotype can be checked by plating the organism on Petri dishes containing LB Agar medium, 100µg/ml Ampicillin and 50µg/ml Kanamycin.

EXAMPLE 3: Construction of the *ED3-PhoA* hybrid genes

Methods

The *ED3-phoA* hybrid genes, coding for hybrid proteins between the ED3 domains of flaviviruses and PhoA, were constructed as follows. Plasmid pEBL1 (see Example 2) was first digested with the restriction enzyme *Sma*I, the completion of the digestion was verified by electrophoresis, and the digested DNA was desalted by size exclusion chromatography on a Microspin G25 column (Amersham-Biosciences). The linear form of pEBL1 was then digested with the *Sal*I enzyme and the restriction cut was monitored by electrophoresis and the appearance of a DNA fragment that corresponded to the cassette of resistance to kanamycin (1252 bp). The *ED3* gene was amplified by PCR with two oligonucleotide primers and the high fidelity polymerase Pfu-Turbo (Stratagene). The primer that hybridized at the 5'-end of the ED3 gene, brought in a *Sal*I site and the primers that hybridized at the 3'-end, ScaI and SpeI sites. The ScaI site (AGT-ACT) was preferred to the SmaI site (CCC-GGG) because the latter introduced a rare codon CCC. The *Sca*I, *Spe*I and *Sal*I sites were absent from the *ED3.DEN1* and *ED3.WN* genes. The PCR products were digested with *Sal*I and *Sca*I. The digestion products were purified by electrophoresis through agarose gels and extraction, and then recombined by ligation. The recombinant plasmids were introduced into the XL1-Blue strain by transformation and the recombinant bacteria, screened for the formation of blue colonies on Xp indicator medium and sensitivity to kanamycine.

The primers that were used to amplify *ED3.DEN1* from plasmid pLB11, had the following sequences, where the restriction sites are underlined:

5'-GCCGGCGGTCGACAAAGGGATGTCATATGTGATGTGCAC-3' (SEQ ID NO:14);

5'-G TTTAGTACTAGTTTTCCCTATGCTGCT TCCCTT C-3' (SEQ ID NO:15).

Similarly, the primers that were used to amplify *ED3.WN,* had the following sequences:

5'-GCCGGCGGTCGACAAAGGAACAACCTATGGCGTCTG-3' (SEQ ID NO:16);

5'GGTGAGTACTAGTTTTGCCAATGCTGCT ACCAGAC-3' (SEQ ID NO:17).

The sequences of the recombinant plasmids, pEBL11 coding for H6-ED3.DEN1-PhoA and pEBL15 coding for H6-ED3.WN-PhoA, were checked with oligonucleotides that hybridized outside of the cloning region in pEBL1:

5'-GCACTGGCACTCTTACCGTTAC-3' (SEQ ID NO:18);

5'-CAGTCTGATCACCCGTTAAAC-3' (SEQ ID NO:19).

EXAMPLE 4: Production and purification of bifunctional ED3-PhoA hybrids

Production and purification

The H6-ED3-PhoA hybrids were produced from plasmids pEBL11 and pEBL15 in strain XL1-Blue. A pre-culture of the producing strain was obtained by inoculation of SB broth (1/10 volume) with an isolated colony and overnight incubation at 37°C. The production was obtained by dilution of the pre-culture in one volume of the same medium to obtain an initial absorbance A_{600nm} = 0.25-0.30, growth at 30°C until A_{600nm} = 1.5-2.0, induction of promoter *ptac* with 0.2 mM IPTG, and further incubation for 2 h at the same temperature. All the subsequent steps were performed at 4°C. The culture was centrifuged 10 min at 5000 rpm. The bacterial pellet was resuspended in 5 mM imidazole, 1 mg/ml polymyxin B sulfate (Sigma-Aldrich) in buffer A (1/40 volume) and the bacterial suspension mildly agitated for 1 h with a magnetic stirrer. The periplasmic extract was collected by centrifugation of the suspension for 10 min at 13 000 rpm and frozen at -20°C. The ED3-PhoA hybrid was purified from the periplasmic extract by affinity chromatography on a column of NiNTA resin (0,6ml/L of culture, Qiagen). The column was loaded with the periplasmic extract and washed with 20 mM imidazole in buffer A (10 volumes of resin). The bound proteins were eluted with a step gradient of 40 to 100 mM imidazole in buffer A. The fractions of purifications were analyzed by SDS-PAGE (12% acrylamide) in reducing conditions. Those that contained H6-ED3-PhoA and were pure at > 90%, were pooled and transferred in PBS buffer by size exclusion chromatography on a P10 column (Amersham biosciences). They were snap-frozen at -80°C either before or after transfer in PBS, indifferently in terms of functional

properties (see Results). The concentrations of the purified H6-ED3-PhoA hybrids were determined by using A_{280nm} and an extinction coefficient, ε_{280nm} = 40 680 $M^{-1}cm^{-1}$ for the monomer, calculated from their amino acid sequences with the subroutine Pepstats of the software suite EMBOSS (Rice et al., 2000).

Indirect ELISA

The indirect ELISAs were performed in microtitration plates with volumes of 200 µL/well. Antibody mAb4E11 was diluted 10 000-fold with PBS. Wells 1 to 11 of a plate were loaded with the antibody solution and well 12 with PBS alone, and the plate was incubated overnight at 4 °C for the reaction of adsorption. The wells were washed with buffer B (3 times), blocked with 3% BSA in buffer B for 3 h at 25 °C, and washed again in buffer B (4 times). The H6-ED3.DEN1-PhoA hybrid (0.2 µM initial concentration) was diluted twofold serially with 1% BSA in buffer B. Wells 1-10 were loaded with the 10 first dilutions of the hybrid, well 11 with the dilution buffer alone, and well 12 with the lowest dilution of the hybrid. The plate was incubated for 1 h at 25 °C for the reaction of capture. The wells were washed as above, and the captured hybrid was revealed by addition of 5 mM (2 mg/ml) pNPP in buffer D. The formation of *para*-nitrophenol was measured after overnight at 4°, using A_{405nm}.

Enzyme activity

The formation of p-nitrophenolate (pNP) from pNPP was monitored at 25 °C in buffer D or E by A_{405nm}. The initial concentration of pNPP (5 mM) was saturating (Le Du et al., 2002) and therefore, the kinetic parameter k_{cat} could be calculated through the equation:

$$dA_{405nm}/dt = k_{cat}E_0\varepsilon_{405nm}(pNP) \quad (4)$$

where dA_{405nm}/dt is the initial rate of formation of pNP; E_0, the total concentration of $(H6\text{-}ED3\text{-}PhoA)_2$ dimer; and $\varepsilon_{405nm}(pNP)$ = 1.78 x10^4 $M^{-1}cm^{-1}$ (Muller et al., 2001). The value of k_{cat} was measured for several values of E_0 and averaged.

Functional properties of the H6-ED3-PhoA hybrids

To evaluate the functionality of the H6-ED3-PhoA hybrids, their phosphatase activity was measured and their recognition by monoclonal antibody mAb4E11 was assayed. The H6-ED3.DEN1-PhoA and H6-ED3.WN-PhoA hybrids

were active for the dephosphorylation of pNPP into pNP, with k_{cat} values in buffer D and 25 °C equal to 190 ± 18 s^{-1} and 154 ± 6 s^{-1} respectively for one molecule of dimer. H6-ED3.DEN1-PhoA bound immobilized mAb4E11 specifically in an indirect ELISA which was revealed by its intrinsic phosphatase activity. These results showed that the PhoA portion of the hybrid was correctly folded and dimeric since the dimeric form of PhoA is 100 fold more active than its monomeric form (Boulanger and Kantrowitz, 2003). They showed that the ED3.DEN1 portion of the hybrid was correctly folded and functional as an antigen since the epitope of mAb4E11 is discontinuous, conformational and included within the ED3.DEN1 domain (Lisova et al., 2007). Because the antigenic property of each hybrid molecule was revealed with its intrinsic enzymatic activity, the results showed that a significant proportion of the H6-ED3.DEN1-PhoA molecules had all the required properties simultaneously, i. e. their PhoA portion was dimeric and active, and their ED3.DEN1 portion was antigenic and in a bivalent state. The two residues in position 7 of the PhoA polypeptide chain are located on the same side in the structure of the PhoA dimer (Le Du et al., 2002). Therefore, the two copies of the ED3 portion in the H6-ED3-PhoA dimers should also be on the same side of the molecule and able to interact with immunoglobulins according to an avidity mode. The above results were sufficient to indicate that the H6-ED3-PhoA hybrids could be used in GAC- and MAC-ELISAs.

The existence of a recognition between H6-ED3.DEN1-PhoA and antibody mAb4E11, whose epitope is discontinuous and conformational, was shown by an indirect ELISA, in which mAb4E11 was immobilized in the wells of a microtiter plate and the binding of the hybrid revealed by its alkaline phosphatase activity. This experiment showed that the two portions ED3.DEN1 and PhoA of each hybrid molecule were simultaneously functional. Therefore, these two portions were properly folded, their essential disulfide bonds had formed in the oxidizing medium of the periplasm, and their assembly was dimeric since PhoA is significantly active only in this oligomerization state. Each molecule of hybrid was dimeric and bifunctional.

The experiment of indirect ELISA showed that it was possible to detect recognition between the ED3.DEN1 domain and antibody mAb4E11 with (H6-ED3.DEN1-PhoA)$_2$. This hybrid could be used to detect interactions between ED3 and other molecules, like inhibitors, other antibodies, receptors, or even whole cells.

The values of the catalytic constants k_{cat} for H6-ED3.DEN1-PhoA and H6-ED3.WN-PhoA confirmed the high activity of the PhoA portion of these hybrid molecules and therefore their dimeric state. The artificial dimerisation of the recombinant ED3 domains through PhoA partially mimicked their multimeric presentation at the surface of the whole viruses and therefore their multivalent mode of interaction with antibodies or other receptors.

EXAMPLE 5: GAC- and MAC-ELISAs

Methods

The capture ELISAs were performed in microtitration plates with a volume of 100 µL/well. The anti-IgG and anti-IgM antibodies were diluted in PBS (final concentrations 1 µg/mL). Wells 1 to 11 of a plate were loaded with the solution of antibody and well 12 with PBS alone. The plate was incubated overnight at 4 °C for the reaction of adsorption. The next morning, the wells were washed with buffer C (3 times), blocked with 3% (w/v) dry milk in buffer C for 1 h at 37 °C, and then washed with buffer C (3 times). The serum under analysis and the control serum were diluted 100 fold with 1% powder-milk in buffer C, then serially; the H6-ED3-PhoA hybrids were diluted in the same buffer (0.5 µM final concentration of monomer). Wells 1-10 were loaded with the 10 first dilutions of the serum, well 11 with the dilution buffer alone, and well 12 with the lowest dilution of the serum. The plate was incubated for 1 h at 37 °C for the reaction of antibody capture. The wells were washed with buffer C (3 times) and then loaded with the solution of H6-ED3-PhoA. The plate was incubated for 1 h at 37 °C for the binding reaction. The wells were washed as above and the bound H6-ED3-PhoA molecules revealed by addition of 5 mM pNPP in buffer E. $A_{405nm}$was measured either after a few hours at 25 °C or overnight at 4 °C. The signal of the serum was considered as significant if its value was at least twice that of the blank controls. The titer of the serum was equal to the maximum dilution factor for which the signal remained significant. The capture ELISAs were performed for the murine serums as for the human serums, except that some washes were extended, the anti-IgM antibody was used at 2.4 µg/mL final concentration, H6-ED3.DEN1-PhoA at 0.2 µM final concentration of monomer, and pNPP in buffer D.

Results

A simplified GAC-ELISA for the quantification of anti-flaviviral IgGs

It was tested if an H6-ED3-PhoA hybrid could detect IgGs, directed against the cognate flavivirus, in the serum of an immunized mouse and thus simplify the protocol of GAC-ELISA which is generally used for such a serology. Therefore, an antibody, directed against the murine IgGs, was immobilized in the wells of a microtitration plate by passive adsorption on the plastics. This immobilized antibody was used to capture the IgGs that were present in the mouse serum. The IgGs that were directed against the ED3 domain, were revealed with the H6-ED3-PhoA hybrid, through the binding of its antigenic portion and the catalytic activity of its PhoA portion (Equation 3).

This assay was performed with the serum of a mouse that had been immunized with DENV1. The serum of a non-immunized mouse, a blank test without anti-IgG antibody, and blank tests without serum were used as controls (Materials and Methods). The formation of pNP from pNPP, catalyzed by the H6-ED3.DEN1-PhoA hybrid and monitored with A_{405nm}, was used as a signal to reveal the binding reaction (Figure 2). The A_{405nm} signal followed a low of saturation as a function of the concentration in immune serum. The titer of the immune serum was > 50000 after an overnight revelation (> 12500 after 2.5 h) in these experiments that were repeated three times independently. The A_{405nm} signal for the non-immune serum did not differ from the blank signal whereas the signal for the immune serum was 2 to 18 fold higher than the blank signal, depending on the concentration, after an overnight revelation (2 to 6 fold after 2.5 h). These results confirmed that both portions of H6-ED3.DEN1-PhoA were simultaneously functional in one molecule of hybrid. They showed that this hybrid could sensitively, quantitatively and specifically assay the presence of IgGs, directed against the ED3.DEN1 domain, in a serum and thus detect an infection by the dengue virus.

A simplified MAC-ELISA for the quantification of anti-flaviviral IgMs

Similarly, it was tested if a H6-ED3-PhoA hybrid could detect IgMs, directed against a flavivirus, in the serum of an immunized mouse and thus simplify

the protocol of MAC-ELISA which is generally used. An antibody, directed against the murine IgMs, was immobilized. This immobilized antibody was used to capture the IgMs that were present in the mouse serum. The IgMs that were directed against the ED3 domain, were revealed with the bivalent H6-ED3-PhoA hybrid (Equation 3).

This assay was performed with the serum of a mouse that had been immunized with the chimeric virus MV_{Schw}-sE_{WNV}, which expresses the secreted form of gpE from WNV. The serum of a mouse that had been immunized with the empty vector MV_{Schw}, a blank test without anti-IgM antibody, and blank tests without sera were used as controls (Materials and Methods). The H6-ED3.WN-PhoA hybrid was used to reveal the binding reactions (Figure 3). The A_{405nm} signal followed a low of saturation as a function of the concentration in immune serum. The titer of the immune serum was > 800 after an overnight revelation (> 400 after 3 h). The A_{405nm} signal for the non-immune serum was at most 1.7 fold higher than the blank signal after an overnight incubation whereas the signal for the immune serum was 2 to 6.4 fold higher than the blank signal, depending on the concentration. These figures were 1.2 fold for the non-immune serum and 2 to 2.6 fold for the immune serum after a revelation of 3h. Note that the signal for the non-immune serum did not differ significantly from the blank signal for relative concentrations of serum $\leq$ 2.5 ‰. These results confirmed that both portions of H6-ED3.WN-PhoA were simultaneously functional in one molecule of hybrid. They showed that this hybrid could sensitively, quantitatively and specifically assay the presence of IgMs, directed against the ED3.WN domain. They suggested that the hybrid could enable one to detect an exposure to WNV precociously (at day 8).

EXAMPLE 6: Discrimination between flaviviruses by the ED3-PhoA hybrids

The specificity of the simplified GAC- and MAC-ELISA according to the invention was tested by performing cross-reactions. The serum of the mouse that had been immunized with the DENV1 virus, was submitted to two parallel GAC-ELISAs that were revealed with either the H6-ED3.DEN1-PhoA hybrid or with H6-ED3.WN-PhoA (Figure 4). Reciproquely, the serum of the mouse that had been immunized with the MV_{Schw}-sE_{WNV} chimeric virus, was submitted to two parallel MAC-ELISAs that were revealed with either the H6-ED3.DEN1-PhoA hybrid or with H6-ED3.WN-PhoA (Figure 5). After an overnight revelation, the cognate signal was

up to 5.4 fold higher than the non-cognate signal in the GAC-ELISA, and up to 3.9 fold higher in the MAC-ELISA. Of course, these figures were much higher when the specific signals (signal of the serum minus signal of the blank) were considered. These results showed that the GAC- and MAC-ELISA, as described here, were specific and that they allowed one to determine the identity of the flavivirus that was involved in the infection or immunization.

EXAMPLE 7: Assay of human serums with the simplified GAC- and MAC-ELISA

The H6-ED3.DEN1-PhoA hybrid was used to test serums from human patients who had been infected with one of the four serotypes DENV1 to DENV4 of the dengue virus. For DENV1, three serum samples, taken between days 9 and 28 after the onset of the symptoms, corresponded to primary infections with the dengue virus; two serum samples, taken at days 13 and 18, corresponded to secondary infections. For DENV2, -3 and -4, the samples were taken between days 8 and 32, and the primary or secondary status of the infection was unknown. These serums had been previously assayed by standard methods of GAC- and MAC-ELISA, with suckling mouse brain extracts as antigens. The following controls were used: an assay in which the immobilized antibody, directed against human IgG or IgM, was omitted; an assay in which the serum was omitted; and two assays with serums of patients who had not been infected by the dengue virus.

The A_{405nm} signal followed a law of saturation as a function of the concentration in serum, for the serums from patients with primary DENV1 infections in the MAC-ELISA (Figure 6) and for the serums from patients with secondary DENV1 infections in the GAC-ELISA (not shown). It increased linearly up to a relative concentration of serum > 2.5 ‰. Therefore, this relative concentration was used for the following of the analysis. A revelation of the assays during 3 h at 25 °C was sufficient.

Among the 20 tested serums, only the three serums that corresponded to primary infections, gave signals that were positive in the MAC-ELISA, i. e. more than twice the signal of the controls; all the other serums gave signals that were identical to the controls (Figure 7A). Therefore, the simplified MAC-ELISA according to the invention could detect a primary infection with

DENV1, and distinguish between infections with DENV1 and the other three serotypes. Four serum samples gave positive signals in the GAC-ELISA: the two samples from patients with a secondary DENV1 infection; one sample (2d) among the six samples from patients with a DENV2 infection; and one sample (4a) among the two samples from patients with a DENV4 infection (Figure 7B). Therefore, the simplified GAC-ELISA according to the invention could detect a secondary infection with DENV1 at day 13 after the onset of symptoms. The patients whose serums 2d and 4a scored as positive in the simplified GAC-ELISA, might have experienced an unnoticed infection with DENV1 previously. No correlation was observed between the day at which each sample was taken and the value of the signal in the simplified MAC- and GAC-ELISAs.

The ratio r of the signals in parallel MAC- and GAC-ELISAs has been used to determine whether an infection by the dengue virus is of the primary or secondary type. Such a ratio for the signals in the simplified capture ELISAs according to the invention (Figure 7C) was calculated. The three serums that corresponded to primary infections by DENV1, had $r > 1.90$. All the serums that corresponded to infections by DENV2, -3 and -4, had $r < 1.4$, except serums 2d and 4a. The serums that corresponded to secondary infections by DENV1, and serums 2d and 4a had $r < 0.4$. Thus, the ratio r could distinguish between primary and secondary infections, and also between primary infections with DENV1 and infections with other DENV serotypes.

The $(H6\text{-}ED3.DEN1\text{-}PhoA)_2$ hybrid was used successfully in a simplified GAC-ELISA to reveal the presence of IgGs, directed against DENV1, in the serum of a mouse that had been hyper-immunized against this virus, or in the serums of human patients who had endured a secondary infection by this virus. The same hybrid was used successfully in a simplified MAC-ELISA to reveal the presence of IgMs, directed against DENV1, in the serums of patients who had endured a primary infection by this virus. The simplified GAC-ELISA enabled us to distinguish between infection by DENV1 and WNV in the mouse. The combination of the simplified GAC- and MAC-ELISAs enabled us to distinguish between an infection by DENV1 and an infection by the three other serotypes of DENV in man, and also between a primary and a secondary infection by DENV1.

Likewise, the (H6-ED3.WN-PhoA)$_2$ hybrid was used successfully in a simplified MAC-ELISA to reveal the presence of IgMs, directed against WNV in the serum of a mouse. This simplified MAC-ELISA enabled us to distinguish between infections by WNV and DENV1. The high specificity and sensitivity of the simplified GAC- and MAC-ELISAs came likely from two factors: the use of the ED3 domain as an antigen and the independence of the detection system, consisting of the fusion with PhoA, towards the nature of the antigen and its interactions with the immunoglobulins of the serum. The specificity of the (H6-ED3-PhoA)$_2$ bifunctional dimers should be higher than those of the antigens and detection systems that have been used up until now.

EXAMPLE 8: Assay of human serums with a simplified MAC-ELISA

In a further series of experiments serums of patients infected by one of the four serotypes DEN1 to DEN4 of the dengue virus or by the yellow fever virus (YFV) were collected and characterized by standard methods of MAC-ELISA (Talarmin et al., 1998) and PCR (Lanciotti et al., 1992). The standard MAC-ELISA used extracts of infected suckling mouse brains as antigens and the PCR used primers that were specific for each viral serotype (Table I). The primer sequences and amplification conditions were as described (Lanciotti et al., 1992). In particular the primer sequences were as follows:Primer D1: 5'-TCAATATGCTGAAACGCGCGAGAAACCG -3' (SEQ ID NO: 26).

Primer D2: 5'- TTGCACCAACAGTCAATGTCTTCAGGTTC -3' (SEQ ID NO: 27).

Primer TS1: 5'- CGTCTCAGTGATCCGGGGG -3' (SEQ ID NO: 28).

Primer TS2: 5'- CGCCACAAGGGGCATGAACAG -3' (SEQ ID NO: 29).

Primer TS3: 5'- TAACATCATCATGAGACAGAGC -3' (SEQ ID NO: 30).

Primer TS4: 5'- CTC TGT TGT CTT AAA CAA GAG A -3' (SEQ ID NO: 31).

Amplification occurred in 100µl volumes containing the following components: 50 mM KCl, 10 mM Tris (pH 8.5), 1.5 mM $MgCl_2$, 0.01% gelatin, 200µM each of the four deoxynucleotide triphosphates, 5 mM dithiothreitol, 50 pmol each of primer, 2.5 Units of rav-2 recombinant RT (Amersham, Arlington Heights, Ill.) and 2.5 Units of Amplitaq polymerase (Perkin Elmer, Norwalk, Conn.). The reactions were allowed to proceed in a thermocycler programmed to incubate for 1 h

at 42°C and then to proceed with 35 cycles of denaturation (94°C, 30 s), primer annealing (55°C, 1 min) and primer extension (72°C, 2 min).

Table I. Number of human serums analyzed by simplified MAC and GAC-ELISAs.

Serum	ELISA	DEN hybrids	YF hybrid
DEN1	MAC	30	18
DEN2	MAC	44	24
DEN3	MAC	38	18
DEN4	MAC	13	13
YF	MAC	19	19
DEN1	GAC	18	0
DEN2	GAC	24	0
DEN3	GAC	18	0

In said Table I, Column 1: flavivirus detected in the serum of human patients by standard diagnostic methods (see text). Column 3: number of serums tested in parallel with the H6-ED3-PhoA hybrids corresponding to the four serotypes of DENV. Column 4: number of serums tested in parallel with the four H6-ED3.DEN-PhoA hybrids and the H6-ED3.YF-PhoA hybrid. The serums in column 4 constituted a sub-set of the serums in column 3.

Four among the 19 serums of patients that were infected with YFV, came from the Institut Pasteur of Cayenne (French Guyana) and corresponded to patients that had been recently vaccinated against YFV, and the remaining 15 serums came from the Institut Pasteur of Dakar (Senegal).

The collected serums were assayed by the simplified MAC-ELISA according to the present invention, with the five corresponding H6-ED3-PhoA hybrids and as previously described (see Example 5). The general format of the simplified MAC-ELISA is the following:

Support-@huIgM :: serum :: $(H6\text{-}ED3\text{-}PhoA)_2$

where @huIgM is an antibody directed against the human IgMs. The inventors considered the signal *A* of a serum assay to be positive, when it was

higher than twice the signal A_c of the control, i. e. $A > 2A_c$. The latter consisted in an assay which was performed in n-plicates ($n \geq 3$) and in which the antibody directed against the human IgMs, was omitted.

Table II gives the proportion of positive signals for each type of serum and hybrid. For each type of serum, the proportion of positive signals was maximal for the cognate hybrid, except for the serums of patients that were infected by DENV4. In this last case, the proportion of positive signals was maximal with the ED3.DEN1-PhoA and ED3.DEN2-PhoA hybrids. The DEN2 and YF serums reacted rarely with non-cognate hybrids. In contrast, the DEN1 serums reacted often with the DEN2 and DEN3 hybrids, and the DEN4 serums with every DEN hybrid. Conversely, for each type of hybrid, the proportion of positive signals was maximal with the cognate serums, except for ED3.DEN4-PhoA which reacted weakly with every kind of serum. In particular, the DEN1 and YF hybrids reacted rarely with the non-cognate serums.

Table II. Analysis of human serums by a simplified MAC-ELISA, using H6-ED3-PhoA hybrids.

Hybrid	Proportion of positive serums (%)				
	DEN1	DEN2	DEN3	DEN4	YF
DEN1	83	11	16	23	0
DEN2	63	73	26	23	4
DEN3	37	9	47	15	17
DEN4	3	7	3	8	15
YF	0	5	0	0	47

In said Table II, Column 1 gives the type of the H6-ED3-PhoA hybrid used in the assay, i. e. the viral origin of its ED3 portion. Columns 2-6 give the proportion of positive serums in the assay for each type of serum and hybrid. The signal A of a serum was considered as positive if higher than twice the control signal A_c ($A \geq 2A_c$) and negative if lower ($A < 2A_c$). The number and properties of the human serums are given in Table I.

Table III gives the mean value of the ratio (serum signal)/(control signal) for each type of serum and each type of hybrid, i. e. $<A/A_c>$. For each type of serum, this mean value was maximal for the cognate hybrid, except for the DEN4 serums. However, for each type of hybrid this mean value was not maximal for the cognate serum, in general.

Table III. Relative signals of human serums in simplified MAC-ELISAs.

Hybrid	Relative signal for serums				
	DEN1	DEN2	DEN3	DEN4	YF
DEN1	10.0	1.9	1.4	1.9	1.3
DEN2	5.3	5.0	2.0	1.8	1.4
DEN3	3.0	2.0	2.6	1.4	1.6
DEN4	1.3	1.6	1.2	1.3	1.7
YF	1.4	1.4	1.6	1.7	2.5

In said Table III, Column 1 gives the type of H6-ED3-PhoA hybrid used in the assay. Columns 2-6 give the mean value of A/A_c for each type of serum and hybrid. See legend to Table II for details.

EXAMPLE 9: Sensitivity and specificity of the simplified MAC-ELISA, using threshold signals

The sensitivity of the simplified MAC-ELISA is given in row 1 of Table IV, for each type of serum and cognate hybrid. This sensitivity was high for the DEN1 and DEN2 serums, medium for the DEN3 and YF serums, and low for the DEN4 serums. If one restricts itself to the four YF serums that came from the Institut Pasteur of Cayenne and corresponded to vaccinated patients, the sensitivity was much higher (four positive signals). The ED3.YF-PhoA hybrid, whose sequence corresponded to the vaccinal strain 17D of YFV, might detect the IgM that are directed against the vaccinal virus better that those that are directed against wild type strains.

Table IV. Sensitivity and specificities of the simplified MAC-ELISAs for human serums.

Property	Param	Detection of				
		DEN1	DEN2	DEN3	DEN4	YF
Sensitivity (%)	$2xA_c$	83	73	47	8	47
Serotype specificity (%)	$2xA_c$	20	78	50	0	na
Serotype specificity (%)	A_{max}	100	97	89	0	na
Group specificity (%)	$2xA_c$	100	94	89	100	89
Viral specificity (%)	A_{max}	100	100	89	0	89

In Table IV, sensitivity in row 1 was defined as the proportion of serums that gave a positive signal when assayed with the cognate H6-ED3-PhoA hybrid (see diagonal in Table II). DEN serotype specificity in row 2 was defined as the proportion of serums that gave negative signals with the three non-cognate DEN hybrids, among those that gave a positive signal with the cognate DEN hybrid. DEN serotype specificity in row 3 was defined as the proportion of serums that gave a higher signal with the cognate DEN hybrid than with the three non-cognate DEN hybrids, among those that gave a positive signal with the cognate hybrid. The DEN serotype specificities in rows 2 and 3 were determined with the serums of Table I, column 3. Group specificity in row 4 was defined as the proportion of DEN serums that gave a positive signal with the cognate DEN hybrid and a negative signal with the YF hybrid; and as the proportion of YF serums that gave a positive signal with the cognate YF hybrid and a negative signal with all four DEN hybrids. Viral specificity in row 5 was defined as the proportion of serums that gave a higher signal with the cognate hybrid than with the non-cognate ones, among those that gave a positive signal with their cognate hybrid. The group and viral specificities in rows 4 and 5 were determined with the serums of Table I column 4. See Table II for other details.

The specificity of the ED3-PhoA hybrids for a DEN serotype in the simplified MAC-ELISAs was calculated as the proportion of serums that gave negative signals with the three non-cognate hybrids ($A < 2A_c$), among serums that gave a positive signal with the cognate hybrid ($A > 2A_c$). This specificity of serotype was

high for the ED3.DEN2-PhoA hybrid, medium for the DEN3 hybrid, low for the DEN1 hybrid and nil for the DEN4 hybrid (Table IV, row 2).

The specificity of the ED3-PhoA hybrids for a viral group in the simplified MAC-ELISAs was calculated on the one hand as the proportion of DEN serums that gave a positive signal with the cognate ED3.DEN-PhoA hybrid and a negative signal with the ED3.YF-PhoA hybrid; and on the other hand as the proportion of YF serums that gave a positive signal with the cognate ED3.YF-PhoA hybrid and a negative signal with all the ED3.DEN-PhoA hybrids. This specificity for a viral group was ≥ 89 % in every case and up to 100 % for the ED3.DEN1-PhoA and ED3.DEN4-PhoA hybrids (Table IV, row 4).

EXAMPLE 10: Specificity of the simplified MAC-ELISA, using the maximal signals

The modular structure of the ED3-PhoA hybrids is such that the intensity of the signal in a simplified MAC-ELISA depends only on the properties of recognition between its ED3 portion and the antibodies of the serum. This property enables one to quantitatively compare the signals obtained for a given serum with ED3-PhoA hybrids that carry different ED3 domains. Therefore, the inventors calculated the proportion of serums that gave a positive signal with the cognate ED3-PhoA hybrid, and a higher signal with the cognate hybrid than with the non-cognate ones. The inventors calculated these proportions for the four ED3.DEN hybrids and then for the five ED3-PhoA hybrids. The serotype specificity, calculated in this way for the four DEN hybrids, was ≥ 89 % for the DEN1, DEN2 and DEN3 hybrids, and nil with the DEN4 hybrid (Table IV, row 3). The viral specificity, calculated for the five hybrids, was also ≥ 89 % except for the DEN4 hybrid (Table IV, row 5).

EXAMPLE 11: Assay of human serums with a simplified GAC-ELISA

Serums of patients infected by one of the three serotypes DEN1, DEN2 and DEN3 of the dengue virus were collected and characterized by standard methods of IgG-specific indirect ELISA and PCR. The indirect ELISA used extracts of infected suckling mouse brains as antigens, and the PCR used primers that were specific for each viral serotype (Table I). The collected serums were assayed by the simplified GAC-ELISA according to the present invention, with the three

corresponding H6-ED3-PhoA hybrids, as previously described (see Example 5). The general format of the simplified GAC-ELISA is the following:

Support-@huIgG :: serum :: $(H6\text{-}ED3\text{-}PhoA)_2$

where @huIgG is an antibody directed against the human IgGs. The inventors considered that the signal of a serum assay was positive when it was higher than twice the signal of the control. The latter consisted in an assay which was performed in n-plicates ($n \geq 3$) and in which the antibody directed against the human IgGs, was omitted. The proportion of positive serums in a simplified GAC-ELISA, performed with the cognate hybrid, was low and at most 29 % (Table V).

Table V. Sensitivity and specificities of the simplified GAC-ELISAs for human serums.

Property	Param	Detection of		
		DEN1	DEN2	DEN3
Sensitivity (%)	$2xA_c$	28	29	17
Serotype specificity (%)	$2xA_c$	60	0	0
Serotype specificity (%)	A_{max}	100	29	33

In Table V, the sensitivity and serotype specificity were defined as in Table IV. The number and properties of the human serums are given in Table I.

The H6-ED3-PhoA hybrids characterised in examples 8-11, have enabled the inventors to recognize recent infections by the dengue viruses or the yellow fever virus precociously, by a simplified MAC-ELISA. The sensitivities were going from high to very high except for the DEN4 virus, in the order DEN1 > DEN2 > DEN3 = YF >> DEN4.

These differences in sensitivity could be due to variable levels of immunogenicity of the ED3 domains, according to the virus. Under this assumption, the ED3.DEN4 domain could be less immunogenic than the ED3 domains from the three other serotypes DEN1-DEN3 or from YFV. Alternatively, the differences in sensitivity could be due to the specific strains, and therefore sequences, of viruses that the inventors used to construct the recombinant hybrids. For example, the simplified MAC-ELISA for the infection by YFV could be improved by having two hybrids at

one's disposal, one corresponding to the 17D vaccine strain and the other one to a wild type strain.

The five tested hybrids had a very good specificity of viral group, i. e. dengue group versus yellow fever group, higher than 89 %. They also had a very good specificity of serotype, higher than 89 % except for the DEN4 hybrid, when assays of a same serum with different hybrids were compared quantitatively. The results suggested that the antibodies directed against ED3.DEN4 recognize epitopes that are shared between flaviviruses.

The simplified GAC-ELISA, performed on human serums with serotypes DEN1-DEN3 of the H6-ED3-PhoA hybrids, had low sensitivities. Whether this conclusion is general and should be extended to other viruses or organisms, remains to be determined. Figures 2 and 4 show the sensitivities for a simplified GAC-ELISA that was performed with the H6-ED3.DEN1-PhoA hybrid on serums from mice that had been immunized with DENV1. The inventors have also shown that a quantitative comparison of the signals in simplified MAC- and GAC-ELISAs performed on human serums and that this could distinguish between primary and secondary infections by DENV1, see Example 7 and figure 7.

Thus, the recombinant H6-ED3-PhoA hybrids can be prepared easily in low safety laboratories. They enable the detection of infections by flaviviruses precociously and allow clinicians to distinguish between groups of viruses or even between serotypes of the dengue virus.

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CLAIMS

1°) A method for the diagnosis or the screening of an arbovirus in a subject or animal host, characterized in that it comprises:

(i) contacting a sample from the subject or animal with a solid support sensitized with an Ig binding protein which is directed against a specific class of Ig molecules of the subject or animal species under consideration and

(ii) incubating the immunocomplex formed in (i) with a detector molecule consisting of a hybrid protein comprising at least an arboviral ED3 domain and an alkaline phosphatase (PhoA), the detection of said immunocomplex being the sign of the presence of an arbovirus in said sample.

2°) The method according to claim 1, characterized in that the Ig binding protein is selected in the group consisting of anti-IgM, anti-IgG and anti-IgA antibodies.

3°) The method according to claim 1 or claim 2, characterized in that said arbovirus is a flavivirus.

4°) The method according to anyone of claims 1 to 3, characterized in that said alkaline phosphatase is selected from the group consisting of: rat, mouse, chicken, bovine, yeast and bacterial alkaline phosphatases.

5°) The method according to claim 4, characterized in that said alkaline phosphatase is the alkaline phosphatase of *E. coli* and comprises SEQ ID NO: 25.

6°) The method according to anyone of claims 1 to 5, characterized in that said hybrid protein further comprises a polypeptide tag.

7°) The method according to claim 6, characterized in that said polypeptide tag is selected in the group consisting of HIS (hexahistidine), c-MYC, HA, VSV-G, HSV, V5 and FLAG.

8°) The method according to anyone of claims 1 to 7, characterized in that said hybrid protein comprises preferably a hexahistidine, a flaviviral ED3 domain and the alkaline phosphatase of *E. coli.*

9°) The method according to claim 5 or 8, characterized in that said alkaline phosphatase of *E. coli* is modified.

10°) The method according to claim 9, characterized in that said alkaline phosphatase of *E. coli* includes two mutations in its active site: D153G and D330N and comprises SEQ ID NO: 24.

11°) The method according to anyone of claims 1 to 10, characterized in that the ED3 domain polypeptide is selected in the group consisting of a yellow fever virus ED3 domain polypeptide, a West Nile virus ED3 domain polypeptide, a Dengue virus ED3 domain polypeptide, a St Louis encephalitis virus ED3 domain polypeptide, a Murray Valley encephalitis virus ED3 domain polypeptide and a Japanese encephalitis virus ED3 domain polypeptide.

12°) A hybrid protein, characterized in that it comprises a polypeptide tag, an arbovirus ED3 domain and an alkaline phosphatase.

13°) The hybrid protein according to claim 12, characterized in that it comprises a hexahistidine, a flaviviral ED3 domain selected from the list comprising: a yellow fever virus ED3 domain polypeptide, a West Nile virus ED3 domain polypeptide, a Dengue virus ED3 domain polypeptide, a St Louis encephalitis virus ED3 domain polypeptide, a Murray Valley encephalitis virus ED3 domain polypeptide and a Japanese encephalitis virus ED3 domain polypeptide; and the alkaline phosphatase of *E. coli*.

14°) The hybrid protein according to claim 12 or claim 13, characterized in that said hybrid protein is preferably in a multimeric form and more preferably in a dimeric form.

15°) The hybrid protein according to claim 12, 13 or claim 14, characterized in that it is selected in the group consisting of $(H6\text{-}ED3.DEN1\text{-}PhoA)_2$ which consists of the sequence SEQ ID NO:2, $(H6\text{-}ED3.DEN2\text{-}PhoA)_2$ which consists of the sequence SEQ ID NO:4, $(H6\text{-}ED3.DEN3\text{-}PhoA)_2$ which consists of the sequence SEQ ID NO:6, $(H6\text{-}ED3.DEN4\text{-}PhoA)_2$ which consists of the sequence SEQ ID NO:8, $(H6\text{-}ED3.WN\text{-}PhoA)_2$ which consists of the sequence SEQ ID NO:10 and $(H6\text{-}ED3.YF\text{-}PhoA)_2$ which consists of the sequence SEQ ID NO:12.

16°) Nucleic acids encoding the hybrid proteins according to anyone of claims 12 to 15.

17°) The nucleic acid according to claim 16, characterized in that it is selected in the group consisting of: SEQ ID NO:1 encoding H6-ED3.DEN1-PhoA

hybrid protein, SEQ ID NO:3 encoding H6-ED3.DEN2-PhoA hybrid protein, SEQ ID NO:5 encoding H6-ED3.DEN3-PhoA hybrid protein, SEQ ID NO:7 encoding H6-ED3.DEN4-PhoA hybrid protein, SEQ ID NO:9 encoding H6-ED3.WN-PhoA hybrid protein and SEQ ID NO:11 encoding H6-ED3.YF-PhoA hybrid protein.

18°) A method of preparing a hybrid protein according to anyone of claims 12 to 15, characterized in that it comprises:

(a) obtaining an expression vector containing the sequence encoding an hybrid protein as defined in anyone of claims 12 to 15 by inserting the sequence coding for an arboviral ED3 polypeptide and preferably flaviviral ED3 polypeptide in the vector pEBL1 (SEQ ID NO:13),

(b) transforming an appropriate *E. coli* strain, preferably the XL1-blue strain with the expression vector obtained in (a),

(c) culturing said modified strains in an appropriate medium and

(d) purifying the tag-ED3-PhoA hybrid protein from the periplasmic extract.

19°) The method according to claim 18, characterized in that the expression vector of step (a) is selected in the group consisting of an expression vector of a hybrid protein as defined in anyone of claims 12 to 15.

20°) The method according to claim 19, characterized in that said expression vector contains the sequence encoding the hybrid protein H6-ED3.DEN1-PhoA (pEBL11, deposited at the CNCM (Collection Nationale de Culture de Microorganismes, 28 rue du Docteur Roux, 75015 PARIS) on April 23, 2007 under the accession number I-3748.

21°) The method according to claim 19, characterized in that said expression vector contains the sequence encoding the hybrid protein H6-ED3.WN-PhoA (pEBL15, deposited at the CNCM (Collection Nationale de Culture de Microorganismes, 28 rue du Docteur Roux, 75015 PARIS) on April 23, 2007 under the accession number I-3749.

22°) Expression vector pEBL1 deposited at the CNCM (Collection Nationale de Culture de Microorganismes, 28 rue du Docteur Roux, 75015 PARIS) on April 23, 2007 under the accession number I-3747.

23°) Expression vector pEBL11 deposited at the CNCM (Collection Nationale de Culture de Microorganismes, 28 rue du Docteur Roux, 75015 PARIS) on April 23, 2007 under the accession number I-3748.

24°) Expression vector pEBL15 deposited at the CNCM (Collection Nationale de Culture de Microorganismes, 28 rue du Docteur Roux, 75015 PARIS) on April 23, 2007 under the accession number I-3749.

25°) A method for screening for arbovirus antibodies and preferably flavivirus antibodies in a subject or an animal, said method comprising:

(i) contacting a sample from said subject or animal with a solid support sensitized with an Ig binding protein which is directed against a specific class of Ig molecules of subject or the animal species under consideration,

(ii) incubating the immunocomplex formed in (i) with a detector molecule consisting of a hybrid protein comprising at least a arboviral ED3 domain and an alkaline phosphatase and

(iii) detecting the presence of said arbovirus antibodies.

26°) A kit for diagnosing and/or screening for arbovirus antibodies and preferably flavivirus antibodies in a subject comprising:

- a solid support sensitized with an Ig binding protein which is directed against a specific class of Ig molecules of the animal species under consideration,
- at least a hybrid protein comprising at least an arbovirus ED3 domain and an alkaline phosphatase as defined in claims 12 to 15,
- at least one positive control, preferably a reference serum from an infected individual and
- at least one negative control, preferably a reference serum from a non-infected individual.

27°) The kit according to claim 26, characterized in that the Ig binding protein is selected in the group consisting of anti-IgM, anti-IgG and anti-IgA, and said hybrid protein comprises a hexahistidine, a viral ED3 domain of an appropriate flavivirus and the alkaline phosphatase of *E. coli*.

28°) The kit according to claim 27, characterized in that the alkaline phosphatase is a modified alkaline phosphatase including two mutations in its active site: D153G and D330N and comprises SEQ ID NO: 24.

29°) Use of a hybrid protein comprising an appropriate antigen of a pathogen and an alkaline phosphatase, for an *in vitro* diagnostic of infections by said pathogen or for studying the epidemiology of said pathogen.

30°) Use of a hybrid protein comprising an appropriate antigen of a pathogen and an alkaline phosphatase, for an *in vitro* validation of a vaccination against said pathogen or an immunogen thereof.

31°) Use of a hybrid protein comprising a protein or a fragment thereof and alkaline phosphatase to study the interaction between said protein or fragment thereof fused with PhoA and molecules, proteins or cells.

32°) A method for the diagnosis of an infection by a pathogen, for validating a vaccination by a pathogen or an immunogen thereof or for studying the epidemiology of said pathogen, characterized in that it comprises:

(i) contacting a sample from a subject or an animal with a solid support sensitized with an Ig binding protein which is directed against a specific class of Ig molecules of the animal species under consideration,

(ii) incubating the immunocomplex formed in (i) with a detector molecule consisting of a hybrid protein comprising an appropriate antigen of a pathogen and alkaline phosphatase, the presence of said immunocomplex being the sign of said infection.

33°) A method for studying the interaction between a protein or a fragment thereof fused to PhoA and molecules, proteins or cells, characterized in that it comprises:

(i) contacting said molecule, protein or cell with a hybrid protein comprising the protein or a fragment thereof fused to PhoA, and

(ii) detecting the complex eventually formed between the protein or a fragment thereof fused to PhoA and said molecule, said protein or said cell.

34°) A method for screening for anti-arbovirus compounds, said method comprising:

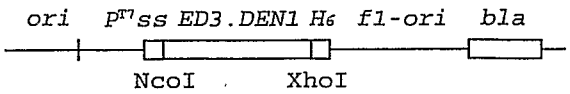
(i) contacting an anti-arbovirus antibody or a receptor of a surface molecule of an arbovirus, eventually bound to a solid support with a hybrid protein comprising an epitope of an arbovirus fused to PhoA,

(ii) detecting the complex formed between said anti-arbovirus antibody or said receptor and said epitope by measuring an appropriate signal, for instance the formation of paranitrophenol,

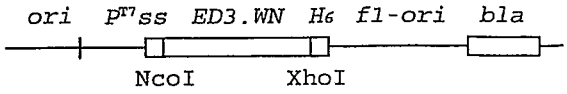
(iii) adding a compound to be tested, and

(iv) detecting if the amount of complex formed between said anti-arbovirus antibody or said receptor and said epitope has decreased in relation to the amount of complex detected in step (ii), by measuring an appropriate signal and comparing the signal obtained with the signal obtained in (ii).

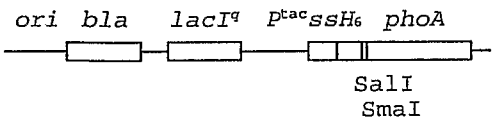
pLB11



pVP5



pLIP5GN-H6



Signal peptide

GTG	AAA	CAA	AGC	ACT	ATT	GCA	CTG	GCA
M	K	Q	S	T	I	A	L	A

CTC	TTA	CCG	TTA	CTG	TTT	ACC	CCT	GTG
L	L	P	L	L	F	T	P	V

↓ Mature PhoA

ACA	AAA	GCC	CGG	ACA	CCA	GAA	ATG	CCC
T	K	A	R	T	P	E	M	P

His-tag

GTC	*GAA*	*CAT*	*CAC*	*CAT*	*CAC*	*CAT*	*CAC*	*GAC*
V	*E*	*H*	*H*	*H*	*H*	*H*	*H*	*D*

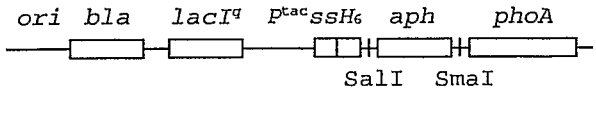
DrdI SalI SacI SmaI

GAT	*GAC*	*GAT*	*AAG*	*GT*	*CGA*	*CGA*	*GCT*	*CCC*
D	*D*	*D*	*K*		*R*	*R*	*A*	*P*

PhoA

GGG	GTT	CTG	GAA	AAC	CGG	...
G	V	L	E	N	R	...

pEBL1



Signal peptide

GTG	AAA	CAA	AGC	ACT	ATT	GCA	CTG	GCA
M	K	Q	S	T	I	A	L	A

CTC	TTA	CCG	TTA	CTG	TTT	ACC	CCT	GTG
L	L	P	L	L	F	T	P	V

↓ Mature PhoA

ACA	AAA	GCC	CGG	ACA	CCA	GAA	ATG	CCC
T	K	A	R	T	P	E	M	P

His-tag

GTC	*GAA*	*CAT*	*CAC*	*CAT*	*CAC*	*CAT*	*CAC*	*GAC*
V	*E*	*H*	*H*	*H*	*H*	*H*	*H*	*D*

DrdI SalI KanR

GAT	*GAC*	*GAT*	*AAG*	*GTC*	*GAC*	/	-	*aph*	-	/
D	*D*	*D*	*K*	*V*	*D*					

SalI SacI SmaI PhoA

GT	*CGA*	*CGA*	*GCT*	*CCC*	*GGG*	GTT	CTG	GAA
	R	*R*	*A*	*P*	*G*	V	L	E

AAC	CGG	...
N	R	...

Figure 1

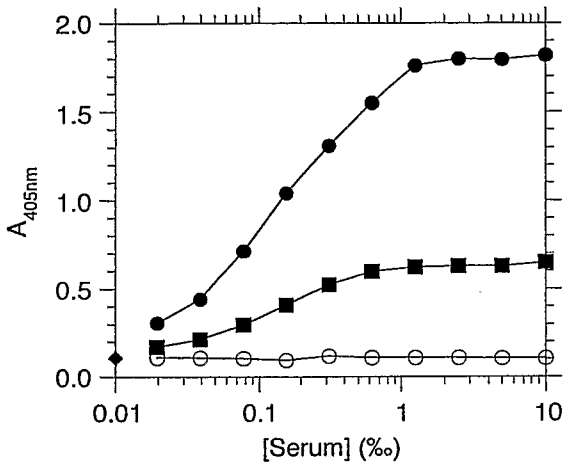


Figure 2

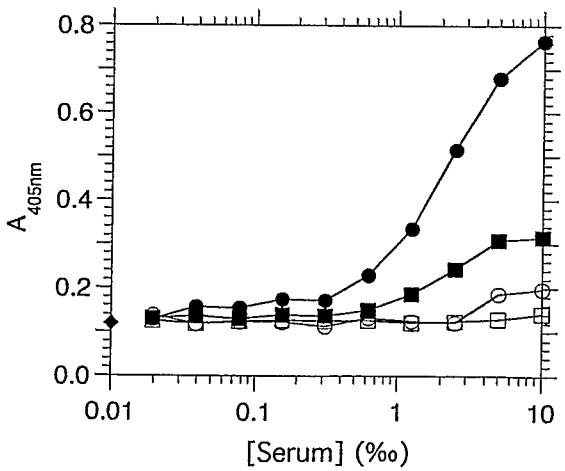


Figure 3

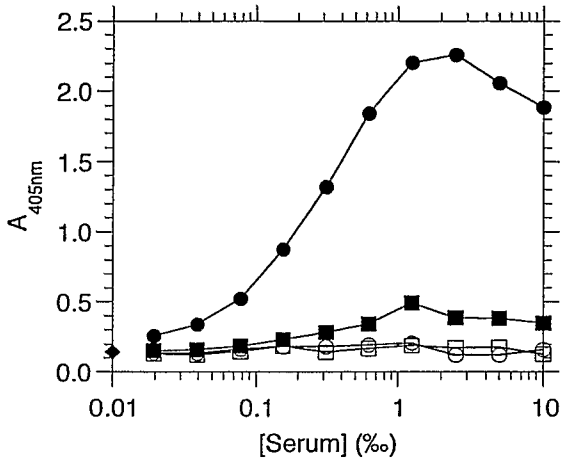


Figure 4

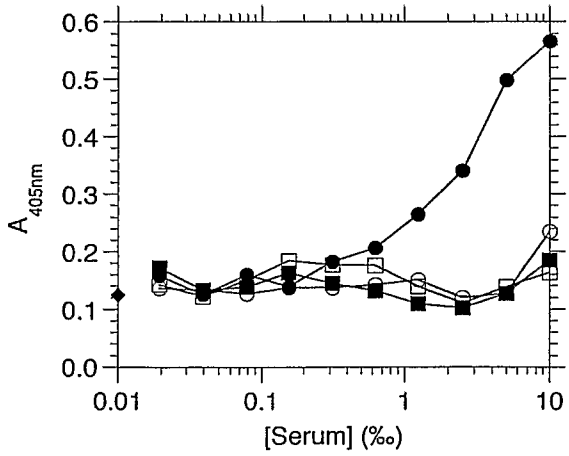


Figure 5

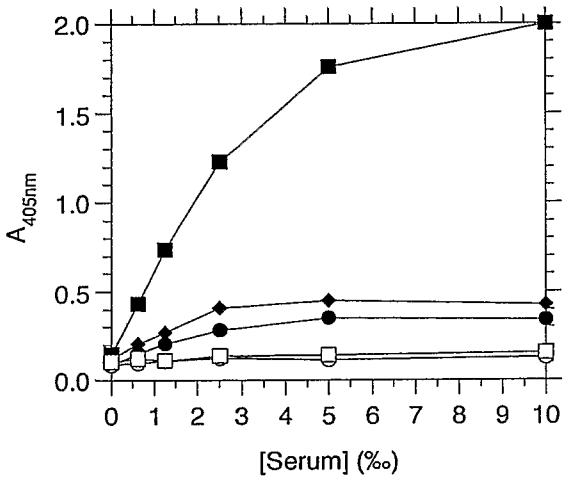


Figure 6

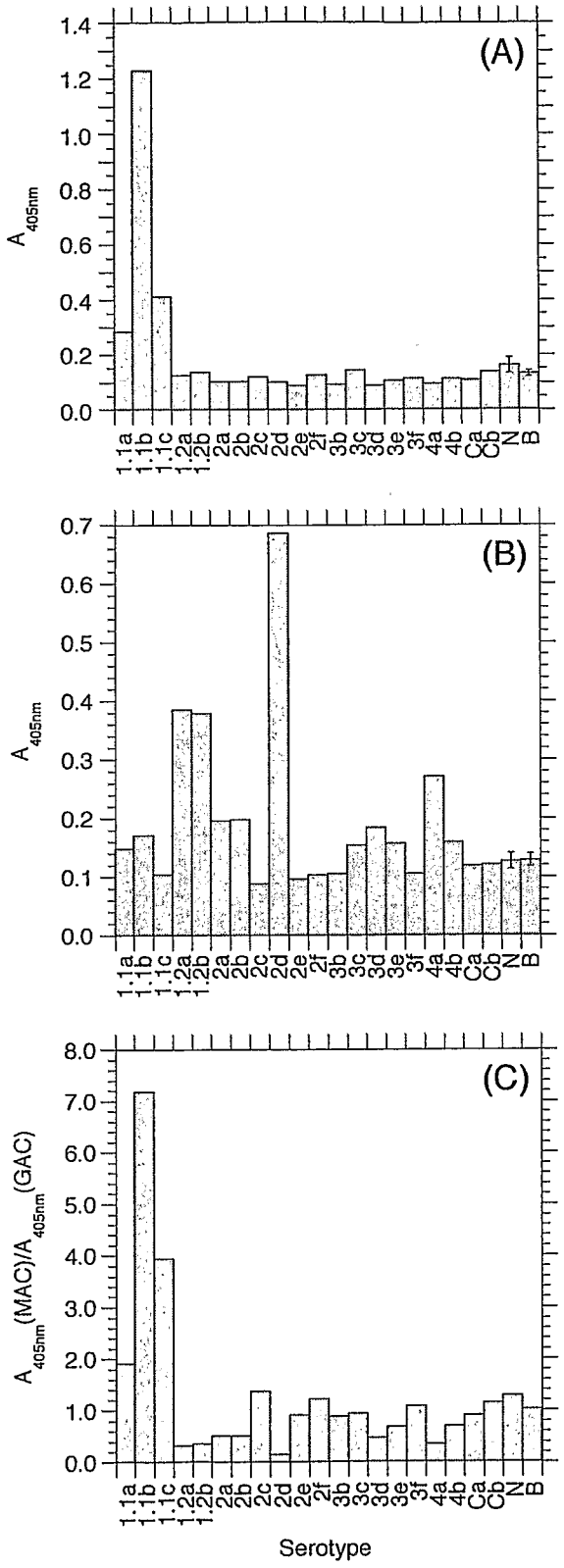

(A)
(B)
(C)
A_{405nm}
A_{405nm} (MAC)/A_{405nm} (GAC)
1.1a
1.1b
1.1c
1.2a
1.2b
2a
2b
2c
2d
2e
2f
3b
3c
3d
3e
3f
4a
4b
Ca
Cb
N
B
Serotype

Figure 7