(12) STANDARD PATENT (11) Application No. **AU 2007327367 B2**
(19) AUSTRALIAN PATENT OFFICE

(54) Title
Immunization protocol against the 4 dengue serotypes

(51) International Patent Classification(s)
A61K 39/12 (2006.01) ***A61P 31/14*** (2006.01)

(21) Application No: **2007327367** (22) Date of Filing: **2007.11.30**

(87) WIPO No: **WO08/065315**

(30) Priority Data

(31) Number	(32) Date	(33) Country
0655255	**2006.12.01**	**FR**

(43) Publication Date: **2008.06.05**
(44) Accepted Journal Date: **2013.05.09**

(71) Applicant(s)
Sanofi Pasteur

(72) Inventor(s)
Lang, Jean;Guy, Bruno;Forrat, Remi;Barban, Veronique

(74) Agent / Attorney
Davies Collison Cave, Level 15 1 Nicholson Street, MELBOURNE, VIC, 3000

(56) Related Art
GUIRAKHOO F., et al, Construction, Safety, and Immunogenicity in Nonhuman Primates of a Chimeric Yellow Fever-Dengue Virus Tetravalent Vaccine, Journal of Virology, 2001, Vol. 75(16), pg. 7290-7304

(12) DEMANDE INTERNATIONALE PUBLIÉE EN VERTU DU TRAITÉ DE COOPÉRATION EN MATIÈRE DE BREVETS (PCT)

(19) Organisation Mondiale de la Propriété Intellectuelle
Bureau international

(43) Date de la publication internationale
5 juin 2008 (05.06.2008)

PCT

(10) Numéro de publication internationale
WO 2008/065315 A1

(51) Classification internationale des brevets :
A61K 39/12 (2006.01) *A61P 31/14* (2006.01)

(21) Numéro de la demande internationale :
PCT/FR2007/052431

(22) Date de dépôt international :
30 novembre 2007 (30.11.2007)

(25) Langue de dépôt : français

(26) Langue de publication : français

(30) Données relatives à la priorité :
0655255 1 décembre 2006 (01.12.2006) FR

(71) Déposant : **SANOFI PASTEUR** [FR/FR]; 2 avenue du Pont Pasteur, F-69367 Lyon Cedex 07 (FR).

(72) Inventeurs: **GUY, Bruno**; 15B rue des Noyers, F-69005 Lyon (FR). **BARBAN, Véronique**; 7 ter rue Jean-Claude Martin, F-69290 Craponne (FR). **FORRAT, Rémi**; 2 rue Pierre Devaux, F-69780 Serezin Du Rhône (FR). **LANG, Jean**; 118 route de Saint-Priest, F-69780 Mions (FR).

(74) Mandataires : **SCHAEFFER, Nathalie** etc.; Sanofi Pasteur, Direction de la Propriété Intellectuelle, 2, avenue du pont Pasteur, F-69367 Lyon cedex 07 (FR).

(81) États désignés *(sauf indication contraire, pour tout titre de protection nationale disponible)* : AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, ZA, ZM, ZW.

(84) États désignés *(sauf indication contraire, pour tout titre de protection régionale disponible)* : ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), eurasien (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), européen (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Publiée :
— *avec rapport de recherche internationale*
— *avant l'expiration du délai prévu pour la modification des revendications, sera republiée si des modifications sont reçues*
— *avec la partie réservée au listage des séquences de la description publiée séparément sous forme électronique et disponible sur demande auprès du Bureau international*

WO 2008/065315 A1

(54) **Title:** IMMUNIZATION PROTOCOL AGAINST THE 4 DENGUE SEROTYPES

(54) **Titre :** METHODE D'IMMUNISATION CONTRE LES 4 SEROTYPES DE LA DENGUE

(57) **Abstract:** The invention relates to a method to induce a protection against the 4 dengue serotypes in a patient, comprising: (a) the administration of a monovalent vaccine comprising a vaccine virus of a first dengue serotype, and (b) the administration of a tetravalent vaccine comprising vaccine viruses of the four serotypes of dengue, wherein the administration (b) is implemented at least 30 days to at most 12 months after the first administration (a).

(57) **Abrégé :** L'invention concerne une méthode pour induire une protection contre les 4 sérotypes de la dengue chez un patient, comprenant : (a) l'administration d'un vaccin monovalent comprenant un virus vaccinal d'un premier sérotype de la dengue, et (b) l'administration d'un vaccin tétravalent comprenant des virus vaccinaux des quatre sérotypes de la dengue, dans laquelle l'administration (b) est mise en œuvre au moins 30 jours à au plus 12 mois après la première administration (a).

Immunization protocol against the 4 dengue serotypes

Method of immunization against the 4 serotypes of dengue fever The invention relates to a method for inducing protection against the 4 serotypes of dengue fever in a patient, comprising:

(a) a first administration of a monovalent vaccine comprising a vaccinal virus of a first serotype of dengue fever,

(b) a second administration of a tetravalent vaccine comprising vaccinal viruses of the four serotypes of dengue fever, and

in which the second administration (b) is made between at least 30 days and not more than 12 months after the first administration (a).

Dengue fevers are caused by four viruses of the flavivirus genus which are of similar serological type but differ from the antigen point of view (Gübler *et al.*, 1988, in: Epidemiology of arthropod-borne viral disease. Monath TPM, editor, Boca Raton (FL): CRC Press: 223-60; Kautner *et al.*, 1997, J. of Pediatrics, 131: 516-524; Rigau-Pérez *et al.*, 1998, Lancet, 352: 971-977; Vaughn *et al.*, 1997, J. Infect. Dis., 176: 322-30). Infection with a serotype of dengue fever may produce a spectrum of clinical disease from non-specific viral syndrome to severe fatal hemorrhagic disease. The incubation period for dengue fever after a mosquito bite is approximately 4 days (from 3 to 14 days). Dengue fever is characterized by a two-phase fever, headaches, pains in various parts of the body, prostration, eruptions and lymphadenopathy (Kautner *et al.*, 1997, J. of Pediatrics, 131: 516-524; Rigau-Pérez *et al.*, 1998, Lancet, 352: 971-977). The viremic period is of the same as the febrile period (Vaughn *et al.*, 1997, J. Infect. Dis., 176: 322-30). Cure of dengue fever is complete after 7 to 10 days, but prolonged asthenia is normal. Reduced leukocyte and platelet numbers frequently occur.

Hemorrhagic dengue fever is a severe febrile disease characterized by homeostasis abnormalities and an increase in vascular permeability which can lead to hypovolemia and hypotension (dengue fever with shock syndrome), often complicated by severe internal bleeding. The mortality rate for hemorrhagic dengue fever can reach 10% without treatment, but is ≤ 1% in most centers with experience of treatment (WHO Technical Guide, 1986. Dengue haemorrhagic fever: diagnosis, treatment and control, p. 1-2. World Health Organization, Geneva, Switzerland).

Routine laboratory diagnosis of dengue fever is based on isolation of the virus and/or the detection of antibodies specific to dengue fever virus.

Dengue is the second most important infectious tropical disease after malaria, more than half of the world's population living in areas where there is a risk of epidemic transmission. There are estimated to be 50-100 million cases of dengue fever every year, 500,000 patients hospitalized for hemorrhagic dengue fever, and 25,000 deaths. Dengue fever is endemic in Asia, the Pacific, Africa, Latin America and the Caribbean. Dengue fever virus infections are endemic in more than 100 tropical countries and hemorrhagic dengue fever has been documented in 60 of these countries (Gubler, 2002, TRENDS in Microbiology, 10: 100-103; Monath, 1994, Proc. Natl. Acad. Sci., 91: 2395-2400). A number of well-described factors would appear to be implicated in dengue fever - population growth, unplanned and uncontrolled urbanization, in particular associated with poverty, an increase in air travel, lack of effective mosquito control and deterioration of sanitary and public health infrastructure (Gubler, 2002, TRENDS in Microbiology, 10: 100-103). Travellers and expatriates are increasingly being warned about dengue fever (Shirtcliffe *et al.*, 1998, J. Roy. Coll. Phys. Lond., 32: 235-237). Dengue fever has been one of the main causes of febrile diseases among American

troops during deployments in tropical areas where dengue fever is endemic (DeFraites *et al.*, 1994, MMWR, 1994, 43: 845-848).

The viruses are maintained within a cycle involving humans and *Aedes aegypti*, a domestic mosquito which bites during the daytime, and prefers to feed on man. Infection in man is initiated by injection of the virus during the blood meal of an infected *Aedes aegypti* mosquito. The salivary virus is mainly deposited in the extravascular tissues. The first category of cells to be infected after inoculation are the dentritic cells, which then migrate to the lymphatic ganglia (Wu *et al.*, 2000, Nature Med., 7: 816-820). After initial replication in the skin and lymphatic ganglia, the virus appears in the blood in the course of the acute febrile stage, generally for 3 to 5 days.

Along with the dentritic cells, monocytes and macrophages are among the first targets of dengue fever virus. Protection against homotypic reinfection is complete and probably lasts a lifetime, but cross-protection between the different types of dengue lasts from less than a few weeks to a few months (Sabin, 1952, Am. J. Trop. Med. Hyg., 1: 30-50). As a consequence, an individual may become infected with a different serotype. A second infection due to dengue fever is in theory a risk factor for the development of severe dengue fever. However, hemorrhagic dengue fever is multifactorial - factors include the strain of virus involved and the age, immune status and genetic predisposition of the patient. Two factors play a major role in the occurrence of hemorrhagic dengue fever - rapid viral replication with a high level of viremia (the severity of the disease being associated with the level of viremia; Vaughn *et al.*, 2000, J. Inf. Dis., 181: 2-9) and a major inflammatory response with the release of high levels of inflammatory mediators (Rothman and Ennis, 1999, Virology, 257: 1-6). There is

no specific treatment against dengue fever. Treatment for dengue fever is symptomatic, with bed rest, control of the fever and pain through antipyretics and analgesics, and adequate drinking. The treatment of hemorrhagic dengue fever requires balancing of liquid losses, replacement of coagulation factors and the infusion of heparin.

Preventive measures are currently based on control of the vector and personal protection measures which are difficult to apply and are costly. No vaccine against dengue fever has at present been approved. Given that the four serotypes of dengue fever are in circulation in the world and that they have been reported as being involved in cases of hemorrhagic dengue fever, vaccination should ideally confer protection against the four serotypes of dengue fever virus.

When immunizing with a tetravalent vaccine, it may happen that the response is induced predominantly against only one or at most 3 serotypes. There is therefore a need for a method which makes it possible to reduce interference between the different serotypes and makes it possible to induce neutralizing antibodies against the 4 serotypes of dengue fever.

The inventors have found that it is possible to generate an immune response comprising antibodies neutralizing the 4 serotypes when the vaccinal formulation which is intended to induce a response against the 4 serotypes is administered after preliminary immunization with an attenuated living vaccine of only one serotype, the second immunization being made 30 days to 12 months after the first administration.

The inventors have in particular shown that tetravalent DEN-1,2,3,4 immunization after monovalent DEN-2 immunization induces responses against the four serotypes in all the monkeys immunized. Conversely, tetravalent immunization alone only induced a

satisfactory response against two out of 4 serotypes, even after a booster.

The immune response generated by the method according to the invention is therefore both quantitatively and qualitatively greater (covers all serotypes).

In accordance with a first object, this invention therefore relates to a method making it possible to induce a neutralizing antibody response against the 4 serotypes of dengue fever in a patient, comprising:

(a) a first administration of a monovalent vaccine comprising a vaccinal virus of a first serotype of dengue fever,

(b) a second administration of a tetravalent vaccine comprising vaccinal viruses of the 4 serotypes of dengue fever, and

in which the second administration (b) is made at least 30 days and not more than 12 months after the first administration (a).

According to a particular embodiment of the method of immunization according to the invention, the said vaccinal virus used in the first administration (a) is selected from the group comprising vaccinal viruses of dengue fever of serotype 1 or 2.

According to another particular embodiment of the method of immunization according to the invention, the said vaccinal virus used in the first administration (a) is selected from the group comprising strains VDV1 and VDV2.

According to another particular embodiment of the method according to the invention, the said vaccinal viruses used in the tetravalent vaccine are selected from the group comprising Chimerivax™ DEN-1,2,3 and 4.

According to another particular embodiment of the method according to the invention the quantity of vaccinal viruses of dengue fever of serotypes 1, 2, 3 and 4 lies within a range from 10^3 to 10^6 $CCID_{50}$.

According to another particular embodiment of the

method according to the invention, the monovalent vaccine comprises 10^4 $CCID_{50}$ of VDV1 or VDV2 and the tetravalent vaccine comprises 10^5 $CCID_{50}$ of Chimerivax™ DEN-1,2,3 and 10^3 $CCID_{50}$ of Chimerivax™ DEN-4.

According to another embodiment of the method according to the invention, the second administration (b) is made 30 to 60 days after the first administration (a).

Another object of the present invention is an immunization kit against dengue fever virus comprising a box containing at least (a) a first container holding a monovalent composition or vaccine comprising a vaccinal virus of a first serotype of dengue fever, (b) a second container holding a tetravalent composition or vaccine comprising vaccinal viruses for the 4 serotypes of dengue fever.

According to one embodiment, the kit according to the invention comprises at least:

(a) a first container holding a monovalent vaccine comprising a VDV1 or VDV2 vaccinal virus,

(b) a second container holding a tetravalent vaccine comprising the 4 Chimerivax™ DEN-1,2,3 and 4.

According to a particular embodiment, the kit according to the invention comprises a monovalent vaccine comprising 10^4 $CCID_{50}$ of VDV1 or VDV2 and a tetravalent vaccine comprising 10^5 $CCID_{50}$ of Chimerivax™ DEN-1,2,3 and 10^3 $CCID_{50}$ of Chimerivax™ DEN-4.

This invention therefore also relates to use of dengue fever vaccinal viruses for the manufacture of a monovalent vaccine and a tetravalent vaccine for immunization against dengue fever virus in which the monovalent vaccine comprises a vaccinal virus of a first serotype of dengue fever, the tetravalent vaccine comprises vaccinal viruses of the 4 serotypes of dengue fever and in which the tetravalent vaccine is administered at least 30 days and not more than 12 months after administration of the monovalent vaccine.

The invention will now be described in more detail

in the description which follows.

Definitions

"Dengue fever viruses" or "DEN" are positive single-strand RNA viruses belonging to the Flavivirus genus of the family of *flaviviridae*. The genome in RNA contains a type I end member at the 5' extremity but has no poly-A tail at the 3' extremity. The organization of the genome comprises the following elements: non-coding region (NCR) 5', structural proteins (capsid (C), pre-membrane/membrane (prM/M), envelope (E)) and non-structural proteins (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5) and NCR 3'. The viral genome RNA is associated with the capsid proteins to form a nucleocapsid. As in the case of flaviviruses, the DEN viral genome codes an uninterrupted coding region which is translated into a single polyprotein.

In the context of this invention, by "vaccinal dengue fever virus" is meant any viral form of dengue fever virus which is capable of inducing a specific immune response comprising neutralizing antibodies, preferably all viral forms of dengue fever virus which can be used in the context of an immunization program in man against infection by a dengue fever virus. By vaccinal dengue fever viruses are therefore meant inactivated viruses, attenuating viruses or recombinant proteins such as the envelope protein of dengue fever virus.

A vaccinal virus is regarded as being "inactivated" if it no longer replicates in permissive cells.

A vaccinal virus is regarded as being "attenuated" if after growth at 37°C or 39°C in Huh-7, VERO and/or C6/36 liver cells the said vaccinal virus has a maximum titer which is at least 10 times less than maximum titer obtained with the wild-type parent strain under the same culture conditions and as measured using the same method for determining titer. A vaccinal virus

which has diminished growth in at least one of the three cell types identified above is therefore regarded as being "attenuated" in the context of this invention.

A vaccinal virus which can be used in man has a positive benefit/risk ratio, the said ratio generally satisfying statutory requirements for obtaining a marketing authorization. A vaccinal dengue fever virus used in the context of this invention is preferably a virus which has been attenuated in such a way that it does not induce the disease in man. Advantageously, the said vaccinal virus only results in side effects of at most moderate intensity (i.e. medium to slight, or zero) in the majority of vaccinated individuals, while retaining its ability to induce a neutralizing antibody response.

Dengue fever vaccinal viruses which can be used in the context of this invention may be cited by way of non-restrictive examples: inactivated vaccinal viruses, attenuated vaccinal viruses such as the attenuated strains VDV-1, VDV-2, the strains described for example in applications WO02/66621, WO0057904, WO0057908, WO0057909, WO0057910, WO02/0950075 and WO02/102828, or chimeras. Chimeric viruses have the special feature that they have the characteristics of attenuated viruses as defined above. All chimeric viruses expressing the envelope protein of a dengue fever virus and inducing an immune response comprising antibodies neutralizing the serotype from which the envelope protein originates may therefore be used in the context of this invention. Mention may be made by way of non-restricting examples of: the dengue fever Chimerivax™ such as described for example in patent application WO 98/37911, dengue/dengue fever chimeras such as described for example in patent applications WO9640933 and WO0160847. The vaccinal virus of serotype 1 dengue fever may for example be the vaccinal strain VDV1 or a Chimerivax™ DEN-1, in particular a YF17D/DEN-1 virus, or again a DEN-1 16007/PDK13 strain.

The vaccinal virus for serotype 2 of dengue fever may for example be the vaccinal strain VDV2 or a Chimerivax™ DEN-2, in particular a YF17D/DEN-2 virus, or again a DEN-2 16681/PDK53 strain. The vaccinal virus of serotype 3 of dengue fever may be a Chimerivax™ DEN-3, in particular a YF17D/DEN-3 virus. The vaccinal virus of serotype 4 of dengue fever may be a Chimerivax™ DEN-4, in particular a YF17D/DEN-4 virus. Reference may be made to the applications identified here for precise description of the strains mentioned and the processes for obtaining them.

"VDV" or "Vero dengue vaccine" denotes an attenuated live dengue fever viral strain adapted to Vero cells (i.e. it is capable of replicating reproducibly at a significant level on Vero cells) and capable of inducing a specific humoral response, including the induction of neutralizing antibodies, in primates and particularly in man.

"VDV-1" is a strain obtained from a wild-type DEN-1 16007 strain which has undergone 11 passes through PDK cells (DEN-1 16007/PDK11) and which has subsequently been amplified in Vero cells at 32°C, the RNA of which has been purified and transfected in Vero cells. The VDV-1 strain has 14 additional mutations in comparison with the DEN-1 16007/PDK13 vaccinal strain (13 passes through PDK - Primary Dog Kidney - cells). The DEN-1 16007/PDK13 strain, also called "LAV1", has been described in patent application EP1159968 in the name of Mahidol University and has been filed with the National Microorganisms Cultures Collection (CNCM) under number I-2480. The complete sequence of the VDV-1 strain is given in sequence SEQ ID NO:1. This strain can easily be reproduced from that sequence. A process for preparing and characterizing the VDV-1 strain has been described in the international patent application filed under number WO2006/134433 in the names of Sanofi-Pasteur and the Center for Disease Control and Prevention.

"VDV-2" is a strain obtained from a wild-type strain DEN-2 16681 which has undergone 50 passes through PDK cells (DEN-2 16681/PDK50), has been plate purified, and the RNA of which has been extracted and purified, before transfection of said strain into Vero cells. The VDV-2 strain has subsequently been obtained by plate purification and amplification in Vero cells. The VDV-2 strain has 10 additional mutations in comparison with the DEN-2 16681/PDK53 vaccinal strain (53 passes through PDK cells), including 4 silent mutations. The DEN-2 16681/PDK53 strain, also known as "LAV2", has been described in patent application EP1159968 in the name of Mahidol University and has been filed with the National Microorganisms Cultures Collection (CNCM) under number I-2481. The complete sequence of the VDV-2 strain is given in sequence SEQ ID NO:2. The VDV-2 strain can easily be reproduced from that sequence. A process for preparing and characterizing the VDV-2 strain has been described in the international patent application filed under number WO2006/134433 in the names of Sanofi-Pasteur and the Center for Disease Control and Prevention.

The VDV 1 and 2 strains are prepared by amplification in Vero cells. The viruses produced are harvested and clarified from cell debris by filtration. The DNA is digested by treatment with enzymes. Impurities are eliminated by ultrafiltration. Infectious titers may be increased by a concentration method. After adding a stabilizer, the strains are stored in lyophilized or frozen form before use and then reconstituted when needed.

By "ChimeriVax™ dengue" or "CYD" is meant a chimeric yellow fever (YF) virus which comprises the skeleton of a YF virus in which the sequences coding for the pre-membrane and envelope proteins have been replaced by those of a DEN virus. Thus, a chimeric YF virus containing the prM and E sequences of a serotype 1 dengue fever strain (DEN-1) is called "CYD-1 or CYD

DEN1". A chimeric YF containing the prM and E sequences of a DEN-2 strain is referred to as "CYD-2 or CYD DEN2". A chimeric YF virus containing the prM and E sequences of a DEN-3 strain is referred to as "CYD-3 or CYD DEN3". A chimeric YF virus containing the prM and E sequences of a DEN-4 strain is referred to as "CYD-4 or CYD DEN4". The preparation of these dengue ChimeriVax™ has been described in detail in international patent applications WO 98/37911 and WO 03/101397, to which reference may be made for a precise description of the processes for their preparation. The chimeras described in the examples have been generated by using prM and E sequences from strains DEN 1 PUO359 (TYP1140), DEN2 PUO218, DEN3 PaH881/88 and DEN 4 1228 (TVP 980). Any dengue fever virus strain may be used to construct chimeras in the context of this invention.

Preferably, the chimeric YF virus comprises the skeleton of an attenuated yellow fever strain YF17D (Theiler M. and Smith H.H. (1937) J. Exp. Med., 65, p. 767-786) (viruses YF17D/DEN-1, YF17D/DEN-2, YF17D/DEN-3, YF17D/DEN-4). Examples of YF17D strains which may be used include YF17D204 (YF-Vax®, Sanofi-Pasteur, Swifwater, PA, USA; Stamaril®, Sanofi-Pasteur, Marcy l'Etoile, France; ARILVAX™, Chiron, Speke, Liverpool, UK; FLAVIMUN®, Berna Biotech, Bern, Switzerland; YF17D-204 France (X15067, X15062); YF17D-204,234 US (Rice *et al.*, 1985, Science, 229: 726-733), or again the related strains YF17DD (Genbank access number U17066), YF17D-213 (Genbank access number U17067) and the strains YF17DD described by Galler *et al.* (1998, Vaccines, 16(9/10): 1024-1028). Any other attenuated yellow fever virus strain which may be used in man may be used to construct chimeras in the context of this invention.

According to a particular embodiment, for each serotype used in the various administrations the vaccinal viruses are present in the vaccine in a quantity from 10^3 to 10^5 $CCID_{50}$.

According to a particular embodiment, vaccinal viruses VDV1 or VDV2 are present in the monovalent vaccine at a level of 10^4 $CCID_{50}$.

According to a particular embodiment, Chimerivax™ DEN-1, 2, 3 are present in the tetravalent vaccine at a level of 10^5 $CCID_{50}$ and Chimerivax™ DEN-4 is present in the tetravalent vaccine at a level of 10^3 $CCID_{50}$.

Each monovalent ChimeriVax™ dengue fever vaccinal virus (serotypes 1, 2, 3 and 4) has been prepared by amplifying each serotype in Vero cells. More specifically, the four viruses are produced separately in adhering Vero cells in a serum-free medium. The viral harvest, clarified from cell debris by filtration, is then concentrated and purified by ultrafiltration and chromatography to remove the DNA from the host cells. After adding a stabilizing agent, the vaccinal strains are stored in a frozen or lyophilized form before use and then reconstituted as needed. The same process is applied to the four chimeras.

A dose, composition or vaccine is "monovalent" when in addition to a pharmaceutically acceptable excipient it contains a vaccinal virus of a single dengue fever serotype. A dose, composition or vaccine is "tetravalent" when it contains vaccinal viruses of the four serotypes of dengue fever. Multivalent compositions are obtained by simple mixing of monovalent compositions.

By "patient" is meant a person (child or adult) who is likely to be infected by dengue fever, in particular a person at risk of infection, such as for example a person travelling in regions where dengue fever is present, or an inhabitant of those regions. The term therefore includes persons who are naïve for dengue fever virus and those who are not naïve.

Tetravalent immunization following initial monovalent immunization

In a first aspect, this invention therefore relates to a method of immunization against dengue fever virus.

The inventors have in fact shown in particular that the administration of 4 serotypes 30 days to 12 months after the first administration of a monovalent vaccine makes it possible to obtain effective protection against the 4 serotypes. The method according to this invention is therefore of very particular interest in the context of an immunization strategy against dengue fever.

According to this invention, the first immunization may be performed using a monovalent composition or vaccine comprising a vaccinal virus of any of the 4 serotypes of dengue fever, the second administration being performed with all 4 vaccinal serotypes. According to a particular embodiment, a serotype 1 or 2 dengue fever vaccinal virus, preferably serotype 2, is used for the first administration. Preferably, the dengue fever vaccinal virus used in the first administration is an attenuated dengue fever virus and is not made up of a chimeric virus. According to a particular embodiment, strain VDV1 or VDV2, preferably strain VDV2, is used as the vaccinal virus in the first administration.

Attenuated living vaccinal viruses are used in the second administration, preferably chimeric viruses expressing antigens for the four serotypes of dengue fever virus, in particular Chimerivax™ DEN1, 2, 3 and 4.

According to particular embodiments, this invention therefore covers the following systems:

- (a) VDV1 (b) CYD DEN-1, 2, 3 and 4
- (b) VDV2 (b) CYD DEN-1, 2, 3 and 4.

In the context of this invention, by "vaccinal composition" is meant a composition comprising an "immunoeffective quantity" of dengue fever vaccinal virus, that is to say a sufficient quantity of dengue

fever vaccinal virus to induce a specific immune response comprising neutralizing antibodies, which may be revealed for example by the seroneutralization test as described in Example 1 below. A serum is regarded as being positive for the presence of neutralizing antibodies when the titer of neutralizing antibodies so determined is not less than 1:10 (unity: 1/dilution).

The quantities of vaccinal strain are commonly expressed in terms of viral plaque forming units (PFU) or doses infecting 50% of the tissue culture or again doses infecting 50% of the cell culture ($CCID_{50}$). For example, compositions according to the invention may contain 10 to 10^6 $CCID_{50}$, in particular 10^3 to 10^5 $CCID_{50}$ of dengue fever vaccinal virus of serotypes 1, 2, 3 or 4 for a monovalent or tetravalent composition. Thus, in the compositions or utilizations according to the invention the doses of dengue vaccinal viruses of serotypes 1, 2, 3 and 4 preferably each lie within a range from 10 to 10^6 $CCID_{50}$, such as 10, 10^2, 10^3, 10^4, 10^5 or 10^6 $CCID_{50}$, in particular within a range from 10^3 to 10^5 $CCID_{50}$. Vaccinal virus may be used at the same or different doses, which can be adjusted in relation to the nature of the vaccinal virus used and the intensity of the immune response obtained.

According to a particular embodiment of a method according to this invention, the quantities of attenuated live vaccinal virus in monovalent and tetravalent compositions or vaccines are 10^3 to 10^5 $CCID_{50}$. According to a particular embodiment, the monovalent vaccine comprises 10^4 $CCID_{50}$ of VDV1 or VDV2, preferably VDV2. According to a particular embodiment, the tetravalent vaccine comprises 10^5 $CCID_{50}$ of Chimerivax™ DEN-1, 2, 3 and 4. According to one advantageous embodiment, the tetravalent vaccine comprises 10^5 $CCID_{50}$ of Chimerivax™ DEN-1, 2 and 3 and 10^3 $CCID_{50}$ of Chimerivax™ DEN-4.

In the context of this invention, the second administration (b) is performed 30 days and not more

than 12 months after administration (a). According to an advantageous embodiment, the second administration is performed 30 days to 60 days after the first administration (a).

The neutralizing antibody response is advantageously durable, that is to say it can be detected in serum up to at least 6 months after the second administration.

Vaccinal viruses are administered in the form of compositions or vaccines which can be prepared by any method known to those skilled in the art. Habitually, viruses, generally in lyophilized form, are mixed with a pharmaceutically acceptable excipient such as water or a phosphate-buffered saline solution, wetting agents or stabilizing agents. By "pharmaceutically acceptable excipient" is meant any solvent, dispersing medium, charge, etc., which does not produce any secondary reaction, for example an allergic reaction, in humans or animals. The excipient is selected on the basis of the pharmaceutical form chosen, the method and the route of administration. Appropriate excipients, and requirements in relation to pharmaceutical formulation, are described in "Remington: The Science & Practice of Pharmacy", which represents a reference work in the field.

Preferably, vaccinal compositions are prepared in injectable form, and may take the form of liquid solutions, suspensions or emulsions. The compositions may in particular comprise an aqueous solution buffered in such a way as to maintain a pH between approximately 6 and 9 (as determined using a pH meter at ambient temperature).

Although it is not necessary to add an adjuvant, the compositions may nevertheless include such a compound, that is to say a substance which increases, stimulates or reinforces the cell or humoral immune response induced by the vaccinal virus administered simultaneously. Those skilled in the art will be able

to select an adjuvant which might be appropriate in the context of this invention from the adjuvants conventionally used in the field of vaccines.

The compositions or vaccines according to the invention may be administered by any means conventionally used in vaccination, for example parenterally (in particular intradermally, subcutaneously or intramuscularly), advantageously subcutaneously. Preferably, the compositions or vaccines are injectable compositions administered subcutaneously, advantageously in the region of the left deltoid or right deltoid.

The volume of vaccine composition administered will depend on the method of administration. In the case of subcutaneous injections, the volume is generally between 0.1 and 1.0 ml, preferably approximately 0.5 ml.

The optimum period for administering all serotypes 1 to 4 is approximately 1 to 3 months before exposure to dengue fever virus. Vaccinations may be administered as a prophylactic treatment against infection by dengue fever virus in adults and children. Target populations therefore include persons who may be naïve (i.e. not previously immunized) or non-naïve with regard to dengue fever virus.

Booster administrations of dengue fever vaccinal viruses of serotypes 1 to 4 may also be used for example between 6 months and 10 years, for example 6 months, 1 year, 3 years, 5 years or 10 years after administration of the second administration (b) according to the invention. Booster administrations will advantageously be performed using the same compositions or vaccines (i.e. the same vaccinal viruses) and preferably under the same conditions of administration (anatomical sites and methods of administration) as used for the 2nd administration (b).

Interference phenomena may be explained by the dominance of one or more serotypes in relation to

others and are therefore independent of the technology used for preparation of the candidate vaccine (from VDV or Chimerivax™). The method according to this invention can therefore be applied in general to all dengue fever vaccinal viruses.

This invention is therefore also intended to cover use of dengue fever vaccinal viruses for the manufacture of a monovalent vaccine and a tetravalent vaccine for immunization against dengue fever virus in which the monovalent vaccine comprises the vaccinal virus of a first serotype of dengue fever, the tetravalent vaccine comprises vaccinal viruses for 4 serotypes of dengue fever, in which the tetravalent vaccine is administered at least 30 days and not later than 12 months after administration of the monovalent vaccine.

For a description of the vaccines and conditions of use in the context of use according to this invention, reference may be made to the description provided in relation to the method of immunization according to the invention.

According to another aspect, this invention has as its object an immunization kit against the four serotypes of dengue fever virus. The kit according to this invention comprises compositions or vaccines as defined above in relation to the method of immunization proposed. The kit according to the invention therefore comprises a box containing various containers holding the compositions or vaccines and advantageously an explanatory brochure including useful information for administration of the said compositions or vaccines.

According to one embodiment, this invention therefore relates to a kit for immunization against dengue fever virus, a box containing at least (a) a first container holding a monovalent vaccine comprising a vaccinal virus of a first serotype of dengue fever, and (b) a second container holding a tetravalent vaccine comprising vaccinal viruses for the 4 serotypes

of dengue fever.

For a description of the vaccines, compositions or dengue fever vaccinal viruses which may be used in the kit according to the invention, reference may be made to the description provided above in relation to the method of immunization according to the invention.

According to a particular embodiment the kit according to the invention comprises at least:

(a) a first container holding a monovalent vaccine comprising a VDV1 or VDV2 vaccinal virus, and

(b) a second container holding a tetravalent vaccine comprising the 4 Chimerivax™ DEN-1, 2, 3 and 4.

According to a particular embodiment, the kit according to the invention comprises at least one monovalent vaccine comprising 10^4 $CCID_{50}$ of VDV1 or VDV2 and a tetravalent vaccine comprising 10^5 $CCID_{50}$ of Chimerivax™ DEN-1, 2, 3 and 10^3 $CCID_{50}$ of Chimerivax™ DEN-4.

The kits according to the invention may contain a single example or several examples of the containers as described above.

If the vaccines used are in lyophilized form, the kit will advantageously comprise at least one additional container holding the diluent which can be used to reconstitute an injectable dose of vaccine. Any pharmaceutically acceptable diluent may be used for this purpose, conventionally water or a phosphate-buffered aqueous solution.

The invention is illustrated by the following example.

Example 1: Immunization against the 4 serotypes of dengue fever virus by successive injection of a monovalent composition followed by a tetravalent composition in monkeys

Viremia and immunogenicity were tested in a monkey model. Viremia in particular has been identified as being one of the factors associated with the virulence

and severity of the disease in man, and therefore constitutes an important parameter which must be taken into consideration. As for immunogenicity, this is a key parameter in the context of evaluating the protection imparted.

1.1 Materials and methods

Experiments on monkeys were carried out in accordance with European Directives relating to animal experiments. The immunizations were performed on cynomolgus monkeys (*Macaca fascicularis*) originating from Mauritania. The monkeys were placed in quarantine for six weeks prior to immunization.

The monkeys were immunized subcutaneously with 0.5 ml of vaccine composition in the arm. After mild anesthesia with ketamine (Imalgene, Merial), blood was collected by puncture of the inguinal or saphenal veins. On days 0 and 28 following each immunization, 5 ml of blood were sampled in order to evaluate antibody responses, while between days 2 and 10 1 ml of blood was sampled in order to evaluate viremia. The blood was collected on ice and preserved on ice until the serum was separated off. In order to do this, the blood was centrifuged for 20 minutes at 4°C and the serum collected was stored at -80°C until the time of the tests.

Measurement of viremia

Post-vaccination viremia was monitored by quantitative real time RT-PCT (qRT-PCR). Two sets of initiators and sensors located in the NS5 gene of the DEN1 and DEN2 strains were used to quantify the RNA of VDV-1 and VDV-2 respectively. A third set of 2 initiators and 1 sensor located in the NS5 gene of the YF virus was used to quantify the RNA of CYD. Finally, 4 sets of initiators and specific sensors for the different CYD serotypes located at the junction of the E (DEN) / NS1 (YF) genes were used to identify the serotype in the samples positive for NS5 YF RNA (see

also Table 1). 7 plasmids containing the region targeted by each PCR, under the control of promoter T7, were transcribed *in vitro* to generate a series of synthetic RNA which were included in each RT-PCT test as an internal reference. The synthetic RNA were determined by spectrophotometry, the quantity of RNA obtained was converted into the number of RNA copies and expressed as GEQ (genome equivalents).

0.140 ml of monkey serum were extracted using the "Nucleospin 96 virus™" RNA extraction kit from Macherey Nagel according to the manufacturer's instructions, and then the purified RNA was eluted with 0.140 ml (0.090 ml, then 0.05 ml) of RNase-free water. In order to avoid repeated freeze/thaw cycles, a first quantification was performed immediately after extraction on 5 µl of the said RNA preparation. The remaining volume was frozen at 70°C.

In addition to the components of the "Qiagen Qauntitect™ probes" RT-PCR quantification kit (Qiagen), the reaction mixtures contained 10 picomoles of each initiator, 4 picomoles of each sensor and 5 µl of RNA in a total volume of 25 µl. In the case of the RNA under test, 5 µl of the purified preparation were added directly to the reaction mixture without a prior dilution stage. The synthetic RNAs were diluted 1/10 in RNAse-free water, and 7 dilutions containing approximately 10 to 10^6 GEQ in 5 µl were quantified in parallel in order to generate a calibration curve.

The quantification reactions were carried out using the ABIPrism 700™ equipment from Applied Biosystem, using the following program: 50°C/30 min, 95°C/15 min, followed by 40 cycles of 95°C/15 sec - 60°C/60 sec.

The quantification limit for viral RNA in this test is 2.9 to 3.3 $\log_{10}$GEQ/ml (800 to 2000 GEQ/ml; 4 to 10 GEQ/reaction), according to PCR targets (standard deviation: +/-0.3 $\log_{10}$).

The correlation between infectious titer and the

quantification of viral RNA was established in parallel with the tests by analyzing 0.140 ml of samples of negative monkey serums (DO) to which a known quantity of infectious particles of the viruses used for immunization (CYD or VDV) had been added. The said control serums were prepared in two dilutions containing approximately 1 PFU and approximately 100 PFU in 5 µl (2.3 and 4.3 log_{10}PFU/ml, respectively).

In the tests used in the examples, the correlation between GEQ and PFU is as follows: GEQ/PFU ratio 2.7 log_{10} (i.e. 1 PFU = 500 GEQ) for sera positive for YF or CYDs; GEQ/PFU ratio 2.5 log_{10} (i.e. 1 PFU = 320 GEQ) for sera positive for VDV1 or VDV2.

The quantification limits are < 3.3 log_{10}GEQ/ml (i.e. < 4 PFU/ml) for YF and CYDs qRT-PCR, and < 2.9 log_{10}GEQ/ml (i.e. < 2.5 PFU/ml) for VDV1 and VDV2 qRT-PCR.

The initiators and sensors used are shown in Table 1 below, in which the sense and anti-sense initiators and the sensor are listed in order for each test.

Table 1

		sequence
YF	YF-NS5 sens	5' GCACGGATGTAACAGACTGAAGA (23 bases)
	YF NS5 anti	5' CCAGGCCGAACCTGTCAT (18 bases)
	YF-NS5	5' Fam- CGACTGTGTGGTCCGGCCCATC -Tamra (22 bases)
CYD1 spe	CYD1- sens	5' CAT TGC AGT TGG CCT GGT AA (20 b)
	CYD1- anti:	5' CTT TGG CAA GAG AGA GCT CAA GT (23 b)
	CYD1-	5' Fam-CCG ATC AAG GAT GCG CCA TCA-Tamra (21 b)
CYD2 spe	CYD2- sens	5' GTG GGA GTC GTG ACG CTG TA (20 b)
	CYD2- anti	5' GTT GAT GGC GCA TCC TTG ATC (21 b)
	CYD2	5' Fam-TGG GAG TTA TGG TGG GCG CCG-Tamra (21 b)
CYD3 spe	CYD3- sens:	5' AAA ACA CTT CCA TGT CAT TTT CAT G (25b)
	CYD3- anti:	5' GTT GAT GGC GCA TCC TTG ATC (21 b)
	CYD3-	5'Fam-TGCGATAGGAATTATCACACTCTATCTGGGAGC-Tamra (33b)
CYD4 spe	CYD4- sens	5' CTT AGT ATT GTG GAT TGG CAC GAA (24 b)
	CYD4- anti:	5' GCG CCA ACT GTG AAA CCT AGA (21 b)
	CYD4-	5'-Fam-AGAAACACTTCAATGGCAATGACGTGCAT-Tamra (29 b)
VDV1 spe	VDV1-NS5 sens	5' TCG CAA CAG CCT TAA CAG C (19 b)
	VDV1-NS5 anti	5' ACT ATC TCC CTC CCA TCC TTC (21 b)
	VDV1-NS5	5' Fam-TTC ACA CCA CTT CCA C-MGB/NFQ (16 b)
VDV2 spec	VDV2-NS5 sens	5' AAT GAC AGA CAC GAC TCC (18 b)
	VDV2-NS5 anti:	5' CCC AAA ACC TAC TAT CTT CAA C (22 b)
	VDV2-NS5	5' Fam-TGG AAG TCG GCA CGT GA-MGB/NFQ (17 b)

Measurement of neutralizing antibodies (seroneutralization test) (SN50)

Conventionally, dengue fever antibodies are measured using the PRNT50 test (test of neutralization by reducing the number of PFU to 50%). As this test is cumbersome and consumes much material, we have developed the SN50 test based on a 50% reduction in the number of units measured in the CCID50 test.

In a 96 well plate, 0.120 ml of each decomplemented serum is added to 0.480 ml of diluent (ISCOVE 4% SVF) in each well. Serial dilutions of a factor 6 are performed by transferring 0.150 ml of serum into 0.450 ml of diluent. 450 µl of viral dilution containing 2.7 $\log_{10}$ CCID50/ml are added to each well so as to obtain 25 CCID50/well. The plate is incubated at 37°C for 1 hour. 0.1 ml of each dilution is then distributed into 6 wells of a 96 well plate in which VERO cells have been seeded 3 days before the start of the experiment at a density of 8000 cells/well in 0.1 ml of ISCOVE 4% SVF medium. After 6 days

incubation at 37°C in the presence of 5% CO_2, the cells are fixed using an ethanol/acetone (70/30) mixture at 4°C for 15 minutes, and then washed 3 times in PBS and incubated for 1 hour at 37°C in the presence of 0.05 ml of a 1/2000 dilution of an anti-flavivirus monoclonal antibody (mAb 4G2 obtained from an ATCC H-B112 hybridoma). The plates are then washed twice and incubated for 1 hour at 37°C in the presence of 0.05 ml of a 1/1000 dilution of an anti-mouse IgG conjugated with alkaline phosphatase. The lysis plaques are revealed by adding 0.05 ml of a stained substrate: BCIP/NBT. The neutralizing antibody titers are calculated using the Karber formula as defined below:

$$Log_{10}SN50 = d + f/N\ (X + N/2),$$

in which:

d: represents the dilution providing 100% neutralization (that is 6 negative replicates, i.e. presenting no signs of infection)

f: represents the dilution factor as log_{10} (e.g. dilution factor of 1:4, f = 0.6)

N: represents the number of replicates/dilution (N = 6)

X: total number of wells having no sign of infection, with the exception of dilution d.

The limit for viral detection is 10 SN50 (i.e. 1.0 log_{10}SN50).

The viral strains used for neutralization were the strains DEN1 16007, DEN2 16681, DEN3 16562 or DEN4 1036.

In the case of the controls, the initial viral dilutions were re-titrated.

The correlation between the neutralizing titer measured in the SN50 test and the neutralizing titer measured conventionally in the PRNT50 test is: $log_{10}PRNT50 = log_{10}SN50 + 0.2$.

1.2 Evaluation of simultaneous immunizations

2 groups of 4 monkeys of equivalent age and weight were immunized (see Table 2).

Immunization was performed subcutaneously in the arm using a 23G1 needle, with a quantity of 10^5 $CCID_{50}$ for each CYD DEN 1 to 4 serotype for the tetravalent vaccine and a quantity of 10^4 $CCID_{50}$ for the monovalent VDV-2.

Table 2: Composition of the groups and immunization protocol

Monkeys		
Groups	Immunizations	
	D0	D56
Group 1	**Monovalent VDV 2**	**Tetravalent Dengue 1234 ChimeriVax**
Group 2	**Tetravalent Dengue 1234 ChimeriVax**	**Tetravalent Dengue 1234 ChimeriVax**

The immunogenicity results obtained after one immunization (D0+28) and two immunizations (D56+28) are shown in Table 3.

The viremia results are provided in Table 4.

Table 3: SN50 neutralizing titer (units 1/dil)

Monkeys			D0+28				D56+28			
ID	Immunizations		DEN-1	DEN-2	DEN-3	DEN-4	DEN-1	DEN-2	DEN-3	DEN-4
	D0	D56								
AM762	VDV 2	CYD 1234	10	501	-	10	63	1005	63	200
AM839			-	802	-	-	80	1271	63	504
AM905			20	158	-	-	318	1010	252	506
AN011			13	1005	-	-	252	1271	319	1010
Geometric mean			11	503	*<10*	<10	142	1131	134	477
AM496	CYD 1234	CYD 1234	50	-	16	32	100	40	80	252
AM645			-	-	13	31	16	-	-	63
AM766			-	-	-	32	20	-	-	80
AM813			25	-	-	13	63	13	20	63
Geometric mean			13	*<10*	<10	25	38	11	14	95

- : titer < 10

Table 4: viremia analyses (units: log10 GEQ/mL)

		First immunization									Booster								
Group	Monkey	D2	D3	D4	D5	D6	D7	D8	D9	D10	D58	D59	D60	D61	D62	D63	D64	D65	D66
1	AM762	<2.7	<2.7	<2.7	<2.7	**4.77**	**4.89**	**4.84**	**4.47**	<2.7	< 3.2	< 3.2	< 3.2	3.11	< 3.2	< 3.2	3.65	3.59	< 3.2
initial VDV 2	AM839	**5.11**	**4.51**	**4.19**	**4.19**	**4.56**	**3.69**	<2.7	<2.7	<2.7	4.98	4.86	4.43	3.79	< 3.2	< 3.2	< 3.2	< 3.2	< 3.2
booster CYD																			
1,2,3,4	AM905	<2.7	<2.7	<2.7	<2.7	**4.16**	**4.31**	**4.16**	**3.50**	**4.14**	< 3.2	< 3.2	3.09	3.42	3.39	< 3.2	< 3.2	4.08	4.22
	AN011	<2.7	<2.7	**3.75**	**4.29**	**4.35**	**4.22**	**3.51**	<2.7	**3.28**	3.56	3.42	< 3.2	< 3.2	3.42	3.37	4.67	5.09	4.97
2	AM496	4.22	3.36	3.71	4.15	3.14	< 3.1	3.58	< 3.1	< 3.1	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2
initial CYD																			
1,2,3,4	AM645	**4.21**	3.61	2,82	3.51	3.65	3.24	< 3.1	3.47	3.44	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2
booster CYD																			
1,2,3,4	AM766	3.97	3.06	3.38	4.19	3.80	3.73	< 3.1	< 3.1	< 3.1	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2
	AM813	4.81	4.60	3.17	< 3.1	< 3.1	< 3.1	< 3.1	< 3.1	< 3.1	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2

CYD1

CYD4

VDV2

In brief, the results can be summarized as follows:

- The method of administration according to this invention brings about a qualitative and quantitative increase in the neutralizing antibody response obtained with a system comprising two identical immunizations with tetravalent vaccine.

- One CYD-1,2,3,4 immunization performed after an initial monovalent VDV2 immunization induces high level responses against the four serotypes in all the monkeys, unlike a system comprising 2 immunizations of tetravalent vaccine.

- As expected, the initial immunization performed with VDV2 induces a response which is almost exclusively directed against serotype 2, with a low level of cross-reactivity against serotypes 1 and 4 in some animals.

- Viremia is observed with VDV2 after initial immunization, and is predominantly caused by CYD-4 after the second administration (b) (group 1). No noteworthy differences were observed in the viremia induced after a first immunization with tetravalent vaccine in naïve animals (group 2). It can therefore be concluded that the system proposed by the invention does not encourage the emergence of viremia of serotypes 1, 3 and 4 after the second administration.

The examples therefore show that the method of immunization according to this invention improves the immunogenicity of the dengue fever vaccinal viruses without adversely affecting the latter's safety.

All the publications cited in the present application are incorporated in their entirety by way of reference.

2007327367 09 Apr 2013

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

2007327367 09 Apr 2013

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. The use of dengue fever vaccinal viruses for the manufacture of a monovalent vaccine and a tetravalent vaccine for immunization against dengue fever virus, comprising:

(a) a first administration of a monovalent vaccine comprising a vaccinal virus for a first serotype of dengue fever, wherein said vaccinal virus is selected from the group consisting of the strains VDV1 and VDV2,

(b) a second administration of a tetravalent vaccine comprising vaccinal viruses for the 4 serotypes of dengue fever, and in which the tetravalent vaccine is administered at least 30 days and not more than 12 months after administration of the monovalent vaccine.

2. The use as claimed in claim 1, in which the vaccinal viruses used in the tetravalent vaccine are CYD DEN-1, 2, 3 and 4.

3. The use as claimed in claim 1 or claim 2, in which the quantity of dengue fever vaccinal viruses of serotypes 1, 2, 3 and 4 lies within a range from 10^3 to 10^6 $CCID_{50}$.

4. The use as claimed in any one of claims 1 to 3, in which the monovalent vaccine comprises 10^4 $CCID_{50}$ of VDV1 or VDV2 and the tetravalent vaccine comprises 10^5 $CCID_{50}$ of CYDDEN-1, 2, 3 and 4.

5. The use as claimed in any one of claims 1 to 3, in which the monovalent vaccine comprises 10^4 $CCID_{50}$of VDV1 or VDV2 and the tetravalent vaccine comprises 10^5 $CCID_{50}$ of CYD DEN-1, 2, 3 and 10^3 $CCID_{50}$ of CYD DEN-4.

6. The use as claimed in any one of claims 1 to 5, in which the second administration (b) is performed 30 to 60 days after the first administration (a).

7. An immunization kit against dengue fever virus comprising a box containing at least:

2007327367 09 Apr 2013

(a) a first container holding a monovalent vaccine comprising a vaccinal virus of a first serotype of dengue fever, wherein said vaccinal virus is selected from the group consisting of the strains VDV1 and VDV2,

(b) a second container holding a tetravalent vaccine comprising vaccinal viruses for the 4 serotypes of dengue fever, when used for sequential administration of the vaccines in (a) and (b), wherein the tetravalent vaccine of (b) is administered at least 30 days and not more than 12 months after administration of the monovalent vaccine of (a).

8. The immunization kit as claimed in claim 7, in which said second container holds a tetravalent vaccine comprising the 4CYD DEN-1,2,3 and 4.

9. The immunization kit as claimed in claim 8, in which the monovalent vaccine comprises 10^4 $CCID_{50}$ of VDV1 or VDV2 and the tetravalent vaccine comprises 10^5 $CCID_{50}$ of CYD DEN-1,2,3 and 4.

10. The immunization kit as claimed in claim 8, in which the monovalent vaccine comprises 10^4 $CCID_{50}$ of VDV1 or VDV2 and the tetravalent vaccine comprises 10^5 $CCID_{50}$ of CYD DEN-1,2,3 and 10^3 $CCID_{50}$ of CYD DEN-4.

11. The immunization kit as claimed in any one of claims 7 to 10, wherein the tetravalent vaccine of (b) is administered 30 to 60 days after administration of the monovalent vaccine of (a).

12. A method for inducing a neutralising antibody response against the 4 serotypes of dengue fever in a patient, comprising:

(a) a first administration of a monovalent vaccine comprising a vaccinal virus of a first serotype of dengue fever, wherein said vaccinal virus is selected from the group consisting of the strains VDV1 and VDV2,

(b) a second administration of a tetravalent vaccine comprising vaccinal viruses of the 4 serotypes of dengue fever, and

2007327367 09 Apr 2013

in which the second administration (b) is made at least 30 days and not more than 12 months after the first administration (a).

13. The method as claimed in claim 12, in which the vaccinal viruses used in the tetravalent vaccine are CYD DEN-1, 2, 3 and 4.

14. The method as claimed in claim 12 or claim 13, in which the quantity vaccinal viruses of dengue fever of serotypes 1, 2, 3 and 4 lies within a range from 10^3 to 10^6 $CCID_{50}$.

15. The method as claimed in any one of claims 12 to 14, in which the monovalent vaccine comprises 10^4 $CCID_{50}$ of VDV1 or VDV2 and the tetravalent vaccine comprises 10^5 $CCID_{50}$ of CYD DEN-1, 2, 3 and 4.

16. The method as claimed in any one of claims 12 to 14, in which the monovalent vaccine comprises 10^4 $CCID_{50}$ of VDV1 or VDV2 and the tetravalent vaccine comprises 10^5 $CCID_{50}$ of CYD DEN-1, 2, 3 and 10^3 $CCID_{50}$ of CYD DEN-4.

17. The method as claimed in any one of claims 12 to 16, in which the second administration (b) is performed 30 to 60 days after the first administration (a).

18. The use as claimed in claim 1, the immunization kit as claimed in claim 7 or the method as claimed in claim 12, substantially as herein described and with reference to any of the Examples and/or sequence listing.

SEQUENCE LISTING

<110> Sanofi Pasteur

<120> Immunization protocol against the 4 dengue serotypes

<130> PM 0608

<160> 2

<170> PatentIn version 3.3

<210> 1
<211> 10735
<212> DNA
<213> Dengue virus

<400> 1

agttgttagt ctacgtggac cgacaagaac agtttcgaat cggaagcttg cttaacgtag 60

ttctaacagt tttttattag agagcagatc tctgatgatc aaccaacgaa aaaagacggg 120

tcgaccgtct ttcaatatgc tgaaacgcgc gagaaaccgc gtgtcaactg tttcacagtt 180

ggcgaagaga ttctcaaaag gattgctctc aggccaagga cccatgaaat tggtgatggc 240

tttcatagca ttcttaagat ttctagccat acccccaaca gcaggaattt tggctagatg 300

gggctcattc aagaagaatg gagcgattaa agtgttacgg ggtttcaaga gagaaatctc 360

aaacatgcta aacataatga acaggaggaa aagatccgtg accatgctcc ttatgctgct 420

gcccacagcc ctggcgttcc atctgacgac acgaggggga gagccgcata tgatagttag 480

caagcaggaa agaggaaagt cacttttgtt caagacctct gcaggtgtca acatgtgcac 540

cctcattgcg atggatttgg gagagttgtg tgaggacacg atgacctaca aatgcccccg 600

gatcactgag gcggaaccag atgacgttga ctgttggtgc aatgccacgg acacatgggt 660

gacctatgga acgtgctctc aaactggcga acaccgacga gacaaacgtt ccgtcgcatt 720

ggccccacac gtggggcttg gcctagaaac aagagccgaa acgtggatgt cctctgaagg 780

tgcttggaaa cagatacaaa aagtagagac ttgggctctg agacatccag gattcacggt 840

gatagccctt tttctagcac atgccatagg aacatccatc acccagaaag ggatcatttt 900

cattttgctg atgctggtaa caccatctat ggccatgcga tgcgtgggaa taggcaacag 960

agacttcgtg gaaggactgt caggagcaac atgggtggat gtggtactgg agcatggaag 1020

ttgcgtcacc accatggcaa aaaacaaacc aacactggac attgaactct tgaagacgga 1080

ggtcacaaac cctgcagttc tgcgtaaatt gtgcattgaa gctaaaatat caaacaccac 1140

caccgattcg agatgtccaa cacaaggaga agccacactg gtggaagaac aagacgcgaa 1200

ctttgtgtgc cgacgaacgt tcgtggacag aggctggggc aatggctgtg ggctattcgg 1260

aaaaggtagt ctaataacgt gtgccaagtt taagtgtgtg acaaaactag aaggaaagat 1320

agctcaatat gaaaacctaa aatattcagt gatagtcacc gtccacactg gagatcagca 1380

ccaggtggga aatgagacta cagaacatgg aacaactgca accataacac ctcaagctcc 1440

tacgtcggaa atacagctga ccgactacgg aacccttaca ttagattgtt cacctaggac 1500

agggctagat tttaacgaga tggtgttgct gacaatgaaa aagaaatcat ggcttgtcca 1560

caaacagtgg tttctagact taccactgcc ttggacctct ggggctttaa catcccaaga 1620

gacttggaac agacaagatt tactggtcac atttaagaca gctcatgcaa agaagcagga 1680

agtagtcgta ctaggatcac aagaaggagc aatgcacact gcgctgactg gagcgacaga 1740

aatccaaacg tcaggaacga caacaatttt cgcaggacac ctaaaatgca gactaaaaat 1800

ggacaaacta actttaaaag ggatgtcata tgtgatgtgc acaggctcat tcaagttaga 1860

gaaagaagtg gctgagaccc agcatggaac tgttctggtg caggttaaat atgaaggaac 1920

agacgcacca tgcaagattc ccttttcgac ccaagatgag aaaggagcaa cccagaatgg 1980

gagattaata acagccaacc ccatagtcac tgacaaagaa aaaccagtca atattgaggc 2040

agaaccaccc tttggtgaga gctacatcgt ggtaggagca ggtgaaaaag ctttgaaact 2100

aagctggttc aagaaaggaa gcagcatagg gaaaatgttt gaagcaactg cccgaggagc 2160

acgaaggatg gccattctgg gagacaccgc atgggacttc ggttctatag gaggagtgtt 2220

cacgtctatg ggaaaactgg tacaccaggt ttttggaact gcatatggag ttttgtttag 2280

cggagtttct tggaccatga aaataggaat agggattctg ctgacatggc taggattaaa 2340

ttcaaggaac acgtcccttt cggtgatgtg catcgcagtt ggcatggtca cactgtacct 2400

aggagtcatg gttcaggcag attcgggatg tgtaatcaac tggaaaggca gagaacttaa 2460

atgtggaagc ggcatttttg tcactaatga agttcacact tggacagagc aatacaaatt 2520

ccaggctgac tcccccaaga gactatcagc agccattggg aaggcatggg aggagggtgt 2580

gtgtggaatc cgatcagcca ctcgtctcga gaacatcatg tggaaacaaa tatcaaatga 2640

attgaaccac atcctacttg aaaatgacat gaaatttaca gtggtcgtgg gagacgttag 2700

tggaatcttg gcccaaggaa aaaaaatgat taggccacaa cccatggaac acaaatactc 2760

gtggaaaagc tggggaaaag ctaaaatcat aggagcggat gtacagaaca ccaccttcat 2820

catcgacggc ccaaacaccc cagaatgccc tgacaatcaa agagcatgga atatttggga 2880

agtagaggac tatggatttg ggattttcac gacaaacata tggttgaaat tgcgtgactc 2940

ctacacccaa gtatgtgacc accggctgat gtcagctgcc attaaggaca gcaaggcagt 3000

ccatgctgac atggggtact ggatagaaag tgaaaagaac gagacatgga agttggcgag 3060

agcctccttt atagaagtta agacatgcat ctggccaaaa tcccacactc tatggagcaa 3120

tggagttctg gaaagtgaaa tgataattcc aaagatatat ggaggaccaa tatctcagca 3180

caactacaga ccaggatatt tcacacaaac agcagggccg tggcacctag gcaagttgga 3240

actagatttc gatttttgtg aaggtaccac agttgttgtg gatgaacatt gtggaaatcg 3300

aggaccatct ctcagaacca caacagtcac aggaaagata atccatgaat ggtgctgcag 3360

atcttgtacg ctacccccccc tacgtttcaa aggggaagac gggtgttggt acggcatgga 3420

aatcagacca gtgaaggaca aggaagagaa cctggtcaag tcaatggtct ctgcagggtc 3480

aggagaagtg gacagctttt cactaggact gctatgcata tcaataatga ttgaagaagt 3540

gatgagatcc agatggagca aaaaaatgct gatgactgga acactggctg tgttcctcct 3600

tcttataatg ggacaattga catggagtga tctgatcagg ttatgtatta tggttggagc 3660

caacgcttca gacaagatgg ggatgggaac aacgtaccta gctttaatgg ccactttcaa 3720

aatgagacca atgttcgccg tcgggctatt atttcgcaga ctaacatcta gagaagttct 3780

tcttcttaca attggcttga gcctggtggc atccgtggag ctaccaagtt ccctagagga 3840

gctgggggat ggacttgcaa taggcatcat gatgttgaaa ttattgactg attttcagtc 3900

acaccagcta tgggctactc tgctatcctt gacatttatt aaaacaactt tttcattgca 3960

ctatgcatgg aagacaatgg ctatggtact gtcaattgta tctctcttcc ctttatgcct 4020

gtccacgacc tctcaaaaaa caacatggct tccggtgctg ttgggatctc ttggatgcaa 4080

accactaccc atgtttctta taacagaaaa caaaatctgg ggaaggaaga gttggcccct 4140

caatgaagga attatggctg ttggaatagt tagtattcta ctaagttcac ttttaaaaaa 4200

tgatgtgccg ctagccggcc cattaatagc tggaggcatg ctaatagcat gttatgtcat 4260

atccggaagc tcagctgatt tatcactgga gaaagcggct gaggtctcct gggaggaaga 4320

agcagaacac tcaggcgcct cacacaacat actagtagag gttcaagatg atggaaccat 4380

gaagataaaa gatgaagaga gagatgacac gctcaccatt ctccttaaag caactctgct 4440

ggcagtctca ggggtgtacc caatgtcaat accagcgacc ctttttgtgt ggtatttttg 4500

gcagaaaaag aaacagagat caggagtgct atgggacaca cccagccccc cagaagtgga 4560

aagagcagtt cttgatgatg gcatctatag aattttgcaa agaggactgt tgggcaggtc 4620

ccaagtagga gtaggagttt tccaagaagg cgtgttccac acaatgtggc acgtcactag 4680

gggagctgtc ctcatgtatc aaggaaaaag gctggaacca agctgggcca gtgtcaaaaa 4740

agacttgatc tcatatggag gaggttggag gtttcaagga tcctggaaca cgggagaaga 4800

agtacaggtg attgctgttg aaccgggaaa aaaccccaaa aatgtacaaa caacgccggg 4860

taccttcaag acccctgaag gcgaagttgg agccatagcc ttagacttta aacctggcac 4920

atctggatct cccatcgtaa acagagaggg aaaaatagta ggtctttatg gaaatggagt 4980

ggtgacaaca agcggaactt acgttagtgc catagctcaa gctaaggcat cacaagaagg 5040

gcctctacca gagattgagg acaaggtgtt taggaaaaga aacttaacaa taatggacct 5100

acatccagga tcgggaaaaa caagaagata ccttccagcc atagtccgtg aggccataaa 5160

aaggaagctg cgcacgctaa tcctagctcc cacaagagtt gtcgcttctg aaatggcaga 5220

ggcactcaag ggagtgccaa taaggtatca gacaacagca gtgaagagtg aacacacagg 5280

aaaggagata gttgacctta tgtgccacgc cactttcacc atgcgcctcc tgtctcccgt 5340

gagagttccc aattataaca tgattatcat ggatgaagca cacttcaccg atccagccag 5400

catagcagcc agagggtaca tctcaacccg agtgggtatg ggtgaagcag ctgcgatctt 5460

tatgacagcc actcccccag gatcggtgga ggcctttcca cagagcaatg caattatcca 5520

agatgaggaa agagacattc ctgagagatc atggaactca ggctatgact ggatcactga 5580

ttttccaggt aaaacagtct ggtttgttcc aagcatcaaa tcaggaaatg acattgccaa 5640

ctgtttaaga aaaaacggga aacgggtgat ccaattgagc agaaaaacct ttgacactga 5700

gtaccagaaa acaaaaaaca acgactggga ctatgtcgtc acaacagaca tttccgaaat 5760

gggagcaaat ttccgggccg acagggtaat agacccaagg cggtgtctga aaccggtaat 5820

actaaaagat ggtccagagc gcgtcattct agccggaccg atgccagtga ctgtggccag 5880

tgccgcccag aggagaggaa gaattggaag gaaccaaaac aaggaaggtg atcagtatat 5940

ttacatggga cagcctttaa aaaatgatga ggaccacgct cattggacag aagcaaagat 6000

gctccttgac aatataaaca caccagaagg gattatccca gccctctttg agccggagag 6060

agaaaagagt gcagctatag acggggaata cagactgcgg ggtgaagcaa ggaaaacgtt 6120

cgtggagctc atgagaagag gggatctacc agtctggcta tcctacaaag ttgcctcaga 6180

aggcttccag tactccgaca gaaggtggtg cttcgatggg gaaaggaaca accaggtgtt 6240

ggaggagaac atggacgtgg agatctggac aaaagaagga gaaagaaaga aactacgacc 6300

tcgctggttg gacgccagaa catactctga cccactggct ctgcgcgagt ttaaagagtt 6360

tgcagcagga agaagaagcg tctcaggtga cctaatatta gaaataggga aacttccaca 6420

acatttgacg caaagggccc agaatgcttt ggacaacttg gtcatgttgc acaattccga 6480

acaaggagga aaagcctata gacatgctat ggaagaactg ccagacacaa tagaaacgtt 6540

gatgctccta gccttgatag ctgtgttgac tggtggagtg acgctgttct tcctatcagg 6600

aagaggtcta ggaaaaacat ctatcggctt actctgcgtg atggcctcaa gcgcactgtt 6660

atggatggcc agtgtggagc cccattggat agcggcctcc atcatactgg agttctttct 6720

gatggtactg cttattccag agccagacag acagcgcact ccacaggaca accagctagc 6780

atatgtggtg ataggtctgt tattcgtgat attgacagtg gcagccaatg agatgggatt 6840

attggaaacc acaaagaaag acctggggat tggccatgta gctgctgaaa accaccacca 6900

tgctacaatg ctggacgtag acctacatcc agcttcagcc tggaccctct atgcagtggc 6960

cacaacaatc atcactccta tgatgagaca cacaattgaa aacacaacgg caaatatttc 7020

cctgacagcc atcgcaaacc aagcagctat attgatggga cttgacaagg gatggccaat 7080

atcgaagatg gacataggag ttccacttct cgccttgggg tgctattccc aagtgaatcc 7140

gctgacactg atagcggcag tattgatgct agtagctcat tacgccataa ttggacctgg 7200

actgcaagca aaagctacta gagaagctca aaaaagaaca gcggctggaa taatgaaaaa 7260

tccaactgtc gacgggattg ttgcaataga cttagatccc gtggtttacg atgcaaaatt 7320

tgaaaaacag ctaggccaaa taatgttgtt gatactttgc acatcacaga ttcttttgat 7380

gcggactaca tgggccttgt gtgaatccat cacattggct actggacctc tgaccactct 7440

ttgggaggga tctccaggaa aattctggaa caccacaata gcggtatcca tggcaaacat 7500

tttcaggggg agttatctag caggagcagg tctggccttc tcattaatga aatctctagg 7560

aggaggtagg agaggcacgg gagcccaagg ggaaacactg ggagaaaaat ggaaaagaca 7620

actaaaccaa ctgagcaagt cagaattcaa tacttacaag aggagtggga ttatggaggt 7680

ggatagatcc gaagccaaag agggactgaa aagaggagaa acaaccaaac acgcagtatc 7740

gagaggaacg gccaaactga ggtggttcgt ggagaggaac cttgtgaaac cagaagggaa 7800

agtcatagac ctcggttgtg gaagaggtgg ctggtcatat tattgcgctg ggctgaagaa 7860

agtcacagaa gtgaaaggat acacaaaagg aggacctgga catgaggaac caatcccaat 7920

ggcgacctat ggatggaacc tagtaaggct gcactccgga aaagatgtat tttttatacc 7980

acctgagaaa tgtgacaccc ttttgtgtga tattggtgag tcctctccga acccaactat 8040

agaggaagga agaacgttac gtgttctgaa aatggtggaa ccatggctca gaggaaacca 8100

attttgcata aaaattctaa atccctatat gccgagcgtg gtagaaactc tggaacaaat 8160

gcaaagaaaa catggaggaa tgctagtgcg aaacccactc tcaagaaatt ccacccatga 8220

aatgtactgg gtttcatgtg gaacaggaaa cattgtgtca gcagtaaaca tgacatctag 8280

aatgttgcta aatcggttca caatggctca caggaagcca acatatgaaa gagacgtgga 8340

cttaggcgct ggaacaagac atgtggcagt agaaccagag gtagccaacc tagatatcat 8400

tggccagagg atagagaata taaaaaatga acataagtca acatggcatt atgatgagga 8460

caatccatac aaaacatggg cctatcatgg atcatatgag gttaagccat caggatcggc 8520

ctcatccatg gtcaatggcg tggtgagatt gctcaccaaa ccatgggatg ttatccccat 8580

ggtcacacaa atagccatga ctgataccac acccttgga caacagaggg tgtttaaaga 8640

gaaagttgac acgcgcacac caaaagcaaa acgtggcaca gcacaaatta tggaagtgac 8700

agccaggtgg ttatggggtt tcctttctag aaacaaaaaa cccagaattt gcacaagaga 8760

ggagtttaca agaaaagtta ggtcaaacgc agctattgga gcagtgttcg ttgatgaaaa 8820

tcaatggaac tcggcaaaag aagcagtgga agacgaacgg ttctgggaac ttgtccacag 8880

agagagggag cttcataaac aggggaaatg tgccacgtgt gtctacaata tgatggggaa 8940

gagagagaaa aaattaggag agttcggaaa ggcaaaagga agtcgtgcaa tatggtacat 9000

gtggttggga gcacgcttcc tagagtttga agcccttggt ttcatgaatg aagatcactg 9060

gttcagtaga gagaattcac tcagtggagt ggaaggagaa ggactccaca aacttggata 9120

catactcaga gacatatcaa ggattccagg ggggaacatg tatgcagatg acacagccgg 9180

atgggacaca agaataacag aggatgatct ccagaatgag gctaaaatca ctgacatcat 9240

ggagcccgaa catgccctgc tggctacgtc aatctttaag ctgacctacc aaaataaggt 9300

ggtaagggtg cagagaccag caaaaaatgg aaccgtgatg gatgttatat ccagacgtga 9360

ccagagaggc agtggacagg ttggaactta tggcttaaac actttcacca acatggaggc 9420

ccaactgata agacaaatgg agtctgaggg aatcttttta cccagcgaat tggaaacccc 9480

aaatctagcc ggaagagttc tcgactggtt ggaaaaatat ggtgtcgaaa ggctgaaaag 9540

aatggcaatc agcggagatg actgtgtggt gaaaccaatt gatgacaggt tcgcaacagc 9600

cttaacagct ttgaatgaca tgggaaaagt aagaaaagac ataccacaat gggaaccttc 9660

aaaaggatgg aatgattggc aacaagtgcc tttctgttca caccacttcc accagctaat 9720

tatgaaggat gggagggaga tagtggtgcc atgccgcaac caagatgaac ttgtggggag 9780

ggccagagta tcacaaggcg ccggatggag cctgagagaa accgcatgcc taggcaagtc 9840

atatgcacaa atgtggcagc tgatgtattt ccacaggaga gacctgagac tggcggctaa 9900

cgctatttgt tcagccgttc cagttgattg ggtcccaacc agccgcacca cctggtcgat 9960

ccatgcccat caccaatgga tgacaacaga agacatgtta tcagtatgga atagggtctg 10020

gatagaggaa aacccatgga tggaggataa gactcatgtg tccagttggg aagaagttcc 10080

atacctagga aagagggaag atcagtggtg tggatccctg ataggcttaa cagcaagggc 10140

cacctgggcc actaatatac aagtggccat aaaccaagtg agaaggctca ttgggaatga 10200

gaattatcta gattacatga catcaatgaa gagattcaag aatgagagtg atcccgaagg 10260

ggcactctgg taagtcaaca cattcacaaa ataaaggaaa ataaaaaatc aaatgaggca 10320

agaagtcagg ccagattaag ccatagtacg gtaagagcta tgctgcctgt gagccccgtc 10380

caaggacgta aaatgaagtc aggccgaaag ccacggtttg agcaagccgt gctgcctgtg 10440

gctccatcgt ggggatgtaa aaacccggga ggctgcaacc catggaagct gtacgcatgg 10500

ggtagcagac tagtggttag aggagacccc tcccaagaca caacgcagca gcggggccca 10560

acaccagggg aagctgtacc ctggtggtaa ggactagagg ttagaggaga ccccccgcgt 10620

aacaataaac agcatattga cgctgggaga gaccagagat cctgctgtct ctacagcatc 10680

attccaggca cagaacgcca gaaaatggaa tggtgctgtt gaatcaacag gttct 10735

<210> 2
<211> 10723
<212> DNA
<213> Dengue virus

<400> 2
agttgttagt ctacgtggac cgacaaagac agattctttg agggagctaa gctcaatgta 60

gttctaacag ttttttaatt agagagcaga tctctgatga ataaccaacg gaaaaaggcg 120

aaaaacacgc ctttcaatat gctgaaacgc gagagaaacc gcgtgtcgac tgtgcaacag 180

ctgacaaaga gattctcact tggaatgctg cagggacgag gaccattaaa actgttcatg 240

gccctggtgg cgttccttcg tttcctaaca atcccaccaa cagcagggat attgaagaga 300

tggggaacaa ttaaaaaatc aaaagctatt aatgttttga gagggttcag gaaagagatt 360

ggaaggatgc tgaacatctt gaataggaga cgcagatctg caggcatgat cattatgctg 420

attccaacag tgatggcgtt ccatttaacc acacgtaacg gagaaccaca catgatcgtc 480

agcagacaag agaaagggaa aagtcttctg tttaaaacag aggttggcgt gaacatgtgt 540

accctcatgg ccatggacct tggtgaattg tgtgaagaca caatcacgta caagtgtccc 600

cttctcaggc agaatgagcc agaagacata gactgttggt gcaactctac gtccacgtgg 660

gtaacttatg ggacgtgtac caccatggga gaacatagaa gagaaaaaag atcagtggca 720

ctcgttccac atgtgcgaat gggactggag acacgaactg aaacatggat gtcatcagaa 780

ggggcctgga aacatgtcca gagaattgaa acttggatct tgagacatcc aggcttcacc 840
atgatggcag caatcctggc atacaccata ggaacgacac atttccaaag agccctgatt 900
ttcatcttac tgacagctgt cactccttca atgacaatgc gttgcatagg aatgtcaaat 960
agagactttg tggaaggggt ttcaggagga agctgggttg acatagtctt agaacatgga 1020
agctgtgtga cgacgatggc aaaaaacaaa ccaacattgg attttgaact gataaaaaca 1080
gaagccaaac agcctgccac cctaaggaag tactgtatag aggcaaagct aaccaacaca 1140
acaacagaat ctcgctgccc aacacaaggg gaacccagcc taaatgaaga gcaggacaaa 1200
aggttcgtct gcaaacactc catggtagac agaggatggg gaaatggatg tggactattt 1260
ggaaagggag gcattgtgac ctgtgctatg ttcagatgca aaaagaacat ggaaggaaaa 1320
gttgtgcaac cagaaaactt ggaatacacc attgtgataa cacctcactc aggggaagag 1380
catgcagtcg gaaatgacac aggaaaacat ggcaaggaaa tcaaaataac accacagagt 1440
tccatcacag aagcagaatt gacaggttat ggcactgtca caatggagtg ctctccaaga 1500
acgggcctcg acttcaatga gatggtgttg ctgcagatgg aaaataaagc ttggctggtg 1560
cacaggcaat ggttcctaga cctgccgtta ccatggttgc ccggagcgga cacacaagag 1620
tcaaattgga tacagaagga gacattggtc actttcaaaa atccccatgc gaagaaacag 1680
gatgttgttg ttttaggatc ccaagaaggg gccatgcaca cagcacttac aggggccaca 1740
gaaatccaaa tgtcatcagg aaacttactc ttcacaggac atctcaagtg caggctgaga 1800
atggacaagc tacagctcaa aggaatgtca tactctatgt gcacaggaaa gtttaaagtt 1860
gtgaaggaaa tagcagaaac acaacatgga acaatagtta tcagagtgca atatgaaggg 1920
gacggctctc catgcaagat ccctttttgag ataatggatt tggaaaaaag acatgtctta 1980
ggtcgcctga ttacagtcaa cccaattgtg acagaaaaag atagcccagt caacatagaa 2040
gcagaacctc catttggaga cagctacatc atcataggag tagagccggg acaactgaag 2100
ctcaactggt ttaagaaagg aagttctatc ggccaaatgt ttgagacaac aatgaggggg 2160
gcgaagagaa tggccatttt aggtgacaca gcctgggatt ttggatcctt gggaggagtg 2220
tttacatcta taggaaaggc tctccaccaa gtctttggag caatctatgg agctgccttc 2280
agtggggttt catggactat gaaaatcctc ataggagtca ttatcacatg gataggaatg 2340
aattcacgca gcacctcact gtctgtgaca ctagtattgg tgggaattgt gacactgtat 2400
ttgggagtca tggtgcaggc cgatagtggt tgcgttgtga gctggaaaaa caaagaactg 2460
aaatgtggca gtgggatttt catcacagac aacgtgcaca catggacaga acaatacaaa 2520
ttccaaccag aatccccttc aaaactagct tcagctatcc agaaagccca tgaagaggac 2580
atttgtggaa tccgctcagt aacaagactg gagaatctga tgtggaaaca aataacacca 2640
gaattgaatc acattctatc agaaaatgag gtgaagttaa ctattatgac aggagacatc 2700
aaaggaatca tgcaggcagg aaaacgatct ctgcggcctc agcccactga gctgaagtat 2760
tcatggaaaa catggggcaa agcaaaaatg ctctctacag agtctcataa ccagaccttt 2820

ctcattgatg gccccgaaac agcagaatgc cccaacacaa atagagcttg gaattcgttg 2880

gaagttgaag actatggctt tggagtattc accaccaata tatggctaaa attgaaagaa 2940

aaacaggatg tattctgcga ctcaaaactc atgtcagcgg ccataaaaga caacagagcc 3000

gtccatgccg atatgggtta ttggatagaa agtgcactca atgacacatg gaagatagag 3060

aaagcctctt tcattgaagt taaaaactgc cactggccaa aatcacacac cctctggagc 3120

aatggagtgc tagaaagtga gatgataatt ccaaagaatc tcgctggacc agtgtctcaa 3180

cacaactata gaccaggcta ccatacacaa ataacaggac catggcatct aggtaagctt 3240

gagatggact ttgatttctg tgatggaaca acagtggtag tgactgagga ctgcggaaat 3300

agaggaccct ctttgagaac aaccactgcc tctggaaaac tcataacaga atggtgctgc 3360

cgatcttgca cattaccacc gctaagatac agaggtgagg atgggtgctg gtacgggatg 3420

gaaatcagac cattgaagga gaaagaagag aatttggtca actccttggt cacagctgga 3480

catgggcagg tcgacaactt ttcactagga gtcttgggaa tggcattgtt cctggaggaa 3540

atgcttagga cccgagtagg aacgaaacat gcaatactac tagttgcagt ttcttttgtg 3600

acattgatca cagggaacat gtcctttaga gacctgggaa gagtgatggt tatggtaggc 3660

gccactatga cggatgacat aggtatgggc gtgacttatc ttgccctact agcagccttc 3720

aaagtcagac caacttttgc agctggacta ctcttgagaa agctgacctc caaggaattg 3780

atgatgacta ctataggaat tgtactcctc tcccagagca ccataccaga gaccattctt 3840

gagttgactg atgcgttagc cttaggcatg atggtcctca aaatggtgag aaatatggaa 3900

aagtatcaat tggcagtgac tatcatggct atcttgtgcg tcccaaacgc agtgatatta 3960

caaaacgcat ggaaagtgag ttgcacaata ttggcagtgg tgtccgtttc cccactgttc 4020

ttaacatcct cacagcaaaa aacagattgg ataccattag cattgacgat caaaggtctc 4080

aatccaacag ctatttttct aacaaccctc tcaagaacca gcaagaaaag gagctggcca 4140

ttaaatgagg ctatcatggc agtcgggatg gtgagcattt tagccagttc tctcctaaaa 4200

aatgatattc ccatgacagg accattagtg gctggagggc tcctcactgt gtgctacgtg 4260

ctcactggac gatcggccga tttggaactg gagagagcag ccgatgtcaa atgggaagac 4320

caggcagaga tatcaggaag cagtccaatc ctgtcaataa caatatcaga agatggtagc 4380

atgtcgataa aaaatgaaga ggaagaacaa acactgacca tactcattag aacaggattg 4440

ctggtgatct caggacttttt tcctgtatca ataccaatca cggcagcagc atggtacctg 4500

tgggaagtga agaaacaacg ggccggagta ttgtgggatg ttccttcacc cccacccatg 4560

ggaaaggctg aactggaaga tggagcctat agaattaagc aaaaagggat tcttggatat 4620

tcccagatcg gagccggagt ttacaaagaa ggaacattcc atacaatgtg gcatgtcaca 4680

cgtggcgctg ttctaatgca taaaggaaag aggattgaac caacatgggc ggacgtcaag 4740

aaagacctaa tatcatatgg aggaggctgg aagttagaag gagaatggaa ggaaggagaa 4800

gaagtccagg tattggcact ggagcctgga aaaaatccaa gagccgtcca aacgaaacct 4860

ggtcttttca aaaccaacgc cggaacaata ggtgctgtat ctctggactt ttctcctgga 4920
acgtcaggat ctccaattat cgacaaaaaa ggaaaagttg tgggtcttta tggtaatggt 4980
gttgttacaa ggagtggagc atatgtgagt gctatagccc agactgaaaa aagcattgaa 5040
gacaacccag agatcgaaga tcacattttc cgaaagagaa gactgaccat catggacctc 5100
cacccaggag cgggaaagac gaagagatac cttccggcca tagtcagaga agctataaaa 5160
cggggtttga gaacattaat cttggccccc actagagttg tggcagctga aatggaggaa 5220
gcccttagag gacttccaat aagataccag accccagcca tcagagctga gcacaccggg 5280
cgggagattg tggacctaat gtgtcatgcc acatttacca tgaggctgct atcaccagtt 5340
agagtgccaa actacaacct gattatcatg gacgaagccc atttcacaga cccagcaagt 5400
atagcagcta gaggatacat ctcaactcga gtggagatgg gtgaggcagc tgggattttt 5460
atgacagcca ctcccccggg aagcagagac ccatttcctc agagcaatgc accaatcata 5520
gatgaagaaa gagaaatccc tgaacgctcg tggaattccg gacatgaatg ggtcacggat 5580
tttaaaggga agactgtttg gttcgttcca agtataaaag caggaaatga tatagcagct 5640
tgcctgagga aaaatggaaa gaaagtgata caactcagta ggaagacctt tgattctgag 5700
tatgtcaaga ctagaaccaa tgattgggac ttcgtggtta caactgacat ttcagaaatg 5760
ggtgccaatt tcaaggctga gagggttata gaccccagac gctgcatgaa accagtcata 5820
ctaacagatg gtgaagagcg ggtgattctg gcaggaccta tgccagtgac ccactctagt 5880
gcagcacaaa gaagagggag aataggaaga aatccaaaaa atgagaatga ccagtacata 5940
tacatggggg aacctctgga aaatgatgaa gactgtgcac actggaaaga agctaaaatg 6000
ctcctagata acatcaacac gccagaagga atcattccta gcatgttcga accagagcgt 6060
gaaaaggtgg atgccattga tggcgaatac cgcttgagag gagaagcaag gaaaaccttt 6120
gtagacttaa tgagaagagg agacctacca gtctggttgg cctacagagt ggcagctgaa 6180
ggcatcaact acgcagacag aaggtggtgt tttgatggag tcaagaacaa ccaaatccta 6240
gaagaaaacg tggaagttga aatctggaca aaagaagggg aaaggaagaa attgaaaccc 6300
agatggttgg atgctaggat ctattctgac ccactggcgc taaaagaatt taaggaattt 6360
gcagccggaa gaaagtctct gaccctgaac ctaatcacag aaatgggtag gctcccaacc 6420
ttcatgactc agaaggcaag agacgcactg gacaacttag cagtgctgca cacggctgag 6480
gcaggtggaa gggcgtacaa ccatgctctc agtgaactgc cggagaccct ggagacattg 6540
cttttactga cacttctggc tacagtcacg ggagggatct ttttattctt gatgagcgca 6600
aggggcatag ggaagatgac cctgggaatg tgctgcataa tcacggctag catcctccta 6660
tggtacgcac aaatacagcc acactggata gcagcttcaa taatactgga gtttttctc 6720
atagttttgc ttattccaga acctgaaaaa cagagaacac cccaagacaa ccaactgacc 6780
tacgttgtca tagccatcct cacagtggtg gccgcaacca tggcaaacga gatgggtttc 6840
ctagaaaaaa cgaagaaaga tctcggattg ggaagcattg caacccagca acccgagagc 6900

aacatcctgg acatagatct acgtcctgca tcagcatgga cgctgtatgc cgtggccaca 6960
acatttgtta caccaatgtt gagacatagc attgaaaatt cctcagtgaa tgtgtcccta 7020
acagctatag ccaaccaagc cacagtgtta atgggtctcg ggaaaggatg gccattgtca 7080
aagatggaca tcggagttcc ccttctcgcc attggatgct actcacaagt caaccccata 7140
actctcacag cagctctttt cttattggta gcacattatg ccatcatagg gccaggactc 7200
caagcaaaag caaccagaga agctcagaaa agagcagcgg cgggcatcat gaaaaaccca 7260
actgtcgatg gaataacagt gattgaccta gatccaatac cttatgatcc aaagtttgaa 7320
aagcagttgg gacaagtaat gctcctagtc ctctgcgtga ctcaagtatt gatgatgagg 7380
actacatggg ctctgtgtga ggctttaacc ttagctaccg ggcccatctc cacattgtgg 7440
gaaggaaatc cagggaggtt ttggaacact accattgcgg tgtcaatggc taacattttt 7500
agagggagtt acttggccgg agctggactt ctcttttcta ttatgaagaa cacaaccaac 7560
acaagaaggg gaactggcaa cataggagag acgcttggag agaaatggaa aagccgattg 7620
aacgcattgg gaaaaagtga attccagatc tacaagaaaa gtggaatcca ggaagtggat 7680
agaaccttag caaaagaagg cattaaaaga ggagaaacgg accatcacgc tgtgtcgcga 7740
ggctcagcaa aactgagatg gttcgttgag agaaacatgg tcacaccaga agggaaagta 7800
gtggacctcg gttgtggcag aggaggctgg tcatactatt gtggaggact aaagaatgta 7860
agagaagtca aaggcctaac aaaaggagga ccaggacacg aagaacccat ccccatgtca 7920
acatatgggt ggaatctagt gcgtcttcaa agtggagttg acgttttctt catcccgcca 7980
gaaaagtgtg acacattatt gtgtgacata ggggagtcat caccaaatcc cacagtggaa 8040
gcaggacgaa cactcagagt ccttaactta gtagaaaatt ggttgaacaa caacactcaa 8100
ttttgcataa aggttctcaa cccatatatg ccctcagtca tagaaaaaat ggaagcacta 8160
caaaggaaat atggaggagc cttagtgagg aatccactct cacgaaactc cacacatgag 8220
atgtactggg tatccaatgc ttccgggaac atagtgtcat cagtgaacat gatttcaagg 8280
atgttgatca acagatttac aatgagatac aagaaagcca cttacgagcc ggatgttgac 8340
ctcggaagcg gaacccgtaa catcgggatt gaaagtgaga taccaaacct agatataatt 8400
gggaaaagaa tagaaaaaat aaagcaagag catgaaacat catggcacta tgaccaagac 8460
cacccataca aaacgtgggc ataccatggt agctatgaaa caaaacagac tggatcagca 8520
tcatccatgg tcaacggagt ggtcaggctg ctgacaaaac cttgggacgt tgtccccatg 8580
gtgacacaga tggcaatgac agacacgact ccatttggac aacagcgcgt ttttaaagag 8640
aaagtggaca cgagaaccca agaaccgaaa gaaggcacga agaaactaat gaaaataaca 8700
gcagagtggc tttggaaaga attagggaag aaaaagacac ccaggatgtg caccagagaa 8760
gaattcacaa gaaaggtgag aagcaatgca gccttggggg ccatattcac tgatgagaac 8820
aagtggaagt cggcacgtga ggctgttgaa gatagtaggt tttgggagct ggttgacaag 8880
gaaaggaatc tccatcttga aggaaagtgt gaaacatgtg tgtacaacat gatgggaaaa 8940

agagagaaga agctagggga attcggcaag gcaaaaggca gcagagccat atggtacatg 9000
tggcttggag cacgcttctt agagtttgaa gccctaggat tcttaaatga agatcactgg 9060
ttctccagag agaactccct gagtggagtg gaaggagaag ggctgcacaa gctaggttac 9120
attctaagag acgtgagcaa gaaagaggga ggagcaatgt atgccgatga caccgcagga 9180
tgggatacaa aaatcacact agaagaccta aaaaatgaag agatggtaac aaaccacatg 9240
gaaggagaac acaagaaact agccgaggcc attttcaaac taacgtacca aaacaaggtg 9300
gtgcgtgtgc aaagaccaac accaagaggc acagtaatgg acatcatatc gagaagagac 9360
caaagaggta gtggacaagt tggcacctat ggactcaata ctttcaccaa tatggaagcc 9420
caactaatca gacagatgga gggagaagga gtctttaaaa gcattcagca cctaacaatc 9480
acagaagaaa tcgctgtgca aaactggtta gcaagagtgg ggcgcgaaag gttatcaaga 9540
atggccatca gtggagatga ttgtgttgtg aaacctttag atgacaggtt cgcaagcgct 9600
ttaacagctc taaatgacat gggaaagatt aggaaagaca tacaacaatg ggaaccttca 9660
agaggatgga atgattggac acaagtgccc ttctgttcac accatttcca tgagttaatc 9720
atgaaagacg gtcgcgtact cgttgttcca tgtagaaacc aagatgaact gattggcaga 9780
gcccgaatct cccaaggagc agggtggtct ttgcgggaga cggcctgttt ggggaagtct 9840
tacgcccaaa tgtggagctt gatgtacttc cacagacgcg acctcaggct ggcggcaaat 9900
gctatttgct cggcagtacc atcacattgg gttccaacaa gtcgaacaac ctggtccata 9960
catgctaaac atgaatggat gacaacggaa gacatgctga cagtctggaa cagggtgtgg 10020
attcaagaaa acccatggat ggaagacaaa actccagtgg aaacatggga ggaaatccca 10080
tacttgggga aaagagaaga ccaatggtgc ggctcattga ttgggttaac aagcagggcc 10140
acctgggcaa agaacatcca agcagcaata aatcaagtta gatcccttat aggcaatgaa 10200
gaatacacag attacatgcc atccatgaaa agattcagaa gagaagagga agaagcagga 10260
gttctgtggt agaaagcaaa actaacatga aacaaggcta gaagtcaggt cggattaagc 10320
catagtacgg aaaaaactat gctacctgtg agccccgtcc aaggacgtta aaagaagtca 10380
ggccatcata aatgccatag cttgagtaaa ctatgcagcc tgtagctcca cctgagaagg 10440
tgtaaaaaat ccgggaggcc acaaaccatg gaagctgtac gcatggcgta gtggactagc 10500
ggttagggga gacccctccc ttacaaatcg cagcaacaat gggggcccaa ggcgagatga 10560
agctgtagtc tcgctggaag gactagaggt tagaggagac ccccccgaaa caaaaaacag 10620
catattgacg ctgggaaaga ccagagatcc tgctgtctcc tcagcatcat tccaggcaca 10680
gaacgccaga aaatggaatg gtgctgttga atcaacaggt tct 10723