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(54) Title: DENGUE SEROTYPE 2 ATTENUATED STRAIN

(57) Abrégé/Abstract:
The invention relates to live attenuated VDV2 (VERO-Derived Vaccine Dengue serotype 2) strains which have been derived from the wild-type dengue-2 strain 16681 by passaging on PDK and Vero cells. The invention further relates to a vaccine composition which comprises a VDV2 strain.

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(54) Title: DENGUE SEROTYPE 2 ATTENUATED STRAIN

(57) Abstract: The invention relates to live attenuated VDV2 (VERO-Derived Vaccine Dengue serotype 2) strains which have been derived from the wild-type dengue-2 strain 16681 by passaging on PDK and Vero cells. The invention further relates to a vaccine composition which comprises a VDV2 strain.

Dengue serotype 2 attenuated strain

The invention relates to new live attenuated VDV2 (VERO-Derived Dengue serotype 2 virus) strains which are derived from the wild-type dengue-2 strain 16681 by passaging on PDK and Vero cells sanitization. The invention further relates to a vaccine composition which comprises such VDV2 strain.

Dengue diseases are caused by four closely related, but antigenically distinct, virus serologic types (Gubler, 1988; Kautner et al., 1997; Rigau-Pérez et al., 1998; Vaughn et al., 1997), of the genus Flavivirus (Gubler, 1988). Infection with a dengue virus serotype can produce a spectrum of clinical illnesses ranging from a non-specific viral syndrome to severe, fatal haemorrhagic disease. The incubation period of dengue fever (DF) after the mosquito bite averages 4 days (range 3-14 days). DF is characterised by biphasic fever, headache, pain in various parts of the body, prostration, rash, lymphadenopathy and leukopenia (Kautner et al., 1997; Rigau-Pérez et al., 1998). The viremic period is the same as of febrile illness (Vaughn et al., 1997). Recovery from DF is usually complete in 7 to 10 days but prolonged asthenia is common. Leukocytes and platelets counts decreases are frequent.

Dengue haemorrhagic fever (DHF) is a severe febrile disease characterised by abnormalities of homeostasis and increased vascular permeability that can lead to hypovolemia and hypotension (dengue shock syndrome, DSS) often complicated by severe internal bleeding. The case fatality rate of DHF can be as high as 10% without therapy, but below 1% in most centres with therapeutic experience (WHO Technical Guide, 1986).

Routine laboratory diagnosis of dengue infections are based on virus isolation and/or the detection of dengue virus-specific antibodies.

Dengue disease is the second most important tropical infectious disease after malaria, with over half of the world's population (2.5 billion) living in areas at risk for epidemic transmission. An estimated 50 to 100 million cases of dengue, 500,000 hospitalised DHF patients and 25,000 deaths occur each year. Dengue is endemic in Asia, the Pacific, Africa, Latin America, and the Caribbean. More than 100 tropical countries have endemic dengue virus infections, and DHF have been documented in

more than 60 of these (Gubler, 2002; Monath, 1994). A number of well described factors appear to be involved in dengue infections: population growth, unplanned and uncontrolled urbanisation particularly in association with poverty, increased air travel, lack of effective mosquito control, and the deterioration of sanitary and public health infrastructure (Gubler, 2002). The awareness of dengue in travellers and expatriates is increasing (Shirtcliffe et al., 1998). Dengue has proven to be a major cause of febrile illness among US troops during deployments in dengue-endemic tropical areas (DeFraites et al., 1994).

The viruses are maintained in a cycle that involves humans and *Aedes aegypti*, a domestic, day-biting mosquito that prefers to feed on humans. Human infection is initiated by the injection of virus during blood feeding by an infected *Aedes aegypti* mosquito. Salivary virus is deposited mainly in the extravascular tissues. The primary cell subset infected after inoculation is dendritic cells, which subsequently migrate to draining lymph nodes (Wu et al., 2000). After initial replication in the skin and draining lymph nodes, virus appears in the blood during the acute febrile phase, generally for 3 to 5 days.

Monocytes and macrophages are with dendritic cells among the primary target of dengue virus. Protection against homotypic reinfection is complete and probably lifelong, but cross-protection between dengue types lasts less than 12 weeks (Sabin, 1952). Consequently a subject can experience a second infection with a different serotype. A second dengue infection is a theoretical risk factor of developing severe dengue disease. However, DHF is multifactorial including: the strain of the virus involved, as well as the age, immune status, and genetic predisposition of the patient. Two factors play a major role in the occurrence of DHF: a rapid viral replication with high viremia (the severity of the disease being related to the level of viremia (Vaughn et al., 2000) and an important inflammatory response with release of high levels of inflammatory mediators (Rothman and Ennis, 1999).

There is no specific treatment against Dengue diseases. The management of DF is supportive with bed rest, control of fever and pain with antipyretics and analgesics, and adequate fluid intake. The treatment of DHF needs correction of fluid loss, replacement of coagulation factors, and infusion of heparin.

Preventive measures presently rely on vector control and personal protection measures, which are difficult to enforce and expensive. No vaccine against dengue is currently registered. Since the 4 serotypes of dengue are circulating worldwide and since they are reported to be involved in cases of DHF, vaccination should ideally confer protection against all 4 dengue virus serotypes.

Live attenuated vaccines (LAVs), which reproduce natural immunity, have been used for the development of vaccines against many diseases, including some viruses belonging to the same genus as dengue (examples of commercially available flavivirus live-attenuated vaccines include yellow fever and Japanese encephalitis vaccines). The advantages of live-attenuated virus vaccines are their capacity of replication and induction of both humoral and cellular immune responses. In addition, the immune response induced by a whole virion vaccine against the different components of the virus (structural and non-structural proteins) reproduced those induced by natural infection.

A dengue vaccine project was initiated in Thailand at the Centre for Vaccine Development, Institute of Sciences and Technology for Development Mahidol University. Candidate live-attenuated vaccines were successfully developed, at a laboratory scale, for dengue serotype 1 (strain 16007, passage 13), serotype 2 (strain 16681, passage 53 = LAV2), and serotype 4 (strain 1036, passage 48) viruses in Primary Dog Kidney (PDK) Cells, and for serotype 3 (strain 16562) in Primary Green Monkey Kidney (PGMK) cells (passage 30) and Fetal Rhesus Lung (FRhL) cells (passage 3). These vaccines have been tested as monovalent (single serotype), bivalent (two serotypes), trivalent (three serotypes), and tetravalent (all four serotypes) vaccines in Thai volunteers. Those vaccines were found to be safe and immunogenic in children and in adults (Gubler, 1997). These LAV 1-4 strains have been described in EP 1159968 in the name of the Mahidol University and were deposited before the CNCM (CNCM I-2480; CNCM I-2481; CNCM I-2482 and CNCM I-2483 respectively).

The Den-2 strain 16681 was recovered from serum of a DHF (Dengue Hemorrhagic Fever) patient in Bangkok in 1964 (Halstead *et al.*, 1970). The original viremic serum had been passaged 4 times on BSC-1 cells (African Green Monkey kidney cells) and 5 times on continuous LLC-MK_2 cells (Rhesus Monkey kidney cells). In

1977, the virus was passaged once *in vivo*, in susceptible monkeys (*Macaca Mulatta*), and then again in LLC-MK_2 cells. Two additional passages in mosquitoes (*Toxorhynchites amboinensis*) were conducted in 1980. Virus attenuation was performed by passages at 32°C on PDK cells (Primary Dog Kidney cells). Attenuation of the strain was checked according to several *in vitro* and *in vivo* markers. Passage 50 fullfilled all these attenuation criteria and was chosen as master seed for vaccine production (1982), at passage 53. DEN-2 PDK53 vaccine candidate was evaluated in humans and found to be strongly immunogenic with no untoward clinical signs and symptoms (Bhamarapravati et al., 1989).

The complete sequence of the Dengue 2 Live-Attenuated Virus strain (LAV2) was established by R. Kinney et al. (CDC, Fort Collins) in 2001. Sequence differences between parent DEN-2 strain 16681 (SEQ ID No.3) and LAV2 (SEQ ID No.38) strain are described in Table 1. Thus, genetic comparison of the wild-type virus strain 16681 and LAV2 strain showed a set of 9 point mutations which could be linked to LAV2 attenuation.

Table 1: DEN-2 16681 and DEN-2 16681/PDK53 (LAV2) Sequence Differences

coordinates		LAV2		16681	
Gene-aa	position	Nt	Aa	nt	aa
Non coding	**Nt-57**	**T**	**-**	**C**	**-**
PrM-29	Nt-524	T	**Val**	A	**Asp**
E-373	Nt-2055	T	Phe	C	Phe
NS1-53	**Nt-2579**	**A**	**Asp**	**G**	**Gly**
NS2A-181	Nt-4018	T	**Phe**	C	**Leu**
NS3-250	**Nt-5270**	**A/T**	**Val/Glu**	**A**	**Glu**
NS3-342	Nt-5547	C	Arg	T	Arg
NS4A-75	Nt-6599	C	**Ala**	G	**Gly**
NS5-334	Nt-8571	T	Val	C	Val

Nucleotide changes modifying the corresponding codon are indicated in bold.

The LAV2 strain which was initially established in 1983 was further rapidly identified as potential vaccine candidate (Bhamarapravati and Yoksan, 1997).

However, at that time, transmission to humans of Spongiform Encephalitis through mammalian cultures was not perceived as a risk and the virus was routinely maintained in Primary Dog Kidney cells (PDK). Furthermore, this LAV2 strain

corresponds to a heterogeneous population. This heterogeneity represents an additional risk due to a potential in vitro or in vivo selection of one of the strain present in the composition.

In view of these increasing concerns, the Applicant decided to set up a sanitization process in order to get rid of any such risks. By transfecting Vero cells with the purified genomic RNA of LAV2, followed by three cycles of amplification in Vero cells, and two successive steps of virus plaque purification the Applicant produced a new Vero-Derived serotype 2 virus (VDV2).

This new VDV2 strain which has been thus derived by transfer to VERO cells and biological cloning differs from the LAV2 strain by sequence, an homogenous plaque size and temperature sensitivity but importantly has conserved some phenotypic and genotypic features of the LAV2 such as e.g. attenuation spots, small plaque phenotype, growth restriction at high temperature and has conserved the immunogenic features of the LAV2 strains. These features make this new strain a valuable vaccine candidate for prophylactic immunization in humans.

The present invention is directed to a live attenuated dengue-2 virus strain which comprises sequence SEQ ID No. 38 wherein at least nucleotides at positions 736, 1619, 4723, 5062, 9191, 10063, and 10507, are mutated, with the proviso that the following nucleotides are not mutated : 57, 524, 2055, 2579, 4018, 5547, 6599, and 8571, wherein said live attenuated dengue-2 virus strain is adapted for growth in VERO cells and induces a specific humoral immune response including neutralizing antibodies in primate.

The present invention is also directed to an immunogenic composition comprising the live attenuated dengue-2 virus strain as defined herein, in a pharmaceutially acceptable carrier.

The present invention is also directed to an isolated nucleic acid which comprises the DNA sequence SEQ ID No. 1 or its equivalent RNA sequence.

The present invention is also directed to an isolated polyprotein encoded by SEQ ID No. 1 or fragments thereof of at least 8 amino acids that comprise at least an arginine at position 9 of M protein, and/or a glutamic acid at position 228 of E protein, and/or threonine at position 69 of NS3 protein, and/or a histidine at position 181 of NS3 protein, and/or a lysine at position 541 of NS5 protein, and/or a threonine at position 832 of NS5 protein, wherein said polyprotein or said fragments thereof induce a specific humoral immune response including neutralizing antibodies in primate.

The present invention is also directed to a fragment of the polyprotein as defined herein which comprises M protein, and/or E protein, and/or NS3 protein and/or NS5 protein.

The present invention is also directed to a dengue-2 virus strain encoded by SEQ ID NO : 1.

Definitions

"Dengue viruses" are positive-sense, single-stranded RNA viruses belonging to the Flavivirus genus of the flaviridae family. In the case of dengue serotype 2 (DEN-2) strain 16681, the entire sequence is 10723 nucleotides long (SEQ ID No.3). The RNA genome contains a type I cap at the 5'-end but lacks a 3'-end poly (A)-tail. The gene organization is 5'-noncoding region (NCR), structural protein (capsid (C), premembrane/membrane (prM/M), envelope (E)) and non structural protein (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5) and 3' NCR. The viral RNA genome is associated with the C proteins to form nucleocapsid (icosahedral symmetry). As with other flaviviruses, the DEN viral genome encodes the uninterrupted open reading frame (ORF) which is translated to a single polyprotein.

Serial passaging of a virulent (disease-causing) strain of dengue-2 results in the isolation of modified virus which are "live attenuated", i.e., infectious, yet not capable of causing disease. These modified viruses are usually tested in monkeys to evaluate their attenuation. However, Humans are the only primates that exhibit signs of clinical

disease. The viruses that cause mild (i.e. acceptable in terms of regulatory purposes as presenting a positive benefit/risk ratio) to low or no secondary effects (i.e. systemic events and/or biological abnormalities and/or local reactions) in the majority of the tested humans but still infect and induce an immune response are called "live attenuated".

The term "LAV" denotes live attenuated Dengue viral strains. In the context of the invention "LAVs" are live attenuated strains initially derived from the Dengue serotype 2 (DEN-2) strain 16681 by passages in Primary Dog Kidney (PDK) Cells. For instance "LAV2/PDK53" is the attenuated strain established after 53 passages of strain 16681 in PDK cells (DEN-2 16681/PDK53). "LAV2/PDK50" is the attenuated strain established after 50 passages of strain 16681 in PDK cells (DEN-2 16681/PDK50). LAV2/PDK53 nucleotide sequence is shown in SEQ ID No.38.

"VDV2" is meant a LAV obtainable by the sanitization process disclosed in the present application. A VDV2 is thus a biological clone (homogeneous) VERO- adapted Dengue serotype 2 virus capable of inducing a specific humoral immune response including neutralizing antibodies in primate especially in humans. The VDV2 strains of the invention can be easily reconstructed starting directly from the here disclosed VDV2 sequences. The induction of a specific humoral immune response can be easily determined by an ELISA assay. The presence of neutralising antibody in the serum of a vaccinee is evaluated by the plaque reduction neutralization test as described in section 4.1.1.2.2. A serum is considered to be positive for the presence of neutralizing antibodies when the neutralizing antibody titer thus determined is at least superior or equal to 1:10.

The terms "mutation" means any detectable change in genetic material, e.g. DNA, RNA, cDNA, or any process, mechanism, or result of such a change. Mutations include substitution of one or more nucleotides. In the context of the instant application, mutations identified in dengue-2 virus genomic sequence or polyprotein are designated pursuant to the nomenclature of Dunnen and Antonarakis (2000). As defined by Dunnen and Antonarakis at the nucleic acid level, substitutions are designated by ">", e.g. "31A>G" denotes that at nucleotide 31 of the reference sequence a A is changed to a G.

Variations at the protein level describe the consequence of the mutation and are reported as follows. Stop codons are designated by X (e.g. R97X denotes a change of

Arg96 to a termination codon). Amino acid substitutions a designated for instant by "S9G", which means that Ser in position 9 is replaced by Gly.

VERO-Derived Dengue serotype 2 viruses (VDV2)

The composition of the previously developed dengue-2 vaccine candidate LAV2 was improved by a sanitization process.

The VERO-Derived Vaccine Dengue serotype 2 (VDV2) disclosed herein uses the DEN-2 16681 virus attenuated by serial passages on PDK cells. VDV2 contains the genomic sequence of the whole live-attenuated DEN-2 virus, and bears the same attenuation spots which have been linked to attenuation as the original LAV2 strain that was tested in humans.

Sanitization of the LAV2 vaccine was performed by removing proteins and introducing only purified viral genomic material into Vero cells. More specifically, sanitization of the strain was performed by purifying and transfecting viral RNA into Vero cells. The process comprises the following steps:

a) extracting and purifying viral RNA from plaque-purified LVA2 strain, e.g. DEN-2 16681/PDK50 viruses;

b) advantageously associating of the purified RNA with cationic lipids;

c) transfecting Vero cell, in particular Vero cell LS10;

d) recovering of the neo-synthesized virus; and

e) purifying a VDV strain by plaque purification and optionally amplifying it in host cells, especially Vero cells.

The Vero cell technology is a well-known technology which has been used for different commercial products (injectable and oral polio vaccines, rabies vaccine). In the present invention qualified Vero cells were advantageously used to guarantee the absence of any risks potentially linked to the presence of adventitious agents. By "qualified VERO cells" is meant cells or cell lines for which culture conditions are known and is such that the said cells are free from any adventitious agents. These include e.g. the VERO cell LS10 of Sanofi Pasteur.

The thus isolated VDV strains are classically stored either in the form of a freezed composition or in the form of a lyophilised product. For that purpose, the VDV can be

mixed with a diluent classically a buffered aqueous solution comprising cryoprotective compounds such a sugar alcohol and stabilizer. The pH before freezing or lyophilisation is advantageously settled in the range of 6 to 9, e.g. around 7 such as a pH of 7.5 +/-0.2 as determined by a pH meter at RT. Before use, the lyophilised product is mixed with a pharmaceutically diluent or excipient such as a sterile NaCl 4‰ solution to reconstitute a liquid immunogenic composition or vaccine.

The Glu variant of LAV2 vaccine strain, at position NS3-250, was selected during transfection and cloning, and positions 5'NC-57 and NS1-53, also identified as critical for attenuation of LAV2 vaccine, were both conserved in VDV2 sequence.

Sequencing, at attenuation-specific loci, of virus recovered after transfection, did not reveal any mutation, compared to SEQ ID No.38. The biologically cloned VDV2 virus exhibits a homogenous plaque phenotype and a remarkable genetic stability with regard to its LAV2 parent as it can be deduced especially from the conservation of the attenuation genotype.

VDV2 (passage 11) strain was sequenced and compared with the serotype 2 Dengue Live Attenuated Virus (LAV2) strain sequence (SEQ ID No.38). A set of 10 nucleotide differences was identified, triggering six amino acid substitutions located in M and Env structural peptides, and also in non-structural peptides NS3 and NS5. None of these differences corresponds to any of the LAV2 attenuation positions.

Table 2: Sequence comparison between LAV2/PDK53 and VDV2 passage 11 strains.

Nucleotides			Amino acids			
Position	LAV2	VDV2	Region	Position	LAV2	VDV2
736	G	C	M	9	G	R
1619	G	A	E	228	G	E
1638	A	G	E	234	K	K
2520	G	A	NS1	33	K	K
4723	**T**	**A**	**NS3**	**69**	**S**	**T**
5062	**G**	**C**	**NS3**	**181**	**D**	**H**
9191	**G**	**A**	**NS5**	**541**	**R**	**K**

Nucleotides			Amino acids			
Position	LAV2	VDV2	Region	Position	LAV2	VDV2
9222	A	G	NS5	551	E	E
10063	**T**	**A**	**NS5**	**832**	**S**	**T**
10507	A	G	3' nc	-	-	-

Grey shading: differences in structural proteins; Bold characters: differences in non-structural proteins.

The invention thus provides for live attenuated dengue-2 virus strains that have been obtained from the wild type virus DEN-2 16681 attenuated by serial passages on PDK cells and then by sanitization on VERO cells. In particular the attenuated strains of the invention comprise at least the identified sequence mutations (non-silent and optionally silent) relative to the nucleotide sequence or polyprotein sequence of the wild-type DEN-2 16681 and LAV2/PDK53 strains.

Accordingly, the invention relates to an isolated live attenuated dengue-2 virus strain which comprises, or consists of, the sequence of LAV2/PDK53 strain (SEQ ID No.38) wherein at least nucleotides at positions 736, 1619, 4723, 5062, 9191, 10063, and 10507, and optionally 1638, 2520, 9222, and 10361, are mutated, with the proviso that the following nucleotides are not mutated: 57, 524, 2055, 2579, 4018, 5547, 6599, and 8571. Preferably, the mutations are substitutions. Preferably, the nucleotide at position 736 is C, the nucleotide at position 1619 is A, the nucleotide at position 4723 is A, the nucleotide at position 5062 is A, the nucleotide at position 9191 is A, the nucleotide at position 10063 A, and the nucleotide at position 10507 is G.

The nucleotide at position 5270 may be A or T, preferably A.

Still preferably, the isolated strain according to the invention comprises the sequence SEQ ID No.38 wherein said sequence comprises at least the mutations 736 G>C, 1619 G>A, 4723 T>A, 5062 G>C, 9191 G>A, 10063 T>A, and 10507 A>G, and optionally the mutation 1638 A>G, 2520 G>A, and/or 9222 A>G.

Hence, a live attenuated dengue-2 virus strain according to the invention may comprise, or consist of, the sequence of wild-type dengue-2 strain 16681 (SEQ ID No.3) wherein said sequence comprises at least the mutations 57 C>T, 524 A>T, 736 G>C,

1619 G>A, 2055 C>T, 2579 G>A, 4018 C>T, 4723 T>A, 5062 G>C, 5547 T>C, 6599 G>C, 8571 C>T, 9191 G>A, 10063 T>A, and 10507 A>G. Preferably, a live attenuated strain according to the invention further comprises the mutation 1638 A>G, 2520 G>A, and/or 9222 A>G by reference to the nucleotide sequence of wild-type strain 16681 (SEQ ID No.3).

The live attenuated dengue-2 virus strains according to the invention may include variant strains that comprise a sequence SEQ ID No.38 mutated at positions 736, 1619, 4723, 5062, 9191, 10063, and 10507, as defined above, and that further comprise a substitution of one or more nucleotides in a given codon position that results in no alteration in the amino acid encoded at that position.

Advantageously, the live attenuated dengue-2 virus strain according to the invention comprises a sequence which differs by a limited number of mutations, e.g. no more than 5, still preferably no more than 2, from SEQ ID No.1.

Preferably, the genomic sequence of a dengue-2 virus strain according to the invention consists of the nucleotide sequence SEQ ID No.1.

The invention also relates to live attenuated dengue-2 strains that may be derived from the VDV2 strain of sequence SEQ ID No.1 by further passages on cells, in particular Vero cells.

The invention also relates to an isolated nucleic acid which comprises, or consists of, the DNA sequence SEQ ID No.1 or its equivalent RNA sequence.

A "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogs thereof, such as phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix.

As used herein, by RNA sequence “equivalent” to SEQ ID No.1 is meant a sequence SEQ ID No.1 wherein deoxythymidines have been replaced by uridines. As SEQ ID No.1 constitutes VDV2 cDNA sequence, the equivalent RNA sequence thus corresponds to the positive strand RNA of VDV2.

The invention further relates to the polyprotein of sequence SEQ ID No.2 and to fragments thereof. SEQ ID No.2 is the sequence of the polyprotein encoded by SEQ ID No.1

A "fragment" of a reference protein is meant a polypeptide which sequence comprises a chain of consecutive amino acids of the reference protein. A fragment may be at least 8, at least 12, at least 20, amino acid long.

Said fragments of the polyprotein of sequence SEQ ID No.2 comprise at least an arginine at position 9 of M protein (position 214 of SEQ ID No.2), and/or a glutamic acid at position 228 of E protein (position 508 of SEQ ID No.2), and/or a threonine at position 69 of NS3 protein (position 1543 of SEQ ID No.2), and/or a histidine at position 181 of NS3 protein (position 1656 of SEQ ID No.2), and/or a lysine at position 541 of NS5 protein (position 1725 of SEQ ID No.2), and/or a threonine at position 832 of NS5 protein (position 3032 of SEQ ID No.2).

According to an embodiment the fragment of the polyprotein encoded by SEQ ID No.1 is or comprises M protein, and/or E protein, and/or NS3 protein and/or NS5 protein.

Immunogenic and vaccine compositions

The invention also relates to an immunogenic composition, suitable to be used as a vaccine, which comprises a VDV2 strain according to the invention.

The immunogenic compositions according to the invention elicit a specific humoral immune response toward the dengue virus comprising neutralizing antibodies.

Preferably, the immunogenic composition is a vaccine.

According to an embodiment, the immunogenic is a monovalent composition, i.e. it elicits en immune response and/or confers protection against Dengue-2 virus only.

According to another embodiment, the invention relates to a multivalent dengue immunogenic composition. Such a multivalent immunogenic composition or vaccine may be obtained by combining individual monovalent dengue vaccines. The immunogenic or vaccine composition may further comprise at least a live attenuated dengue virus of another serotype. In particular, the immunogenic or vaccine composition may comprise a VDV2 according to the invention in combination with at least a live attenuated dengue virus selected from the group consisting of serotype 1, serotype 3, and serotype 4.

Preferably, the immunogenic or vaccine composition may be a tetravalent dengue vaccine composition, i.e. a vaccine composition that comprises a VDV2 according to the invention in combination with a live attenuated dengue-1 virus strain, a live attenuated dengue-3 virus strain and a live attenuated dengue-4 virus strain.

Live attenuated dengue-1, dengue-3 and dengue-4 virus strains have been described previously. Reference may be made to the live-attenuated vaccines that were developed by Mahidol University by passaging dengue serotype 1 (strain 16007, passage 13; LAV1), and serotype 4 (strain 1036, passage 48, LAV4) viruses in Primary Dog Kidney (PDK) Cells, and for serotype 3 (strain 16562) in Primary Green Monkey Kidney (PGMK) cells (passage 30) and Fetal Rhesus Lung (FRhL) cells (passage 3) (LAV3). The nucleotide sequences of LAV1 (SEQ ID No.40), LAV3 (SEQ ID No.41), and LAV4 (SEQ ID No.42) are shown in the annexed sequence listing.

Advantageously, a live attenuated dengue-1 strain may correspond to a VDV1 strain which has been obtained from the LAV1 strain developed by Mahidol by the process of sanitization according to the invention. In particular a live attenuated dengue-1 strain (VDV1) may comprise, and advantageously consists of the sequence SEQ ID No.39.

Immunogenic compositions including vaccines may be prepared as injectables which can correspond to liquid solutions, suspensions or emulsions. The active immunogenic ingredients may be mixed with pharmaceutically acceptable excipients which are compatible therewith.

The immunogenic compositions or vaccines according to the present invention may be prepared using any conventional method known to those skilled in the art. Conventionally the antigens according to the invention are mixed with a pharmaceutically acceptable diluent or excipient, such as water or phosphate buffered saline solution, wetting agents, fillers, emulsifier stabilizer. The excipient or diluent will be selected as a function of the pharmaceutical form chosen, of the method and route of administration and also of pharmaceutical practice. Suitable excipients or diluents and also the requirements in terms of pharmaceutical formulation, are described in Remington's Pharmaceutical Sciences, which represents a reference book in this field.

Preferably, the immunogenic composition or vaccine corresponds to an injectable composition comprising an aqueous buffered solution to maintain e.g. a pH (as determined at RT with a pH meter) in the range of 6 to 9.

The composition according to the invention may further comprise an adjuvant, i.e. a substance which improves, or enhances, the immune response elicited by the VDV2 strain. Any pharmaceutically acceptable adjuvant or mixture of adjuvants conventionally used in the field of human vaccines may be used for this purpose.

The immunogenic compositions or vaccines according to the invention may be administered by any conventional route usually used in the field of human vaccines, such as the parenteral (e.g. intradermal, subcutaneous, intramuscular) route In the context of the present invention immunogenic compositions or vaccines are preferably injectable compositions administered subcutaneously in the deltoid region.

Method for immunizing

The invention further provides for a method of immunizing a host in need thereof against a dengue infection which comprises administering the host with an immunoeffective amount of a vaccine composition according to the invention.

A "host in need thereof" denotes a person at risk for dengue infection, i.e. individuals travelling to regions where dengue virus infection is present, and also inhabitants of those regions.

The route of administration is any conventional route used in the vaccine field. The choice of administration route depends on the formulation that is selected. Preferably, the immunogenic composition or vaccine corresponds to an injectable composition administered via subcutaneous route, advantageously in the deltoid region.

The amount of LAV or VDV, in particular VDV2, in the immunogenic compositions or vaccines may be conveniently expressed in viral plaque forming unit (PFU) unit or Cell Culture Infectious Dose 50% ($CCID_{50}$) dosage form and prepared by using conventional pharmaceutical techniques. For instance, the composition according to the invention may be prepared in dosage form containing 10 to 10^6 $CCID_{50}$, or 10^3 to 10^5 $CCID_{50}$ of LAV or VDV, for instance a dose of 4 ± 0.5 $\log_{10}$ $CCID_{50}$ of VDV2 strain for a

monovalent composition. Where the composition is multivalent, to reduce the possibility of viral interference and thus to achieve a balanced immune response (i.e. an immune response against all the serotype contained in the composition), the amounts of each of the different dengue serotypes present in the administered vaccines may not be equal.

An "immunoeffective amount" is an amount which is capable of inducing a specific humoral immune response comprising neutralising antibodies in the serum of a vaccinee, as evaluated by the plaque reduction neutralization test as described in section 4.1.1.2.2; a serum being considered to be positive for the presence of neutralizing antibodies when the neutralizing antibody titer thus determined is at least superior or equal to 1:10.

The volume of administration may vary depending on the route of administration. Subcutaneous injections may range in volume from about 0.1 ml to 1.0 ml, preferably 0.5 ml.

The optimal time for administration of the composition is about one to three months before the initial exposure to the dengue virus. The vaccines of the invention can be administered as prophylactic agents in adults or children at risk of Dengue infection. The targeted population thus encompasses persons which are naïve as well as well as non-naïve with regards to dengue virus. The vaccines of the invention can be administered in a single dose or, optionally, administration can involve the use of a priming dose followed by a booster dose that is administered, e.g. 2-6 months later, as determined to be appropriate by those of skill in the art.

The invention will be further described in view of the following figures and examples.

FIGURES

Figure 1 is a summary of History of VDV2 seed.

Figure 2 is a flow chart that summarises the developed manufacturing process that gives rise to the Filled Product (monovalent), "ready to use" doses.

Figure 3 is a diagrammatic representation of VDV2 genome map. The above arrow is the polyprotein coding sequence. The below arrows represent mature peptides coding sequence. The vertical bars symbolize the nucleotidic variations between wild-type dengue 2 strain 16681 and LAV2 strain. The stars designate the nucleotidic variations between LAV2 and VDV2.

Figure 4 shows plaque size analysis after 7 days of incubation at 37°C for dengue-1 viruses LAV2, VDV2, and strain 16681.

Figure 5 is a graphic analysis showing plaque size distribution for dengue-2 viruses LAV2, VDV2, and strain 16681.

Figure 6 is a summary of Trial Design for assessment of safety of VDV2 monovalent in healthy flavivirus-naïve adults.

EXAMPLES

Example 1: SANITIZATION

1.1 Viral RNA purification

The RNA purification and transfection process was performed as follows. DEN-2/PDK50 suspension was resuspended in 0.5 ml of water and diluted in order to contain at least 3 x 10^4 and up to 3 x 10^7 $TCID_{50}$ or PFU of virus per milliliter. One unit of benzonase diluted in 0.01 ml of William's medium was added to 0.5 ml of virus, in order to digest DNA or RNA molecules from cellular origin, and the solution was incubated for 2 hours at 4°C on an agitator. At the end of incubation step, 0.65 ml of a denaturing buffer containing guanidium chloride, detergent (SDS), and βmercaptoethanol (RTL-βmercaptoethanol buffer, provided in the kit RNeasy Mini kit, Qiagen Ref. 74104) were added and proteins were extracted once with phenol/chloroform (1/1) vol/vol and once with chloroform vol/vol, followed by centrifugation for 5 min at 14,000 rpm at room temperature. After each extraction, the aqueous phase was collected, taking care not to collect material (white precipitate) at the interface, and transferred to a clean 1 ml-Eppendorf tube. The RNA solution was then applied onto a QIAgen column following the recommendations of the manufacturer (RNeasy minikit, QIAgen), in order to remove traces of solvent, and eluted with 0.06 ml of nuclease-free H2O water. The presence of

viral RNA was confirmed by quantitative RT-PCR, using a reference curve established with known quantities of virus, in $TCID_{50}$/ml.

1.2 Transfection of Vero cells with purified RNA

Transfection was performed using lipofectamine (LF2000 Reagent, Life Technologies), a mixture of cationic lipids that associate to RNA through charge interactions and allows transfer of the complexes into the cytoplasm of the cells by fusion with the cell membrane. The optimal quantity of LF2000 reagent was determined in a preliminary experiment by incubating Vero cells, plated 16 to 24 hours before (0.3-0.5 x 10^6 cells per well in a 6 wells plate) with increasing doses (5 to 20 µl) of lipofectamine. Cells were then incubating 4 to 5 hours at 32°C, 5% CO_2, before replacing the medium by fresh culture medium without FCS, and the incubation was continued overnight at 32°C. Toxicity (round, refringent or floating cells, homogeneity of the cell monolayer) was checked regularly for 48 hours, under an inverted microscope. The highest dose of lipofectamine that was not toxic under these conditions was 10 µl and was chosen for RNA transfection.

Four transfections were carried out in parallel, using ¼ of the RNA preparation (about 2 X 10^4 log eq$TCID_{50}$, according to qRT-PCR). Twenty-five microliters of viral RNA solution were diluted in 500 µl of OptiMEM medium (GIBCO) containing 15 µl of LF2000 Reagent (a mixture of cationic lipids that associate to RNA through charge interactions, and allow transfer of the complexes into the cytoplasm of the cells by fusion with the cell membrane). 200 ng of yeast tRNA were added as carrier in 2 out of the 4 reactions.

The 4 transfection mixes were allowed to precipitate for 10 min at room temperature before addition to 6-wells plates of confluent Vero cells, and incubation at 36°C. After four hours, transfection mix was removed and cells were rinsed once in PBS. Three milliliters of post-transfection medium (Williams, GIBCO) were added, and incubation was continued for 5 days at 32°C. Culture medium was then replaced by 3 ml of Dengue infection medium (Williams supplemented with 10 mM $MgSO_4$).

A focus of cells presenting typical cytopathic effects (round, refringent cells) was detected at day 8 post-transfection in 1 out of the 2 wells transfected in presence of

tRNA. Release of virus in the supernatant of these cells was confirmed by qRT-PCR. Eleven days post-transfection, marked cytopathic effects were detected in this only well, while the supernatant of the three other transfected-wells remained negative.

The viral solution recovered after transfection was re-named TV100 (instead of 16681 PDK50/Vero-2) and exhibited an infectious titer of 5.8 log$TCID_{50}$/ml after dilution at ½ in an aqueous buffered solution comprising cryoprotective agents (pH= 7.5).

1.3 Characterization of viruses recovered after transfection

Spot sequencing of specific loci important for attenuation was performed by R. Kinney (CDC, Fort Collins). Data are presented in Table 3.

Table 3: Sequencing of transfected virus at attenuation-specific positions

Virus	5'-NC-57 Nt 57	NS1-53 Nt 2579 (aa)	NS3-250 Nt 5270 (aa)
DEN-2 16681	C	G (Gly)	A (Glu)
DEN-2 PDK53	T	A (Asp)	T/A (Val/Glu)
TV100	T	A (Asp)	A (Glu)

VDV2 has retained the important attenuating loci at 5'NC-57 and NS1-53, and the wild-type 16681 locus of the NS3-250-Glu variant in the PDK53 vaccine. The NS3-250-Glu/Val mix in the PDK53 vaccine was observed to be stable between passages PDK45 and PDK53 suggesting that selection has occurred in Vero cells. Previous analysis of DEN-2 vaccine isolated from serum of a vaccinee had demonstrated that this selection could also occur in humans.

Viral plaques diameters were measured in Vero cells. Briefly, Vero cells were plated at a density of 1.000.000 cells/cm^2 in culture medium containing 4 % of FBS. After overnight incubation, the medium was removed and cells were infected with serial twofold or fivefold dilutions of virus. After 1,5 hour at 37°C 5% CO_2, the inoculum was removed and cells were incubated at 37°C 5% CO_2 in Mimimal Eagle Medium (MEM) containing 1,26% methylcellulose and 10% FBS. After 11 days of incubation, plates were fixed 20 minutes in cold acetone at –20°C and revealed by immuno-coloration with

a flavivirus-specific mAb, diluted at 2.5 µg/ml. Viral plaques were measured using an image analysis software (Saisam/Microvision).

VDV2 was compared to LAV2 16681/PDK50 seed (Table 4) and exhibited similar homogeneous small plaques of 1-3 mm diameter.

Table 4: Plaques size of LAV2 16681/PDK50 and VDV2

Step	Virus	LP/MP	SP
Before transfection	LAV2 PDK50	0	319
After transfection	Uncloned VDV2	0	183

LP/MP: Number of Large/Medium Plaques in 6 wells
SP: Number of Small Plaques in 6 wells

1.4 Plaque-purifications

Three additional amplification passages (P2 to P4) were performed on the virus recovered after transfection. Biological cloning by plaque-purification was performed on P3 andP4 passaged virus (named LST 003 and LST 007, respectively).

Briefly, Vero cells were plated in 6-well plates and infected with serial dilutions of virus, in order to get between 1 and 20 plaques by plate. After 1,5 hour at 37°C 5% CO_2, the inoculum was removed and cells were incubated under 3 ml of solid medium composed of MEM-10% FCS pre-heated at 42°C and mixed extemporaneally with 2 % of melted agarose equilibrated at 42°C. The medium was allowed to solidify at room temperature for 30 min; under flow hood, and plates were incubated in inverted position for 10 days at 32°C – 5% CO_2. A second layer of the same medium supplemented with 0.01% of neutral red was then added and plates were incubated for an additional night at 32°C. Six well-isolated small plaques were picked under sterile conditions using a micro-pipet equipped with an 0.1 ml tip, and transferred into sterile tubes containing 0.2 ml of MEM-4% FCS : three from P3 passage (identified as clones 31, 32 and 33), and three from P4 passage (identified as clones 71, 72 and 73). The suspension was homogenised by vortexing, serially diluted in the same medium, and immediately used to infect 6-well plates of Vero cells. The protocol was repeated and a second picking of two SP was done on clones 32, 33, 71 and 72, and one SP on clone 31. Each picked plaque was diluted in 1 ml of medium, before amplification on Vero cells, in T25 cm^2

flasks. Culture medium was collected at day 6 post-infection, diluted with the same volume of an aqueous buffered solution comprising cryoprotective agent (pH 7.5) and frozen at –70°C. All these steps were performed at 32°C.

Plaque purified virus were named 311, 321, 322, 331, 332, 341, 342, 351, 352, 711, 712, 721 and 722, respectively.

Infectious titers were determined on Vero cells at the end of the first amplification (see below)

Clone 311: 3.95 $LogCCID_{50}$/ml

Clone 321: 5.20 $LogCCID_{50}$/ml

Clone 322: 5.45 $LogCCID_{50}$/ml

Clone 331: 5.55 $LogCCID_{50}$/ml

Clone 332: 4.95 $LogCCID_{50}$/ml

Clone 341: 2.80 $LogCCID_{50}$/ml

Clone 342: 4.85 $LogCCID_{50}$/ml

Clone 351: 5.35 $LogCCID_{50}$/ml

Clone 352: 5.50 $LogCCID_{50}$/ml

Clone 711: 5.45 $LogCCID_{50}$/ml

Clone 712: 5.65 $LogCCID_{50}$/ml

Clone 721: 5.30 $LogCCID_{50}$/ml

Clone 722: 5.60 $LogCCID_{50}$/ml

A second amplification on Vero cells was carried out for three clones: clones 331, 352, and 722. Culture supernatants were collected at day 8 post-infection, diluted at ½ with an aqueous buffered solution comprising cryoprotective agent (pH 7.5) and named **TV331**, **TV352** and **TV722**.

1.5 Characterization of cloned virus

After the 1st amplification, all amplified viruses exhibited same plaque size phenotype and titers equivalent to, or higher than 5 $logCCID_{50}$/ml (except clones 311 and 341 which were significantly lower). Sequencing at attenuation-specific positions was performed on 6 clones from the 1st amplification (clones 321, 331, 351, 352, 711, 721) and the three clones from the 2nd amplification, and revealed no mutation.

In absence of any significant difference between the clones, TV722 was selected and amplified in VERO cells in order to generate a VDV2 vaccine candidate strain.

Table 5: Sequencing at attenuation-specific spots of DEN-2 viruses

		5'-UTR	prM	E	NS1	NS2a	NS3		NS4A	NS5
Step/cell	Virus	57	524	2055	2579	4018	5270	5547	6599	8571
Wild-type/PGMK	16681	C	A	C	G	C	A	T	G	C
Vaccine/PDK	PDK53	T	T	T	A	T	A/T	C	C	T
2nd plaque-purification/VERO	TV 321	T	T	T	A	T	A	C	C	T
	TV 331	T	T	T	A	T	A	C	C	T
	TV 342	T	T	T	A	T	A	C	C	T
	TV 352	T	T	T	A	T	A	C	C	T
	TV 711	T	T	T	A	T	A	C	C	T
	TV 722	T	T	T	A	T	A	C	C	T
2nd amplification/VERO	TV722PM	T	T	T	A	T	A	C	C	T

Nucleotides position are indicated below each gene and referred to published sequence of DEN-2 16681 strain.

In conclusion, a total number of 11 passages was necessary to obtain a biological clone of DEN-2 166681/PDK50 adapted on VERO cells.

Further characterizations have been performed then by determining VDV2 passage 11 complete sequence and phenotypic testing.

Example 2: SEQUENCING

The complete sequence of the virus was generated according to the following strategy. Viral genomic RNA was purified. The full genome was amplified by 16 overlapping RT-PCR reactions. Each PCR was designed so that sequencing tags were added on each DNA strand. This allowed simpler sequence reactions, all driven by a single pair of universal sequencing primers. Each PCR product was individually sequenced on both DNA strands. All results were reassembled to reconstruct the full VDV2 genome.

2.1 Materials

2.1.1 Viruses

The viruses to which it is referred here are DEN-2 16681; LAV-2/PDK53; VDV2, the sequences of which are given in the attached sequence listing.

The complete genome sequence of these viruses is 10723 nucleotides in length.

2.1.2 Primers

All primers have been designed in Seqweb bioinformatics package (Accelrys), primer design module (Table 6).

Table 6: list of RT-PCT and sequencing primers

Name	Primers sequences	NtStart	NtEnd	Primer length	RT-PCR length	Overlap
D2 01 +	GTTTTCCCAGTCACGACacgtggaccgacaaagacag (SEQ ID No.4)	13	32	37	978	-32
D2 01 -	AACAGCTATGACCATGttcctcctgaaacccttcc (SEQ ID No.5)	991	972	36		371
D2 02 +	GTTTTCCCAGTCACGACatcacgtacaagtgtcccc (SEQ ID No.6)	583	601	36	949	
D2 02 -	AACAGCTATGACCATGagcaacaccatctcattgaag (SEQ ID No.7)	1532	1512	37		163
D2 03 +	GTTTTCCCAGTCACGACtgcaaccagaaaacttggaatacac (SEQ ID No.8)	1325	1349	42	948	
D2 03 -	AACAGCTATGACCATGgctccatagattgctccaaagac (SEQ ID No.9)	2273	2251	39		203
D2 04 +	GTTTTCCCAGTCACGACcccagtcaacatagaagcagaacc (SEQ ID No.10)	2025	2048	41	878	
D2 04 -	AACAGCTATGACCATGccaaagccatagtcttcaacttcc (SEQ ID No.11)	2903	2880	40		155
D2 05 +	GTTTTCCCAGTCACGACatcatgcaggcaggaaaac (SEQ ID No.12)	2707	2725	36	949	
D2 05 -	AACAGCTATGACCATGaccataaccatcactcttccc (SEQ ID No.13)	3656	3636	37		240
D2 06 +	AACAGCTATGACCATGaccataaccatcactcttccc (SEQ ID No.14)	3368	3386	36	930	
D2 06 -	AACAGCTATGACCATGgctctctccagttccaaatc (SEQ ID No.15)	4298	4279	36		146
D2 07 +	GTTTTCCCAGTCACGACaagaaccagcaagaaaaggag (SEQ ID No.16)	4113	4133	38	868	
D2 07 -	AACAGCTATGACCATGcaccattaccataaagacccac (SEQ ID No.17)	4981	4960	38		226
D2 08 +	GTTTTCCCAGTCACGACttgaaccatcatgggcggac (SEQ ID No.18)	4715	4734	37	910	
D2 08 -	AACAGCTATGACCATGtcctgcttttatacttggaacgaac (SEQ ID No.19)	5625	5601	41		208
D2 09 +	GTTTTCCCAGTCACGACaagcccatttcacagaccc (SEQ ID No.20)	5375	5393	36	920	
D2 09 -	AACAGCTATGACCATGtcaatttcttcctttccccttc (SEQ ID No.21)	6295	6274	38		158
D2 10 +	GTTTTCCCAGTCACGACgagaggagaagcaaggaaaac (SEQ ID No.22)	6096	6116	38	923	
D2 10 -	AACAGCTATGACCATGagggacacattcactgagg (SEQ ID No.23)	7019	7001	35		233
D2 11 +	GTTTTCCCAGTCACGACacagagaacaccccaagac (SEQ ID No.24)	6750	6768	36	929	
D2 11 -	AACAGCTATGACCATGtccacttcctggattccac (SEQ ID No.25)	7679	7661	35		308
D2 12 +	GTTTTCCCAGTCACGACacaagtaatgctcctagtcctc (SEQ ID No.26)	7332	7353	39	935	
D2 12 -	AACAGCTATGACCATGttcactgatgacactatgttcc (SEQ ID No.27)	8267	8246	38		211
D2 13 +	GTTTTCCCAGTCACGACgtcatcaccaaatcccacag (SEQ ID No.28)	8016	8035	37	937	
D2 13 -	AACAGCTATGACCATGgcttcttctctcttttcccatc (SEQ ID No.29)	8953	8931	39		140
D2 14 +	GTTTTCCCAGTCACGACaaggtgagaagcaatgcag (SEQ ID No.30)	8773	8791	36	937	
D2 14 -	AACAGCTATGACCATGtggaaatggtgtgaacagaag (SEQ ID No.31)	9710	9690	37		209
D2 15 +	GTTTTCCCAGTCACGACgcattcagcacctaacaatcac (SEQ ID No.32)	9641	9482	39	9335	
D2 15 -	AACAGCTATGACCATGggcatttatgatggcctgac (SEQ ID No.33)	10396	10377	36		-
D2 16i +	ccatggaagctgtacgc (SEQ ID No.34)	10480	10496	64	234	
D2 16i -	AACAGCTATGACCATGtgattcaacagcaccattcc (SEQ ID No.35)	10714	10695	36		-28

2.2 Methods

2.2.1 Viral RNA purification

From previous experience, a minimal of 1000 $DICC_{50}$ is required to get a positive RT-PCR reaction in the next steps. This means that a mimimum virus titer of 10^4 $DICC_{50}$/mL is necessary. Virus genomic RNA was purified using QIAamp viral RNA mini kit (Qiagen), according to the manufacturer's recommendations. Briefly, a volume of 140 µl from a crude viral sample was incubated in the presence of the lysis solution, and loaded onto a kit column. After washing steps, the purified viral RNA was eluted by 60 µl of sterile nuclease-free water containing 1 µl (40 units) of RNAse inhibitor (RNAse Out, Sigma).

2.2.2 Reverse transcription

Viral RNA was reverse transcribed into cDNA by a reverse transcriptase (reverse iT) from ABGene. Again, standard operating conditions were applied, using 10 µl of purified RNA, in a final reaction volume of 20 µl. The reaction was initiated by hybridization of the minus strand primers. One RT reaction per PCR was performed. The cDNA synthesis was obtained by 45 min incubation at 47°C.

2.2.3 PCR

All PCR were performed with Expand High Fidelity PCR system (Roche diagnostics), using all 16 pairs of primers (+) and (-) from Table 6. PCR conditions were the following ones:

RT		2µl	PCR program			
10x buffer		2.5µl	Denaturation	94°C	2 min	
dNTP (10mM)	mix	2µl	Denaturation	94°C	15 sec	
Primers		0.8µl each	Hybridization	55°C	30 sec	40 cycles
H2O		16.4µl	Elongation	68°C	1 min	
Enzyme		0.5µl	Elongation	68°C	5 min	

All PCR products were controlled by electrophoresis on agarose gel.

2.2.4 Sequencing

The major part of the sequence reactions has been outsourced to Genome Express. Genome extremities, ambiguities, some inter-PCR junctions, and regions not sequenced by Genome Express for technical reasons were performed in-house.

- Sequencing at Genome Express: PCR products were shipped at +4°C, and sequencing results were received as informatic sequence files. Text file, quality files and chromatograms are available for each individual sequence. After sequence alignment, all discrepancies were checked on the chromatogram, and corrected if identified as sequence algorithm errors.
- In-house sequencing: Sequence reactions were performed on thermocycler PTC-200 (MJ Research), with Sequitherm Excell II LC kit (Epicentre). Each PCR product was sequenced on both strands independently in a single reaction. Reactions were loaded onto a sequence electrophoresis gel. Run and analysis of sequence were performed on the automated sequencer Gene ReadIR 4200 (Li-Cor).

- Sequence reaction

		PCR program			
DNA	up to 200/250 ng				
Reaction buffer	7.2µl	Denaturation	92°C	2 min	
Primers (1-2 pM)	1.5µl each	Denaturation	92°C	15 sec	30 cycles
Enzyme	1µl	Hybridization	50°C	30 sec	
H_2O	up to20µl	Elongation	70°C	1 min	
		Elongation	70°C	10 sec	

Addition of 3µl of denaturating/loading buffer.

Denaturation of samples 3 min at 95°C and ice cooling just before samples loading.

- Sequence electrophoresis

Electrophoresis parameters		Gel parameters	
Voltage	1500 V	Gel hight	41 cm
Current	35 mA	Gel thickness	0.2 mm
Power	40 W	Temperature	45°C
Run time	9H00	Scan speed	3

2.3 Results

All PCR fragments were sequenced from both ends using a common PCR added tail, i.e. a specific motif which has been added at 5' end of all primers:

5' primers: M13SEQ-GTTTTCCCAGTCACGAC (SEQ ID No.36)

3' primers: M13REV-AACAGCTATGACCATG (SEQ ID No.37)

M13-SEQ and -REV sequences correspond to universal M13 primers motifs (New England Biolabs references).

For final contig assembly, a quick analysis was performed in Vector NTi, in ContigExpress module (Informax). The LAV2 reference sequence was compared with all individual sequencing results. In such conditions, all results could be aligned at the right place on the complete genome, even when some regions were still missing contig assembly, giving a quick visualization of the overall genome alignment.

2.3.1 Complete VDV2 sequence assembly

The final sequence alignment was performed in Vector NTi, AlignX module (Informax). The classical multiple sequence alignment algorithm ClustalW (Thompson et al., 1994) was used by the software to build the global alignment. All the sequence results were aligned together with the LAV2 reference sequence, thus allowing for a better reconstruction of the genome. Any discrepancy in the sequence with regard to the reference required a confirmation on another independent sequence reaction. The complete sequence of VDV2 is shown in SEQ ID No.1.

Some ambiguities are often found in single sequences, especially near sequence extremities. This is inherent to the somewhat poor quality of the reaction at both ends of any PCR fragment. Such poor quality sequences were excluded from the alignment, until two other independent sequence reactions were available from other PCR products. Discrepancy towards the reference was not taken into account in the final alignment when not confirmed in at least two independent other PCR sequences matching the consensus. Conversely, any discrepancy confirmed on two independent sequences was kept in the final sequence.

Table 7 summarizes the characteristics of each individual sequence reaction, indicating start, end and length. Overlaps between adjacent PCR are also indicated, as well as differences with regard to the reference sequence in the last column.

Table 7: Dengue VDV2 individual sequences characteristics

Name	Start	End	Size	Overlap	Comments
D2 01 +	33	365	332	0	2 sequences
D2 01 -	619	79	540	5	2 sequences
D2 02 +	614	1334	720		736 G>C (M9-G>R)
D2 02 -	1488	654	834	127	736 G>C (M9-G>R)
D2 03 +	1361	2135	774		1619 G>A (E228 G>E); 1638 A>G (E234K s)
D2 03 -	2227	1416	811	179	1619 G>A (E228 G>E); 1638 A>G (E234K s)
D2 04 +	2048	2774	726		2520 G>A (NS1-33K s)
D2 04 -	2866	2210	656	133	2520 G>A (NS1-33K s)
D2 05 +	2733	3495	762		
D2 05 -	3619	2819	800	251	
D2 06 +	3393	4196	803		
D2 06 -	4257	3368	889	78	
D2 07 +	4179	4830	651		4723 T>A (NS3-69 S>T)
D2 07 -	4851	4223	628	130	4723 T>A (NS3-69 S>T)
D2 08 +	4742	5506	764		5062 G>C (NS3-181 DD>H)
D2 08 -	5582	4721	861	188	5062 G>C (NS3-181 DD>H)
D2 09 +	5394	6100	706		
D2 09 -	6669	5979	690	545	
D2 10 +	6124	6996	872		
D2 10 -	6983	6148	835	218	
D2 11 +	6778	7567	789		
D2 11 -	7649	6781	868	317	
D2 12 +	7365	8236	971		
D2 12 -	8241	7332	909	191	
D2 13 +	8050	8797	747		
D2 13 -	8819	8147	672		
D2 14 +	8707	9700	903	22	9191 G>A (NS5-541 R>K); 9222 A>G (NS5-551E s)
D2 14 -	9654	8804	850	199	9191 G>A (NS5-541 R>K); 9222 A>G (NS5-551E s)
D2 15 +	9501	10285	784		10063 T>A (NS5-832 S>T)
D2 15 -	10347	9702	645	187	10063 T>A (NS5-832 S>T)
D2 16i +	10486	10687	201		10507 A>G
D2 16i -	10694	10160	534	0	10507 A>G

The two extremities of the genome could not be sequenced from PCR amplification, because cDNA synthesis and PCR DNA reaction required oligonucleotides complementary to the ends of the genome. During the amplification

step, these oligonucleotides are incorporated into the PCR fragment. The sequence result is that of the synthetic oligonucleotide, and not that of the virus itself. PCR from both ends of the virus genome did work properly, suggesting that the viral sequence was not significantly different from the oligonucleotide sequence (if it had been the case, PCR amplification should have failed or at least should have been of poor quality). We were not able to distinguish them from all other PCR amplifications. So, in the reconstructed genome, both genome ends were considered as identical to oligonucleotide sequences (and also identical to the reference). At 5' end, the sequence is that of nucleotides 1 to 32. At 3' end, the sequence is that of nucleotides 10695 to 10723.

2.3.2 Sequence comparison

Ten nucleotide differences have been detected with regard to the parent LAV2 genomic sequence. VDV2 vaccine strain is derived from LAV2, through virus sanitization and passage from dog to monkey cells.

Differences between LAV2 and VDV2 can have several origins. First, cloning steps can select a viral subpopulation that is not 100% identical to the major sequence previously detected in LAV2. Second, LAV2 has been produced on PDK cells, whereas VDV2 has been made on Vero cells. Such passage from dog to monkey cells is known to potentially induce virus changes that reflect adaptation to the new cell line. Third, as for all RNA viruses, the lower viral RNA polymerase fidelity triggers a higher genomic mutation rate than DNA polymerases do.

In term of sequences, all 9 nucleotide positions which have been linked to viral attenuation of LAV2 are conserved in VDV2 passage 11.

Furthermore, sequence comparison between VDV2 passage 9 and passage 11 showed the occurrence of two mutations between passages 9 and 11 which are linked to differences in phenotype, viremia and immunogenicity.

Table 8: Sequence comparison between LAV2/PDK53 strain and VDV2 passages 9 and 11 strains

Nucleotides				Amino acids				
Position	LAV2	VDV2		Region	Position	LAV2	VDV2	
		Passage 9	Passage 11				Passage 9	Passage 11
736	G	**G**	**C**	M	9	G	**G**	**R**
1619	G	A	A	E	228	G	E	E
1638	A	G	G	E	234	K	K	K
2520	G	A	A	NS1	33	K	K	K
4723	T	A	A	NS3	69	S	T	T
5062	G	C	C	NS3	181	D	H	H
5270	A/T	A	A	NS3	250	E/V	V	V
9191	G	**G**	**A**	NS5	541	R	**R**	**K**
9222	A	G	G	NS5	551	E	E	E
10063	T	A	A	NS5	832	S	T	T
10507	A	G	G	3' nc	-	-	-	-

Bold: sequence differences between VDV2 passage 9 and passage 11/

When performing sequence alignment between all available Genbank serotype 2 Dengue genomic sequences, it appears that only two positions are shared by other Dengue 2 strains (1638 and 2520), both silent at amino acid level. All other positions are specific to the VDV2 passage 11 strain, triggering an amino acid substitution (Table 8). Concerning amino acid changes, the four changes in non-structural peptides appear relatively conservative, from a biochemical point of view, whereas the two changes in M and in the envelope bring modification both in charge and hydrophobicity.

Example 3: CHARACTERIZATION

The objective of these studies was to assess whether changes in attenuation markers occurred through passages.

The flow chart shown on Figure 2 summarises the developed manufacturing process that gives rise to the Filled Product (monovalent), "ready to use" doses

Briefly, after 2 successive passages on Vero cells of the VDV2 passage 8, the respective working seeds were obtained. The final virus cultivations are also conducted by infection of a Vero cell suspension. The viruses produced are then harvested. DesoxyRiboNucleic Acid (DNA) is digested according to an enzymatic treatment. Impurities are removed by ultrafiltration. Infectious titers are enhanced by a concentration step. An aqueous buffered solution comprising cryoprotective agents (pH = 7.5) is added and this 0.22-µm filtrated mixture is then diluted at the targeted dose within the same solution. The active substance is then filled into glass vials, freeze-dried, and stored before use.

3.1 Phenotypic Markers

Table 9 presents data from three phenotypic assays performed on DEN-2 16681 wt strain, DEN-2 16681/PDK53 vaccine strain, VDV2 passage 9 and VDV2 passage 11 (last adaptation passage) : temperature-sensitivity (Ts), growth curves on monkey (Vero) and mosquito (C6/36) cells and neurovirulence in Newborn mice (data obtained at CDC). Reduced mouse neurovirulence (reduced mortality and longer average survival time (AST)), restricted –growth at 39°C and restricted replication on C6/36 are currently accepted by the scientific community as attenuation criteria for Dengue viruses. Vero-adapted passages exhibit clear Ts profile, and are more restricted than DEN2/PDK53. Final adaptation passage is restricted by about 3 log in this assay. Temperature sensitivity was also confirmed by viral growth curves. On Vero cells, similar replication levels were observed with all tested viruses. On mosquito cells, viral growth of Vero-adapted viruses was clearly restricted (about 3 log) compared to wt DEN2, and slightly restricted (about 0.5 log) compared to DEN2-PDK53. Surprisingly, mouse neurovirulence of Vero-adapted viruses was close to neurovirulence of wt DEN2, and

significantly higher than neurovirulence of DEN2/PDK53 vaccine. These data point out the low predictive value of this say, with regard to viral strain attenuation (cf clinical data).

Plaque size distribution of VDV2 passages 9 and 11, DEN2/PDK53 and wtDEN2 are compared to figure 5. Wt DEN2 exhibits heterogenous profile with 95 % of plaques with a size homogeneous profile, with a major population (81 %) of plaques < 0.6 mm and a minor population (12 %) of 1-2 mm plaques. This profile is close to, but distinct from DEN2-PDK53 profile. Noteworthy, the intermediate adaptation passage, VDV2 P9, exhibits a more heteregenous profile, with a major population (70 %) of 1-2 mm plaques, and a minor population (25 %) of plaques < 0.6 mm. These data demonstrate that VDV2 strain was not yet fully adapted at passage 9, and that the two additional passages were required for obtention of a homogeneous population replicating stably in Vero cells.

Table 9 : Summary of DEN-2 viral phenotypes

Temperature sensitivity (Percent titer reduction at 39°C)$_{Fold\text{-}reduction}$						Growth curves (Peak log_{10} pfu/ml) Vero-LS10		Neurovirulence in newborn Swiss Webster mice	
Virus	Score	Day 3	Day 4	Day 5	Day 6	Titer	at Day	$Mortality_n$	AST (S.D.)
D2-16681	+	n.d.	**92.7**$_{13.7}$	n.d.	**92.2**$_{12.8}$	7.5	8	100.0%$_{16}$	12.2 (1.5)
D2-PDK53	+	n.d.	**96.6**$_{29.4}$	n.d.	**99.7**$_{333.3}$	7.3	8-10	43.75%$_{16}$	16.0 (2.4)
VDV2 P9	+	n.d.	**99.94**$_{1666.7}$	n.d.	**99.97**$_{3333.3}$	7.5	8-10	100.0%$_{16}$	10.9 (0.7)
VDV2 P11	+	n.d.	**99.92**$_{1250.0}$	n.d.	**99.88**$_{833}$	7.5	10	100.0%$_{16}$	10.9 (0.6)

N: number of animals.

Example 4: IMMUNOGENICITY, VIREMIA, AND TOXICOLOGY IN MONKEYS

The most solid and numerous data that can be obtained in monkeys concern immunogenicity and viremia. Viremia, in particular, has been identified as one of the

factors associated with virulence and disease severity in humans, and then constitute an important parameter to consider. Obviously, immunogenicity is a key parameter when testing vaccines.

Inventors have established minimal/maximal values for viremia and immunogenicity.

Table 10: Minimal requirements for responses induced by Dengue vaccine candidates in monkeys, as measured in Vero or LLC-MK2 cells by plaque assay (these cells being considered equivalent in such an assay)

Viremia mean duration (days) (all serotypes being considered)	**Viremia mean peak titer** (log 10 pfu) (all serotypes being considered)	**Mean neutralizing titer** Day 30 (for each serotype) PRNT 50
≤3 days	≤1.5-2	≥80

pfu: plaque forming unit

PRNT 50: Plaque Reduction Neutralization Titer 50 (titre corresponding to a reduction of 50% of plaque number)

4.1 Pre-Clinical Pharmacology, Pharmacokinetics, and Product Metabolism in Animals

4.1.1 Material and methods

4.1.1.1 Monkey experiments

Monkey experiments were carried out according to European guidelines regarding animal experiments.

Immunizations were performed on cynomolgus monkeys (*Macaca fascicularis*) originating from Mauritius (CRP Le Vallon). Monkeys were quarantined for 6 weeks in the animal facility of Sanofi Pasteur before immunization.

Monkeys were immunized by subcutaneous (SC) route in the arm with vaccines in a volume of 0.5 ml (see each respective section). After light anesthesia with ketamine (Imalgene, Merial), blood was collected by puncture of the inguinal or saphene veins. At days 0 and 28, 5 ml of blood were sampled for evaluating antibody responses while

between days 2 and 10 only 1 ml of blood was sampled for evaluating viremia. Blood was collected on ice and kept on ice until serum separation. To do so, blood was centrifuged for 20 minutes at 4°C, and serum collected and stored at –80°C until testing in Rich Kinney's laboratory. Shipment to USA was performed in dry ice.

4.1.1.2 Viremia and neutralizing antibody responses (Plaque Reduction Neutralization Test, PRNT)

All analyses were performed in the laboratory of R. Kinney in CDC, Fort Collins, USA. Serum samples were shipped and stored at –80°C until the time of testing. At the time of first thawing, the samples were tested for viremia, and a 1:5 dilution of the serum was made. The 1:5 serum dilutions were inactivated for 30 min at 56°C before testing for neutralizing antibodies.

4.1.1.2.1 Viremia

0.125 ml of serum was added to 0.125 ml of diluent (RPMI medium) in the first well of 96-well plate and serial 10-fold dilution series were done, transferring 0.025 ml into 0.225 ml of diluent for each dilution. 0.2 ml of $10^{0.3}$-$10^{5.3}$ dilution series was plated in 6-well plate of Vero cells (virus was adsorbed at 37°C for 1.5 hour, overlayed with 4 ml of agarose lacking neutral red, overlayed 6-7 days later with 2 ml of agarose containing neutral red, and plaques counted). The limit of virus detection was = 10 PFU/ml. For controls stock DEN-16681 PDK-53 (LAV2) vaccine was plated.

4.1.1.2.2 PRNT (Plaque Reduction Neutralization Test)

Neutralizing antibodies were quantified as described in Huang et al. (2000). Briefly, 0.2 ml of heat-inactivated, 1:5 dilution of serum was added to the first well of 96-well plate and serial 2-fold dilution series were made, transferring 0.1 ml into 0.1 ml of diluent (RPMI medium) for each dilution. This resulted in a 1:10 - 1:320 serum dilution series. 0.1 ml of DEN virus (60-160 PFU; parental DEN2 16681 virus) was added to each serum dilution well for a total of 0.2 ml of serum-virus mixture. 96-well plates were incubated overnight at 4°C. 0.1 ml of serum-virus mixtures (containing 30-80 PFU of input virus) were plated in 6-well Vero plates (as indicated above in the Viremia section)

and plaques were counted after staining with neutral red. Multiple back titrations of the input viruses at 2-fold, 1-fold, and 0.5-fold test concentrations provided direct experimental determination of the input PFU, which was the basis for determining 50% ($PRNT_{50}$) and 70% ($PRNT_{70}$) endpoint antibody titers. A negative serum result should have a neutralizing antibody titer of < 1:10. Sera showing neutralization titers of 320 were retested at dilutions 1:80 - 1:2560 for determination of endpoint titer.

4.1.2 Evaluation of Monovalent VDV2 Candidate at passage 9 in Monkeys

Purification/selection of VDV2 candidate has been conducted as described in example 1. The selected clones (based on phenotypic markers and sequence) have been tested after 9 passages in cell culture in Sanofi Pasteur on male cynomolgus macaques (*Macaca fascicularis*, mean weight 3.1 kg) originating from CRP Le Vallon, Mauritius.

After immunization on D0, viremia was followed from D2 to D10, and immunogenicity measured at D0 and D28. All viruses and vaccines, when in liquid form, were kept at –70°C.

LAV2 : titre : $10^{3,93}$ $DICC_{50}$ / ml; lyophilized, resuspended in 0.5 ml of PBS (containing Ca^{2+} and Mg^{2+}; $CaCl_2.2H_2O$ 0.133 g/l; $MgCl_2.6H_2O$, 0.1g/l) and administered in totality.

Passage VDV2 DEN2-TV722 (2 plaque purifications + 1 amplification) : Titre : $10^{5,6}$ $DICC_{50}$ /ml; liquid, diluted at $10^{5,3}$ pfu / ml in PBS (containing Ca^{2+} and Mg^{2+}; $CaCl_2.2H_2O$ 0,133 g/l; $MgCl_2.6H_20$, 0,1g/l); 0.5 ml administered.

Injection was done by SC route in the arm with a 23G1 needle, at a 10^5 $DICC_{50}$ dose for VDV2.

The results are as presented in Table 11. Titration at day 28 were carried out in triplicate for both $PRNT_{70}$ or and $PRNT_{50}$.

The comparison between VDV2 and LAV2 showed clear differences in viremia, with high viremia of short duration for VDV2 in 3/4 monkeys compared to LAV2, and significant immunogenicity for both types (rather lower for VDV2). This viremia may be considered as too high for VDV2 at this pre-master level after only a few passages on Vero cells. However, wild type DEN-2 (and other types too) induce viremia of longer

duration (6 to 7 days) and intensity (up to 5 logs plaque forming units [pfu]) (Monath et al., 2000; Bray et al., 1996).

Table 11: VDV2 passage 9 immunogenicity

		Neutralizing Antibody Titer				**Viremia** (PFU/ml in Vero cells)									
Serum	Group	Day (-15)		Day 28		Day -15	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
		$PRNT_{70}$	$PRNT_{50}$	$PRNT_{70}$	$PRNT_{50}$										
AD 097	LAV DEN-2	<10	<10	80/80/160	320/160/320	0	0	0	0	0	0	5	0	50	20
AC 170		<10	<10	160/80/320	320/160/640	0	0	0	0	0	5	5	0	0	0
AD 677		<10	<10	1280/640/2560	2560/1280/2560	0	5	0	0	10	50	0	5	0	0
AC 182		<10	<10	320/320/320	640/1280/1280	0	0	5	0	15	5	0	5	0	0
AC 658	VDV DEN-2	<10	<10	160/160/160	320/160/640	0	550	35	0	0	0	0	0	0	0
AC 512		<10	<10	160/80/160	160/160/160	0	1650	35	0	5	0	0	0	0	0
AD 608		<10	<10	160/320/160	320/320/320	0	1700	60	100	0	0	0	0	0	0
AD 132		<10	<10	80/80/80	80/160/160	0	70	10	0	50	10	100	0	0	0
Virus	Exp#1	Exp#2	Exp#3												
DEN-2	60PFU	54PFU	46PFU												

4.1.3 Evaluation of Monovalent VDV2 Candidate at passage 11

As immunogenicity of the vaccines had been tested at the passage 9, a further experiment was designed to test the monovalent passage after two additional passages (passage 10).

Male *Macaca fascicularis* monkeys were used as before, originating from C.R.P. Le Vallon, Ile Maurice (24 monkeys, mean weight 3.4 kg).

Passage 11 VDV2; Batch : Titre : 8,07 log10 $DICC_{50}$ /ml

Placebo: PBS with Ca^{2+} and Mg^{2+}

VDV3: VERO-Derived Vaccine Dengue serotype 3 strain, obtained by sanitization of LAV3 on Vero cells.

VDV4: VERO-Derived Vaccine Dengue serotype 4 strain, obtained by sanitization of LAV4 on Vero cells.

Vaccines were diluted at $10^{5.3}$ $DICC_{50}$ /ml in PBS (containing Ca^{2+} and Mg^{2+}; $CaCl_2.2H_2O$ 0.133 g/l; $MgCl_2.6H_2O$, 0.1g/l); 0.5 ml administered by SC route in the arm with a 23G1 needle, corresponding to a dose of 10^5 $DICC_{50}$.

Viremia and immunogenicity have been measured as usual in CDC by R Kinney. The results are shown in Table 12.

VDV2 passage 11 monovalent vaccine induced a significant immune response, while viremia was low or absent. The absent/low VDV2-induced viremia is to be considered in light of the previous experiment in which the passage 9 VDV2 induced high early viremia. Some evolution between passages 9 and 11 suppressed this high viremia while immunogenicity was maintained. VDV2 therefore constitutes an acceptable candidate.

It is to be noted that in the same experiment, 4 monkeys were vaccinated with a tetravalent formulation involving the same VDV2 passage 11 vaccine; no viremia was detected for VDV1 and VDV2 while VDV3 and VDV4 induced viremia.

Two other experiments involved the administration of VDV2, alone or in combination with the other serotypes.

In the first one (tetravalent study; 5-log of each serotype), no viremia was detected for VDV2, and VDV1, while high levels of viremia were detected for VDV3 and VDV4.

In the second experiment, VDV2 passage 11 was administered alone or within a tetravalent combination including VDV1. When administered alone, VDV2 passage 11 induced a low viremia (peak 40) in only 1 out of 4 monkeys while the 3 others were negative. When present within tetravalent formulations, VDV2 induced no or dramatically lower viremia than VDV3 and VDV4, even though VDV2 was administered at 4 log while VDV3 and VDV4 were administered at 2 log. This demonstrates the higher safety of VDV2, as far as viremia is concerned. Monovalent VDV2 thus fulfilled the success criteria initially defined in monkeys.

Table 12: passage 11 VDV2 immunogenicity and viremia

		Neutralizing Antibody Titer				**Viremia** (PFU/ml in Vero cells)								
		Day (-14)		Day 29		Day	Day	Day	Day	Day	Day	Day	Day	Day
Monkey	Group	$PRNT_{50}$	$PRNT_{70}$	$PRNT_{50}$	$PRNT_{50}$	-14	3	4	5	6	7	8	9	10
AE 971	VDV DEN-2	-	-	180	80	0	0	0	0	0	0	0	0	0
AE 990		-	-	160	50	0	15	0	0	5	0	0	0	0
AE 998		-	-	905	508	0	0	0	0	0	0	0	0	0
AF 182		-	-	285	101	0	0	0	0	0	0	0	0	0
Geo Mean	homologous	-	-	293	119									
AE 538	Placebo	-/-/-/-	-/-/-/-	2.5/-/2/2	-/-/-/-	0	0	0	0	0	0	0	0	0
AE 548		-/-/-/-	-/-/-/-	-/-/1/2	-/-/-/-	0	0	0	0	0	0	0	0	0
AE 556		-/-/1.5/2	-/-/-/-	1/-/-/-	-/-/-/-	0	0	0	0	0	0	0	0	0
AE 572		-/-/1.5/5	-/-/1.5/2	5/-/-/2	-/-/-/-	0	0	0	0	0	0	0	0	0
Geo Mean		-/-/1.2/3	-/-/1/1	2/-/1.2/1.6	-/-/-/-									
		D1/D2/D3/D4	D1/D2/D3/D4	D1/D2/D3/D4	D1/D2/D3/D4									

4.2 Toxicology of VDV2

4.2.1 Neurovirulence Tests in Monkeys

The objective of this test was to demonstrate the lack of neurotropic properties in monkeys (Ph. Eur. 2.6.18) of the attenuated 2 dengue virus seed produced by Sanofi Pasteur.

10 cynomolgus monkeys from Mauritius were inoculated with VDV2 passage 9 by the intracerebral route ($10^{7.10}$ $CCID_{50}$/ in the thalamus of each hemisphere). At the end of the test, the monkeys were sacrificed and perfused with formaline solution. Tissue samples were taken from the brain of each monkey (medulla oblongata, pons and cerebellum, midbrain, thalamus including the left and the right parts, the left and the right of the cerebral cortex). Sections were cut at a thickness of 8 µm and stained by eosin and gallocyanin.

No histopathological signs of pathogenicity were observed in the monkey brains injected with serotype 2 primary virus seed.

4.2.2 GLP Toxicity Study in the Cynomolgus Monkey after 1 Subcutaneous Administration of VDV2 Followed by a 28-Day Observation Period

The objective of this GLP study was to assess the interactions between VDV2 passage 9 and other Dengue vaccine candidates. The 1st step of the study was to assess the safety and immunogenicity of VDV2 prior to the administration of another vaccine candidate.

One human dose of VDV2 (approximately 10^4 $CCID_{50}$ per dose) was administered subcutaneously on Day 0 to cynomolgus monkeys (4 males and 4 females). A control group of 2 males and 2 females received the vehicle (4‰ NaCl).

Mortality, clinical condition, body weight, and food consumption were monitored throughout the study. Body temperature was taken once pre-test, daily from the day of each administration and during 2 days after. Blood samples were taken for clinical laboratory determinations once pre-test and on Days 8 and 27.

There were no effects on clinical signs, body weight, food consumption, dermal reactions, body temperature, haematology, clinical chemistry, or organ weights. No deaths were reported during the study.

In conclusion, the subcutaneous administration of VDV2 to the cynomolgus monkey (*Macaca fascicularis*) at the test doses did not adversely affect the health of the monkeys as assessed by in-life clinical observations and clinical pathology.

Example 5: SAFETY OF MONOVALENT VDV2 IN HEALTHY, FLAVIVIRUS-NAIVE ADULTS AGED 18 TO 40 YEARS

The aim of this phase 1 trial is to document the safety, viremia, and immunogenicity profiles of monovalent VDV2 passage 11at a virus concentration of 10^4 $CCID_{50}$ compared to Stamaril® (used as control group) in flavivirus-naive adults. Single injections are given, with follow-up at 6 and 12 months. For safety precaution, sequential inclusions are performed in the study.

Enrollment and vaccinations are therefore staggered; a 1st cohort (n=4 per group, total n=12) have been vaccinated. The safety data collected up to Day 28 have been reviewed by an Independent Data Monitoring Committee (IDMC) and by the Royal Adelaide Hospital Investigational Drugs Subcommittee (IDSC) before deciding to proceed with the vaccination of the remaining subjects (n=8per group, total n=16). A schematic representation of the trial design is provided in figure 6.

After administration of the vaccine the patient are regularly submitted to various clinical examination and testing. A summary of this follow up is given in Table 13 below.

The enrolled population consists of adults aged 18 to 40 years (i.e. the day of the 18th birthday to the day before the 41st birthday) on day of inclusion who are flaviviruses-naïve [persons presenting vaccination against flavivirus diseases (e.g. yellow fever, Japanese encephalitis, dengue fever); or history of flavivirus infection (confirmed either clinically, serologically or microbiologically) or previous residence in or travel to areas with high dengue infection endemicity (whatever the duration), or residence in or travel to North Queensland for 2 weeks or more) were excluded]

Table 13: Flow chart for follow up

Visit Number	V01	V02	V03	V04	V05	V06	V07	V08	V09	V10	V11	V12
Trial timelines[¤]	D0	D2	D4	D6	D8	D10	D12	D14	D16	D28	D180	D365
Time Windows						±1d	±1d			±4d	±15d	±30d
Clinical Examination	√	√	√	√	√	√	√	√	√	√	√	√
Vital signs (BP, pulse rate)	√											
Oral temperature	√	√	√	√	√	√	√	√	√	√		
Blood Sampling:												
-Serology HBV/HCV/HIV												
-Biological Safety												
-Viremia												
-Immunogenicity	√		√		√		√		√	√		
-Cytokines in serum	√	√	√	√	√	√	√	√	√			
-PBMCs for T cell	√								√	√	√	√
(subset)	√	√	√	√	√	√	√	√	√	√		
-immediate surveillance	√									√		
Local & systemic events	√									√		
	√											
	√	√	√	√	√	√	√	√	√	√	√	√

V: visit - D: day

¤ Time intervals between visits will be calculated from the date of study vaccination which might differ from the date of visit (e.g. in case a temporary exclusion criterion is met). V06 and V07 must be done with at least 1-day interval.

The products tested are:

The vaccine evaluated is a lyophilised product in a vial that is reconstituted extemporaneously with the diluent provided separately:

Active ingredient: 4 ±0.5 $\log_{10}$ $CCID_{50}$ of monovalent Vero dengue virus serotype 2 (VDV2 passage 11) per 0.5 mL dose;

Diluent: Sterile NaCl 4‰ solution for vaccine reconstitution.

The reconstituted vaccine, i.e 0.5 mL of NaCl 4‰ solution of monovalent VDV2, should be used immediately or be maintained until use +2°C and +8°C.

The 0.5 mL vaccine dose is administered subcutaneously in the deltoid region.

The control vaccine Stamaril®, is a yellow fever vaccine produced by Aventis Pasteur. Stamaril® is presented as a lyophilised, avian-leukosis-free, stabilised product to be reconstituted with a diluent immediately before use. (Active ingredient: Live attenuated yellow fever virus (17D strain): ≥ 1,000 mouse Lethal Dose 50% (LD_{50})/Diluent: Sterile NaCl 4‰ solution).

The control vaccine is administered subcutaneously in the deltoid region.

No subject had clinically significant syndrome related to vaccination. One subject had a transient fever (<38 °C). One subject had a local reaction (induration). No serious adverse event related to vaccination was observed.

All subjects have antibodies response 28 days after vaccination against dengue 2 (titer between 1888 and 6393)

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CLAIMS

1. A live attenuated dengue-2 virus strain which comprises sequence SEQ ID No. 38 wherein at least nucleotides at positions 736, 1619, 4723, 5062, 9191, 10063, and 10507, are mutated, with the proviso that the following nucleotides are not mutated : 57, 524, 2055, 2579, 4018, 5547, 6599, and 8571, wherein said live attenuated dengue-2 virus strain is adapted for growth in VERO cells and induces a specific humoral immune response including neutralizing antibodies in primate.

2. The dengue-2 virus strain according to claim 1, wherein the primate is a human.

3. The dengue-2 virus strain according to claim 1 or 2, wherein at least a nucleotide is further mutated at a position consisting of positions 1638, 2520, 9222, or 10361.

4. The dengue-2 virus strain according to any one of claims 1 to 3, wherein SEQ ID No. 38 comprises the mutations 736 G>C, 1619 G>A, 2520 G>A, 4723 T>A, 5062 G>C, 9191 G>A, 9222 A>G, 10063 T>A, and 10507 A>G.

5. The dengue-2 virus strain according to any one of claims 1 to 4, which further comprises a substitution of one or more nucleotides in a given codon position which excludes an alteration in the amino acid encoded at that position.

6. The dengue-2 virus strain according to any one of claims 1 to 5, which comprises SEQ ID No. 1.

7. An immunogenic composition comprising the live attenuated dengue-2 virus strain as defined in any one of claims 1 to 6, in a pharmaceutially acceptable carrier.

8. The immunogenic composition according to claim 7, which is a monovalent vaccine composition.

9. The immunogenic composition according to claim 7, which is a multivalent dengue vaccine composition.

10. The immunogenic composition according to claim 9, which comprises a live attenuated dengue-1 virus strain which comprises sequence SEQ ID No. 39.

11. The immunogenic composition according to any one of claims 7 to 10, which contains 10 to 10^5 $CCID_{50}$ live attenuated dengue-2 virus strain as defined in any one of claims 1 to 5.

12. An isolated nucleic acid which comprises the DNA sequence SEQ ID No. 1 or its equivalent RNA sequence.

13. An isolated polyprotein encoded by SEQ ID No. 1 or fragments thereof of at least 8 amino acids that comprise at least an arginine at position 9 of M protein, and/or a glutamic acid at position 228 of E protein, and/or threonine at position 69 of NS3 protein, and/or a histidine at position 181 of NS3 protein, and/or a lysine at position 541 of NS5 protein, and/or a threonine at position 832 of NS5 protein, wherein said polyprotein or said fragments thereof induce a specific humoral immune response including neutralizing antibodies in primate.

14. The isolated polyprotein according to claim 13, wherein the primate is a human.

15. A fragment of the polyprotein as defined in claim 13 or 14 which comprises M protein, and/or E protein, and/or NS3 protein and/or NS5 protein.

16. A dengue-2 virus strain encoded by SEQ ID NO : 1.

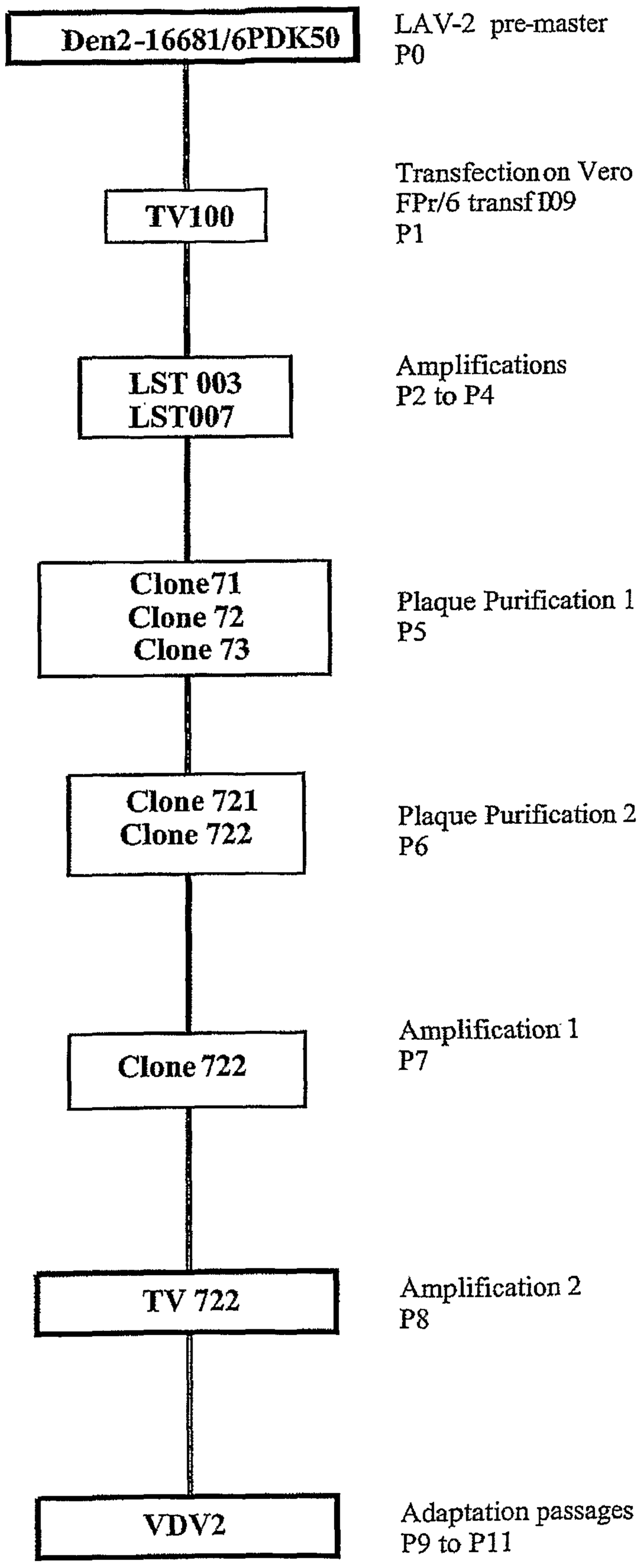
Den2-16681/6PDK50
LAV-2 pre-master
P0
TV100
Transfection on Vero cells
FPr/6 transf D09
P1
LST 003
LST007
Amplifications
P2 to P4
Clone71
Clone 72
Clone 73
Plaque Purification 1
P5
Clone 721
Clone 722
Plaque Purification 2
P6
Clone 722
Amplification 1
P7
TV 722
Amplification 2
P8
VDV2
Adaptation passages
P9 to P11

FIG.1

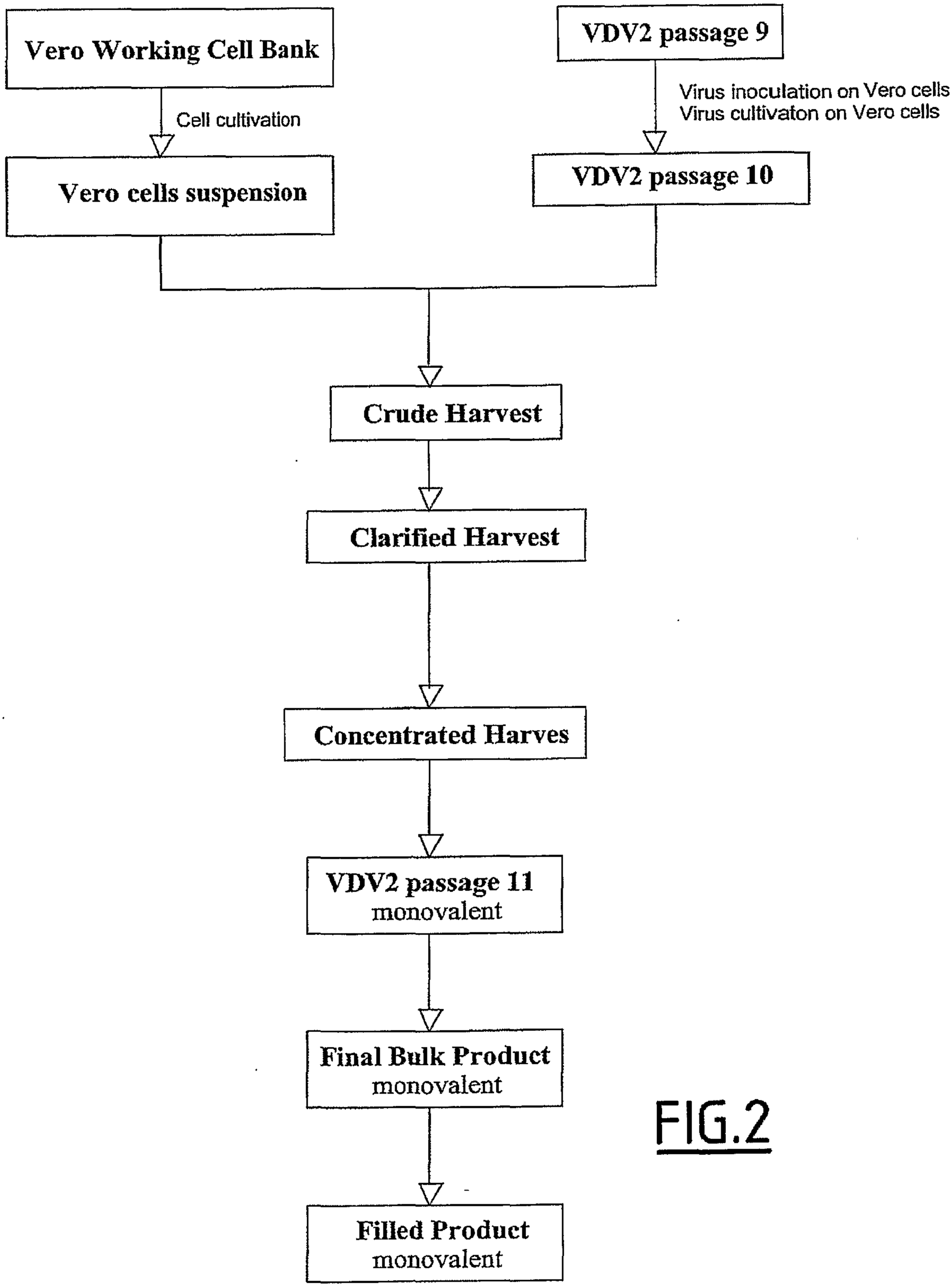
Vero Working Cell Bank
Cell cultivation
Vero cells suspension
VDV2 passage 9
Virus inoculation on Vero cells
Virus cultivaton on Vero cells
VDV2 passage 10
Crude Harvest
Clarified Harvest
Concentrated Harves
VDV2 passage 11
monovalent
Final Bulk Product
monovalent
Filled Product
monovalent

FIG.2

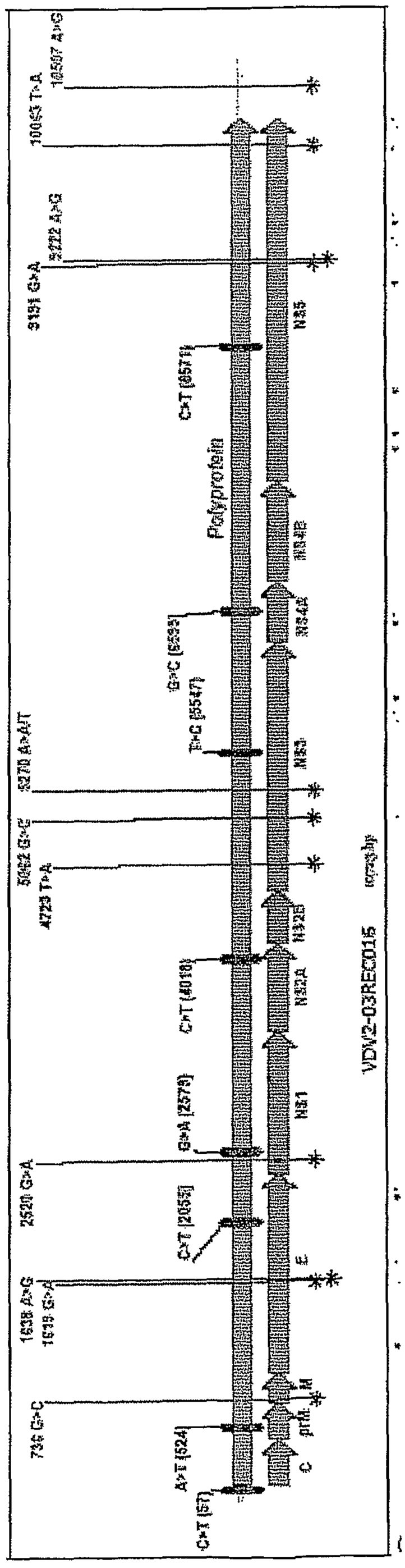

FIG.3

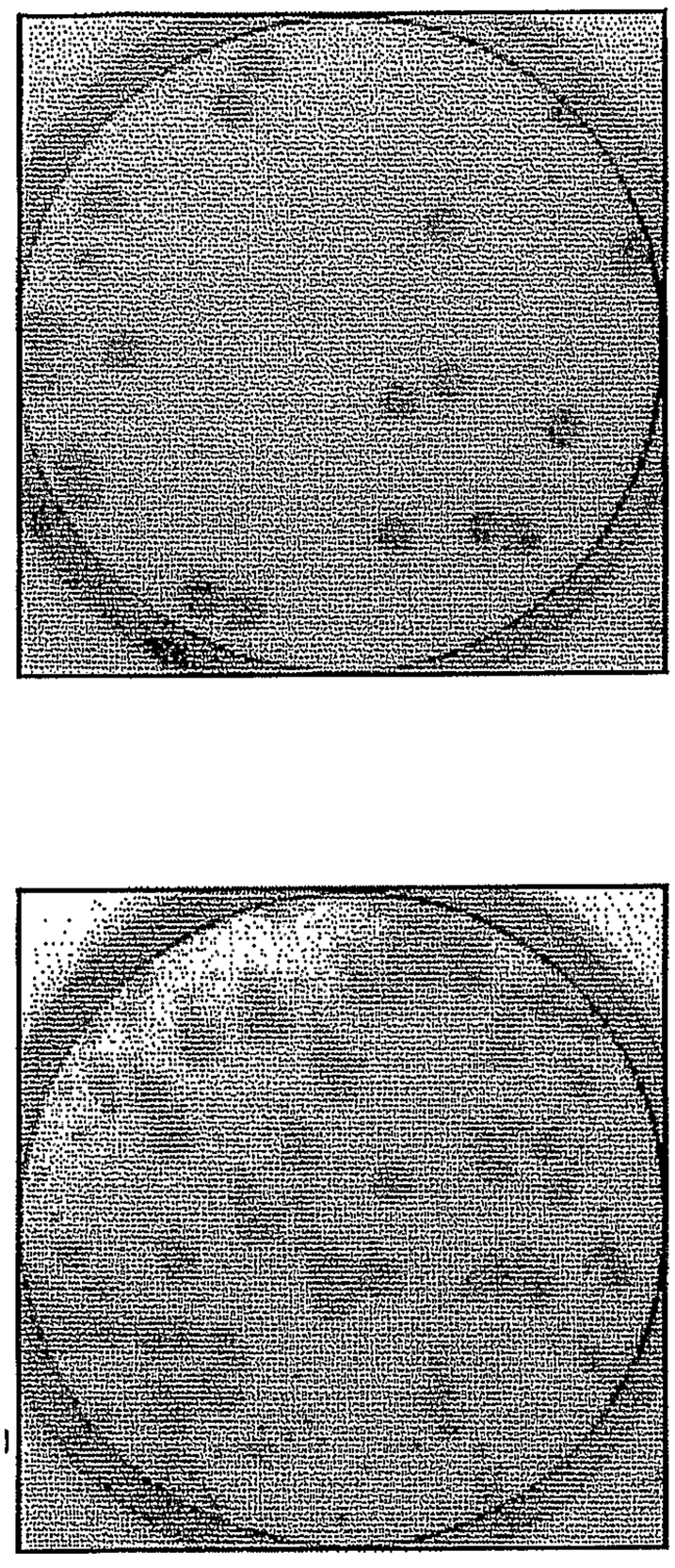

FIG.4

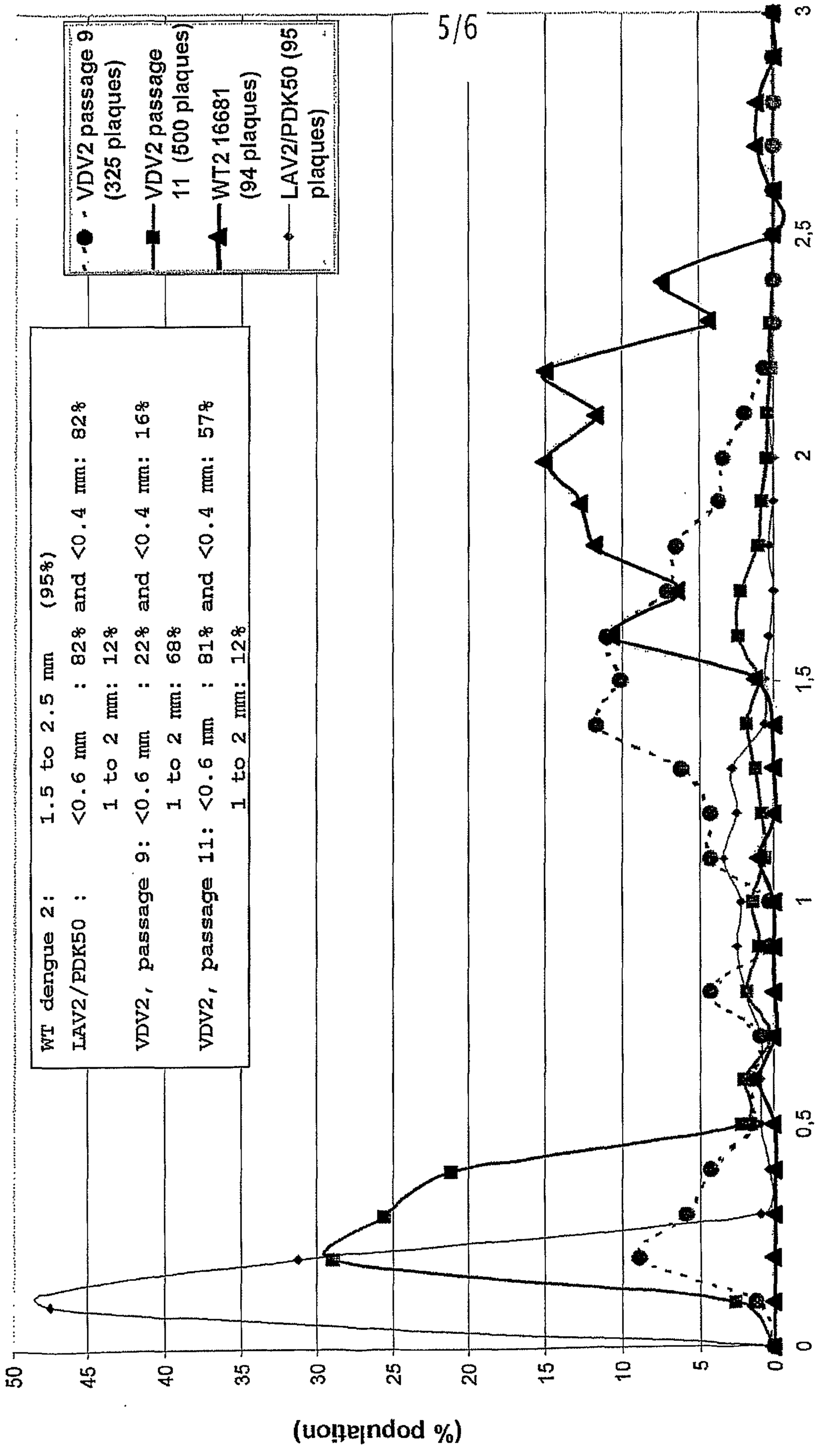
VDV2 passage 9 (325 plaques)
VDV2 passage 11 (500 plaques)
WT2 16681 (94 plaques)
LAV2/PDK50 (95 plaques)
(95%)
% and <0.4 mm: 82%
%
% and <0.4 mm: 16%
%
% and <0.4 mm: 57%
%
WT dengue 2: 1.5 to 2.5 mm
LAV2/PDK50 : <0.6 mm : 82%
1 to 2 mm: 12%
VDV2, passage 9: <0.6 mm : 22%
1 to 2 mm: 68%
VDV2, passage 11: <0.6 mm : 81%
1 to 2 mm: 12%
Plaque diameter (mm)
(% population)
0
5
10
15
20
25
30
35
40
45
50
0,5
1
1,5
2
2,5
3

FIG.5

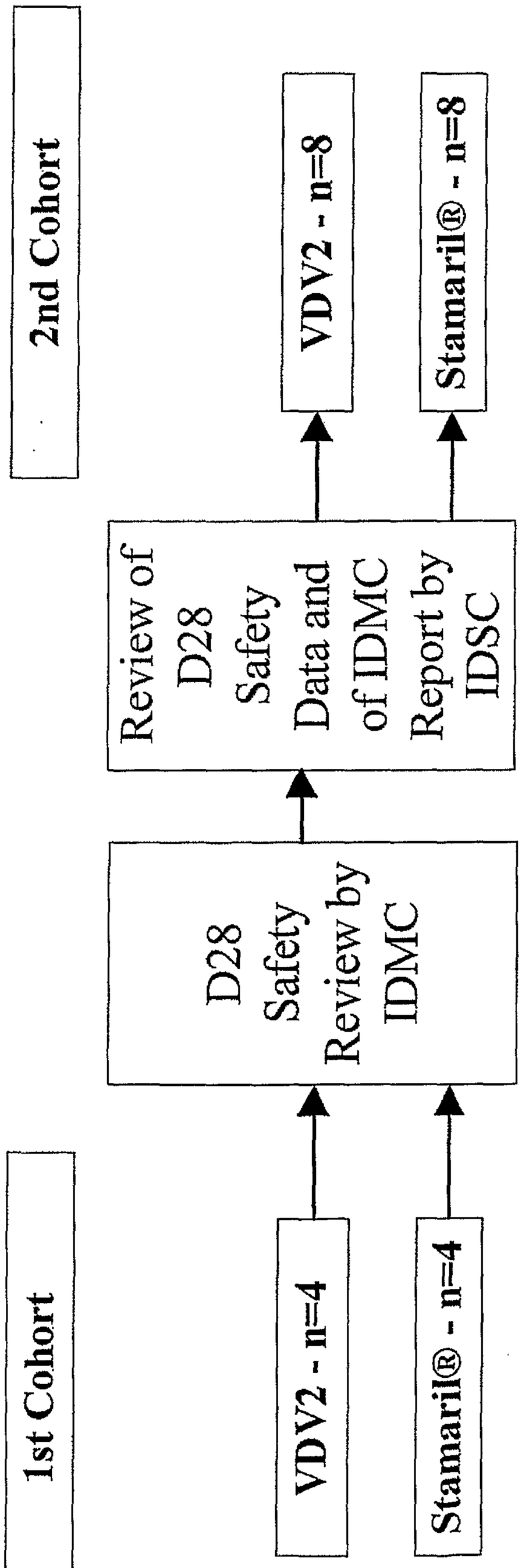
1st Cohort
VDV2 - n=4
Stamaril® - n=4
D28 Safety Review by IDMC
Review of D28 Safety Data and of IDMC Report by IDSC
2nd Cohort
VDV2 - n=8
Stamaril® - n=8

FIG.6