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Complementary use of mass spectrometry and cryo-electron microscopy to assess the maturity of live attenuated dengue vaccine viruses

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ABSTRACT

Dengue virus (DENV) infection is a global health threat with the potential to affect at least 3.6 billion people living in areas of risk. No specific curative treatments against dengue disease are available and vaccines are currently the only way to prevent the disease. The tetravalent dengue vaccine developed by Sanofi Pasteur has demonstrated significant efficacy in phase III studies and is now licensed in several countries for the prevention of disease in dengue-seropositives over 9 years of age. The vaccine is composed of four recombinant, live, attenuated vaccines (CYD 1–4) based on a yellow fever vaccine 17D (YFV 17D) backbone, each expressing the pre-membrane (prM) and envelope (E) genes of one of the four DENV serotypes. Virus maturity could impact the biological activity of the vaccine viruses. To address this question, the maturity of the four vaccine viruses used in phase III clinical studies was assessed by two complementary techniques: mass spectrometry (MS) and cryo-electron microscopy (cryoEM). MS assessed viral maturity at the molecular level by quantifying specifically the prM, and M proteins. CryoEM provided information at the particle level, allowing visualizing the different phenotypes of viral particles: spiky (immature), smooth/bumpy (mature), and mixed (partially mature). Results of the two assays used in this study show that all four CYD dengue vaccine viruses present in lots used in phase III efficacy trials, display in the majority a mature phenotype.

1. Introduction

Dengue virus (DENV) infection is a global health threat with the potential to affect at least 3.6 billion people living in areas of risk in more than 125 countries of the tropics and subtropics [1]. The disease can present as a mild self-limiting illness, dengue fever (DF), or as the more severe forms of the disease; dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [2]. An estimated 390 million dengue infections occur annually, of which 96 million are symptomatic with the remainder, 294 million infections, asymptomatic but nonetheless contributing to the circulating dengue reservoir. The incidence of dengue has increased 30-fold over the last 50 years, accelerated by globalization, urbanization, and climate change, but the true magnitude of the disease burden likely continues to be underestimated [3]. No specific curative treatments against dengue disease are available and vaccines are currently the only way to prevent disease. The tetravalent dengue vaccine developed by Sanofi Pasteur (CYD-TDV) [4] demonstrated significant efficacy in the first two years (active phase) of phase III studies conducted in Latin America [5] and Asia [6,7]. It is now licensed in several countries and was recently approved by the European Medicine Agency (EMA). The vaccine is indicated in the dengue-seropositive population over 9 years of age, in agreement with the 2018 WHO position [8]. CYD-TDV is composed of four recombinant, live, attenuated vaccines (CYD-1 to 4) based

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on a yellow fever vaccine 17D (YFV 17D) backbone, each expressing the pre-membrane (prM) and envelope (E) genes of one of the four DENV serotypes [4].

DENV is a member of the genus of flaviviruses, which are small, enveloped, icosahedral viruses with a single copy of a positive-strand RNA genome, and is composed of an outer glycoprotein shell and an internal host derived lipid bilayer. Inside the latter is an RNA-protein core consisting of genome RNA and capsid proteins (C). The glycoprotein shell is well defined and consists of 180 copies each of an envelope (E) and premembrane/membrane protein (prM/M). The viral envelope maturation process occurs during transport through the Trans-Golgi Network (TGN) of the infected cells. In immature particles, the E protein forms heterodimers with prM protein, the M protein precursor, that extend as trimer spikes $(E:prM)_3$ from the particle surface, conferring the "spiky" virion morphology observed in cryo-electron microscopy (cryoEM). Then, the viral envelope protein E undergoes conformational changes triggered by the low pH in the lumen of the TGN. The trimeric spikes $(E:prM)_3$ of the immature particle change to dimers $(E:prM)_2$ and expose the prM protein cleavage site. The pr protein is cleaved from prM by a host encoded furin protease, to form a "smooth", mature virion as observed by cryoEM. Once cleaved, the pr protein remains associated with the E protein, until release into the extra-cellular environment, in which neutral pH allows pr release [9]. Yu et al. [10] proposed the hypothesis that pr occurs as a cap-like or chaperone structure that prevents E peptide-mediated premature fusion before virus is released. Maturation can be an incomplete process and partially mature viruses, prM-containing virus characterized by structural features of both mature and immature particles, can often be observed [11]. An additional conformational change has been revealed for some DENV-2 strains when these viruses are exposed to temperature higher than 33 °C [12]. Mature viruses which have a smooth form exhibit a bumpy form with a larger diameter when exposed at 37 °C, and it is likely that the bumpy form is present in humans [13].

It was suggested that maturity could play a critical role in the infectivity of DENV [14]. Nevertheless, it remains unclear if maturity can also impact the immunogenicity, safety and protection triggered by live vaccine viruses. It was observed that vaccine efficacy varied according to serotype in the Sanofi-Pasteur phase III clinical studies, with a higher efficacy afforded by vaccine serotypes 3 and 4 as compared to serotypes 1 and 2 [6–8]. Among other parameters and potential causes for such differences [15], it was also of interest to investigate virus maturity in the lots used in these trials. A previous study has been conducted with human monoclonal antibodies targeting key conformational epitopes, showing the presence of such epitopes on CYD dengue viruses; CYD-2 maturity was furthermore documented in that investigations [16]. To complement that study and further address the structure of the CYD viruses, the maturity of the four vaccine viruses that were used in phase III clinical studies was assessed by two complementary techniques: mass spectrometry (MS) and cryoEM. The first technique assessed viral maturity at the molecular level by quantifying specifically the prM, and M proteins [17]. The second provides information at the particle level by allowing visualizing the different phenotypes of viral particles: spiky (immature), smooth/bumpy (mature), and mixed (partially mature).

2. Materials and methods

2.1. Samples

Chimeric Yellow Fever Dengue (CYD) vaccine batches of serotypes 1, 2, 3 or 4, produced from Vero cell cultures, were used for MS experiments. Sample concentrations were estimated to be between 45 and 307 μg/ml of total protein established using the Bradford assay. Virus concentrations were determined by reverse-transcription quantitative PCR, and expressed through log genome-equivalent values, found to be between 10.2 and 11.5 particles/ml [34] (Table 1). CYD viruses of serotypes 1, 2, 3 or 4 were further purified from vaccine batches by PEG precipitation and were used for cryo-EM experiments.

2.2. Mass spectrometry-based assessment of virus population mean maturity

Samples were submitted to single or double digestion, either with sodium deoxycholate trypsin digestion protocol or high pressure assisted gluC or gluC/aspN double digestion protocol using Barocycler® instrumentation [17,35,36]. AQUA® peptide standards [21] were added before digestion with sodium deoxycholate (DOC) protocol, while they were added at the beginning of the process for the double digestion. After desalting, sample peptide mixtures were analyzed with targeted MS coupled to liquid chromatography, and data were processed with the open source software Skyline [37] (Table 2) (see details in SI materials and methods).

2.3. CryoElectron microscopy

A quantifoil copper grid with circular holes (Ted Pella, Redding CA, USA) was covered with a fine layer of carbon and then submitted to glow discharge. A 5 μl droplet of virus sample was deposited onto the copper side of the grid. The excess of sample was blotted with filter paper and the grid was flash frozen in liquid ethane using a grid-plunging device (EMGP, Leica Microsystems, Vienna, Austria) at 15 °C and under conditions of 70% relative humidity. Specimens were maintained at a temperature of approximately −170 °C, using a 626 cryo holder (Gatan, Pleasanton CA, USA) and observed with a FEI Tecnai F20 electron microscope operating at 200 kV and at a nominal magnification of 29,000x under low-dose conditions. Images were recorded with a 2 k × 2 k USC 1000 slow-scan CCD camera (Gatan). Pixel size was 0.37 nm. Due to the heterogeneity of the viral particle surface, they were classified into smooth, bumpy, partially spiky or full-spiky by a single, blinded human operator.

3. Results

3.1. Mass spectrometry-based maturity determination at molecular level

Due to the low concentration of virus in monovalent vaccine bulk (Table 1), an MS technique was chosen to evaluate mean viral population maturity at the molecular level. The combination of stable isotope dilution (SID) and selected reaction monitoring (SRM) are currently considered as the gold standard methods for MS quantification in the proteomics field, and used in many applications such as metabolic engineering [18] and systems biology [19]. To ensure sensitivity and specificity, SRM was chosen as it confers two levels of selection, achieved by filtering predefined peptide ions and specific fragment ions [20]. Combined with SRM analysis, the SID strategy ensured accuracy and precision, using heavy peptide standards [21].

During the maturation process, once cleaved, protein pr is released in the extracellular environment, and eliminated during virus vaccine purification process. Therefore, it appeared judicious to estimate the virus maturity by the ratio of protein prM divided by the sum of protein M and protein prM. To ensure exclusive protein prM quantification, only junction peptide could be used, which was positioned astride protein pr and protein M. However, this junction peptide contained a trypsin cleavage site identical to the

Table 1
Characteristics of the samples analyzed in the study. Total protein concentrations and viral concentrations are indicated. Protein concentrations were quantitated by classical Bradford assay, while virus concentrations were determined by reverse-transcription quantitative PCR, and expressed as log genome-equivalent (Geq) values.

Serotype	Sample name	Phase III clinical trial	[Total proteins] (μg/ml)	Log Geq (particles/ml)
CYD1	FDV01131	CYD14/CYD15	124.5	11.5
	FDV01151		151.6	11.3
	FDV01168		134.0	11.1
CYD2	FDV01219	CYD14/CYD15	87.0	10.2
	FDV01181		45.5	10.8
	FDV01198		124.5	10.8
CYD3	FDV01228	CYD14/CYD15	103.5	10.2
	FDV01252		103.5	10.6
	FDV01319		62.5	10.4
CYD4	FDV01254	CYD14/CYD15	307.5	10.8
	FDV01286		130.5	11.0
	FDV01318		235.5	10.8

furin cleavage site. As a consequence, trypsin digestion of immature forms of the virus led to the release of C-terminus (C-ter) peptide of protein pr and N-terminus (N-ter) peptide of protein M, both identical to peptides released from mature virus. Thus, it becomes impossible to distinguish peptides released from immature and mature virions (Fig. 1).

To overcome this problem, gluC enzyme digestion was appropriate, since cleavage occurs after glutamate amino -acids and at a site different than the furin cleavage site [17]. Junction peptide subsequently allowed protein prM to be quantified, linked to the immature portion of the overall virus population in monovalent vaccine bulk. N-ter peptide of protein M released after furin cleavage was selected to quantify protein M, linked to the mature portion of the overall virus population in monovalent vaccine bulk (Fig. 1). Considering that only one junction peptide and one protein M peptide (for each serotype) can be used for quantification, methionine containing peptides were included (despite being routinely avoided in quantification experiments, owing to their oxidation properties) [20]. This was the case for junction peptide of serotype 2 (CYD2), serotype 3 (CYD3) and serotype 4 (CYD4). To avoid quantification errors due to methionine-containing peptides, an oxidation protocol was used, based on performic acid properties (SI material and methods). Di-oxidized peptides were monitored, but also mono-oxidized to ensure they were present in insignificant amounts. For serotypes 1 and 3, another difficulty was encountered. GluC enzyme digestion resulted in junction peptides which were too long, with poor ionization capabilities and consequently low MS signal. A double digestion was thus performed, applying sequentially gluC then aspN enzymes, which cut after glutamate amino-acids and before aspartate amino-acids, respectively (SI material and methods).

To assess gluC (CYD2 and CYD4) and gluC/aspN (CYD1 and CYD3) pipeline consistency, protein E was also quantified. The concentration value obtained for protein E was compared with the sum of concentrations obtained for proteins prM and M (theoretically they must be in equal molarity based on the cryo-EM structural data) [9]. Furthermore, concentrations obtained for protein E with gluC protocol [E_{gluC}] were compared with those obtained using a trypsin protocol [E_{tryp}]), as described in a previous study [22]. The list of all monitored peptides is summarized in Table 2.

Accurate quantification of three phase III clinical monovalent batches for each serotype (CYD 1–4) was performed (Table 3), with trypsin and gluC or gluC/aspN digestion. To assess the consistency of the results, the sum of the concentrations of prM and M proteins were compared to protein $E_{gluc/aspN}$ concentrations for each sample (Δ1 calculation). Theoretical expectations were confirmed (Table 3 and SI Raw quantification results dataset), since for all samples, calculated deviations were less than 25% with a median value at 12.8%. Moreover, for each sample $E_{gluc/aspN}$ concentrations were compared with values obtained for the concentrations of protein E with trypsin protocol (E_{tryp}) (SI Table 1 and SI Raw quantification results dataset). Once again, values were found to be very close with deviations lower than 20% (Δ2 calculation) (Table 3 and SI Raw quantification results dataset). Finally, for each sample, the relevance of E_{tryp} determined concentration was assessed by comparison with determined values of protein prM/M (trypsin). Results were consistent, since deviation did not exceed 15% (Δ3 calculation; SI Table 1 and SI Raw quantification results dataset) with exception for CYD2 serotype. For the latter, deviation was found to be around 20%, probably due to overestimation of protein prM/M caused by adsorption of the SVALVPHVGMGLETR standard

Table 2
List of gluC, gluC/aspN, and trypsin peptides monitored for MS-based quantification of sample CYD1, CYD2, CYD3, and CYD4.

Serotypes & enzyme	Proteins	Peptides
CYD1 (gluC/aspN peptides)	PrM	DKRSVALAPHVGLGLE
	M	SVALAPHVGLGLE
	E	GAMHTALTGATE
CYD2 (gluC peptides)	PrM	KRSVALVPHVGMGLE
	M	SVALVPHVGMGLE
	E	KDSPVNIE
CYD3 (gluC/aspN peptides)	PrM	DKRSVALAPHVGMGL
	M	SVALAPHVGMGL
	E	GAMHTALTGATE
CYD4 (gluC peptides)	PrM	KRSVALTPHSGMGLE
	M	SVALTPHSGMGLE
	E	GAMHSALAGATE
CYD1 (trypsin peptides)	Pr/PrM	FHLTTR
		SLLFK
	PrM/M	VETWALR
	E	TEVTNPAVLR
		VITANPIVTDK
CYD2 (trypsin peptides)	Pr/PrM	FHLTTR
		SLVFK
	PrM/M	SVALVPHVGMGLETR
	E	LITVNPIVTEK
		ETLVTFK
CYD3 (trypsin peptides)	Pr/PrM	FHLTSR
	PrM/M	VETWALR
	E	LITANPVVTK
		TEATQLATLR
CYD4 (trypsin peptides)	Pr/PrM	GRPLLFK
	PrM/M	VESWILR
	E	VPIEIR
		EVALLR

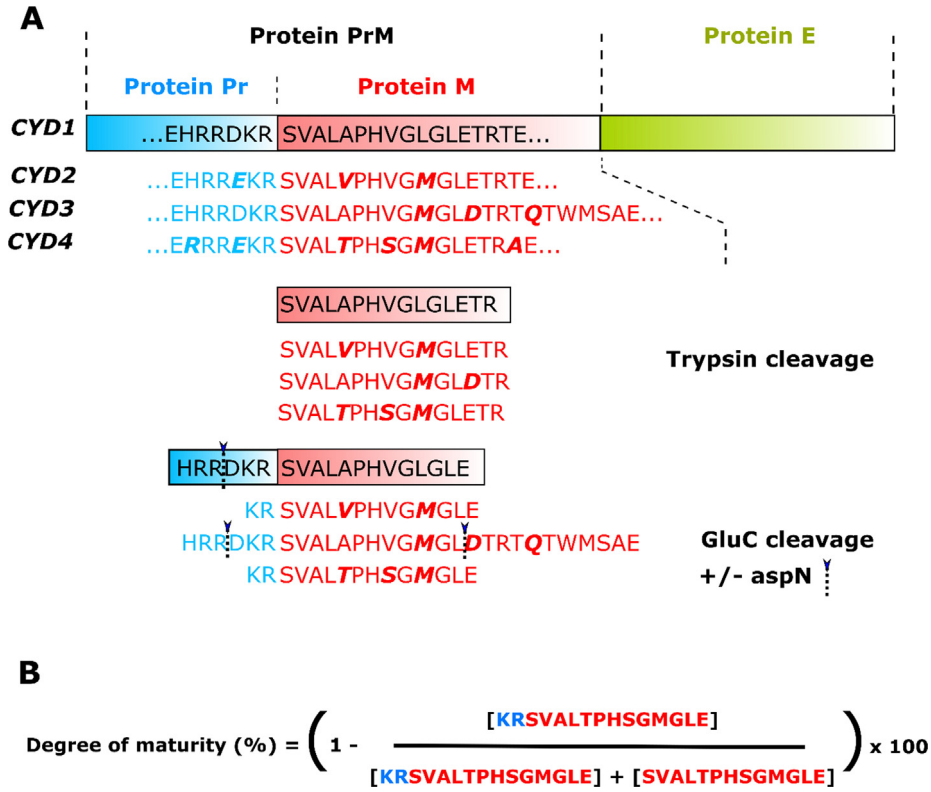

Fig. 1. Schematic representation of the mass spectrometric approach used for analyzing the maturity of CYD1, CYD2, CYD3, and CYD4 (A) peptides released after trypsin, gluC or gluC/aspN digestions (B) equation used for the calculation of the global degree of virus population maturity (CYD4 sequence is taken as an example).

Table 3
Absolute quantification results obtained with gluC and gluC/aspN high pressure-assisted digestion protocol for CYD2/CYD4 and CYD1/CYD3 samples respectively. Samples were analyzed as full-process technical triplicates and deviation calculated. Percent maturity at population level was calculated using the following equation: (1 – ([prM] / ([M] + [prM]))) * 100. Δ1 and Δ2 were calculated to assess the consistency of the method. E_{tryp} values were obtained using trypsin digestion protocol as described in previous study (20), with full-process technical triplicates (SI Virus quantification with DOC protocol and SI Raw quantification results dataset).

Serotype	Batch	[E_{tryp}] nM	[prM] nM	[M] nM	[$E_{gluC/aspN}$] nM	[prM] + [M] nM	Maturity %	Δ1* %	Δ2* %
CYD1	FDV01131	101.5 +/− 25.6	18.4 +/− 0.2	61.8 +/−5.3	86.1 +/− 4.4	80.2 +/− 5.4	77	6.8	15.2
	FDV01151	63.2 +/− 15.7	10.6 +/− 0.8	37.9 +/− 1.9	49.0 +/− 2.1	48.5 +/− 2.6	78	1.0	22.4
	FDV01168	39.4 +/− 10.0	5.6 +/− 0.5	20.6 +/− 1.3	33.0 +/− 0.5	26.2 +/− 1.2	78	20.6	16.2
CYD2	FDV01181	13.8 +/− 1.8	0.7 +/− 0.2	11.3 +/− 2.7	11.2 +/− 2.5	11.9 +/− 2.9	95	6.7	19.0
	FDV01198	20.1 +/− 1.4	2.1 +/− 0.1	13.2 +/− 0.7	16 +/− 0.5	15.2 +/− 0.7	86	4.9	20.3
	FDV01219	5.6 +/− 0.4	0.6 +/− 0.1	4.7 +/− 0.2	4.7 +/− 0.1	5.3 +/− 0.2	89	11.3	15.1
CYD3	FDV01228	7.5 +/− 0.1	1.7 +/− 0.2	5.6 +/− 0.4	5.2 +/− 0.5	7.2 +/− 0.5	77	3.6	0.9
	FDV01252	24.9 +/− 0.6	6.2 +/− 0.1	19.1 +/− 0.4	16.9 +/− 2	25.3 +/− 0.4	75	25.1	18.8
	FDV01319	11.7 +/− 0.4	3.0 +/− 0.4	7.8 +/− 0.6	6.7 +/− 1.4	10.8 +/− 1.0	73	14.3	7.2
CYD4	FDV01254	25 +/− 1.4	8.1 +/− 0.2	22.4 +/− 0.1	25.4 +/− 0.8	30.6 +/− 0.3	73	20.5	1.3
	FDV01286	64.1 +/− 5.2	21.8 +/− 0.3	52.8 +/− 2.3	59.7 +/− 0.1	74.5 +/− 2.5	71	24.9	7.0
	FDV01318	25.0 +/− 0.7	8.5 +/− 0.2	22.1 +/− 0.1	25.1 +/− 1.0	30.6 +/− 0.2	72	22.0	0.3

*Δ1 = | [prM] + [M] − [$E_{gluC/aspN}$] | / [$E_{gluC/aspN}$] * 100.
*Δ2 = | [$E_{gluC/aspN}$] − [E_{tryp}] | / [E_{tryp}] * 100.

during the long storage period. All the quantification results were obtained with heavy peptide standards [21] (Table 2), and calibration curves were performed for each, in a range encompassing the expected viral peptides concentrations. The linearity range, accuracy and LLOQ (low limit of quantification) were deduced from these calibration curves to ensure the robustness of the method (SI material and methods and SI Raw quantification results dataset). Moreover, considering a theoretical average of 180 protein E monomers per viral particle [9], a mean molecular mass of 54 kDa for E protein and the number of viral particles per sample based on Geq determination, E protein concentration should be in the range of 1–2 μg/ml for 10^{11} viral particles/ml. This value is close to that obtained by MS for the different batches.

According to these quantitative values, the MS-based mean maturity of the virus population was estimated for each sample, and results are summarized in Table 3. For all serotypes, maturity at the population level was found to be at least 70% for all batches (77% < CYD1 < 78%, 86% < CYD2 < 95%, 73% < CYD3 < 77%, and 71% < CYD4 < 73%). Considering the consistency of MS-based results (see Δ1, Δ2 and Δ3 calculation) it was possible to determine with confidence the viral population mean maturity degree at a molecular level. Besides determining mean maturity at the population level

by mass spectrometry, it was also of interest to investigate viral maturity at the particle level, and this was done by the cryoEM method.

3.2. Cryo-electron microscopy based maturity determination at particle structural level

CryoEM was employed to visually assess the distribution of particles in terms of maturity and also to confirm the findings from the MS assay. The same clinical batches as those analyzed by MS were observed by cryoEM. Virus particles exhibited the same morphology for all four serotypes. Representative images of each serotype, shown in Fig. 2, revealed well distributed spherical and electron-dense particles with a 50–60 nm diameter. They contained a dense interior likely ascribed to the core capsid of the yellow fever virus moiety. The lipid bilayer was not visible due to the presence of a dense layer of envelope proteins. It was notable that these virus particles were the most abundant particles visible on the grid. Other particles with smaller diameters and liposome-like particles were scarcely encountered, indicative of the homogeneity of the virus samples. Images show, whatever the analyzed phenotype was (Fig. 2), the presence of spiky (Fig. 2**I**) and smooth/bumpy particles (Fig. 2**E and F**). The smooth surfaced particles are close to 50 nm in diameter and appear similar to that of the mature

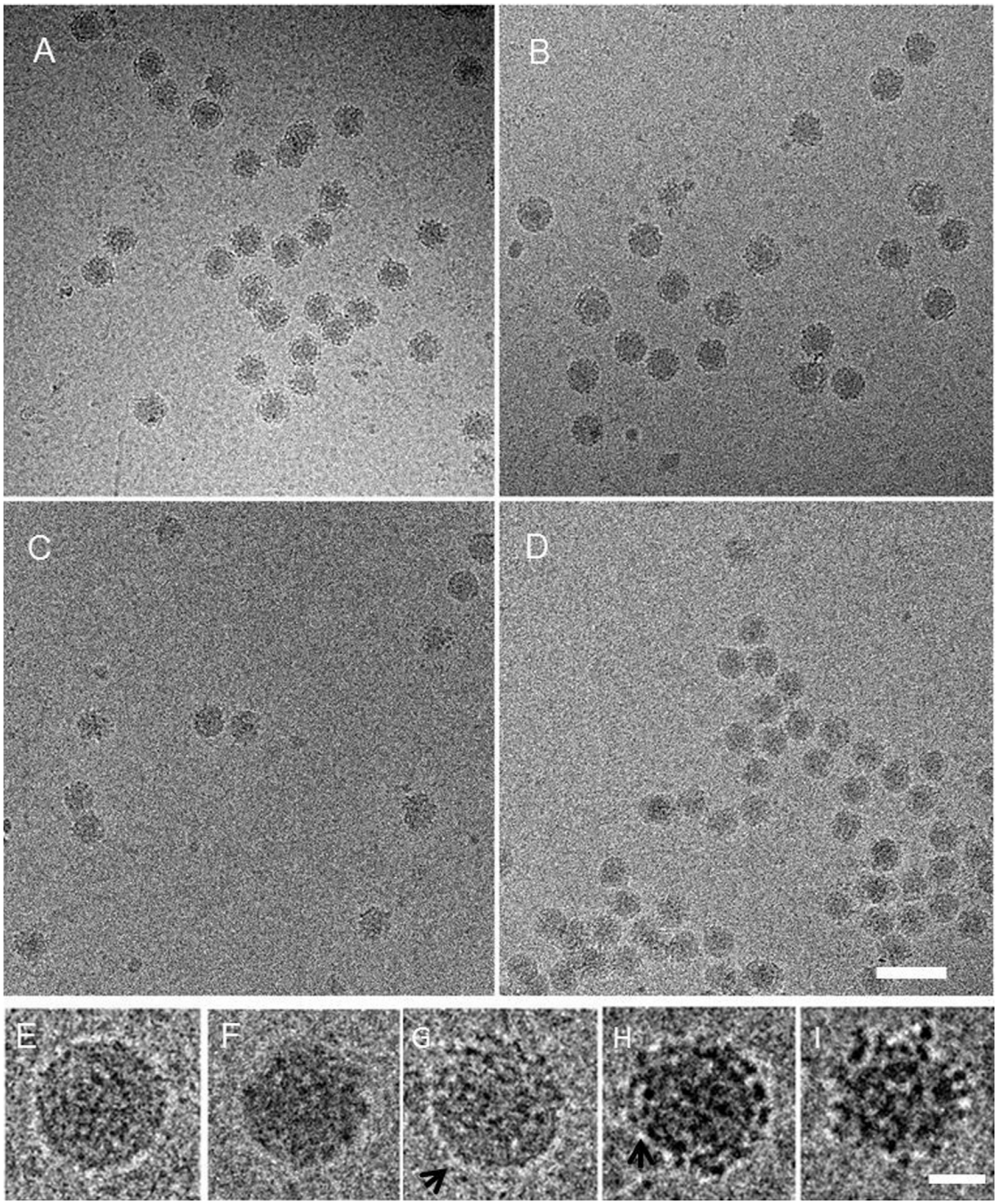

Fig. 2. CryoEM images of the different CYD batches. The different virus populations are represented: smooth, bumpy, partially spiky, spiky; corresponding to different maturation and structural states of the virus. Scale bar 100 nm low magnification and 20 nm high magnifications. A_CYD1 FDV01151; B_CYD2 FDV01181; C_CYD3 FDV01252; D_CYD4 FDV01286; E_smooth/F_bumpy/G-H_partially spiky/I_spiky (from CYD2 serotype).

Table 4
Percentages of the different populations (smooth/bumpy, partially spiky, and spiky) of CYD batches as analyzed by cryoelectron microscopy. Summary of mass spectrometry and cryoelectron microscopy results.

Batches	Mature (smooth + bumpy) %	Partially mature % (partially spiky)	Immature % (spiky)	Empty-not classified	Mass spectrometry Maturity %
CYD1					
FDV01131	69	1	27	3	77
FDV01151	65	0	30	5	78
FDV1168	63	18	17	2	78
CYD2					
FVD01219	62	0	26	12	89
FDV01198	87	3	6	4	86
FVD01181	74	10	1	15	95
CYD3					
FVD01228	52	44	1	3	77
FDV01252	52	6	20	22	75
FVD01319	42	56	0	2	73
CYD4					
FDV01254	72	15	1	12	73
FDV01286	59	34	6	1	71
FDV01318	70	26	1	3	72

DENV [23]. The bumpy particles reveal protrusions at their surface and have a diameter of ~52–55 nm; similar to those described by Zhang et al. [12]. The spiky particles show large spikes of about 5 nm long protruding from the surface that can be differentiated visually from the rough surface of the bumpy particles exhibiting a thin and continuous protein layer. Image processing demonstrates that particles with spikes are composed by a heterogeneous population having smooth domains (Fig. 2**G and H** white arrows) as described by Plevka et al. [11]. Based on these cryoEM observations, the envelope glycoproteins of the CYD vaccine particles share similar morphological characteristics with wild-type dengue virus [24], with both mature and immature structural features.

Due to the important structural heterogeneity it was not possible to accurately quantify the percentage of each subpopulation (smooth, bumpy, partially spiky or full-spiky particles) whether through unsupervised or supervised classification approaches.

Nevertheless, blind manual analysis of a large number of particles for each serotype by a human operator indicated that most particles are smooth, partially-smooth or bumpy meaning that the population is mainly composed of mature, or partially-mature, particles. This is coherent with results from MS studies (Table 4). The hypothesis of a majority mature vaccine population is therefore confirmed for each serotype.

4. Discussion

In order to assess the maturity of the CYD vaccine viruses, we developed two complementary assays: MS and cryoEM. MS provides information on the mean maturity of the viral population at the molecular level by quantifying the ratio of prM protein to the sum of prM plus M proteins. A special enzymatic design based on gluC/aspN digestion allowed quantifying of the "junction peptide", which is positioned astride protein pr and protein M, for all serotypes. Quantification of E protein after gluC/aspN and trypsin digestions also ensured results were consistent (equal molarity for E and prM + M proteins). CryoEM gives information on the maturity of individual viral particles, by visual inspection, at a phenotypic level providing an estimation of smooth/bumpy, partially spiky, and spiky particles. Both assays were in good agreement with each other regarding the phase III lots tested in the present study. Results from these assays show that all four CYD dengue vaccine viruses, including vaccine lots used in the recent Sanofi-Pasteur phase III efficacy trials, present in the majority a partially mature and mature phenotype. A recent publication from Tsai et al. [25] showed that potent neutralizing human monoclonal antibodies preferentially target mature dengue viruses, and results obtained here with CYD dengue viruses are positive in this regard.

Different morphologies of wild type DENV (compact "smooth" and expanded "bumpy" mature virus, fully spiky virus, and partially spiky virus) have been described by Lok et al. [26]. While the infectivity of these different DENV forms is variable, one can propose that a vaccine formulation should be representative of the overall diversity observed for wild type DENV, in order to stimulate protection against these different forms, as observed here for the CYD viruses. It appears nevertheless from literature that DENV2 may be a particular serotype regarding its structure and particular ability to form bumpy structures and "breathe"; neutralizing this serotype may thus require a different quality and/or level of antibodies, possibly targeting different epitopes (fully accessible or cryptic); this needs further investigations. Bumpy forms are observed for all 4 CYD serotypes, which is somehow different to what is observed with wild type viruses, DENV2 being prominent in this regard. Whether this is linked to the CYD yellow fever core and the way it interacts with DENV prM/E requires further investigation.

One has to consider different parameters and limitations when trying to establish a link between results obtained in the study and observed vaccine efficacy with the same lots, or to compare these results with previously published data obtained with viruses produced at laboratory scale.

Firstly, maturation depends on furin content, which differs from one cell substrate to another, and furthermore production/culture conditions can dramatically impact maturation levels for a given cell substrate [24,27]. In this regard, lots analyzed here where produced in large fermenters under optimized Vero cell culture and production conditions, which most likely differ substantially from small scale culture at research level. Vero clones used in different laboratories may also present differences in this regard.

Second, a potential limitation when trying to establish a link between results obtained in the study and observed vaccine efficacy is whether the structure of the CYD DENV lots, used for immunization, reflects the structure of *de novo* CYD viruses produced after vaccination *in vivo*. In fact, the amount of viral protein per dose is in the ng range (taking into account non-infectious particles), which is insufficient to trigger a detectable response against any serotype. This is what we previously observed in a monkey model when injecting inactivated vaccine preparations, even preparations containing a log10 greater cell culture infectious dose 50% (CCID) per serotype than the current formulation (unpublished data). This suggests that at least local replication is required to

induce significant immunity. Therefore, the conformation of the viruses produced by the vaccinee's cells following immunization could be more important regarding induced immunity, and these viruses are likely to be more mature after replication in dendritic cells/monocytes than those produced in cell lines such as Vero [28].

Third, can one nevertheless compare serotype-specific efficacy and the corresponding maturation levels for the vaccine lots? Some apparent hierarchy could be observed in the maturity of these lots, with CYD2 being the most mature as compared to the other three serotypes, while no apparent differences in maturity are seen between CYD1, 3 and 4. On the other hand, efficacy afforded in the efficacy trials by CYD1 and CYD2 in particular was lower than the one afforded by CYD3 and CYD4 [15]. This raises the question of an inverse relationship between efficacy and vaccine virus maturation levels. One could propose that vaccine viruses should ideally present the same continuous array of diverse structures as found with wild type viruses, from immature to mature forms. However, data are still limited, and one cannot conclude at the present time whether CYD viruses do present significant differences in maturity between serotypes; therefore, one cannot convincingly link maturity and protection in this study.

Furthermore, regarding immunogenicity of vaccine lots, when dissecting further serotype specificity using depletion assays, responses against DENV 4 are dominant, while responses against the three other serotypes are in the majority cross-reactive [29]. Therefore, it still seems that vaccine virus maturity, comparable between CYD 1, 3 and 4 serotypes in the present study, does not explain such differences at the antibody level in seronegatives. In previously exposed individuals, receiving the vaccine may act in part as a booster of previously induced responses [29], thus generating broadly cross-neutralizing responses, making the impact of vaccine structure more difficult and complex to assess. This point is to be considered too, as vaccine efficacy demonstrated in the efficacy trials was superior in seropositive individuals, in whom the vaccine is indicated. In this regard, one can nevertheless discuss the potential impact of vaccine maturity in pre-immune individuals, who are indeed the target of the CYD vaccine. Actually, pre-existing cross-reactive antibodies directed against E or prM (targeting thus immature particles) could enhance viral vaccine infectivity of some serotypes and mediate an antibody-dependent enhancement (ADE)-like phenomenon at the vaccine level and thus increase its overall infectivity and subsequent immunogenicity. This has been observed for the yellow fever vaccine in presence of heterologous antibodies directed against Japanese encephalitis virus [30]. However, should this occur for CYD viruses, this did not result in increased viremia or reactogenicity [15], and would then take place only locally in draining lymph nodes. This again deserves more investigations and is also further discussed below.

Finally, the question of whether virus maturity can be linked to some safety issues is an important point for discussion. It has been previously proposed that antibodies, largely against prM found in immature particles, can sensitize individuals to more severe disease through ADE, concerning here wild type viruses upon infection subsequent to vaccination [31]. However, the link between anti-prM levels, ADE and disease outcome has been questioned by other authors [32] suggesting that virus maturity may not play a critical role in pathogenesis. The significant maturity observed in the vaccine lots tested suggests that such a risk of ADE, if it exists, would be low. This is further supported by the studies we performed in FcγR + cells with sera from vaccinees, that demonstrated no link between the level of protection and *in vitro* ADE activity [33]. As previously addressed, one has also to consider that the vaccine is indicated in the dengue-seropositive population, in whom the vaccine acts in a large part as a booster, increasing and broadening responses to confer more potent and long-lasting protection [29]. The quality of the vaccine-induced responses in the primed populations will then mostly reflect the nature of the pre-existing responses induced by prior wild type dengue contact. In this regard, the potential impact on safety of the conformation/maturity of the injected vaccine, whichever it is, will be even lower than in naïve populations.

In conclusion, the results of the two complementary assays used in this study, MS at the molecular level and cryoEM at the phenotypic level, show that all four CYD dengue vaccine viruses, including vaccine lots used in the phase III efficacy trials, present in the majority a mature phenotype. With the limitations inherent to this study, it does not appear that a firm link can be established between differential immunogenicity/protection and maturity, but some points deserve further explorations, in particular the conformation of virus vaccine particles produced *in vivo* after immunization.

Author contributions

Olivier Lambert, Devika Sirohi, Richard Kuhn, Bruno Guy, Frederic Ronzon and Catherine Manin designed research with the methods developed by Devika Sirohi and Richard Kuhn at Purdue University which were adapted with modifications for analysis of vaccine lots. Mathieu Trauchessec, Pierre Bonnafous, Yves Berard, Fabienne Barriere, Celine Huillet, Sergio Marco, Devika Sirohi and Victoria Hedrick performed research. Mathieu Trauchessec, Olivier Lambert, Richard Kuhn, Sergio Marco, Bruno Guy, Frederic Ronzon and Catherine Manin wrote the paper. All authors approved the final version of the paper.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Conflicts of interest

Olivier Lambert, Pierre Bonnafous, Devika Sirohi, Victoria Hedrick and Richard Kuhn received funding from Sanofi Pasteur. Mathieu Trauchessec, Yves Berard, Fabienne Barrière, Celine Huillet, Sergio Marco, Bruno Guy, Frederic Ronzon and Catherine Manin were Sanofi Pasteur employees.

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Appendix A. Supplementary material

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