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# Virus-like particles displaying envelope domain III of dengue virus type 2 induce virus-specific antibody response in mice

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ABSTRACT

*Objective:* Currently, dengue represents one of the most significant arboviral disease worldwide, for which a vaccine is not yet available. Persistent challenges in live viral dengue vaccines have sparked a keen interest in exploring non-replicating dengue vaccines. We have examined the feasibility of using the methylotrophic yeast *Pichia pastoris* to develop a chimeric vaccine candidate displaying the dengue virus type-2 (DENV-2) envelope domain III (EDIII), implicated in host receptor binding and in the induction of virus-neutralizing antibodies, on the surface of non-infectious virus-like particles (VLP)-based on the Hepatitis B virus core antigen (HBcAg).

*Methods:* We designed a fusion antigen by inserting DENV-2 EDIII into c/e1 loop of HBcAg. A codon-optimized gene encoding this fusion antigen was integrated into the genome of *P. pastoris*, under the control of the Alcohol Oxidase 1 promoter. The antigen was expressed by methanol induction and purified to near homogeneity by $Ni^{2+}$ affinity chromatography. The purified antigen was characterized physically and functionally to evaluate its ability to assemble into VLPs, and elicit DENV-2-specific antibodies in mice.

*Results:* This fusion antigen was expressed successfully to high yields and purified to near homogeneity. Electron microscopy and competitive ELISA analyses showed that it formed VLPs in which the EDIII moiety was accessible to different EDIII-specific antibodies. These VLPs were immunogenic in mice, stimulating the production of antibodies that could specifically recognize DENV-2 and neutralize its infectivity. However, virus-neutralizing antibody titers were modest.

*Conclusions:* Our data show: (i) insertion of EDIII into the c/e1 loop of HBcAg does not compromise particle assembly; and (ii) the chimeric VLPs elicit a specific humoral response against DENV-2. The strategy of displaying dengue virus EDIII using a VLP platform will need further optimization before it may be developed into a viable alternative option.



## 1. Introduction

Dengue disease, which is prevalent in several countries of the world, collectively representing roughly half the global population, is caused by any of four closely related serotypes of dengue viruses, DENV-1, -2, -3 and -4. There is no drug to treat DENV infections and a vaccine is yet to be licensed. While the major thrust in the vaccine development field is on live attenuated viral vaccines, efforts are underway to explore non-replicating subunit vaccines as well, for their obvious safety advantage [1]. In this context, the ~100 amino acid (aa) residue DENV envelope domain III (EDIII), stabilized by a single S-S bridge [2], has emerged as a potential subunit vaccine candidate [1,3]. Several key reasons underlie this emergence. This domain located at the C-terminus of the DENV envelope (E) protein is exposed and accessible on the virion surface, implicated in virion binding to the host cell surface receptor and contains multiple type- and sub-type specific neutralizing epitopes. Consequently, EDIII has been the focus of many groups who have successfully expressed it, fused to a whole range of carriers, and demonstrated its ability to induce virus-neutralizing antibodies [1,3].

In the context of recombinant subunit vaccines, initiatives seeking to utilize carriers with the capacity to self-assemble into virus-like particles (VLPs) have come into focus with the recent success of the VLP-based vaccine for human papilloma virus [4]. Several viral structural proteins, expressed in recombinant systems, have an inherent ability to generate non-infectious, non-replicating VLPs and thus combine the advantages of whole virus vaccines and recombinant subunit vaccines [5]. In this regard, the 183 aa residue hepatitis B virus core antigen (HBcAg) is well-documented as a very promising VLP carrier [6]. Recombinant HBcAg, expressed in either prokaryotic or eukaryotic hosts, assembles into VLPs of two sizes, 30 nm and 34 nm, containing 180 and 240 copies of the monomer, respectively. Importantly, it has been

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documented that a variety of antigens, from bacterial, viral and protozoan pathogens, fused to HBcAg form chimeric VLPs. These insertions have been at three sites, the N and C termini and an internal region, known as the c/e1 loop, which projects from the VLP surface. While the N-terminal site can accommodate insertions of up to 50 aa long, the C-terminal site can accept much larger inserts (>100 aa long polypeptides). However, in contrast to the N-terminal inserts, the C-terminal inserts tend not to be well-displayed on the surface of the chimeric VLPs. Consequently, the C-terminal inserts are not as immunogenic as the N-terminal inserts. Interestingly, the c/e1 loop, which represents the major immunodominant region, not only accepts large antigen inserts, but can also display them on the surface of the chimeric VLPs [6]. A survey of the literature focusing on >100 aa long antigen inserts in the c/e1 loop of HBcAg revealed several examples ranging in length from 108 to 256 aa [7–15]. Most of these assemble into VLPs when expressed in *E. coli* (Supplemental Table S1).

Using an *E. coli* expression system, we recently showed that DENV-2 EDIII (EDIII-2, 104 aa long), inserted into the c/e1 loop of HBcAg, could also be displayed on the surface of chimeric VLPs [15]. Unexpectedly, the chimeric VLPs generated from the *E. coli*-expressed HBcAg-EDIII-2 fusion antigen elicited low titers of DENV-2 neutralizing antibodies. As the structural and antigenic integrity of EDIII depends on a single S-S linkage [2], it is likely that the reducing environment in the *E. coli* host cell which is not conducive to efficient formation of S-S bonds may have compromised the ability of EDIII to elicit high titer virus-neutralizing antibodies. This study was undertaken to examine if using a eukaryotic expression host instead of *E. coli* would circumvent this issue. To this end, we expressed the chimeric HBcAg-EDIII-2 antigen in the methylotrophic yeast, *Pichia pastoris*, because of its well-documented high expression potential [16], its utility in expressing S-S linked viral antigens [17,18] and the observations that HBcAg expressed using this yeast assembles into VLPs [19]. This work showed that *P. pastoris* offers an alternate host for the creation of HBcAg-based chimeric VLPs capable of displaying DENV-2 EDIII on its surface. Further, the chimeric VLPs generated using *P. pastoris* appeared to be relatively more efficient, than their *E. coli*-expressed counterparts, in stimulating DENV-2-specific neutralizing antibodies.

## 2. Materials and methods

The ~0.8 kilobase *Pp-HBcAg-EDIII-2* gene (GenBank accession number JQ723012), codon optimized for expression in *P. pastoris*, encoding ~31 kilodalton (kDa) chimeric Pp-HBcAg-EDIII-2 protein (Fig. 1a) was obtained by chemical synthesis (Geneart AG, Regensburg, Germany). This gene was cloned into *P. pastoris* plasmid pPICZa, integrated into the genome of *P. pastoris* host strain KM71H using zeocin selection and purified by Ni-NTA affinity chromatography under denaturing conditions essentially as described before [17]. Samples containing Pp-HBcAg-EDIII-2 antigen were characterized by immunoblot analyses using in-house EDIII-specific mAb 24A12 [17], anti-His mAb, or anti-HBcAg mAb ab8638, using a protocol described earlier [15]. Protein estimation was done using BCA method with BSA as the standard [20]. The purified protein was dialysed against 20 mM sodium bicarbonate buffer, pH 9.2 and its ability to assemble into VLPs was assessed by Electron Microscopy (EM) as described previously [15]. Groups ($n = 6$) of 4–6 week old Balb/C mice were immunized intraperitoneally with different antigens (Pp-HBcAg-EDIII-2 and its precursors HBcAg and EDIII-2) formulated in alum (20 μg antigen coated on 500 μg alum in 100 μl) on days 0, 30 and 90. Sera were obtained one week after the final immunization for analysis of antibody titers. Animal experiments were performed in accordance with Government of India animal ethics guidelines after approval by the Institutional Animal Ethics Committee. Competitive ELISA to probe the surface-accessibility of the EDIII moiety on the chimeric VLPs using mAb 24A12 [17], anti-EDIII-T antiserum [18] and anti-Pp-HBcAg-EDIII-2 antiserum, indirect ELISA to assess EDIII-2- and DENV-2-specific antibody titers in sera of immunized mice, indirect immunofluorescence assay to evaluate the ability of anti-Pp-HBcAg-EDIII-2 polyclonal serum to recognize and bind to infectious DENV-2, and plaque

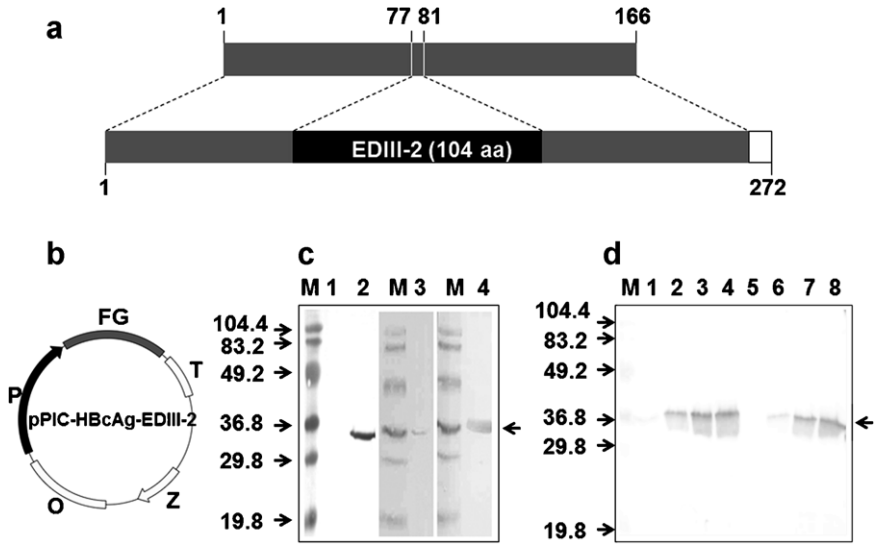


**Fig. 1.** Design and expression of chimeric Pp-HBcAg-EDIII-2 antigen. (a) Schematic representation of the C-terminally truncated HBcAg antigen (top) and the Pp-HBcAg-EDIII-2 chimeric antigen (bottom). The numbers in black indicate the aa residue numbers of the two antigens. The empty box at the C-terminal end of the fusion antigen represents the polyhistidine tag for affinity purification. (b) Map of the expression vector integrated into *P. pastoris*. Abbreviations are as follows. P: *AOX1* promoter; FG: synthetic fusion gene insert encoding the chimeric antigen, shown in panel 'a'; T: transcription terminator; Z: zeocin selection marker; O: plasmid origin of replication. (c). Immunoblot analysis. Total lysates of cultures of a *P. pastoris* clone harboring the plasmid shown in 'b' were analyzed before (lane 1) and after methanol induction (lanes 2, 3 and 4) for recombinant chimeric antigen expression in Western blots using mAbs specific to the DENV EDIII moiety (lane 2), the HBcAg moiety (lane 3) and the polyhistidine tag (lane 4). (d). Induction optimization. Logarithmically growing cultures of the *P. pastoris* clone were either induced for a fixed duration (72 h) at varying methanol concentrations of 0.5% (lane 1), 1% (lane 2), 1.5% (lane 3) and 2% (lane 4), or for varying durations of 0 h (lane 5), 24 h (lane 6), 48 h (lane 7), and 72 h (lane 8), at a fixed methanol concentration (2%). Induced cultures were lysed and analyzed in Western blots using mAb 24A12. In both panels 'c' and 'd', pre-stained protein markers were run in lanes marked 'M'. Their sizes (in kDa) are shown to the left of the panels. The arrow to the right of these two panels indicates the position of the Pp-HBcAg-EDIII-2 antigen.