

5. Discussion

5. Discussion

Dengue, an arthropod-borne, febrile illness, is rapidly spreading across the world. It has become a global burden with forty percent of world's population at the risk of contracting this disease. It can be caused by any of the four dengue virus serotypes, DENV-1, -2, -3 and -4, borne by female *Aedes* mosquito. Dengue illness, in most of the cases, is either asymptomatic or associated with mild fever, and hence, goes unnoticed. Such cases have played a critical role in spreading the virus, and hence the disease, to other geographical areas (Chastel, 2012). In most of the symptomatic cases, dengue appears to be a self-limited illness. On the other hand, a small fraction of symptomatic cases progress to severe form of the disease, characterized by dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which may even lead to death. Due to non-availability of an antiviral, the treatment is largely supportive in nature (Kyle and Harris, 2008).

Currently, a licensed vaccine against dengue is not available. The major hassles in developing a potential vaccine candidate against dengue have been the lack of an appropriate animal model, antibody-dependent enhancement and the requirement to generate balanced immune response against all four DENV serotypes. Though, live-attenuated dengue vaccine candidates are in the most advanced phase of clinical trials, concerns over their safety still remain. Therefore, subunit vaccine candidates are gaining importance because of being safer. One such subunit vaccine candidate, based on dengue envelope protein, has reached clinical phase I (Murphy and Whitehead, 2011).

It is important to simultaneously develop other vaccine candidates, which may have better efficacy over the current vaccine candidates in clinical trials. They also ensure the continuous availability of potential candidates in case of failure of vaccine candidate in clinical trials. All these efforts may reduce the overall time required to develop a successful dengue vaccine candidate. Moreover, these next generation vaccine candidates may also be used with the current vaccine candidates in clinical phase in heterologous prime-boost immunization strategy to further improve their efficacy, or reduce the dose and immunization schedule (Schmitz *et al.*, 2011).

The present study focuses on presentation of domain III of DENV-2 envelope (EDIII-2) on the surface of Hepatitis B core antigen (HBcAg) virus-like-particles (VLPs) and on immunological evaluation of the chimeric antigen. The study involves two proteins, HBcAg and HBcAg-based EDIII-2 chimera (HBcAg-EDIII-2), expressed and purified from eukaryotic host, *Pichia pastoris*.

The rationale for this work is based on four important factors.

First, in the field of dengue vaccine development research, there has recently been an increasing awareness to explore non-replicating candidates as potential dengue vaccine candidates, especially, after witnessing the problems associated with live-attenuated virus vaccine candidates. While, an insect-cell-expressed recombinant DENV Envelope-based vaccine candidate has reached phase I trials, domain III of DENV envelope (EDIII) has emerged as a promising vaccine immunogen for a variety of reasons. It is the receptor-binding domain and is well exposed on the surface of dengue virus; contains dengue neutralizing epitopes and anti-EDIII antibodies are efficient virus blockers (Batra *et al.*, 2010; Swaminathan *et al.*, 2010).

Secondly, virus-like-particles (VLPs) are supramolecular, non-infective and non-replicative structures, with an intrinsic quality of being safe and immunogenic. A number of subunits assemble to form a VLP, resulting in repetitive display of antigenic epitopes, in close proximity, on its VLP architecture. Hence, VLPs are highly immunogenic. The small size of VLPs also contributes significantly to the feature of enhanced immune response generated by VLPs (Jennings and Bachmann, 2008).

Thirdly, Hepatitis B core antigen (HBcAg) possesses an intrinsic quality of assembling into VLPs. It is a highly flexible protein, since it can accept the incorporation of foreign epitopes into its structure and yet retain its potential to assemble into VLPs. Thus, it has been widely explored as a carrier for the display of vaccine antigens. Currently, two vaccine candidates with HBcAg as VLP carrier, one for malaria (Gregson *et al.*, 2008), and the other for influenza (Fiers *et al.*, 2009), are in clinical development.

Fourthly, from the perspective of high level production of viral antigens, for use as vaccines, the yeast *P. pastoris*, which combines key advantages of the prokaryotic and eukaryotic hosts (Macauley-Patrick *et al.*, 2005), is recognized as an ideal system. It has

been used for the expression of HBcAg, which assembles into VLPs (Freivalds *et al.*, 2011), and S-S linked viral antigens (Batra *et al.*, 2010).

Thus, we sought to integrate all this information to create chimeric HBcAg-EDIII-2 VLPs, using the *P. pastoris* expression system, in order to increase the immunogenicity of dengue virus envelope domain III for its development into a potential dengue vaccine candidate.

Only the N-terminal segment of HBcAg is required for its assembly into VLPs (Pumpens and Grens, 2001). Hence, C-terminally truncated version of carrier protein, HBcAg, without any insert, was expressed in *P. pastoris*, under the control of methanol-inducible *AOXI* promoter, and purified by denaturing Ni-NTA chromatography. The protein, after dialysis, was found to form VLPs through electron microscopy.

The chimeric protein, HBcAg-EDIII-2, was also designed using C-terminally truncated HBcAg. The Most Immunodominant Region (MIR), or c/e1 loop, of HBcAg is the most well exposed and immunogenic region of HBcAg (Pumpens and Grens, 2001). Hence, MIR was selected as the most suitable site for insertion of EDIII-2 to enable its display on the surface of the chimeric VLPs, and thus, increase its immunogenicity. This chimeric antigen, HBcAg-EDIII-2, was expressed in *P. pastoris*, under the control of the methanol-inducible *AOXI* promoter. An analysis of the relative proportion of the chimeric antigen in the soluble and insoluble fractions of a native lysate showed most of the expressed protein to be associated with the latter. This is consistent with our earlier observation regarding the expression of DENV-2 EDIII without a fusion partner (Batra *et al.*, 2010), and is presumably a reflection of the efficient expression, arising from the use of a codon-optimized gene, which tends to favor the association of recombinant protein with the insoluble phase. Interestingly, and consistent with this notion, a recent report describes the purification of HBcAg VLPs from the soluble fraction of *P. pastoris* lysate, using a wild type gene without codon optimization (Freivalds *et al.*, 2011).

As the chimeric HBcAg-EDIII-2 antigen was insoluble, it was purified from the extract of lysed cell pellet, under denaturing conditions, followed by removal of the denaturing agent by dialysis. Preliminary analysis by Sephacryl S300 chromatography indicated that, following removal of the denaturing agent, the purified chimeric antigen assembled into

higher order structures. This was confirmed by electron microscopic analysis, which revealed the presence of characteristic VLPs in the purified preparation. Considering that the recombinant chimeric antigen had to be purified under denaturing conditions, these data reflect the strong propensity of the HBcAg-EDIII-2 antigen to self-assemble into VLPs, following the removal of denaturant.

Chimeric VLPs, HBcAg and monomeric EDIII-2 purified from *P. pastoris* (Batra *et al.*, 2010) were immunized in Balb/c mice using alum as the adjuvant. The animals were boosted twice and a substantial boosting effect was observed for HBcAg-EDIII-2 antigen. Sera, post-boost 2, were evaluated for EDIII-2, HBcAg and HBcAg-EDIII-2-specific antibodies. HBcAg-EDIII-2 antiserum, unlike HBcAg antiserum, elicited high titer EDIII-2-specific antibodies, and hence, was concluded to be immunogenic. Interestingly, it was observed that the anti-EDIII-2 and anti-HBcAg-EDIII-2 titers of EDIII-2 antiserum were higher than that of HBcAg-EDIII-2 antiserum. One of the probable reasons behind this pattern could be the fact that the same amount of EDIII-2 and HBcAg-EDIII-2, which possesses relatively lesser molar fraction of functional EDIII-2 moiety, as compared to EDIII-2 monomer, were used for immunization. Immunization with the two antigens in amounts with comparable molar ratios for EDIII-2, could enhance the ELISA titer of HBcAg-EDIII-2 antigen. The other reason could be inefficient display of EDIII-2 moiety on the surface of chimeric VLP, which was analyzed by competitive assay. Importantly, it was found that the EDIII-2 moiety of the chimeric antigen was accessible to anti-EDIII antibodies (anti-EDIII mAb, HBcAg-EDIII-2 antisera and antisera raised against EDIII tetraivalent protein) in solution, indicating that it was presumably surface exposed akin to that in the wild type DENV-2 virion.

Since, presence of high titer anti-EDIII-2 antibodies, in EDIII-2 and HBcAg-EDIII-2 antisera, were revealed through ELISA, immunofluorescence assay was performed to determine if these antibodies could recognize DENV-2. Interestingly, it was observed that even though the titers of EDIII-2 antiserum were higher than HBcAg-EDIII-2 antiserum, its efficiency to recognize DENV-2 was fairly low. Hence, it was concluded that HBcAg-EDIII-2 could elicit antibodies capable of recognizing DENV-2. Next, the presence of neutralizing antibodies, against DENV-2, in HBcAg-EDIII-2 antiserum was determined by Plaque Reduction Neutralization Test (PRNT). It was observed that

HBcAg-EDIII-2 antiserum could neutralize DENV-2, with PRNT₅₀ titer of 1:70. Under the same experimental setup, EDIII-2 antiserum exhibited much lower PRNT₅₀ titer (~1:25). The PRNT₅₀ titer of each antiserum was mock-corrected to calculate their effective titers. The corrected titer of only HBcAg-EDIII-2 was significant (~1:40). Though, the PRNT titers of HBcAg-EDIII-2 were not very high, it may still provide protection against dengue in an animal challenge experiment, since PRNT₅₀ titer of 1:10 is, generally set as the limit for probable protective immunity (Khanam *et al.*, 2007). Additionally, it can be used with other viral or subunit vaccine candidates in a prime/boost strategy.

In conclusion, this work demonstrates that (i) HBcAg-EDIII-2 fusion antigen could be successfully expressed in eukaryotic expression host *P. pastoris*; (ii) the fusion antigen could assemble into chimeric virus-like-particles (VLPs) with (iii) the EDIII-2 moiety, inserted in the c/e1 loop, being accessible on the surface of the chimeric VLPs. The HBcAg-EDIII-2 VLPs (iv) were immunogenic, since they could elicit high titer antibodies, capable of (v) recognizing the virus and also (vi) neutralizing it. Collectively, these findings underline the potential utility of HBcAg as a VLP platform for DENV EDIII display.