6. Summary

6. Summary

- An effort was made to display the receptor binding domain of DENV-2 envelope protein, domain III (EDIII-2), on the surface surface of Hepatitis B core antigen (HBcAg) VLP, to enhance its immunogenicity.
- The chimeric protein, HBcAg-EDIII-2, was designed to contain EDIII-2 in the Most Immunogenic Region (MIR) of HBcAg. MIR is also known to expose the inserted epitopes better.
- The *HBcAg-EDIII-2* gene was artificially synthesized from Geneart.
- *HBcAg* gene, encoding HBcAg, without any insert, was also artificially synthesized from Geneart.
- *P. pastoris* was selected as the expression host of choice for the expression of HBcAg and HBcAg-EDIII-2.
- Both the genes, *HBcAg* and *HBcAg-EDIII-2*, were cloned in the *P. pastoris* expression vector, *pPICZa*, under strong and inducible *AOX1* promoter.
- Mut^S strain of *P. pastoris*, KM71H, was transformed with the recombinant plasmid, in order to integrate the gene into the host genome. Selection of the transformants was made on the basis of Zeocin-resistance.
- The transformants were screened for the expression of desired protein by methanol-based induction.
- Positive transformants, expressing the desired protein, for both, HBcAg and HBcAg-EDIII-2, were obtained. One of the clones was selected for further studies.
- Expression of HBcAg-EDIII-2 was optimized with respect to duration of induction and concentration of methanol as feed.
- The induced biomass of the clone expressing HBcAg-EDIII-2 was prepared, under optimized conditions, at shake-flask level. Induced biomass of the clone expressing HBcAg was also prepared similarly.

- Biomass of the HBcAg-EDIII-2 expressing clone was lysed in native lysis buffer. The proteins associated with the membranes were extracted in a denaturing extraction buffer. The chimeric protein was found to be present, only in the membrane extract, leading to the conclusion that it is a membrane-associated protein.
- Both the proteins were purified, to near homogeneity, by denaturing Ni-NTA chromatography. Both the proteins were dialysed against bicarbonate buffer to allow the proteins to attain near-native conformations.
- HBcAg-EDIII-2 protein was subjected to a preliminary size exclusion chromatography. Its elution in the void volume indicated that it, probably, existed as large-sized higher order structure.
- Its capability of assembling into higher order structure was, further, confirmed through electron-microscopic visualization. HBcAg-EDIII-2 was found to form virus-like-particles (VLPs) of ~40nm in size. HBcAg was also evaluated, through electron microscopy, to form ~30nm sized particles.
- The exposure of EDIII-2 moiety on the surface of HBcAg-EDIII-2 VLP was critical in order to elicit a robust immune response. This feature was evaluated through competitive ELISA, which revealed that EDIII-2 moiety was, indeed, exposed on the surface of chimeric VLP.
- HBcAg-EDIII-2 VLPs, along with its precursor components, HBcAg and EDIII-2, were immunized in Balb/c mice, in order to evaluate their immunogenicity. Mice, after being boosted twice with the antigen, were bled one week after the last boost. The sera obtained were evaluated for titers against HBcAg, EDIII-2 and HBcAg-EDIII-2. HBcAg-EDIII-2 elicited high titer antibodies against, both, EDIII-2 and HBcAg-EDIII-2. Hence, it was concluded to be immunogenic.
- On the basis of ELISA titers, it was inferred that EDIII-2 elicited a higher titer immune response than HBcAg-EDIII-2.
- A specific dilution of these sera was incubated with DENV-2 infected BHK-21 cells, to detect the presence of DENV-2-specific antibodies by

immunofluorescence assay. It was observed that unlike EDIII-2 antiserum, HBcAg-EDIII-2 antiserum could recognize the virus efficiently.

- The presence of dengue virus neutralizing antibodies in the sera was evaluated through Plaque Reduction Neutralization Test (PRNT). In this assay, sera dilutions were pre-incubated with the virus before infecting Vero cells, with the idea that the virus-neutralizing antibodies in the sera would neutralize the virus, reducing the number of plaques formed. The sera dilution leading to 50% reduction in plaque formation was calculated as $PRNT_{50}$. HBcAg-EDIII-2 antiserum gave a higher $PRNT_{50}$ titer than EDIII-2 antiserum.
- In nutshell, the following inferences were drawn. First, eukaryotic expression host, *P. pastoris*, could be used for the expression of HBcAg-EDIII-2 protein. Secondly, HBcAg-EDIII-2 could assemble into VLPs with EDIII-2 moiety exposed on its surface. The insertion of ~100aa EDIII-2 at c/e1 loop of HBcAg did not adversely affect its assembly, highlighting again the exceptional flexibility of HBcAg. Thirdly, HBcAg-EDIII-2 was immunogenic and could elicit dengue virus neutralizing antibodies.