5. SUMMARY AND CONCLUSION

Rabies, also known as hydrophobia in man, is an acute and the only communicable viral disease of man that is always fatal, despite intensive care. In India, every year approximately 1.1 to 1.5 million people receive post exposure treatment for dog bites, and more than 30,000 deaths are reported by national authorities. Since India is highly endemic to rabies and the disease being transmitted through various warm blooded animals, particularly carnivorous animals, it has become mandatory to control the spread of rabies infection.

Post exposure vaccination is the cost effective approach for controlling rabies infection. The rabies vaccines currently in use are derived from neural tissues of rabbits, sheep, goats, mice or rats or by growing rabies virus in embryonated duck eggs or in cell cultures such as primary cells (hamster kidney, dog kidney, fetal calf kidney, pig kidney and chick embryo fibroblasts) or diploid cell lines (human lung-WI-38, human lung-MRC5 and Rhesus monkey lung-FRhL-2) or continuous cell lines (monkey kidney- Vero, hamster kidney- BHK-21, hamster kidney- Nil-2, dog kidney and pig kidney). The tissue culture derived rabies vaccines have been found to be more potent and safe compared to the conventional brain tissue vaccine. Further, immunization requires fever injections of smaller volume with relatively no side effects. The continuous Vero cell line, derived from kidneys of African green monkey has been extensively used for large scale production of rabies vaccine. However, the final purified rabies vaccine prepared from established continuous Vero cells should be free from the risk associated with substrate cellular DNA and calf serum proteins (WHO Tech. Rep. Ser., No. 673, 1982 and 747, 1987). As per WHO recommendations, the purified Vero cell derived rabies vaccine should not contain more than 100pg of residual cellular DNA and one part per million of calf serum proteins.
The glycoprotein (70.5 - 80 KDa) found on the virion envelope of rabies virus is associated with the virus-neutralizing antibody and considered to be the most important antigen for immunization. The nucleoprotein (N) having a molecular weight of 55 KDa, M1 phosphoprotein of 37 to 40 KDa and the matrix protein M2 of 21 to 26 KDa have also been attributed towards total immunity. Hence, the purification process is mainly directed towards the retention of maximal amount of these viral proteins in the order of their antigenicity.

The present research work was carried out to optimize the processes of purification of Vero cell derived human rabies vaccine. The viral harvests were prepared by propagating Vero cells (ATCC) in roux/ roller bottles, infected with PV-11 rabies virus. The concentrated and β-propiolactone (BPL) inactivated viral harvests were purified by zonal centrifugation and by column chromatography as well. The column chromatography technique using DEAE -Sepharose CL-6B column was standardized for cost effective purification. An alternative rapid in vitro single radial immuno-diffusion potency assay method was improvised for Vero cell-derived rabies vaccine in place of in vivo NIH potency test involving many laboratory animals. The immunogenicity analysis for the purified rabies vaccine tested in animals and human volunteers by RFFIT method was carried out for validation. For the quantification of substrate DNA the usual radioactive labeling method was replaced using non radioactive biotin labeling method duly standardized and validated. The technique of counter immuno-electrophoresis using specific immune serum raised against calf serum proteins in rabbits for the quantification of calf serum protein in the final vaccine was standardized and suitably applied.

For the present study, samples from fifteen batches of concentrated and inactivated (20X material) rabies virus materials were subjected to single zonal centrifugation. The fractions between 13 and 21, containing concentrated virus material were pooled and tested for haemagglutination
(HA), serum protein and residual cellular DNA. In all these batches, the DNA content and residual serum protein content were on an average found to be more than 1000 pg and 80 ng respectively. Hence, each of three samples of virus material were individually treated with tween-80, tween-80 and protamine sulfate, double the quantity of tween-80 and protamine sulfate and DNase I along with tween-80 and protamine sulfate were subjected to single run zonal centrifugation. However, there was no considerable improvement in the removal of residual cellular DNA and serum protein, and the level of virus recovery was also reduced to about 95%, 60.9% and 55% respectively in the first three treatments. In case of DNase I along with tween-80 and protamine sulfate treated material, the virus recovery on an average was reduced to 55.5%, inspite of the fact that the level of impurities was found to be well within the permissible limit.

For the optimal recovery of rabies viral antigen an additional zonal centrifugation run was tried. Fractions pooled in the first run were diluted with vaccine PBS to 20-fold and centrifuged to band the virus for the second time in the similar manner as in the case of first run, which resulted in the final virus recovery of 69.3% containing <40 ng of residual serum and < 100 pg of cellular DNA. To enhance the virus recovery, the floatation second run was carried out on all the fifteen samples already subjected to first centrifugation run at 32,000 rpm for 8 h, by filling the rotor with 1,400 mL vaccine PBS and 1,800 mL of virus material containing 60% sucrose. The vaccine PBS buffer flow was maintained throughout the run at a flow rate of 4.5 L/h. Each fraction was tested at OD 280 nm and OD 260 nm for protein and DNA respectively. The fraction between 9-19 were pooled and tested for HA, calf serum protein and residual cellular DNA. In all the fifteen experiments the resultant purified virus material contained <100 pg of substrate DNA and <40 ng of
serum protein. The average virus recovery was found to be 95% of the recovery recorded after normal single run.

Additional fifteen samples of concentrated rabies virus materials were subjected to single step column chromatography purification using DEAE-Sepharose CL-6B as column matrix. The method of packing of column matrix, total surface area of the column and the thickness of the column were studied in detail for optimal purification, based on OD, HA and SRD values for different elutions. The elutions of serum protein, virus antigen and residual cellular DNA were found to be at 0.1 M, 0.3 M and 0.6 M NaCl in phosphate buffer pH7.5, respectively. The thickness of the matrix column was analyzed from 1 cm to 2.5 cm. When the matrix thickness was maintained at 2-2.5 cm, fractions in the 0.3 M NaCl elutions were found to contain maximum SRD potency unitage (on an average 8.5 IU/mL). During 0.6M NaCl elution, the loss of viral antigen to a tune of 4.3 SRD unitage (average) was noticed. Hence the matrix thickness of 2.5 cm and eluent of 0.3 M NaCl were maintained as optimum. The maximal amount of viral antigen obtained during 0.3M DEAE-Sepharose CL-6B column elution was found to be more in terms of number of doses per batch of virus material, when compared to zonal centrifugation run. Moreover the viral antigen recovery was low when the matrix material was tightly packed despite low level of residual cellular DNA.

The final purified vaccine doses were calculated based on the values of HA, SRD and NIH potency values. It was found that on an average, the chromatography purification technique yielded 50% extra number of doses when compared with the values for zonal purified virus materials. It is emphasized that the number of final vaccine doses after purification is an important factor when mass scale production of rabies vaccine is evaluated on cost basis. At the same time the levels of residual cellular DNA and serum protein were also monitored so that the limit did not exceed 100pg and 1 ppm respectively.
The in vitro SRD assay was standardized and employed successfully for the estimation of potency unitage for the final purified (zonal and column) rabies vaccine in addition to the usual NIH in vivo potency assay. In all the twelve samples of zonal purified rabies viral antigen, the SRD values obtained for the test and reference vaccine were comparable and found to be proportionately higher than the NIH values. Hence it is ascertained that the standardized SRD in vitro method is a definite alternative method to NIH in vivo method, which involves many live animals and infectious virus material, posing hazards to the users. The zonal and column purified rabies virus antigen as analyzed by SDS-PAGE test revealed the presence of immunogenic G, N, M1 and M2 proteins. The level of immunogenicity in animals and human volunteers injected with these purified rabies vaccines was assessed by RFFIT test in the context of SRD and NIH values and found to be significantly comparable.

For the estimation of residual cellular DNA the usual radioactive labeling method and nonradioactive biotin labeling assay method were used and the results were compared. The nonradioactive biotin labeling assay method was found to be equally good in the context of accuracy, time consumption and resolution. Hence, the radioactive labeling method involving regulations and potential hazards can be dispensed with.

For the quantification of residual calf serum protein, the counter immunoelectrophoresis method was standardized using hyper immune serum raised against calf serum protein in rabbits and used successfully. It was also noticed that the final purified rabies vaccine containing human albumin (1%) and maltose (5%) did not undergo any major structural changes during lyophilization and after reconstitution as analyzed by FTIR method.
Thus the Vero cell-derived rabies vaccine can be purified cost effectively by a single step column chromatography purification technique containing loosely packed DEAE- Sepharose CL-6B (thickness of 2-2.5 cm). This method yielded extra number of doses to an extend of 50.6% containing permissible level of residual cellular DNA (<100pg) and calf serum protein (<1ppm). The quantitative potency assay on the final purified rabies vaccine can be easily carried out using SRD in vitro technique instead of NIH in vivo test which is time consuming. The estimation of residual cellular DNA can be quantitatively carried out using nonradioactive biotin labeling method in lieu of radioactive labeling method.