# EXPRESSION OF GLYCOPROTEIN OF RABIES VIRUS IN EUKARYOTIC CELLS

**K.G. TIRUMURUGAAN**
**(DPV- 96009)**

*Thesis submitted in partial fulfillment*
*for the requirements of the degree of*

**DOCTOR OF PHILOSOPHY**
**IN**
**ANIMAL BIOTECHNOLOGY**

*to the*
**TAMIL NADU VETERINARY AND ANIMAL SCIENCES UNIVERISTY**

**DEPARTMENT OF ANIMAL BIOTECHNOLOGY**
**MADRAS VETERINARY COLLEGE**
**TAMIL NADU VETERINARY AND ANIMAL SCIENCES UNIVERSITY**
**CHENNAI-7**
**DECEMBER 2001**

**TAMIL NADU VETERINARY AND ANIMAL SCIENCES UNIVERSITY**
**MADRAS VETERINARY COLLEGE, CHENNAI-7**

# CERTIFICATE

This is to certify that the thesis entitled **"EXPRESSION OF GLYCOPROTEIN OF RABIES VIRUS IN EUKARYOTIC CELLS"** submitted in the partial fulfillment for the requirements of the degree of **DOCTOR OF PHILOSOPHY in ANIMAL BIOTECHNOLOGY** to the Tamil Nadu Veterinary and Animal Sciences University, is a record of bonafide research work carried out by **K.G. TIRUMURUGAAN** under my supervision and guidance and that no part of the thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles or prizes and that the work has not been published in part or full in any scientific or popular journal or magazine

Date: 07-12-2001

Place: Chennai-7

**(Dr. R. JAYAKUMAR)**
**CHAIRMAN**

**APPROVED**

**CHAIRMAN** : **Dr. R. JAYAKUMAR**

**MEMBERS** : **1. Dr. K. NACHIMUTHU**

**2. Dr. K.S. PALANISWAMI**

**3. Dr. S. NEDUNCHELLIYAN**

**EXTERNAL EXAMINER** :

**DATE** : 26/6/02

(Dr. K. THANGARAJ)

Om Santhana Devi Amman Thunai

# Dedicated to

## The Lord of Seven Hills

## and

## My Family Members

# CURRICULUM VITAE

| | |
|---|---|
| **Name** | **K.G. TIRUMURUGAAN** |
| **Date of birth** | 20-08-1970 |
| **Place of birth** | Kamayagoundanpatti |
| **Major field of specialisation** | **ANIMAL BIOTECHNOLOGY** |
| **Educational status** | B.V.Sc., - passed out in the year 1993, Madras Veterinary College.<br><br>M.V.Sc., - passed out in the year 1995, Madras Veterinary College. |
| **Marital status** | Married |
| **Permanent address** | No.3, $14^{th}$ street, Tansinagar,<br>Velachery, Chennai- 600 042. |
| **Publications** | Research articles - 13<br>Popular articles - 2 |
| **Membership** | |
| **Professional society** | Member of **Tamil Nadu Veterinary Council** |
| **Other organizations** | Life member **Indian society for Veterinary Immunology and Biotechnology**<br><br>Life member **Association for the promotion of DNA fingerprinting and other DNA technologies** |

# ABSTRACT

# ABSTRACT

| | | |
|---|---|---|
| *Title* | : | **EXPRESSION OF GLYCOPROTEIN OF RABIES VIRUS IN EUKARYOTIC CELLS** |
| *Name and Degree* | : | **K.G. TIRUMURUGAAN**<br>Ph.D., in Animal Biotechnology |
| *Chairman* | : | **Dr.R. JAYAKUMAR,**<br>Professor,<br>Department of Animal Biotechnology,<br>Madras Veterinary College,<br>Chennai-7. |
| *University* | : | **Tamil Nadu Veterinary and Animal Sciences University** |
| *Year* | : | **2001** |

Rabies is a lethal disease caused by rabies virus, which belongs to the Rhabdoviridae family. Rabies continues to be a serious problem in both developed and developing countries due to the reservoir of rabies virus in wild life and domestic animals. An attempt had been made in this study to express the glycoprotein gene of rabies virus in eukaryotic cells. 28 brain samples from suspected cases of rabies were obtained from field cases. Direct fluorescent antibody test and monoclonal antibody screening revealed that 12 samples belonged to the classical rabies group (serotype I). The samples were then subjected to five serial passages in murine neuroblastoma cell line. A dog sample that adapted well was labeled as RVD4 and the infected cells were used for extracting total RNA.

Primers were designed with built in restriction enzyme sites for amplification of the full-length glycoprotein gene. PCR with this primer amplified a product of 1592-bp. The specificity of

amplification was checked by nested PCR that amplified a 627-bp product. The full-length glycoprotein gene was cloned in to pTARGET™ mammalian 'T' expression vector. Colony hybridization was done to screen the recombinant colonies. Plasmid was extracted from the recombinant colonies. The presence and orientation of the insert was checked by digestion with *BamHI*, *BamHI/KpnI* and *EcoRI*. Two positive colonies pT17 and pT29 were sequenced with four primers to confirm the orientation as well as to obtain the full-length sequence. The full-length glycoprotein gene sequence was aligned with available vaccine and field isolates sequences to determine its phylogenetic relationship.

The conceptually translated glycoprotein sequence was checked for its reading frame The polypeptide was equivalent in size and organization to previously characterized rabies glycoprotein and coded for 524 amino acids with the mature peptide having 505 amino acid. The characteristics of the derived glycoprotein sequence were predicted in-silico. The recombinant plasmid was transfected into Vero and Murine Neuroblastoma cell lines. The expression of glycoprotein was tested by indirect immunofluorescence test on fixed and live non-permeabilized cells. The surface fluorescence indicated that in these cells the recombinant protein was processed, transported and anchored into the plasma membrane. This study is an initial attempt to clone and express the glycoprotein of local isolate of rabies virus in eukaryotic cells.

**Key words:** Rabies virus– Glycoprotein gene – PCR- Cloning- Expression – *In-vitro* expression.

# CONTENTS


**ACKNOWLEDGEMENTS**
**LIST OF FIGURES**
**LIST OF TABLES**
**LIST OF PLATES**

| | | |
|---|---|---|
| **I** | **INTRODUCTION** | 1 |
| **II** | **REVIEW OF LITERATURE** | 3 |
| 2.1 | Rabies As A Disease | 4 |
| 2.2 | Characteristics Of Rabies Virus | 5 |
| 2.3 | Molecular Biology Of The Rabies Virus | 6 |
| 2.3.1 | The Leader Rna | 6 |
| 2.3.2 | The Nucleoprotein (N) | 7 |
| 2.3.3 | The Phosphoprotein (M1) | 8 |
| 2.3.4 | The Matrix Protein (M) | 8 |
| 2.3.5 | The Glycoprotein (G) | 9 |
| 2.3.6 | The Polymerase Protein (L) | 9 |
| 2.3.7 | The Intergenic Region | 9 |
| 2.4 | Viral Proteins Involved In Host Immune Response | 10 |
| 2.5 | The Properties Of Glycoprotein | 10 |
| 2.6 | Structural Requirements For Immunogenic Activity Of Glycoprotein | 11 |
| 2.7 | Potential Sites Of Glycosylation In The Glycoprotein | 12 |
| 2.8 | Mapping And Defining Antigenic Sites Of Rabies Virus Glycoprotein | 14 |
| 2.9 | *In-Vitro* Expression Of Rabies Virus Glycoprotein | 15 |
| **III** | **MATERIALS METHODS** | |
| **3.1** | **MATERIALS** | 21 |
| 3.1.1 | Biologicals | 21 |
| 3.1.1.1 | Rabies Virus Isolates | 21 |
| 3.1.1.2 | Hyperimmune Serum | 21 |

| | | |
|---|---|---|
| 3.1.1.3 | Antinucleocapsid Antibody Conjugate | 21 |
| 3.1.1.4 | Monoclonal Antibody Conjugate | 22 |
| 3.1.1.5 | Murine Neuroblastoma cell line (N2a) | 22 |
| 3.1.2 | For Rna Isolation | 22 |
| 3.1.2.1 | Distilled Water Treated With DEPC | 22 |
| 3.1.2.2 | Solution – D | 22 |
| 3.1.2.3 | Sodium acetate | 23 |
| 3.1.2.4 | Water saturated phenol | 23 |
| 3.1.3 | Determination of integrity of the RNA samples by denaturing formaldehyde gel electrophoresis | 23 |
| 3.1.3.1 | Formaldehyde gel running buffer | 23 |
| 3.1.2.2 | Gel loading buffer | 24 |
| 3.1.4 | For Complementary DNA synthesis (cDNA) | 24 |
| 3.1.5 | For Polymerase Chain Reaction | 24 |
| 3.1.6 | Purification of PCR products | 25 |
| 3.1.7 | Cloning of Glycoprotein and Recombinant Selection | 26 |
| 3.1.7.1 | Cloning Vector | 27 |
| 3.1.7.2 | Transformation and Recombinant Selection | 27 |
| 3.1.7.3 | Plasmid extraction | 28 |
| 3.1.7.4 | Restriction enzyme digestion and Recombinant selection | 29 |
| 3.1.7.5 | Molecular weight markers | 29 |
| 3.1.7.6 | Colony Hybridization, Southern blotting and Hybridization | 30 |
| 3.1.8 | Automated Nucleotide Sequencing | 32 |
| 3.1.9 | Softwares | 32 |
| 3.1.9.1 | Automated sequencing | 32 |
| 3.1.9.2 | Sequence analysis | 33 |
| 3.1.9.2.1 | Phylogenetic analysis of the obtained glycoprotein gene sequence | 33 |

| | | |
|---|---|---|
| 3.1.9.2.1.1 | Clustal X version 1.7 Documentation | 33 |
| 3.1.9.2.1.2 | PHYLIP (Phylip Inference Package) | 33 |
| 3.1.9.2.1.3 | MEGA (Molecular Evolutionary Genetic Analysis) | 33 |
| 3.1.10 | In-vitro expression | 34 |
| 3.1.10.1 | Open reading frame and protein sequence | 34 |
| | determination | 34 |
| 3.1.10.1.1 | Genetool Lite | 34 |
| 3.1.10.1.2 | SEQAID II | 34 |
| 3.1.10.2 | Expression of rabies glycoprotein in Eukaryotic cells | 34 |
| 3.1.10.2.1 | Cell lines | 34 |
| 3.1.10.2.2 | Medium for growth of cell lines | 35 |
| 3.1.10.2.3 | Antibiotic stock solution | 35 |
| 3.1.10.2.4 | Trypsin stock solution | 35 |
| 3.1.10.2.5 | Phosphate buffered saline | 36 |
| 3.1.10.2.6 | CELLFECTIN R reagent | 36 |
| 3.1.10.2.7 | Anti-goat rabbit IgG coupled with FITC | 37 |
| **3.2** | **METHODS** | |
| 3.2.1 | Isolation, Identification And Adaptation Of Local Isolate Of Rabies Virus | 37 |
| 3.2.1.1 | Collection and preservation of brain samples from suspected cases of rabies | 37 |
| 3.2.1.2 | Preparation of impression smears | 37 |
| 3.2.1.3 | Fluorescent Antibody Test | 38 |
| 3.2.1.4 | Detection of serotype variation by monoclonal antibody screening | 38 |
| 3.2.1.5 | Murine neuroblastoma cell culture | 39 |
| 3.2.1.5.1 | Isolation and identification of rabies virus in murine neuroblastoma cell line | 39 |
| 3.2.2 | Amplification of full-length glycoprotein gene and cloning in expression vector | 40 |

| | | |
|---|---|---|
| 3.2.2.1 | Sterilizatin of plastic wares and solutions | 40 |
| 3.2.2.2 | Preparation of Total RNA from infected neuroblastoma cells | 41 |
| 3.2.2.3 | Determination of integrity of RNA samples by denaturing fomaldehyde gel electrophoresis | 42 |
| 3.2.2.4 | Complementary DNA synthesis | 42 |
| 3.2.2.5 | Polymerase chain reaction for amplification of full-length glycoprotein gene | 43 |
| 3.2.2.5.1 | Designing of primers | 43 |
| 3.2.2.5.2 | Polymerase chain reaction | 44 |
| 3.2.2.5.3 | Agarose gel electrophoresis to check the amplified full-length glycoprotein gene | 45 |
| 3.2.2.5.4. | Purification of the full-length glycoprotein gene product | 46 |
| 3.2.2.6 | Cloning of the glycoprotein gene into pTARGET™ mammalian expression T vector | 47 |
| 3.2.2.7 | Competant cell preparation | 48 |
| 3.2.2.8 | Transformation and Recombinant Selection | 49 |
| 3.2.2.9 | Colony hybridization | 50 |
| 3.2.2.9.1 | Digoxigenin labeling of the internal 627-bp glycoprotein product | 50 |
| 3.2.2.9.2 | Transfer of colonies to nylon membrane | 51 |
| 3.2.2.9.3 | Pre-hybridization | 52 |
| 3.2.2.9.4 | Post- hybridization washes | 52 |
| 3.2.2.9.5 | Immunological detection | 53 |
| 3.2.2.10 | Plasmid extraction | 53 |
| 3.2.2.11 | Restriction enzyme digestion of the recombinant plasmid | 54 |
| 3.2.2.12 | Southern blotting and hybridization | 55 |
| 3.2.2.12.1 | Transfer of restricted plasmid to nylon membrane | 55 |

| | | |
|---|---|---|
| 3.2.2.12.2 | Hybridization and immunological detection | 56 |
| 3.2.2.13 | Automated sequencing of the cloned glycoprotein gene | 56 |
| 3.2.2.13.1 | Preparation of plasmids for sequencing | 56 |
| 3.2.2.13.2 | Automated sequencing | 57 |
| 3.2.2.13.2.1 | Sequencing reaction set-up | 57 |
| 3.2.2.13.2.2 | Cycle sequencing conditions | 58 |
| 3.2.2.13.2.3 | Preparation of the samples for gel loading | 58 |
| 3.2.2.13.2.4 | Sequence data collection | 59 |
| 3.2.2.14 | Nucleotide sequence analysis | 60 |
| 3.2.2.14.1 | Phylogenetic analysis of the obtained glycoprotein gene sequence | 60 |
| 3.2.2.15 | *In-vitro* Expression | 60 |
| 3.2.2.15.1 | Open reading frame (ORF) and protein sequence determination | 61 |
| 3.2.2.15.2 | Predicted characteristics of the conceptually translated glycoprotein | 61 |
| 3.2.2.15.3 | Expression of the cloned rabies glycoprotein in eukaryotic cells | 61 |
| 3.2.2.15.3.1 | Transfection | 61 |
| 3.2.2.15.3.2 | Indirect Fluorescent Antibody Test to check expression | 63 |
| **IV** | **RESULTS** | |
| 4.1 | Isolation, identification and adaptation of local isolate of rabies virus | 64 |
| 4.1.1 | Sample collection and examination by fluorescent microscopy | 64 |
| 4.1.2 | Detection of serotype variation by monoclonal antibody screening | 64 |
| 4.1.3 | Adaptation of rabies virus in murine neuroblastoma cell line | 66 |

| | | |
|---|---|---|
| 4.2 | Amplification of full-length glycoprotein gene and cloning in expression vector | 66 |
| 4.2.1 | Preparation of Total RNA | 66 |
| 4.2.2 | Polymerase chain reaction for amplification of full-length glycoprotein gene | 67 |
| 4.2.3 | Cloning of the glycoprotein gene into pTARGET™ mammalian expression T vector | 67 |
| 4.2.3.1 | Purification of the full-length glycoprotein gene product | 67 |
| 4.2.3.2 | Cloning in pTARGET ™ mammalian expression vector | 68 |
| 4.2.3.3 | Colony hybridization | 68 |
| 4.2.3.4 | Plasmid extraction | 68 |
| 4.2.3.5 | Restriction enzyme digestion of the recombinant plasmid | 69 |
| 4.2.3.6 | Southern blotting and hybridization | 70 |
| 4.3 | Sequencing | 70 |
| 4.3.1 | Preparation of the plasmids for sequencing | 70 |
| 4.3.2 | Sequencing of the recombinant plasmid | 71 |
| 4.4 | Sequence analysis | 71 |
| 4.4.1 | Phylogenetic analysis of the obtained glycoprotein sequence with software CLUSTAL X | 71 |
| 4.5 | *In-vitro* expression | 73 |
| 4.5.1 | Open reading frame (ORF) and protein sequence determination | 73 |
| 4.5.2 | Protein sequence alignment and variation estimation | 75 |
| 4.5.3 | Predicted characteristics of the conceptually translated glycoprotein | 81 |
| 4.5.4 | Expression of the cloned rabies glycoprotein in eukaryotic cells | 86 |

| | | |
|---|---|---|
| 4.5.4.1 | Transfection | 86 |
| 4.5.4.2 | Indirect FAT to check expression | 86 |
| **V** | **DISCUSSION** | |
| 5.1 | Isolation, identification and adaptation of local isolate of rabies virus | 88 |
| 5.1.1 | Sample collection and examination by fluorescent microscopy | 88 |
| 5.1.2 | Detection of serotype variation by monoclonal antibody screening | 90 |
| 5.1.3 | Adaptation of rabies virus in neuroblastoma cell line | 91 |
| 5.2 | Amplification of full-length glycoprotein gene and cloning in expression vector | 91 |
| 5.2.1 | Preparation of total TRNA | 92 |
| 5.2.2 | Polymerase chain reaction for amplificatin of full-length glycoprotein gene | 92 |
| 5.2.3 | Cloning into pTARGET™ mammalian Expression vector and Recombinant selection | 92 |
| 5.2.4 | Restriction enzyme digestion of the recombinant plasmids | 93 |
| 5.3 | Sequence analysis | 93 |
| 5.3.1 | Phylogenetic analysis of the obtained glycoprotein sequence with software CLUSTAL X | 94 |
| 5.4 | *In-vitro* expression | 95 |
| 5.4.1 | Open reading frame (ORF) and protein sequence determination | 96 |
| 5.4.2 | Predicted characteristics of the conceptually translated glycoprotein | 96 |
| 5.4.3 | Expression of the cloned rabies glycoprotein in eukaryotic cells | 97 |
| 5.5 | **Conclusion** | 100 |
| **VI** | **SUMMARY** | |
| | **REFERENCES** | |

## ACKNOWLEDGEMENTS

I wish to offer my deep sense of gratitude and appreciation to my most respectful guide **Dr.R. Jayakumar, Ph.D.**, Professor, Department of Animal Biotechnology. I am in indeed greatly indebted to him for his constructive comments, advice and supreme guidance in the accomplishment of this research project.

I take this opportunity to place on record my deep sense of gratitude to **Dr.K.Nachimuthu, Ph.D.**, Dean i/c, Faculty of Basic Sciences for his healthy suggestions as a member of the advisory committee.

I am greatly obliged to **Dr.K.S. Palaniswami, Ph.D.**, Professor and Head, Department of Microbiology for his healthy suggestions as a member of the advisory committee.

I take this opportunity to express my deep sense of profound gratitude to **Dr.S.Nedunchelliyan, Ph.D.**, Professor and Head, Department of Preventive Medicine for his healthy suggestions as a member of the advisory committee.

I am highly indebted to **Dr.A. Mahalinga Nainar,** Ph.D., Professor and head, Department of Animal Biotechnology for his valuable suggestions and guidance.

I am highly thankful to **Dr. N. Daniel Joy Chandran**, Ph.D., Professor and Head, Vaccine Research Centre- Bacterial Vaccines, Centre

for Animal Health Studies for continuing support and encouragement during the study.

I take this opportunity to place on record my deep sense of gratitude to **Dr.G.Rajavelu**, Ph.D., Director, Centre for Animal Health Studies, his encouragement and continuing support during the study.

The author expresses his sincere indebtedness and thanks to **Dr.P.Ramadass,**Ph.D., **Dr.V.Thiagarajan,**Ph.D., **Dr.K. Kumanan,** Ph.D., **Dr.G.Dhinakaraj,**Ph.D., **Dr.K.Kalaimathi,**Ph.D., **Dr.A.Palanisamy,**Ph.D, **Dr.M.Parthiban,**Ph.D. and other staffs of the Department of Animal Biotechnology for their timely help and critical suggestions.

The author is highly greatful to **Dr. Murali Manohar,** Ph.D., Professor and Head and other staffs Department of pathology for their help rendered in collection of the samples during postmorterm.

I take this opportunity to thank **Dr. Andrew Chandramohan**, Associate professor and Head i/c, and other staff of the Central Instrumentation Facility for timely help in allowing to use the sensitive equipments required for this study.

I am greatly obliged to **Dr.V.D. Padmanaban**, Editor, IVJ and Former Registrar, TANUVAS for his suggestions and help rendered during the study.

I am very much pleased to convey my heartful thanks to **Mrs. K.P. Sheela, Mrs. K. Amutha, Dr. P. Balakrishnan, Mr.S. Balakrishnan, Mr.M. Chandrasekar** and other research fellows of the Department of Animal biotechnology for their help rendered.

I wish to express my sincere thanks to **The Director, Spic Science foundation**, for their help in developing primers for amplifying the glycoprotein gene of rabies virus.

I wish to place on record my sincere thanks to **Dr.Laljit Singh**, Director, CCMB and his research team members **Dr.K. Thangaraj, Mr. Subramanian and Mr. Reddy** for their extensive co-operation and the timely help rendered in sequencing of the cloned product.

I and grateful to the **Centre for Disease Control, Atlanta** for their help in supplying neuroblastoma cell line for this study.

I wish to express my heartful thanks to **Dr.A. Senthil Kumar, Dr.T. G. Prahakar, Dr.S. Manoharan, Dr.S.Ramesh, Dr. A. Ramesh, Dr. N. Vengadabady, Dr. C. Sreekumar,** for their co-operation and help during the research work.

My sincere thanks are due to **Dr. R. Manickam**, **Dean**, Madras Veterinary College for his valuable help rendered during this study.

I am very much thankful to The **Vice-Chancellor**, TANUVAS, for permitting and providing all the facilities needed to carryout this work at MVC.

Finally my heartiest thanks to **my parents, wife and my son** for their ever ending support and above all to **The Almighty** for keeping in good health and the confidence **He** had given me carry out any difficult task.

# LIST OF FIGURES


| Figure No. | Title | Page No. |
| --- | --- | --- |
| 1. | Sequence data generated by automated sequencing of the cloned glycoprotein gene | 72 |
| 2. | Phylogenetic grouping of RVD4 with other vaccine and street isolate sequences using CLUSTAL X software and NJ method | 74 |
| 3. | Phylogenetic grouping of RVD4 with other vaccine and street isolate sequences using MEGA software and Jukes cantor method of distance estimation | 74 |
| 4. | Glycoprotein gene sequence of local isolate (RVD4) of rabies virus aligned with the amino acid coded | 76 |
| 5. | Different open reading frames depicting the length of polypeptide coded | 77 |
| 6. | Conceptually translated RVD4 glycoprotein sequence aligned with other vaccine virus glycoprotein sequences | 79 |
| 7. | Conceptually translated RVD4 glycoprotein sequence aligned with other predicted street isolate and mokola glycoprotein sequences | 80 |
| 8. | Feature map of RVD4 depicting the comparison of the amino acids constituting the antigenic domains of RVD4 with other field isolates | 82 |
| 9. | Feature map of RVD4 depicting the comparison of the amino acids constituting the antigenic domains of RVD4 with other vaccine strains | 83 |
| 10. | Feature map of RVD4 glycoprotein gene sequence | 84 |
| 11. | Hydropathicity profile of RVD4 glycoprotein (determined SEQAID II) depicting the stretch of hydrophobic amino acids constituting signal peptide and transmembrane domain | 85 |

# LIST OF TABLES


| Table No. | Title | Page No. |
|---|---|---|
| 1. | Details of field samples screened for rabies virus antigen using Antinucleocapsid antibody and panel of monoclonal antibodies | 65 |
| 2. | Comparison of conceptually translated amino acid sequence of RVD4 with various field and vaccine strains | 78 |

# LIST OF PLATES


| Plate No. | Title | Page No. |
|---|---|---|
| 1. | Direct FAT (with antinucleocapsid antibody FITC) on brain impression smear from dog showing specific fluorescence (100 X) | 64-65 |
| 2. | Direct FAT (with monoclonal antibody C 15-2) on brain impression smear from dog showing specific fluorescence (100 X) | 64-65 |
| 3. | Formaldehyde agarose gel (1%) electrophoresis of total RNA extracted from RVD4 infected murine neuroblastoma cell lysate | 67-68 |
| 4. | Agarose gel (1%) electrophoresis of PCR amplified glycoprotein gene of local isolae | 67-68 |
| 5. | LB agar ampicillin/X-gal/IPTG plates showing wild type blue and recombinant white colonies | 68-69 |
| 6. | Colony hybridization using 627-bp glycoprotein gene product as probe (purple colour signals indicate the location of recombinant colonies) | 68-69 |
| 7. | Agarose gel (1%) electrophoresis of pTarget ™ vector and recombinant pTarget vector | 69-70 |
| 8. | Southern blotting and Hybridization with digoxigenin labelled 627 bp product as probe | 70-71 |
| 9. | Feature map of RVD4 depicting the comparison of the amono acids constituting the antigenic domains of RVD4 with other field isolates | 86-87 |

# LIST OF ABBREVIATIONS

| | |
|---|---|
| RNA | Ribonucleic acid |
| PCR | Polymerase chain reaction |
| cDNA | Complementary DNA |
| DNA | Deoxy ribonucleic acid |
| PM | Pitman-Moore virus |
| CVS | Challenge Virus Standard |
| PV | Pasteur Virus |
| ERA | Evlyn Rokitniki Abelesth |
| SAD | Street-Alabama-Dufferin |
| LEP | Low Egg Passaged |
| HEP | High Egg Passaged |
| N | Nucleoprotein |
| NP | Nonstructural protein |
| M | Matrix protein |
| G | Glycoprotein |
| L | Large Polymerase protein |
| CHV | Canine herpes virus |
| SV40 | Simian virus 40 |
| EBL | European bat lyssavirus |
| FAT | Fluorescence antibody test |
| FITC | Fluorescein isothiocyanate |
| PHYLIP | Phylogeny Inference Package |
| MEGA | Molecular Evolutionary Genetic Analysis |
| MEM | Minimum essential medium |

| | |
|---|---|
| FBS | Foetal bovine serum |
| Mab | Monoclonal antibody |
| DEPC | Diethyl pyro-carbonate |
| CMV | Cytomegalo virus |
| NJ | Neighbour joining |
| JCD | Jukes cantor distance |
| DMEM | Dulbecco's minimum essential medium |
| BSA | Bovine serum albumin |
| RVD4 | Rabies virus dog 4 |
| ORF | Open reading frame |

# INTRODUCTION

## INTRODUCTION

Rabies is a lethal disease caused by rabies virus, which belongs to the Rhabdoviridae family. Rabies continues to be a serious problem in both developed and developing countries due to the reservoir of rabies virus in wild life and domestic animals. The disease causes a fatal encephalomyelitis in all warm-blooded animals, and there is no treatment once its symptoms have appeared. Protection against lethal infection can be achieved by pre- or, frequently, post-exposure vaccination.

There are quite a few vaccines currently in use for humans, domestic and wild animals. However, limited access to high quality cell culture-based anti-rabies vaccines, coupled with their high cost and lack of a cold chain are responsible for the deaths of more than 50,000 people and millions of animals in developing countries. In India, approximately 30,000 people die of rabies annually and it counts between 7,00,000 and 1 million post exposure treatment per year (Sehgal, S. 1997).

Vaccines that can be produced and purified by inexpensive procedures and also stored at room temperature are ideally suited for eradication of the disease in developing countries. In addition due to the ethical issue gaining importance, there will certainly be difficulty in preparing the nervous tissue vaccine in sheep, which is commonly available, both for human and animal use.

Over the last decade, the tools of molecular biology have broadened the ways in which the immune system has been investigated,

providing new means for identification of antigenic determinants for many infectious diseases, and has also created new tools for the production of candidate vaccines. In this context the use of plasmid DNA as a vaccine assumes great significance, since it can be both produced at a very low cost and stored at room temperature. Inoculation of animals with purified plasmid DNA encoding antigenic proteins results in the transfection of host cells followed by the expression of vector encoded foreign proteins, leading to the stimulation of a specific immune response including T helper cells, cytolytic T cells and antibodies. This methodology known as DNA vaccination, genetic immunization or nucleic acid immunization is a viable alternative to attenuated virus-or recombinant protein-based vaccines.

Hence with this view in mind the study was undertaken as an initial attempt to express the glycoprotein gene of rabies virus *in-vitro* in eukaryotic cells with the following objectives:

a) **To isolate and identify a suitable local strain of rabies virus for vaccine production.**
b) **To clone the Glycoprotein sequence of rabies virus in expression vectors.**
c) **To express the Glycoprotein of rabies in eukaryotic cells.**

The results of the study would help in future for stable expression of the rabies glycoprotein in eukaryotic cells and thereby enabling to purify the protein in large scale. In addition the results of the study would also favour in developing a DNA vaccine utilizing the glycoprotein of a local isolate.

# REVIEW OF LITERATURE

# CHAPTER II
# REVIEW OF LITERATURE

Rabies is a lethal disease causing threat to both animals and humans, and transmission usually takes place by bite of infected domestic or/and wild carnivores. After a relatively long incubation period, nervous signs form the most prominent features before death occurs. The dramatic symptomology and a nearly hundred percent fatality rate of rabies had attracted the attention of many research workers from the first modern microbiologists.

India is a very large country with the land area of about 33 million square km, livestock population of 195 million and dog population of 20 million. Rabies occurs in all parts of Indian sub-continent except Andaman and Nicobar and Lakshwadeep group of islands. Human beings, domestic animals and wild animals are victims of the disease throughout the year. It has been estimated that 30,000 people die of rabies every year and also accounts for 1:1000 hospital admissions every year in India. About 7,00,000 persons undertake antirabies treatment following exposure. Dogs are responsible for 96 per cent of the cases. Mortality in livestock and pet animals is far more than the number available due to lack of exact reports (Sehgal, 1997). Three major factors contribute for the present situation. One is the increase in the dog population in the country, which has touched the figure of 20 million. The second factor being the increase in the density of human population which has resulted in increased human-dog contact and the third being the virtual absence of any effective comprehensive rabies control programmes in the country. Control of this disease is hampered by cultural and economic realities. The high cost of imported modern

tissue culture vaccines is the main reason for which nervous tissue vaccines derived products are still being used throughout the world.

## 2.1 RABIES AS A DISEASE

Democritus has described rabies in animals during 500 B.C. Aristotle (4th century B.C.) recognized rabies as a disease of animals transmitted from the bite of a mad dog. Celsus, a Roman writer, (1st century A.D.) described clinical picture of rabies, perhaps for the fist time, in humans. Pierre Victor Galtier (1846-1908) and subsequently Louis Pasteur were the first to undertake a scientific approach to the disease in an attempt to find an effective vaccine. Pasteur in 1881 demonstrated the neurotropism of virus. In 1885 Pasteur successfully used antirabies vaccine to a 9 year-old boy Joseph Meister, who was bitten by a rabid dog. Negri in 1903 demonstrated intra cytoplasmic, acidophilic inclusions called "Negri" bodies in neurons of dogs, cats and rabbits experimentally infected with rabies virus.

Sir David Semple, previous Director of Central Research Institute, Kausauli, India, described the practical aspects of rabies in 1919 and a method for preparation of inactivated carbolised rabies vaccine (Sharma and Adlakha, 1994). But the use of animal brain tissue may occasionally cause allergic encephalomyelitis leading to neuroparalysis in vaccinated individuals. Levaditi (1913) first applied the tissue culture technique to study the rabies virus. Kissling and Reese (1953) first adapted rabies to a non-nervous tissue culture system. This paved the way for the development of tissue culture vaccines. Koprowski (1966) described the use of human diploid cell cultures for the production of safe efficacious rabies vaccine

Habel and Kaprowski (1955) had conducted trials on human subjects confirmed that the combined use of antirabies serum and vaccine afforded more protection than the vaccine alone.

## 2.2 CHARACTERISTICS OF RABIES VIRUS

Rabies virus isolates from clinical cases are referred to as street viruses. The street virus, by serial passages in mammals, produces fixed virus. Many of the rabies virus strains studied throughout the world have been derived from a rabid cow, isolated by Louis Pasteur in 1884. After several passages (more than 1500) in rabbit brain, the virus became "fixed" and further adaptation to various hosts and cell types have led to the actual Pitman-Moore (PM), Challenge Virus Standard (CVS) and Pasteur virus (PV) strains. Other fixed strains derived from independent isolates include the Street-Alabama-Dufferin (SAD) and Evelyn Rokitniki Abelesth (ERA) strains from dog and the Flury LEP and HEP strains from a young girl (Clark and Wiktor, 1972). In addition, Kelev strain, isolated from a dog in 1950, in Israel, also has been used in vaccine production for veterinary use.

Almeida et al. (1962) observed the rabies virus through electron microscope and described the precise morphology (bullet shape) of the virus. The virus measures 100-300 nm in length and 75 nm in diameter. The virion is bounded by a lipoprotein membrane envelope from which spike like projections, 5-10 nm long, extend to the outside. The nucleocapsid or ribonucleoprotein core exhibits helical symmetry with a diameter of 30-70 nm.

Organic solvents, detergents and media having pH below 4 and above 10, readily inactivate the virus. It is rapidly inactivated at

$50^oC$. The virus is sensitive to UV-light and X-ray irradiation. The virus has varying incubation periods ranging from 10 days to 1-2 months, rarely several years.

## 2.3 MOLECULAR BIOLOGY OF THE RABIES VIRUS

Since 1981, when the glycoprotein mRNA of the ERA strain became the first rabies gene to be cloned and sequenced (Anilionis et al., 1981), considerable advances have been made in determining the sequence of the rabies genome. With the development of Polymerase Chain Reaction (PCR), it has now become possible to amplify any rabies gene. PCR amplification coupled with direct sequence analysis has currently lead to a dramatic increase in our fundamental knowledge of the Lyssa viruses. The virus comprises of a single unsegmented negative strand RNA genome of about 12000 nucleotides long. The genome can be divided into five gene regions. Each gene encodes one of the five species of viral protein designated as N, M1, M2, G and L (Coslett et al., 1980).

The nucleoprotein (N) is the major protein associated with nucleocapsid, while the spikes or surface projections consists of glycoprotein (G). The surface protein (M2) is membrane associated, anchored to the inner side of the viral envelope. The polymerase protein (L) and phospoprotein (M1) occur in small quantities in the virion where they are associated with N protein (Tordo and Poch, 1988; Morimoto et al., 1989).

### 2.3.1 THE LEADER RNA

This is a small RNA (57-58 ribonucleotides), which is very rich in Adenine (A) residues transcribed at the 3' end of the genome.

During transcription it carries the promoter of encapsidation and cleaves it from the distal mRNA transcripts (Grinnell et al., 1985).

### 2.3.2 THE NUCLEOPROTEIN (N)

The nucleoprotein constitutes the major component of the internal helical nucleocapsid of the rabies virus. Most of the rabies related viruses were identified according to their reactivity with antinucleocapsid monoclonal antibodies (Wiktor and Koprowski, 1980).

The amino acid sequence of rabies virus N protein has been deduced from the primary nucleotide sequence of PV, CVS, ERA and SAD-B19 strains. The amino acid sequence of each of the viral N protein is 450 amino acids long (Tordo et al., 1986), which is phosphorylated on a serine residue in position 389 (Dietzschold et al., 1987).

Analysis with monoclonal antibodies has revealed a high degree (98 - 99.6per cent) of homology between the different N proteins of these fixed rabies strains that correlates with their antigenic similarity to each other and to several field rabies virus strains (Wunner et al, 1988). Three antigenic sites have been characterized along the protein, but only two of them are mapped. Site I and III involve the stretches of amino acids in position 374-383 and 313 to 337 respectively.

The nucleoprotein may also be involved in immunity, since recent studies on N protein had been shown to protect animals against a peripheral challenge with infectious virus. Celis et al. (1989) identified a T-cell epitope on the N protein using cloned T-cell

hybridomas and synthetic peptides containing amino acid sequences of the N protein. The T-helper epitopes have been mapped to amino acids in position 404-418 serving as immunodominant when coupled with linear epitope of glycoprotein (Dietzschold et al., 1990).

### 2.3.3 THE PHOSPHOPROTEIN (NP or M1)

This highly hydrophilic protein is 297 amino acids long. It presents different phosphorylation states (Tuffereau et al., 1985) and possesses numerous serine and threonine amino acids, which anchor the phosphate residues. Phosphorylation provides an overall negative charge, which is increased by the very high content of acidic amino acids (aspartate and glutamate). Recently, immunodominant cytotoxic T epitopes and T-helper epitopes were identified in position191-206 (Larson et al., 1991).

### 2.3.4 THE MATRIX PROTEIN (M2)

This is a 202 amino acid long polypeptide and plays an intermediate role between the ribonucleocapsid and the viral membrane and is also believed to play a role in morphogenesis of the virus. This region seems to be involved in the host immune response to rabies, since a major antigenic determinant was recently located between the residues 1 and 72 (Hiramatsu et al., 1992). A central 19-residue segment appears sufficiently hydrophobic to anchor the protein into the virion membrane (Tordo et al., 1986)

### 2.3.5 THE GLYCOPROTEIN (G)

The glycoprotein (G) is the best studied of the rabies proteins and is 524 amino acids long. This protein forms the 10-nm long peplomers on the external surface of the virus membrane. It is responsible for the induction and binding of virus-neutralizing antibodies. Each peplomer consists of three associated Glycoprotein (Gaudin et al., 1992). The glycoprotein is also able to confer protection against lethal infection with rabies virus (Wunner et al., 1983). The first 19 amino acids of the predicted sequence constitutes the hydrophobic signal peptide and initiates the translocation of the nascent protein through the rough endoplasmic reticulum before being cleaved into the mature protein (Lai et al., 1981). The $20^{th}$ lysine of the deduced peptide sequence then becomes the $NH_2$ terminal amino acid of the mature glycoprotein. The deduced sequence also has an uninterrupted hydrophobic domain of 22 amino acids, bounded by lysine at 439 and two arginine residues at 462 and $463^{rd}$ positions near the carboxy terminus.

### 2.3.6 THE POLYMERASE PROTEIN (L PROTEIN)

This giant protein, which is 2142 amino acids long, occupies more than half of the rabies genome.

### 2.3.7 THE INTERGENIC REGION

In the genomic sequence of rabies virus, two non-coding intergenic regions are present between M2 - G and G - L genes. During transcription alternative termination occurs at the intergenic region. The G – L intergenic region is called pseudogene ($\psi$ gene). On the basis of analysis of pseudogene region, Tordo et al, and

(1986) suggested that rabies have an evolutionary link with unsegmented negative-strand RNA virus genomes.

## 2.4 VIRAL PROTEINS INVOLVED IN HOST IMMUNE RESPONSE

Although all the viral proteins show antigenicity, they do not play a role in protection (Celis et al., 1990). The purified Glycoprotein has been shown to protect against an intracerebral challenge with rabies, while the purified ribonucleocapsid only protects against a peripheral challenge (Dietzschold et al., 1987).

The Glycoprotein is the only rabies antigen that consistently induces virus-neutralizing antibodies (Wiktor et al., 1973). This property mainly depends on the preservation of its three-dimensional structure, although linear neutralizing epitope has been identified (Bunschoten et al., 1989). T cell response is thought to play an important role in the immune response to rabies (Kawano et al., 1990). The glycoprotein also shares the capacity to induce a cellular immune response involving both T-helper cells and cytotoxic T cells with the N and M1 proteins. Thus, studies suggest that the Glycoprotein is the most important antigen for immunization, and is the best candidate to increase the protection spectrum of vaccines, notably to distant rabies-related viruses (Bourhy et al., 1993).

## 2.5 THE PROPERTIES OF GLYCOPROTEIN

Rabies virus glycoprotein (G) is a type I membrane glycoprotein. It is a trimer that forms a spike extending 8.3nm from the viral membrane (Gaudin et al., 1992). The glycoprotein gene codes for 524 amino acids with the mature peptide having 505 amino acid

(Anilionis et al., 1981). Its amino acid sequence indicates that it has three potential N-linked oligosaccharide acceptor sites, of which only one or two are glycosylated, depending on the strain. The ectodomain contains 14 cysteines forming disulfide bridges.

Glycoprotein is responsible for adsorption of the virus onto the host cell and therefore determines the tissue tropism of the virus. After attachment and internalization of the virus via the endocytic pathway, G mediates low pH-induced fusion of the viral envelope with the endosomal membrane (Whitt et al., 1991).

The pH threshold for fusion is 6.3±0.1 and preincubation in the absence of target membranes below pH 6.75 leads to inhibition of viral fusion properties. Gaudin et al. (1993) reported that readjusting the pH to above 7 could reverse loss of fusion properties

## 2.6 STRUCTURAL REQUIREMENTS FOR IMMUNOGENIC ACTIVITY OF GLYCOPROTEIN

Glycoprotein can assume at least three different states; the Native (N) state detected at the viral surface above pH7.0; the activated hydrophobic state (A), which interacts with the target membrane as a first step of the fusion process and the fusion-inactive conformation (I). In the absence of the transmembrane domain, the ectodomain of G folds in a monomeric Inactive-like conformation and is not able to adopt the Native confirmation (Wojczyk et al., 1995).

The transmembrane domain also favours folding of the ectodomain and thereby facilitating oligomerization. It also acts in a more

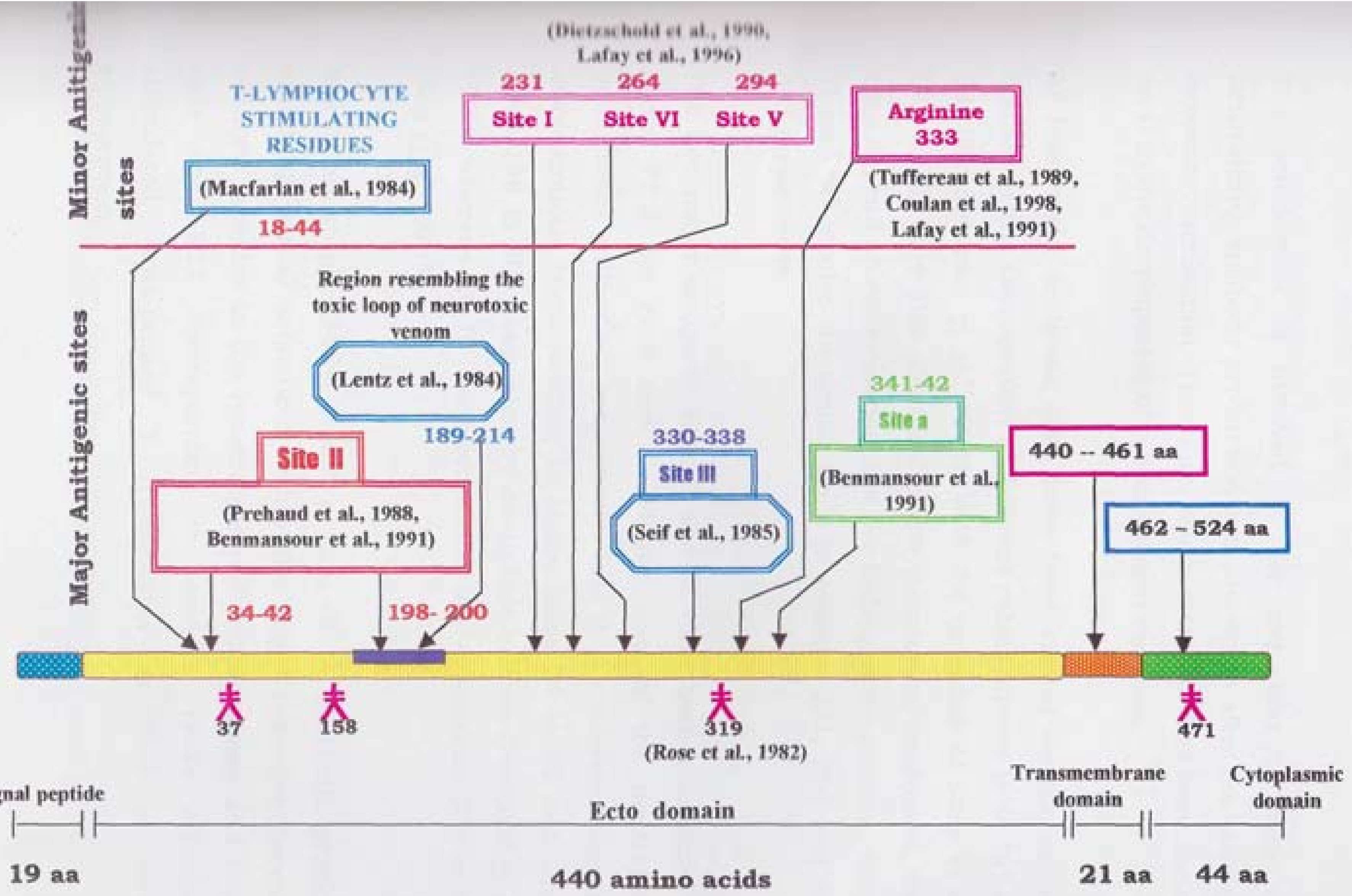
specific manner by nucleating the oligomerization process (Gaudin et al. 1990).

## 2.7 POTENTIAL SITES OF GLYCOSYLATION IN THE GLYCOPROTEIN

The glycosylation and fatty acylation takes place during its transport from the rough endoplasmic reticulum to the Golgi apparatus and to the cytoplasmic membrane. N- linked glycosylation plays a critical role in the expression of most cell surface and secreted proteins and is often required for protein stability, antigenicity and biological function (Shakin Eshleman et al., 1996). Even though several potential N-linked glycosylation sites exist along the Lyssa virus Glycoprotein, only Asn 319 is sufficient for correct folding and transport of the Glycoprotein.

N-linked glycosylation generally occurs at the sequon Asn-X-Ser or Asn-X-Thr, where X is any amino acid except proline. Bause et al. (1982) provided direct confirmation that Trp, Asp and Glu at the X position inhibit core glycosylation and the sequeon Asn-Cys-Ser in rabies glycoprotein is fully glycosylated.

The ERA strain of rabies virus has three possible sequence specific sites for oligosaccharide attachment at asparagine residues 37, 247 and 319. In CVS strain, the middle glycosylation signal is located at position 204, whereas in Kelev strain, a base mutation resulting in substitution of aspartic acid at position 247 abolishes the middle glycosylation site. One glycosylation site 319 appears to be of major importance because it is present in all Lyssa virus strains sequenced up to now (Rose et al., 1982).


(Dietzschold et al., 1990,
Lafay et al., 1996)
231
264
294
Site I
Site VI
Site V
Arginine
333
(Tuffereau et al., 1989,
Coulan et al., 1998,
Lafay et al., 1991)
Minor Anitigenic sites
T-LYMPHOCYTE
STIMULATING
RESIDUES
(Macfarlan et al., 1984)
18-44
Region resembling the
toxic loop of neurotoxic
venom
(Lentz et al., 1984)
189-214
Major Anitigenic sites
Site II
(Prehaud et al., 1988,
Benmansour et al., 1991)
34-42
198-200
330-338
Site III
(Seif et al., 1985)
341-42
Site a
(Benmansour et al.,
1991)
440 -- 461 aa
462 – 524 aa
37
158
319
(Rose et al., 1982)
471
Signal peptide
Ecto domain
Transmembrane
domain
Cytoplasmic
domain
19 aa
440 amino acids
21 aa
44 aa

## 2.8 MAPPING AND DEFINING ANTIGENIC SITES OF RABIES VIRUS GLYCOPROTEIN

The ectodomain is involved in the induction of both viral neutralizing antibody production and protection after pre- and post exposure vaccination. Therefore, much attention has been focused on G in the development of rabies subunit vaccines.

At least eight antigenic sites have been located on the external domain of the Glycoprotein of different rabies strains (I-VI, "a" and G1) (Benmansour et al., 1991). With the exception of sites VI and G1, which were also identified under denaturing conditions, these sites depend on conformation and the folding of the protein. Sites I, VI and "a" involve the amino acids in position 231, 264 and 342-343 respectively.

The two major antigenic sites II and III have been recognized by about 72.5 per cent and 24 per cent of the neutralizing monoclonal antibodies, respectively. Site II is conformational and discontinuous corresponding to amino acids 34 to 42 and amino acids 198 to 200 associated by disulphide bridges (Prehaud et al., 1988), whereas site III is conformational and continuous 330 to 338 (Seif et al., 1985).

Macfarlan et al., (1984) tested a total of 14 synthetic peptides matching partial sequences within the larger cyanogen bromide reduced peptides in the lymphocyte proliferation assay and found only one, R21 corresponding to residues 18-44 effectively stimulated virus-primed T lymphocytes as that of intact glycoprotein.

The Glycoprotein is also involved in the pathogenesis of rabies and is believed to assume at least part of the neurotropism of the virus. The neurotropism seems to directly relate to the maintenance of lysine 330 and arginine 333 in site III as they play a key role in recognition of neuronal receptors (Tuffereau et al., 1989). Mutants with other amino acids in this position cannot infect certain types of neurons, presumably because they are unable to recognize the receptor (Lafay et al., 1991).

Thus Lyssa virus glycoprotein molecules can be split structurally and immunologically into two different parts; the COOH half carrying site III and anchoring the protein to the membrane and the $NH_2$ half carrying the site II, which stabilizes the conformation of site III. A limited flexibility is authorized at the site II – site III junction, at between amino acids 247 and 257 (Perrin et al., 1999).

**2.9 IN-VITRO EXPRESSION OF RABIES VIRUS GLYCOPROTEIN**

The process for the present rabies vaccine production includes hazardous steps of handling large amounts of infectious materials and hence it has long been desired to develop new low cost rabies vaccines with high efficacy and safety. The most popular and promising approaches to develop new vaccines are to clone viral genes to which the genetic engineering technologies are applied. In this effort a variety of prokaryotic and eukaryotic systems have been tried expressing rabies virus glycoprotein.

Yelverton et al. (1983) used a vector containing the Glycoprotein cDNA for the direct expression of the complete sequence of the Glycoprotein of CVS-11 rabies strain in Escherichia coli. Lathe et al. (1984) used derivatives of the M13 bacterial plasmids to direct the

expression of the rabies Glycoprotein cDNA in E.coli. However, the Glycoprotein expressed in E.coli was extremely insoluble, and did not react with anti-Glycoprotein monoclonal antibodies directed against conformational determinants. This Glycoprotein also failed to confer protection against rabies.

Although induction of rabies neutralizing antibody is considered to be essential for protection against rabies, it appears that cellular immunity is also required (Wiktor, T.J. 1977) and correct presentation of the glycoprotein is necessary for the induction of specific cytotoxic T cells capable of destroying rabies-infected cells (Mcfarlan et al., 1986).

The approach that has been more successful in addressing the essential immunological requirements has been the use of live virus vectors. Kieny et al. (1984) altered the rabies glycoprotein cDNA by site-directed mutagenesis, removed the poly (dG) tail, aligned with an early vaccinia virus promoter sequence and inserted within a cloned copy of vaccinia thymidine kinase gene. The protein expressed reacted with monoclonal antibodies against Glycoprotein in a pattern identical to that of native rabies Glycoprotein. A live recombinant vaccine was developed which induced high titres of rabies virus-neutralizing antibodies and conferred protection against challenge with street rabies virus strains (Wiktor et al., 1984).

Taylor et al. (1988) cloned the glycoprotein in fowl poxvirus and canary poxvirus to develop a recombinant vaccine and also compared the protective efficacy. They also showed that foreign genes could be authentically synthesized, processed and presented

on the infected cell surface without infectious progeny virus being produced.

Xuan et al., (1998) constructed a recombinant Canine Herpesvirus (CHV) expressing the glycoprotein of rabies by inserting the gene within the thymidine kinase gene of CHV YPH strain under the control of the human cytomegalovirus immediate early promoter. The biological and immunological properties of the protein have been studied and it was concluded that CHV recombinant expressing Glycoprotein can be used as a vaccine to control canine rabies and also that CHV may also be useful to develop live recombinant vaccines against other infectious diseases.

Prevec et al. (1990) inserted the cDNA of the rabies virus Glycoprotein of the ERA strain between the promoter and poly-A addition site of the early region of Simian Vacuolating virus (SV40) and placed this construct into the deleted E3 region of human adenovirus type 5. The recombinant virus not only elicited high virus neutralizing titres but also protected against challenge.

The insect baculovirus Autographa californica (AcNPV) is a very potent expression system for foreign genes, because the yields of the expressed protein are very high and the biological properties of the expressed material are conserved (Luckow et al., 1988). The Glycoprotein cDNA was inserted into the transfer vector pAcYM1 such that the cDNA was under the control of the AcNPV polyhedron promotor (Prehaud et al., 1989). Co-transfection of Spodoptera frugiperda cells with this recombinant transfer vector and AcNPV DNA resulted in recombinant virus that exhibited a polyhedron-negative phenotype and expressed the rabies Glycoprotein in those

cells. This system not only offered high expression of the protein but also favoured effective purification methods.

Sakamoto et al. (1999) used G cDNA of Nishigahara strain of rabies virus (seed strain for dog vaccine production in Japan) to transfect yeast cells and observed that two forms of glycoprotein were expressed yGI (66kDa) and yGII (56kDa). They also reported that only yGII component (constituting only 1per cent of the total Glycoproteins) reacted with G Mabs and induced protective immunity in guinea pigs. Their results also suggest that most Glycoprotein processed in yeast cells, result in abnormal folding and multimer formation and mostly deprived of the C-terminal protein.

The full length cDNA of the Glycoprotein of ERA strain was inserted into the eukaryotic shuttle vector PsG5 and then stably transfected into wild-type Chinese Hamster Ovary (CHO) cells and mutant CHO cells defective in glycosylation (Burger et al., 1991). They used this system to study the role of N-linked glycosylation in the intracellular transport and antigencity of Glycoprotein.

DNA-based immunization with a plasmid encoding the antigen responsible for inducing protection seems to be more cost-effective than classical techniques involving cell culture. Xing et al. (1994) cloned the glycoprotein gene in eukaryotic expression vectors with Simian Virus 40 (SV40) early promotor and termed it pSG5rab.gp. Mice immunized intramuscularly with the pSG5rab.gp developed rabies virus specific cytolytic cells, lymphokines secreting T helper cells of the TH1 subunit, and rabies virus neutralizing antibodies. It also protected the mice against subsequent challenge.

Biswas et al. (1999) cloned the rabies Glycoprotein into expression plasmid devoid of immunostimulatory sequences (bacteria harbouring kanamycin resistant (kanR)) present in the ampicillin resistance (ampR) and immunized mice intramuscularly. They showed that the mice were protected against a subsequent lethal intracerebral rabies virus challenge.

Biswas et al. (2001) demonstrated for the first time that co-inoculation of DNA rabies vaccine (DRV) and cell culture-derived human or veterinary inactivated rabies virus vaccine induces higher anamnestic antibody response than DRV in mice as well as cattle.

Beagles have been immunized by intramuscular (i.m.) injection with a plasmid encoding the rabies virus (PV strain) glycoprotein. Neutralizing antibodies against both wild-type rabies virus and European Bat Lyssa viruses (EBL1 and EBL2) were detected after a single injection and a boost, but the levels of neutralizing antibodies against EBL1 were low. Moreover, all vaccinated dogs were protected against a lethal challenge with a wild-type dog rabies strain. This is one of the first studies to demonstrate that DNA vaccines can protect dogs, and opens important perspectives for rabies control (Perrin et al., 1999).

The Glycoprotein cDNA of the ERA strain of rabies including the signal peptide was placed under the control of the 35S promotor of cauliflower mosaic virus (McGarvey et al., 1995). Tomato tissues were transformed by Agarobacterium tumefaciens mediated transformation and the resulting G-protein expressed was of the molecular mass 62 and 60 kDa. The expressed protein was capable of being immunoprecipitated by different polyclonal antisera to G-protein and also detected by Monoclonal antibody in western blot.

Modelska et al. (1998) used engineered plant virus particles containing rabies virus sequence to immunize mice intra peritonealy or orally (gastric intubation or by oral feeding) to mount a local and systemic immune response. The study also supports the potential of plants as oral delivery vehicles and plant-produced antigens as vaccine material.

# MATERIALS

# CHAPTER III
# MATERIALS AND METHODS

## 3.1 MATERIALS

### 3.1.1 BIOLOGICALS

#### 3.1.1.1 Rabies Virus Isolates

Rabies suspected brain samples were collected in sterile vials at postmortem at the Department of Pathology, Madras Veterinary College, Chennai-7. Impression smears were also taken in slides at the time of sample collection. The samples that tested positive were stored at -70°C for RNA isolation. A sample obtained from dog which tested positive used in this study.

#### 3.1.1.2 Hyperimmune serum

Hyperimmune serum against rabies virus was raised as per standard procedures and available at the Rabies unit, Department of Animal Biotechnology, Madras Veterinary College, Chennai-7 was used.

#### 3.1.1.3 Antinucleocapsid antibody conjugate

Lyophilized, adsorbed rabies antinucleocapsid antibody conjugated with fluorescein isothiocyanate received from Diagnostic Pastuer, France was used in Direct Fluorescent Antibody test.

### 3.1.1.4 Monoclonal antibody conjugates

Monoclonal antibody conjugates **W**502-2, **C**15.2 and **W**422-5 obtained from Centre for Disease Control, Atlanta; USA was used to confirm the isolates as Lyssa virus serotype I.

### 3.1.1.5 Murine Neuroblastoma cell line (N2a)

Murine neuroblastoma cell line generously supplied as a gift by the Centre for Disease Control (CDC), Atlanta, USA was used in this study.

### 3.1.2 For RNA Isolation

### 3.1.2.1 Distilled water treated with DEPC

Triple glass distilled water was treated with 0.1 per cent Diethyl pyrocarbonate and was either stirred for 6 hours or left at 37 °C incubator overnight, dispensed in sterile containers and sterilized at 121 °C for 15 min.

### 3.1.2.2 Solution – D

| | | |
|---|---|---|
| Guanidine thiocyanate (USB, USA) | : | 4 M |
| Sodium citrate (pH 7.0) (Sigma, USA) | : | 25 mM |
| N-lauryl sarcosine (Sigma, USA) | : | 0.5 % |
| 2- Mercaptoethanol (GIBCO- BRL, USA) | : | 0.1 M |

Solution-D was prepared by dissolving 250 g of Guanidine thiocyanate in 293 ml of DEPC treated water followed by the addition of 17.6 ml of 0.75 M sodium citrate pH 7.0, 26.4 ml of 10

per cent sarcosyl at 65 °C. 2-mercaptoethanol was added at 0.1M level just prior to use.

**3.1.2.3 Sodium acetate (2 M)**

To 13.608 g of sodium acetate, 30 ml of DEPC treated water was added and stirred to mix. The pH was adjusted to 4.0 with Glacial acetic acid and then the volume was made upto 50 ml with DEPC treated water.

**3.1.2.4 Water saturated phenol**

Melted phenol was equilibrated with equal volume of DEPC treated water and water was extracted from phenol phase after stirring vigorously. This procedure was repeated until the phenol phase reached pH 4.0. The saturated phenol was stored at4 °C and was used for RNA extraction.

**3.1.2.5 70 per cent ethanol**

30 ml of DEPC-treated water was added to 70 ml of Ethyl alcohol (99 per cent) to make 100 ml of 70 per cent ethanol.

**3.1.3 Determination of integrity of the RNA samples by denaturing Formaldehyde gel electrophoresis**

**3.1.3.1 Formaldehyde gel running buffer (10 X)**

| | | |
|---|---|---|
| MOPS | : | 0.2 M |
| Sodium acetate | : | 50 mM |
| EDTA (pH 8.0) | : | 10 mM |

### 3.1.3.2 Gel loading buffer

| | | |
|---|---|---|
| Glycerol | : | 50 per cent |
| EDTA pH 8.0 | : | 1mM |
| Bromophenol blue | : | 0.25 per cent |
| Xylene Cyanol FF | : | 0.25 per cent |

### 3.1.4 For Complementary DNA (cDNA) synthesis

THERMOSCRIPT ™ Reverse Transcriptase -GIBCO BRL, NY, USA
0.1 M Dithiothreitol -GIBCO BRL, NY, USA
5 X first strand buffer -GIBCO BRL, NY, USA
(Tris HCl (pH 8.3) 250 mM, KCl 375 mM, $MgCl_2$ 15 mM)
RNase Out (40U/ µl) -GIBCO BRL, NY, USA
10mM 2-deoxynucleoside 5-triphosphate mix-GIBCO BRL, NY, USA
Random Hexamers $p(dN)_6$(50 ng / µl) -GIBCO BRL, NY, USA
Ribonuclease H (2 U/ µl) -GIBCO BRL, NY, USA
Nuclease Free water -GIBCO BRL, NY, USA

### 3.1.5 Polymerase Chain Reaction to amplify the full-length Glycoprotein gene

Primers were designed to amplify the full length Glycoprotein gene of Rabies virus. The primers were also designed to have built in Restriction Enzyme sites to enable directional cloning of the PCR products.

**Primer 1 (Forward)**

Bam HI site

5'- GCG GAT CC**A** **TG**G TTC CTC AGG CTC TCC TG -3'

Start codon

**Primer 2 (Reverse)**

Kpn I site

5'- TCC GGT ACC **TCA** CAG TCC GGT CTC ACC CCC - 3'

Stop codon

An internal primer pair was also selected to amplify a 627 bp pair region within the full -length glycoprotein gene for an initial confirmation.

**Internal Primer 1 (Sense) (position 564 to 583)**

5' - GGA ATT GCT CAG GAG TAG CG - 3'

**Internal Primer 2 (antisense) (position 1200 to 1181)**

5' - TAA GAC ATT GCC GTC AGG TC - 3'

Taq DNA Polymerase (5 U / µl) -Genetix, New Delhi
10 X PCR buffer, minus $MgCl_2$ -Genetix, New Delhi
(200 mM Tris-HCL, pH 8.4)
50 mM Magnesium chloride -Genetix, New Delhi
10mM dNTP mix -GIBCO BRL, NY, USA

**3.1.6 Purification of PCR products**

**Low Melting Point Agarose** - GIBCO BRL, NY, USA

**GFX ™ PCR DNA and Gel Band Purification Kit (Amersham pharmacia biotech, USA)**

The kit has the following components

Capture buffer - buffered solution containing acetate and chaotrope.

GFX ™ Columns- Microspin columns pre-packed with a glass fiber matrix.

Collection tubes - 2-ml capless microcentrifuge tubes.

Wash buffer - Tris-EDTA buffer with absolute ethanol added to a final concentration of 80 per cent before use

Nuclease free water

**50 X Tris Acetate EDTA buffer**

| | | | |
|---|---|---|---|
| Tris Base | - | 242 | g |
| Glacial acetic acid | - | 57.1 | ml |
| 0.5 M EDTA (pH 8.0) | - | 100 | ml |
| Distilled water to | - | 1000 | ml |

**Molecular Weight Marker**

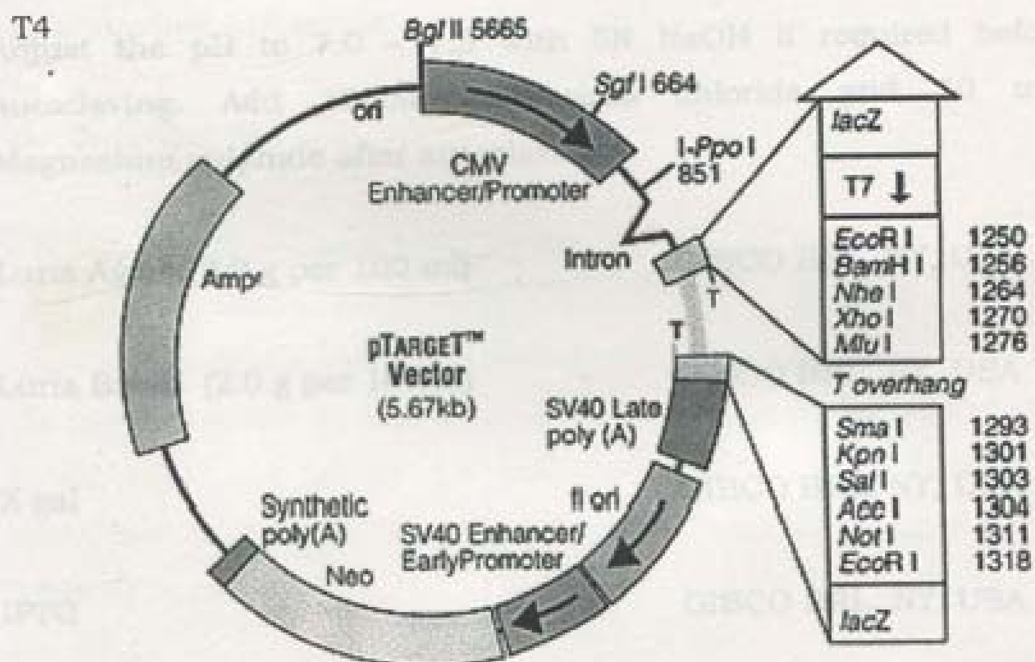
λ DNA double digested with *EcoRI* and *Hind III* - Bangalore genei, INDIA

**3.1.7 Cloning of Glycoprotein Gene and Recombinant Selection**

### 3.1.7.1 Cloning Vector

pTargeT™ Vector Mammalian Expression T-Vector -
Promega Corporation, USA

T4




DNA ligase (3 Weiss units / µl) - Promega Corporation, USA

Purified full-length glycoprotein PCR product adjusted to a concentration of 50ng / µl.

### 3.1.7.2 Transformation and Recombinant selection

*Escherichia coli* DH5α cells - GIBCO BRL, NY, USA

Competent Cells Preparation Kit (B) (Bangalore Genei Pvt. Ltd, Bangalore, INDIA)

**SOB medium**

| | | |
|---|---|---|
| Tryptone | - | 20 g |
| Yeast Extract | - | 5 g |
| 5M Sodium chloride stock solution | - | 2 ml |
| 3M Potassium chloride stock solution | - | 830 µl |
| Distilled water upto | - | 1000 ml |

Adjust the pH to 7.0 – 7.3 with 5N NaOH if required before autoclaving. Add 10mM magnesium chloride and 10 mM Magnesium sulphate after autoclaving.

| | | |
|---|---|---|
| Luria Agar (3.2 g per 100 ml) | - | GIBCO BRL, NY, USA |
| Luria Broth (2.0 g per 100ml) | - | GIBCO BRL, NY, USA |
| X gal | - | GIBCO BRL, NY, USA |
| IPTG | - | GIBCO BRL, NY, USA |

Prepare a stock solution of 20mg /ml of X gal and 20mg/ ml of IPTG separately. To 800 µl of IPTG stock solution add 3.8 ml of distilled water, mix well and add 400 µl of X gal stock solution. Use 250 µl of the X gal – IPTG mix per plate for blue and white selection.

**3.1.7.3 Plasmid Extraction**

**1. Solution I**

| | | |
|---|---|---|
| Glucose | - | 50mM |
| Tris-Hcl (pH8.0) | - | 25mM |
| EDTA (pH8.0) | - | 10mM |

**2. Solution II**

Prepared stock solutions of the following

| | | |
|---|---|---|
| Sodium Hydroxide (NaOH) | - | 10N |
| Sodium dodecyl sulphate | - | 10per cent |

Prepare 0.2 N NaOH and 1per cent SDS freshly from the stock solution just before use

**3. Solution III**

| | | |
|---|---|---|
| Potassium Acetate (5M) | - | 60 ml |
| Glacial Acetic Acid | - | 11.5 ml |
| Water upto | - | 28.5 ml |

To 5M potassium acetate solution add the quantity of glacial acetic acid, adjust the pH to 4.8 and then make it upto 100ml. The resultant solution is 3M with respect to Potassium and 5 M with respect to acetate.

**4. QIA Miniprep plasmid kit** - Qiagen

### 3.1.7.4 Restriction Enzyme Digestion of Recombinant Plasmids

Restriction enzymes *Bam HI, Kpn I* and *Eco RI* obtained along with the Tango buffer from MBI Fermentas were used in this study.

### 3.1.7.5 Molecular weight markers

| | | |
|---|---|---|
| 1 kb plus DNA ladder | - | GIBCO BRL, NY, USA |
| Supercoiled DNA ladder | - | GIBCO BRL, NY, USA |

### 3.1.7.6 Colony Hybridization, Southern blotting Colony Hybridization

**1. Probe**

Rabies Glycoprotein internal 627-bp product was labeled and used as probe

2. Nylon membrane (Hybond N+) - Amersham, USA

**3. Denaturing solution**

| | | |
|---|---|---|
| Sodium hydroxide | - | 0.5 M |
| Sodium chloride | - | 1.5 M |

**4. Neutralization Solution**

| | | |
|---|---|---|
| Tris-HCl (pH 7.5) | - | 1.0 M |
| Sodium chloride | - | 1.5 M |

**5. 20 X SSC**

| | | |
|---|---|---|
| Sodium chloride | - | 175.3 g |
| Sodium citrate | - | 88.2 g |
| Water upto | - | 1 lit |

**6. Dig DNA labeling and Detection kit, Boehringer Mannehim, Germany**

**For DNA labeling**

Hexanucleotide mix

10 mM deoxy nucleotide mix

Klenow Enzyme

0.2 M EDTA pH 8.0
4 M lithium chloride
70 per cent ethanol
10 per cent Sodium dodecyl sulfate
20 X SSC

**Buffer 1**

| | | |
|---|---|---|
| Maleic acid | - | 0.1 M |
| Sodium chloride | - | 0.15 M |

pH adjusted to 7.5 with 0.1 N sodium hydroxide and autoclaved

**Blocking reagent stock solution**

Blocking reagent supplied in the powder form was dissolved in buffer 1 to a final concentration of 10 per cent (W/V) with shaking and heating. The stock solution was autoclaved and stored at – 20°C

**Hybridization buffer**

| | | |
|---|---|---|
| 20 X SSC | - | 25 ml |
| Blocking reagent (10 per cent stock solution) | - | 10 ml |
| N-lauryl Sarcosine | - | 0.1 g |
| Sodium dodecyl sulfate (10per cent) | - | 200 μl |
| Water upto | - | 100ml |

store at – 20°C

**Primary wash buffer** - 2x SSC and 0.1 per cent SDS
**Secondary wash buffer** - 0.1x SSC and 0.1 per cent SDS

**Buffer 2**

Blocking stock solution diluted 1:10 in buffer (final concentration of 1 per cent blocking reagent)

**Buffer 3**

| | | |
|---|---|---|
| Tris-Hcl | - | 100 mM |
| Sodium chloride | - | 100 mM |
| Magnesium chloride | - | 50 mM |
| pH adjusted to | - | 9.5 |

**Buffer 4**

| | | |
|---|---|---|
| Tris-HCl | - | 10 mM |
| EDTA | - | 1 mM |
| pH adjusted to | - | 8.0 |

**Colour-substrate solution (freshly prepared)**

| | | |
|---|---|---|
| NBT solution | - | 45 µl |
| X-phosphate solution | - | 35 µl |
| Buffer 3 | - | 10 ml |

### 3.1.8 Automated Nucleotide Sequencing

| | | |
|---|---|---|
| Sequencing kit | - | Perkin Elmer Inc, USA |
| ABI prism ™ 3700 automated capillary sequencer | - | PE Applied Biosystems, USA |

### 3.1.9 Software's

#### 3.1.9.1 Automated sequencing

ABI prism ™ sequence data collection software - PE Applied Biosystems, USA

ABI prism ™ auto assembler software - PE Applied Biosystems, USA

### 3.1.9.2 SEQUENCE ANALYSIS

#### 3.1.9.2.1 Phylogenetic analysis of the obtained glycoprotein gene sequence

**3.1.9.2.1.1 CLUSTAL X** version 1.7 Documentation

Multiple sequence alignment programme (Thompson et al., 1994) available at web site ftp/ ebi.ac.uk/pub/software.

**3.1.10.2.1.2 PHYLIP (Phylogeny Inference Package)**

PHYLIP version 3.6 for windows by Joe Felsentein, Department of Genetics, University of Washington, Seattle, Washington 98195, USA (email : joe@genetics.washington.edu) and available at web site http://evolution.genetics.washington.edu/phylip.html. This package also contains several programmes for phylogenetic analysis using different methods. This also has programmes for the phylogenetic tree output like DRAWGRAM for plotting phenograms etc., and DRAWTREE for plotting unrooted tree diagrams with many options for tree manipulation and previewing. These programmes were also used in this study

**3.1.10.2.1.3 MEGA (Molecular Evolutionary Genetic Analysis)**

MEGA version 1.02 was used for estimating evolutionary distances and construction of phylogenetic trees. The software is available from Sudhir Kumar, Institute of Molecular Evolutionary Genetics,

328 Mueller Laboratory, The Pennsylvania State University Park, PA 16802, USA. Email: imeg@psuvm.psu.edu (Kumar et al., 1993).

The programmes Clustal X, MEGA and PHYLIP were used for phylogenetic analysis of the derived glycoprotein nucleoprotein sequence as well as the conceptually translated glycoprotein sequence.

### 3.1.10 *IN-VITRO* EXPRESSION

#### 3.1.10.1 Open reading frame and Protein sequence determination

**3.1.10.1.1 GeneTool Lite** from http://www.DoubleTwist.com was also used for finding the open reading frames (both in tabular and graphical form) and conceptual protein sequence determination by translation of nucleotide sequence.

**3.1.10.1.2 SEQAID II** package version 3.81 (Rhoads and Roufa, 1991), available at ftp.ebi.ac.uk/pub/software/DOS was also used for finding the open reading frames (both in tabular and graphical form) and conceptual protein sequence determination by translation of nucleotide sequence. This Programme was also to determine the hydopathicity plot of derived amino acid sequence.

#### 3.1.10.2 Expression of rabies glycoprotein in eukaryotic cells

**3.1.10.2.1 Cell Lines**

Cell line Vero obtained from the National Facility for Cell and Tissue Culture (NFATCC) and maintained in the Central Tissue Culture laboratory, Dept. of Animal Biotechnology, MVC, Chennai-7 and

Neuroblastoma cell line generously supplied by CDC, Atlanta, USA was used in the study.

### 3.1.10.2.2 Medium for Growth of Cell lines

The growth medium was prepared in Triple glass distilled water. One litre of the medium contains

| | | |
|---|---|---|
| Dulbeco's Modified Eagle's Medium (Sigma, USA) | : | 1 bottle |
| Foetal Bovine Serum | : | 50 ml |
| Antibiotic stock solution | : | 10 ml |

Sodium bicarbonate 3.6 gm and L-glutamine 400mg were added to the medium, final volume made upto 1 litre, sterilized by filtration through 0.45 um membrane filter and checked for sterility.

The growth medium for transfected cells was prepared without the addition of antibiotic

### 3.1.10.2.3 Antibiotic stock solution

A stock solution of antibiotics was prepared as follows:

| | | |
|---|---|---|
| Benzyl penicillin (Alembic) | : | 10 lakh units |
| Streptomycin sulphate (Sarabhai) | : | 1 g |

Dissolve the contents in autoclaved triple distilled water, volume made upto 100 ml and stored.

### 3.1.10.2.4 Trypsin stock solution

Trypsin stock solution (0.25 per cent) was prepared in autoclaved triple glass distilled water and sterilized by filtering through 0.45 um syringe filter and stored at 4 °C.

**3.1.10.2.5 Phosphate buffered saline (PBS) 1 X**

| | | |
|---|---|---|
| Sodium chloride | : | 8.0 g |
| Potassium chloride | : | 0.2 g |
| Disodium hydrogen phosphate | : | 1.15 g |
| Potassium dihydgrogen phosphate | : | 0.2 g |

Dissolve the contents in water, the pH adjusted to 7.2 and volume adjusted to 1 litre with distilled water. Sterilized by autoclaving at 121 °C and 15 lbs for 15 min.

**3.1.10.2.6 CELLFECTIN$^{R}$ reagent** - GIBCO BRL, NY, USA

CELLFECTIN Reagent is a 1:1.5 (M/M) liposome formulation of the cationic lipid N, $N^{I}$, $N^{II}$, $N^{III}$ -Tetramethyl- N, $N^{I}$, $N^{II}$, $N^{III}$ -tetrapalmitylspermine (TM-TPS) and dioleoyl phosphatidylethanolamine (DOPE) in membrane filtered water. It is supplied 1 ml quantity in concentration of 1mg/ml.

**3.1.10.2.7 Anti Goat rabbit IgG couple with Fluorescein isothiocyanate - SIGMA, USA.**

# METHODS

## 3.2. METHODS

### 3.2.1. ISOLATION, IDENTIFICATION AND ADAPTATION OF LOCAL ISOLATE OF RABIES VIRUS

#### 3.2.1.1 Collection and preservation of brain samples from suspected cases of rabies

The brain was removed from the skull during postmortem of rabies suspected animals and dissected longitudinally to separate the two hemispheres. The cerebellum and medulla were detached from the hemispheres. A longitudinal incision was made externally in the posterior third of each hemisphere, about 1.5 cm from the mid line. The incision was continued through the greymatter and white matter until a narrow space, the lateral ventricle, is reached. The hippocampus major was seen as a semi-cylindrical white glistening body bulging laterally on either side from the ventricle floor. Using sterile scissors, parts of hippocampus and cerebellum were dissected out and transferred to sterile storage vials and stored in the deep freezer (-70°C). Brain samples were collected from 28 rabies suspected animals.

#### 3.2.1.2 Preparation of impression smears

Impression smears were made on fresh, clean microscopic slides. With a pair of scissors, small transverse sections of 2-3 mm in thickness were cut from hippocampus and/or cerebellum and placed on a piece of clean blotting paper with the cut surface facing upward. The cut surface was then gently touched with blotting paper to remove the blood and cerebrospinal fluid. A clean microscopic slide was then touched against the cut surface of the section and pressed gently downwards with just enough pressure

exerted to create a slight spread of the exposed surface of tissue against the slide.

#### 3.2.1.3 Fluorescent Antibody Test (FAT)

Direct FAT was carried out as per the method described by Dean & Abelseth (1973). Fluorescein isothiocyanate (FITC) tagged rabies antinucleocapsid antibody conjugate was used in the test to detect the antigen. Positive and negative control smears were also included along with the test samples.

- The smears were fixed in acetone at -20°C for 30 min and then briefly air-dried.
- To each smear sufficient quantity of antibody conjugate (50µl) was added and incubated at 37°C for 30 min in a moist chamber.
- Slides were then rinsed twice in water for 5 min each at room temperature.
- The slides were examined under fluorescent microscope with few drops of glycerol saline.

A dog sample that tested positive by direct FAT with antinucleocapsid conjugate was processed for infecting murine neuroblastoma cell line.

#### 3.2.1.4 Detection of serotype variation by monoclonal Antibody screening

A panel of three monoclonal antibodies namely **W** 502-2 (which reacts with rabies and non rabies Lyssa viruses), **C** 15-2 (which reacts only with rabies virus) and **W** 422-5 (which reacts with Lagos

bat, Mokola and Duvenhage viruses but does not react with rabies virus) were used to detect any possible strain variation as per the method described by Jayakumar et al., (1993).

Monoclonal antibody screening of the samples tested positive with antinucleocapsid conjugate were done by direct FAT. Three impressions were made in a clean glass slide for each sample. After drying, the smears were fixed in cold acetone at -20°C for 30 min. The slides were then air-dried. FITC-Mab conjugates were applied to the smears and incubated at 37°C for 30 min in a moist chamber. The smears were then gently washed in tap water, air dried and mounted in 25 per cent glycerol saline and examined under fluorescent microscope.

The dog sample that confirmed to be rabies by the monoclonal reactive pattern was used for infecting neuroblastoma cells.

#### 3.2.1.5 Murine neuroblastoma cell culture

The cells were grown in minimum essential medium (MEM) supplemented with 10 per cent fetal bovine serum (FBS). On confluence the monolayer was detached with 0.25 per cent trypsin and the cells were suspended in growth medium and sub cultured with a split ratio of 1:3.

##### 3.2.1.5.1 Isolation and identification of rabies virus in murine neuroblastoma cell line

Isolation and identification of rabies virus in murine neuroblastoma cells was carried out as per the method of Jayakumar et al., (1994). A 10 per cent suspension of rabies positive brain sample was

prepared in MEM with 2 percent FBS. Antibiotics benzyl penicillin 150 units/ml and streptomycin 150 µg/ml were added to the final suspension. The suspension was centrifuged at 5000 rpm for 20 min, the supernatant collected and the vials freeze thawed thrice and stored at –70°C.

500 µl of the supernatant fluid was used to infect neuroblastoma monolayer in 6 well plates. After virus adsorption for 1 hour at 37°C, the inoculum was discarded, fresh maintenance medium was added and the plate was incubated at 37°C. At 72 and 96 hrs post-infection, the cells were fixed by adding cold 80 per cent acetone. Direct FAT was performed on the cells with antinucleocapsid antibody FITC conjugate for the presence of specific fluorescent foci. The positive samples were used to infect N2a in $25cm^2$ flask and used for RNA extraction.

## 3.2 AMPLIFICATION OF FULL LENGTH GLYCOPROTEIN GENE AND CLONING IN EXPRESSION VECTOR

### 3.2.2.1 Sterilization of plastic wares and solutions

All plastic wares used were fresh ones for RNA isolation techniques. Fresh micro centrifuge tubes and PCR tubes were dispensed in sterile containers using gloved hands, wrapped with autoclavable plastic covers and autoclaved for 15 min at 121°C. Care was taken not to touch or bring the plastic ware into contact with bare hands or other materials.

All solutions and buffers were prepared with 0.1per cent DEPC treated distilled water which were autoclaved for 15 min at 121°C to remove excess DEPC.

### 3.2.2.2 Preparation of Total RNA from infected neuroblastoma cells

Total RNA from the infected cells was carried out as per Chomczynski and Sacchi (1987) with minor modifications.

1. To an infected 25cm$^2$ flask 2 ml of solution D was added and thoroughly rinsed. The lysate was collected in eppendorf tubes. To each ml of solution D the following were added in order
   i. 0.1ml of 2 M sodium acetate pH4.0
   ii. 1 ml of water-saturated phenol.
   iii. 0.2 ml of chloroform-isoamyl alcohol.

2. The sample was further homogenized, kept on ice for 10 min and transferred into DEPC treated centrifuge tubes.
3. The sample was centrifuged at 10,000-rpm for 20 min at 4$^oC$.
4. The aqueous phase (containing RNA) was transferred to fresh centrifuge tubes and mixed with an equal volume of isopropanol and kept at room temperature for 10 minutes.
5. The precipitate was collected by centrifugation at 10,000 rpm for 20 min at 4$^oC$. The supernatant was carefully decanted and discarded.
6. The pellet was washed twice with 0.5 ml of 75 per cent ethanol, recentrifuged and vaccum dried briefly.
7. The RNA pellet was stored in 0.5ml of 75 per cent alcohol at -70$^oC$ until use or resuspended in nuclease free water for immediate use.

### 3.2.2.3 Determination of integrity of RNA samples by denaturing formaldehyde gel electrophoresis

RNA samples were denatured with both formaldehyde and formamide before electrophoresis. Formaldehyde was also added to the gel in order to maintain the denatured state of RNA sample during electrophoresis. Formaldehyde gel electrophoresis was done according to the method described by Farrell (1993).

A 1 per cent gel was prepared as follows: 0.5 gm of agarose was melted in 42.3 ml of DEPC water and cooled to 55-60°C. To this 5 ml of 10 X MOPS buffer and 2.7 ml of 37 per cent formaldehyde were added (this will result in a 1per cent agarose gel in 1 X MOPS with 0.66M formaldehyde). After further cooling of the agarose solution to 45°C the gel was cast.

RNA samples for electrophoresis were prepared by mixing the RNA sample in RNA loading buffer in 1:5 ratio. The RNA sample was then denatured at 65°C for 10 min and snap cooled on ice. The samples wee loaded on to the gel immersed in 1 X MOPS running buffer. At the end of electrophoresis the gel was visualized under ultra violet transilluminator and photographed using Polaroid film.

### 3.2.2.4 Complementary DNA (cDNA) synthesis

Complementary DNA was synthesized with the total RNA isolated from the positive brain sample (dog brain – tested by direct FAT as well as monoclonal antibody screening) using THERMOSCRIPT ™ Reverse Transcriptase and random hexamers according to the manufactures instruction.

Briefly RNA (18μl) was denatured with random hexamers (2μl) at 70oC for 10 min and snap cooled on ice for 5 min. The reaction mixture was then assembled containing

| | | |
|---|---|---|
| 5x RT buffer | : | 8 μl (1X) |
| Dithiothreitol (0.1M) | : | 2 μl |
| dNTP mix (10mM) | : | 4 μl |
| RNase Out | : | 2 μl |
| THERMOSCRIPT ™ RT | : | 2 μl |
| Nuclease free water | : | 2 μl |
| Total | : | 20ul reaction |

To the reaction mixture the denatured RNA sample was added and incubated at 25°C for 10 minutes followed by 60 minutes at 42°C. The first strand cDNA synthesized was incubated at 85°C for 5 minutes and then treated with RNase Out at 37°C for 30 minutes to remove the RNA template strand from the cDNA-RNA hybrid. The cDNA was used for PCR for the glycoprotein gene (1592 bp) or stored at –70°C for future use.

### 3.2.2.5 POLYMERASE CHAIN REACTION FOR AMPLIFICATION OF FULL LENGTH GLYCOPROTEIN GENE

#### 3.2.2.5.1 Designing of primers

All the sequences of the rabies available in Gene bank were downloaded from the website http://www.ncbi.nlm.nih.gov/entrez. The glycoprotein full-length sequence of vaccine viruses and street viruses were selected and converted in to FASTA format and stored in a single file. These sequences were loaded in to the clustal programme and aligned to find out the homology regions. It was ascertained using this programme that there was no variation

between the available sequences both at the start and the end of the glycoprotein gene. Sequences corresponding to the areas 3317 to 3338 and 4892-4872 the rabies genome near the start and stop codon were selected as primers with restriction sites added to the primer for directional cloning. An internal primer set corresponding to the nucleotides 564 to 583 and 1180 to 1200 was also used for a second PCR to confirm the amplified gene.

**3.2.2.5.2 Polymerase chain reaction**

The Polymerase chain reaction for the glycoprotein gene (1592 bp full length including restriction sites approx) was carried out in a 100 µl reaction. The reaction mixture consisted of 2.5 µl of cDNA (synthesized in a 40 µl reaction) and 1.67 mM $MgCl_2$ for amplification of the full-length glycoprotein gene.

**PCR mix for 100 µl volume**

| | | |
|---|---|---|
| 10x PCR buffer (minus Mg) | : | 10.0 µl |
| 50mM $MgCl_2$ | : | 3.0 µl |
| 10mM dNTP mix | : | 2.0 µl |
| Forward primer (50 pmol) | : | 1.0 µl |
| Reverse primer (50 pmol) | : | 1.0 µl |
| Taq DNA Polymerase (5 IU/ µl) | : | 0.8 µl |
| cDNA template | : | 2.5 µl |
| Nuclease free water | : | 79.5 µl |

**PCR programme for amplification of full-length glycoprotein gene**

The amplification was carried out with an initial denaturation at 94°C for 3 min followed by a step-up PCR as per the conditions given below:

| | |
|---|---|
| 94°C for 45 sec | 30 cycles |
| 55.5°C for 50 sec + 0.2°C per cycle | |
| 72°C for 2 min | |

A final extension hold at 72°C for 7 min was given followed by ramping to 4°C. Five µl of the amplified product was checked in a 1 per cent agarose gel with DNA molecular weight standards.

### 3.2.2.5.3 Agarose Gel Electrophoresis to check the amplified full-length glycoprotein gene

The Polymerase chain reaction for checking the amplified glycoprotein gene (627 bp internal product) was carried out using the internal set of primers in a 100 µl reaction. The reaction mixture consisted of 0.2 µl of amplified full-length glycoprotein PCR product and 1.5 mM $MgCl_2$ for amplification.

PCR mix for 100 µl

| | | |
|---|---|---|
| 10x PCR buffer (with 15 mM $MgCl_2$) | : | 10.0 µl |
| 10mM dNTP mix | : | 2.0 µl |
| Internal Forward primer (50 pmol) | : | 1.0 µl |
| Internal Reverse primer (50 pmol) | : | 1.0 µl |
| Taq DNA Polymerase (5 IU/ µl) | : | 0.8 µl |
| Glycoprotein PCR product | : | 0.2 µl |
| Nuclease free water | : | 85.0 µl |

The amplification was carried as per the PCR conditions given below:

94°C for 30 sec
55.5°C for 45 sec    30 cycles
72°C for 50 sec

A final extension hold at 72°C for 7 min was given followed by ramping to 4°C. Five µl of the amplified product was checked in a 1 per cent agarose gel with DNA molecular weight standards.

**3.2.2.5.4 Purification of full-length glycoprotein gene product**

1. The full-length glycoprotein PCR product (approx 1.6 kb) was electrophoresed in a 1 per cent LMP gel and the band was cut out.

2. The gel band (approx 10 mg) was placed in a 1.5 ml eppendorf tube to which 300 µl capture buffer was added.

3. The tube was closed, mixed by vortexing vigorously and incubating at 60°C until the agarose is dissolved.

4. During the incubation process, a GFX column was prepared by snapping off the bottom closure and placing the column in a collection tube.

5. After the agarose is completely dissolved, centrifuge briefly to collect the same to the bottom of the tube, transfer to the prepared GFX column and incubated for 1 min.

6. The column was centrifuged at full speed for 30 seconds. The flow through was discarded by emptying the collection tube and placing the GFX column back inside the collection tube.
7. 500 μl of wash buffer was added to the column, centrifuged briefly at full speed for 30 seconds.

8. The collection tube was discarded and 50 μl of elution buffer (10m M Tris-Hcl, pH 8.0 or Nuclease free water) was added to the column, incubated at room temperature for 1 min and centrifuged at full speed for 1 min to collect the purified DNA.

9. The eluted DNA was checked for purity and concentration by electrophoresing 1μl of the eluted DNA in 1 per cent agarose gel.

#### 3.2.2.6 Cloning of the glycoprotein gene into pTARGET™ mammalian expression T vector

The pTARGET™ vector carries the human cytomegalovirus (CMV) immediate-early enhancer/ promotor to promote constitutive expression of cloned DNA inserts in mammalian cells. This vector also contains the neomycin phosphotransferase gene, a selectable marker for mammalian cells. The pTARGET™ vector can be used for transient expression or for stable expression by selecting transfected cells with the antibiotic G0418.

To clone the glycoprotein gene into the pTARGET™ mammalian expression T vector a ratio of 1:3 of insert to vector was used.

The vector was briefly centrifuged to collect the contents at the bottom of the tube. The ligation reaction was set up as described below in a 0.5 ml eppendorf tube:

| | | |
|---|---|---|
| PTarget T vector (60ng) | : | 1 µl |
| T4 DNA Ligase buffer (10X) | : | 1 µl |
| T4DNA ligase (3 Weiss units / µl) | : | 1 µl |
| Insert (amplified and purified glycoprotein gene) | : | 5 µl |
| Water | : | 2 µl |

The mixture was incubated overnight in a water bath at 4°C for ligation and aliquot of the mixture was used for transformation.

**3.2.2.7 COMPETENT CELL PREPARATION**

1. *Escherichia coli DH5α* cells were streaked onto SOB plate from the stock and incubated at 37°C overnight.

2. 10-12 moderately sized colonies from the SOB plate were inoculated in to 100 ml of SOB broth in a 1 litre conical flask on the next day.

3. The broth was incubated in an orbital shaker incubator at 37°C with the rotary shaking speed of 200 rpm. When the optical density (OD) reaches 0.3 at $A_{600}$ the growth was arrested by chilling on ice. The flask was chilled for 20 minutes.

4. The entire culture was transferred into a tube and centrifuged at 3500 rpm for 15 min at 4°C.

5. The supernatant was discarded. Keeping the tube on the ice, the bacterial pellet was resuspended in 33.3-ml ice cold Solution A.

6. The cells were kept on ice for 20 minutes and centrifuged at 3500 rpm for 15 min at 4°C.

7. The supernatant was discarded and the pellet was resuspended in 5-6 ml of the ice cold Solution A (resuspension was done very gently as the cells are very fragile at this stage).

8. The suspension was left on ice for 10 minutes and the desired volume aliquoted and used for transformation.

### 3.2.2.8 Transformation and Recombinant selection

1. LB ampicillin/IPTG/X-Gal plates were prepared prior to the transformation and were equilibrated at room temperature prior to plating.
2. The tubes containing the ligation reaction were centrifuged briefly to collect the contents at the bottom of the tube. 5 μl of the ligation reaction was transferred to a sterile eppendorf tube.
3. 100 μl of freshly prepared competent cells was transferred to the tube, gently flicked to mix and placed on ice for 30 minutes.
4. The cells were subjected to a heat shock at 42°C for 50 sec and immediately returned to ice for 1-2 min.
5. 900 μl of room temperature LB broth was added to the cells and incubated for 1 hr at 37°C with shaking.

6. The cells were spread on to LB agar plate with ampicillin/X gal/ IPTG and incubated at 37°C overnight for selection of the recombinants

**3.2.2.9 Colony hybridization**

**3.2.2.9.1 DIG Labeling of the internal 627 bp glycoprotein**

The labeling was performed as prescribed in the DIG DNA labeling detection kit, Boehringer Mannheim, Germany.

1. The internal 627 bp of the glycoprotein gene was purified using GFX tm PCR DNA and gel band purification kit.

2. Approximately 3 μg of the purified template was diluted to a total volume of 15 μl and denatured by heating for 10 minutes in a boiling water bath and snap cooling on ice.

3. The following were added on ice:

| | | |
|---|---|---|
| Hexanucleotide mix | - | 2 μl |
| Deoxy nucleotide triphosphate | - | 2 μl |
| Klenow enzyme | - | 1 μl |

4. The mixture was centrifuged briefly and incubated for 1 hr at 37°C.

5. 2 μl of 0.2 M EDTA, pH 8.0 was added to stop the reaction. 2.5 μl of 4 M Lithium and 75 μl of absolute ethanol were added, left at - 70°C for 30 min to precipitate the DNA.

6. The tube centrifuged in a microfuge at top speed for 15 min, the pellet washed with 70 per cent ethanol and air-dried.

7. The pellet was resuspended in 50 µl of TE, electrophoresed to check for the concentration and used as a probe for colony hybridization.

**3.2.2.9.2 Transfer of colonies to nylon membrane**

1. A piece of nylon membrane was placed with the help of a blunt end forceps on the master plate and a mark was made on the plate with pen to denote the position of the notch.

2. The plate was left undisturbed for 5 min for the colonies to adsorb to the filter. The plate was returned to the incubator and incubated overnight for the colonies to grow.

3. A sheet of plastic wrap was spread on a flat surface and two separate pools of 0.75 ml of denaturing solution was made.

4. The membrane was lifted from the master plate and placed in the pool of denaturing solution with the colony side up.

5. The membrane was left for 5 min and transferred to the second pool and left again for 5 min.

6. Two pools 0.75ml of neutralizing solution was made and the membrane was placed 5 min each in both the pools of neutralizing solution.

7. The membrane was then transferred to two pools 0.75ml of 2X SSC and left for 5 min in each pool and air-dried.

8. The membrane was placed in a Whatmann paper and fixed by UV cross-linking for 15 seconds.

**3.2.2.9.3 PRE- HYBRIDIZATION**

Proteinase K 500 µl (2mg/ml) was placed in a petri dish. The fixed membrane was placed on this solution with the colony side up and incubated for 1 hour at 37°C.

The membrane was then sandwiched between two moistened sheets of Whatmann paper and slight pressure was applied.

The membrane was incubated with prehybridization buffer for 30 min at 60°C in Amersham hybridization oven.

The digoxigenin labelled glycoprotein 627bp probe (5µl) was denatured in a boiling water bath for 5 min and snap cooled on ice.

The denatured probe was then added to the membrane with prehybridization buffer and incubated overnight at 60°C in hybridization oven.

**3.2.2.9.4 Post hybridization washes**

The blot was washed for 2 x 5 minutes in ample Primary wash buffer at room temperature.

The blot was again washed for 2 x 15 min in secondary wash buffer at 64°C with constant agitation.

### 3.2.2.9.5 Immunological detection

After hybridization and stringency washes, the membrane was rinsed briefly in wash buffer.

The blot was incubated for 30 min in 10 ml of blocking solution.

Anti-DIG-AP conjugated (1:5000 dilution) was diluted in blocking solution and further incubated for 30 min.

The blot was washed for 2 x 15 min in 25 ml of washing buffer and equilibrated in detection buffer.

The blot was then immersed in freshly prepared substrate solution and allowed for colour development.

When the desired spots were achieved, washing the membrane for 5 min in water stopped the reaction.

### 3.2.2.10 Plasmid extraction

Plasmid was extracted as per the method of Birboin (1983) from the positive colonies as detected in colony hybridization.

1. From overnight cultures of the colonies in LB broth 1.5 ml was transferred into 1.5ml microfuge tubes.
2. The tubes were centrifuged for 5 min at 10,000 rpm and the medium was carefully aspirated.

3. 100 µl of Solution I was added and the cells were resuspended by vortexing.
4. 200 µl of Solution II was added and the tubes inverted 4-6 times or till the solution turns somewhat clear and viscous.
5. 150 µl of ice cold Solution III was added and mixed by inversion.
6. The tubes were centrifuged for 5 min at 12,000 rpm in a microfuge.
7. The supernatant was carefully transferred into a fresh 1.5 ml microfuge tube, 1 ml of absolute ethanol added, mixed well and incubated at –70°C for 10-15 min.
8. The tubes were centrifuged for 10 min at 12,000 rpm and the supernatant discarded carefully. The pellet was washed with 0.5 ml 70per cent ethanol and air dried
9. The pellet was resuspended in 50 µl of LTE buffer with RNase and incubated at room temperature for 20 min.
10. The plasmids were electrophoresed in a 1per cent agarose gel in TAE buffer along with supercoiled DNA ladder and control plasmids to determine their size.

### 3.2.2.11 Restriction enzyme digestion of the recombinant plasmids

The plasmid extracted from the white colonies was subjected to digestion with the following enzymes to check the presence of the insert as well also to ascertain the orientation of the insert.

1. *BamHI*
2. *BamHI* and *KpnI* and
3. *EcoRI*

### 3.2.2.12 Southern blotting and hybridization

### 3.2.2.12.1 Transfer of restricted plasmid to nylon membrane

The recombinant plasmid was digested with the enzymes *BamHI*, *BamHI/ KpnI* and *EcoRI*. The restricted plasmid was electrophoresed on 1per cent agarose gel in TAE buffer along with undigested control plasmid.

The gel was photographed and processed for southern transfer as follows:

1. The gel was placed in depurination solution for 10 minutes with mild shaking and transferred to the denaturing solution.

2. The gel was left in the denaturing solution for 10 min, rinsed briefly in distilled water and transferred to the neutralizing solution and left for 10 min.

3. The gel was then transferred to 20X SSC for equilibration before vaccum transfer.

4. The vaccum transfer assembly was prepared with a sheet of Whatmann paper below and nylon membrane (equilibrated with 20X SSC) on the top. The processed gel was placed on the membrane and the transfer was performed for 1 hour.

5. The membrane was removed and air-dried, while the gel stained to assess the transfer efficiency.

6. The membrane was fixed in a UV cross linker for 15 seconds.

#### 3.2.2.12.2 Hybridization and immunological detection

The membrane was hybridized with digoxigenin labeled 627 bp glycoprotein product and processed for immunological detection as per the Digoxigenin labeling and detection kit instructions as described above.

### 3.2.2.13 AUTOMATED SEQUENCING OF THE CLONED GLYCOPROTEIN GENE

#### 3.2.2.13.1 Preparation of plasmids for sequencing

1. The recombinant plasmid was inoculated into ampicillin Luria broth and grown overnight. Plasmid was extracted using the QIAprep[R] Spin Miniprep kit following the manufacturer's instructions.

2. The bacterial cells were pelleted and the cells were resuspended completely in 250μl Buffer P1.

3. 250μl of buffer P2 was added and the tube gently inverted 4-6 times followed by addition of 350μl of buffer N3 and inversion of the tube gently for 4-6 times.

4. The tubes were centrifuged at 10,000 rpm for 10 min and the supernatant was transferred to QIAprep spin column.

5. The column was washed by centrifugation with 500 μl buffer PB, followed by 750 μl of PE and discarding the flow-through.

6. The plasmid was finally eluted in 30ul of nuclease free water. The plasmid thus obtained was checked by agarose gel electrophoresis and restriction digestion for the presence of the insert. The concentration of plasmid was adjusted to 100ng per μl for automated sequencing.

### 3.2.2.13.2 Automated sequencing

Direct sequencing of the plasmid was carried out by cycle sequencing using Bigdye Terminator Ready Reaction kit with fluorescent-labelled dye-terminators incorporation method as per manufacturers protocol. The sequenced samples were analyzed by using capillary gel forming ABI Prism 3700 DNA sequencer and analyzer.

#### 3.2.2.13.2.1 Sequencing reaction set up

The recombinant plasmid samples (pT17 and pT29) to be sequenced was approximately adjusted to 200ng (2ul) for each sequencing reaction. Four sequencing reactions were performed with the following four primers to assess the orientation of the cloned glycoprotein.

1. Primer 1 (Forward)
2. Internal Primer 1 (forward)
3. Internal Primer 2 (reverse)
4. pTarget sequencing primer

A master mix was assembled as given below:

| | | |
|---|---|---|
| Plasmid (200 ng) | - | 2.0 μl |
| Big dye mix | - | 1.8 μl |
| Nuclease free water | - | 0.2 μl |
| Total | - | 4.0 μl |

4 μl of master mix was placed in each tube of the 96 well templates and 1 μl of the primer (10 μM) was mixed and placed in Perkin Elmer Gene Amp PCR system 9600.

**3.2.2.13.2.2 Cycle sequencing conditions**

The cyclic sequencing conditions employed were an initial denaturation step of 96°C for 1 min followed by 25 cycles of:

94°C for 10 seconds
50°C for 5 seconds
60°C for 4 minutes
4°C forever

**3.2.2.13.2.3 Preparation of the samples for gel loading**

The extended products after cycle sequencing were purified and prepared for gel loading as given below:

To 5μl of the sequencing termination in each tube, 1ul of 3M sodium acetate and 25 μl of absolute ethanol was added, tapped slightly for mixing and incubated at room temperature for 10 min.

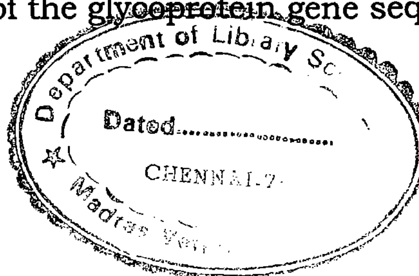
The tubes were then centrifuged for 10 min at 4500 rpm at room temperature. The supernatant was discarded by tapping down and 100 μl of 70 per cent ethanol was added. The tubes were than centrifuged for 10 min at 4500 rpm at room temperature and the supernatant discarded. The pellet was allowed to air dry and finally resuspended in 10 μl of 50 per cent deinonized formamide.

The 96 well templates was then placed in the ABI Prism 3700 DNA sequencer which automatically forms the capillaries for electrophoresis and loads 1 μl of each sample in different capillaries for the sequencing reaction. The results of sequencing were automatically read and stored in different files as chromatograms.

**3.2.2.13.2.4 Sequence data collection**

The sequencing gel run data was collected as a batch run file by the ABI Prism 3700 data collection software. The electropherogram was displayed with peaks of fluorescent labelled dye-terminators (ddATP – Green, ddCTP – Blue, ddGTP – Black and ddTTP – Red) on the 'Y" axis with respective bases on top of each peak on the X-axis. The nucleotides (N) that could not be assigned to any specific base was corrected by aligning and checking the reverse primer generated sequence of the same sample

The analyzed sequences obtained from each primer were directly stored onto text files separately. The reverse primer generated sequences were reverse complemented and aligned with the forward primer generated sequences using the software "Auto-assembler" to generate the complete sequence of the glycoprotein gene sequenced.


Department of Library Sc.
Dated
CHENNAI-7.
Madras Univ.

### 3.2.2.14 Nucleotide sequence analysis

#### 3.2.2.14.1 Phylogenetic analysis of the obtained glycoprotein gene sequence

The glycoprotein sequence of the fixed viruses and street isolates of rabies virus available as on date in Gene bank along with the sequence of RVD4 were written onto a single text file in FASTA format and used as input file for the programme CLUSTAL X 1.8. The sequences were aligned and written as an alignment file. The conceptually translated amino acid sequence was also aligned as described above. Neighbour-Joining (NJ) tree as described by Saitou and Nei (1987) was derived from the distance calculated. The tree-viewing programmes Drawgram or Drawtree of the PHYLIP package was used read the phylogenetic tree

Molecular Evolution and Genetic Analysis (MEGA) software V 1.02 was used to estimate distances and construct phylogenetic trees using two different distance estimation methods viz., Jukes-Cantor Distance (JCD) (Jukes and Cantor, 1969) and Kimura 2-Parameter distance (K2-PD) (Kimura, 1980). This software was also used to find out the varying amino acids between the fixed and vaccine viruses and also to test the tree obtained by clustal X programme

### *3.2.2.15 IN-VITRO* EXPRESSION

The nucleotide sequence obtained using the four different primers was analyzed using various softwares for evaluation of the open reading frame and conceptual translation of nucleotide to protein sequence. Software's were also used to find out the nucleotide and amino acid variation with the other published sequences.

### 3.2.2.15.1 Open reading frame (ORF) and protein sequence estimation

The Gene Tool Lite programme was used to find out the open reading frames of the nucleotide sequence data obtained in all three reading frames. The reading frame without any stop codons and giving a full-length protein was conceptually translated into protein sequence using the same software package. The graphic output of the ORF's was also written to separate files for the glycoprotein sequence. Also the nucleotide sequence aligned with protein sequence data was written to separate text files.

### 3.2.2.1.5.2 Predicted Characteristics of the cloned glycoprotein

The full-length glycoprotein sequence obtained after alignment was stored as a text file in FASTA format. The nature of the glycoprotein namely the hydropathicity plot was generated using the SeqAid programme.

### 3.2.2.15.3 Expression of rabies glycoprotein in eukaryotic cells

### 3.2.2.15.3.1 Transfection

1. The recombinant plasmids were transfected into Vero cells and Neuroblastoma cells as per the method of Burger et al., (1991) and McDonald et al., (2001) with minor modifications.

2. The cells (Vero as well as Neuroblastoma cells) were seeded at a concentration of 1-2 X $10^5$ cells per 60 mm tissue culture plate in 4 ml of Dulbecco's Modified Eagle Medium (DMEM) with 5 per cent fetal calf serum (FCS).

3. The cells were incubated at 37°C in a $Co_2$ incubator for 24 hours so that the cells reach 30-50 per cent confluence.

4. The following solutions were prepared in two-milliter eppendorf tube as follows:

5. Solution A: For each transfection 2 μg of the recombinant plasmid was diluted in 100 μl of serum free DMEM.

6. Solution B: For each transfection 5-20μl CELLFECTIN reagent was diluted in 100 μl of serum free DMEM.

7. The two solutions were mixed gently and incubated at room temperature for 30 min.

8. The cells were washed once with 2 ml of serum free growth medium without antibacterial agents.

9. 1.8 ml of serum free growth medium was added to each tube containing the CELLFECTIN reagent-DNA complexes, mixed gently and overlaid onto the cells.

10. The cells were incubated for overnight at 37°C in a $Co_2$ incubator.

11. The DNA containing medium was replaced with 4 ml of growth medium (DMEM) with 5 per cent of FCS and the cells were incubated for another 24 hrs at 37°C in a $Co_2$ incubator.

12. The cells were then subjected to indirect fluorescent antibody test with and without permeabilization to check the expression

### 3.2.2.15.3.2 Indirect Fluorescent Antibody Test to check expression

The transfected cells were subjected to indirect FAT for checking the expression

For surface fluorescence, live unfixed cells were incubated with the primary antibody (antirabies goat hyperimmune serum) diluted in 1:100 in DMEM containing 30mg/ml of bovine serum albumin (DMEM-BSA) for 45 min at 4°C.

For cytoplasmic staining, cells were first permeabilized with 80per cent acetone for 20 min at 4°C and then air-dried before incubation with primary antibody.

The cells were washed three times with DMEM-BSA and the cells were incubated for 45 min at 4°C with the secondary antibody (FITC labeled rabbit antigoat whole IgG molecule) diluted 1:50 in DMEM-BSA.

The cells were washed three times with DMEM-BSA and then covered with 50per cent glycerol in phosphate buffered saline (pH 7.4) and a cover slip and examined under fluorescent microscope (Nikon, Japan) for the fluorescence there by indicating expression.

# RESULTS

# CHAPTER IV
# RESULTS

## 4.1 ISOLATION, IDENTIFICATION AND ADAPTATION OF LOCAL ISOLATE OF RABIES VIRUS

### 4.1.1 Sample collection and examination by fluorescent microscopy

Brain samples (hippocampus major and cerebellum) were collected from 28 rabies-suspected animals. Out of the 28 samples, 12 samples were found to be positive for rabies by direct FAT using rabies antinucleocapsid antibody conjugate. Positive samples revealed areas of brilliant green fluorescence (Plate1) and negative samples were identified by the absence of specific fluorescence. All the samples tested by direct FAT were also subjected to monoclonal antibody (Mabs) screening.

### 4.1.2 Detection of serotype variation by monoclonal antibody screening

All the 28 brain samples were screened by direct FAT using three different monoclonal antibodies (Mabs). Again of the 28 samples, 12 samples gave positive reaction, as revealed by the presence of specific fluorescence, with both W502-2 and C15-2 Mabs (Plate 2). None of the samples tested gave positive reaction with the monoclonal antibody W422-5 (Table 1). This clearly indicates that all the isolates belonged to the classical rabies group (serotype 1) and not to rabies related viruses.

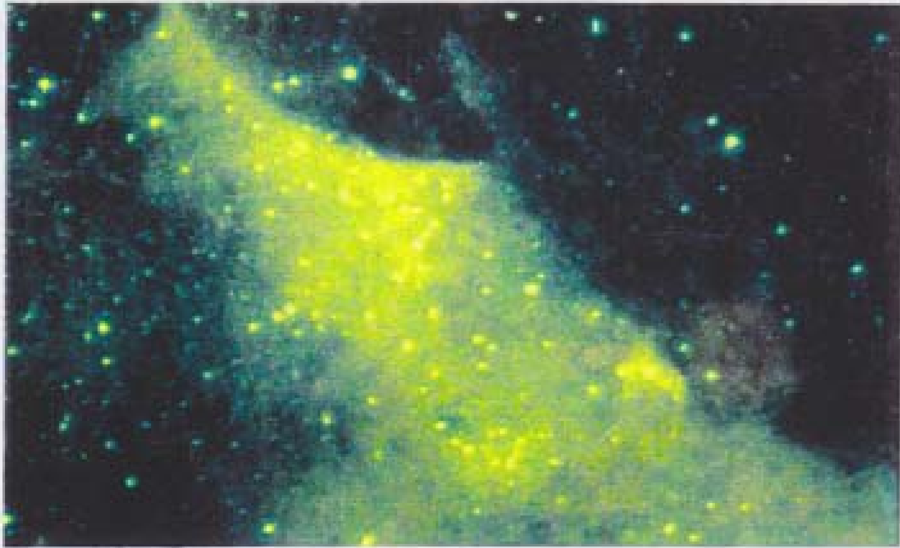


**Plate 1.** Direct FAT (with antinucleocapsid antibody FITC) on Brain impression smear from Dog showing specific fluorescence **100 X**

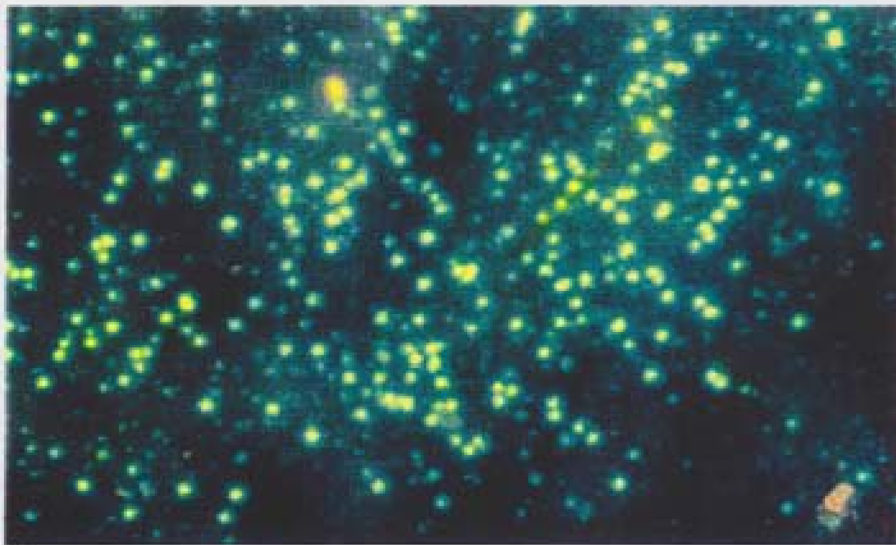


**Plate 2.** Direct FAT (with monoclonal antibody C15-2) on Brain impression smear from Dog showing specific fluorescence **100 X**

## Table 1

**Details of field samples screened for Rabies virus antigen using antinucleocapsid antibody and panel of monoclonal antibodies**

| Source of sample | No.Of samples screened | Number of samples positive with | | | |
|---|---|---|---|---|---|
| | | Antinucleocapsid antibody (FITC) | W502-2 | C15-2 | W422-5 |
| Dogs | 10 | 6 | 6 | 6 | Nil |
| Cattle | 12 | 3 | 3 | 3 | Nil |
| Goat | 6 | 3 | 3 | 3 | Nil |
| Total | 28 | 12 | 12 | 12 | Nil |

### 4.1.3 Adaptation of rabies virus in murine neuroblastoma cell line.

The 12 samples that were positive by FAT with antinucleocapsid and monoclonal antibody screening were used for infecting murine neuroblastoma cell line. Five blind passages were done. None of the samples produced visible cytopathic effect (CPE) during any of the 5 passages. Fluorescent microscopic examination of infected cover slip cultures of all the isolates were done at the fifth passage. Only one dog sample revealed multiple fluorescent foci, 72-96 hours post infection. Since the study required a local isolate of rabies virus form which the glycoprotein gene need to be isolated, the dog sample alone was processed. This dog sample was designated as RVD4 and was amplified by subsequent passages in N2a cell line until 8 passages at which time they showed 100 % fluorescence. The infected cells at the 8$^{th}$ passage were used for total RNA extraction.

## 4.2 AMPLIFICATION OF FULL LENGTH GLYCOPROTEIN GENE AND CLONING IN EXPRESSION VECTOR

### 4.2.1 Preparation of Total RNA

Total RNA was extracted from the infected neuroblastoma cells 48 hours post infection. The RNA pellet, obtained when subjected to formaldehyde agarose gel electrophoresis, revealed two distinct bands of 28 S and 18 S rRNA when viewed in an UV transilluminator (Plate 3). The presence of the clear bands indicated the integrity of the RNA sample extracted.

### 4.2.2 Polymerase chain reaction for amplification of full-length glycoprotein gene

Polymerase chain reaction to amplify the full-length glycoprotein gene was performed with the primer set 1 and 2 (Forward and Reverse). Five µl aliquot of the amplified product was electrophoresed in a 1% agarose gel along with DNA molecular weight marker (λ *EcoRI* / *Hind III* double digest). It was found to lie between 1904 bp and 1584 bp of the molecular weight marker (Plate 3). This amplified product that was 1592-bp (about 1.6kb) was purified and used for cloning in expression vector and sequencing.

The amplified full-length glycoprotein gene product was subjected to a nested PCR with the internal primer pair. Five µl aliquot of the amplified product was electrophoresed in a 1% agarose gel along with DNA molecular weight marker (λ *EcoRI* / *Hind III* double digest). The amplicon was found to lie between 831 bp and 564 bp of the molecular weight marker (Plate 4). This PCR amplified product of 627-bp size was used as a probe in colony and southern hybridization for screening recombinants.

### 4.2.3 Cloning of the glycoprotein gene into pTARGET™ mammalian expression T vector

#### 4.2.3.1 Purification of full-length glycoprotein gene

The full-length glycoprotein gene amplified by PCR was run in a 1 % LMP agarose gel and the 1.6-kb fragment excised. The fragment was eluted from the gel using GFX ™ PCR DNA and Gel Band Purification Kit. The purified product was run in 1 % agarose gel to check the purity. The purified product was seen as a single band on

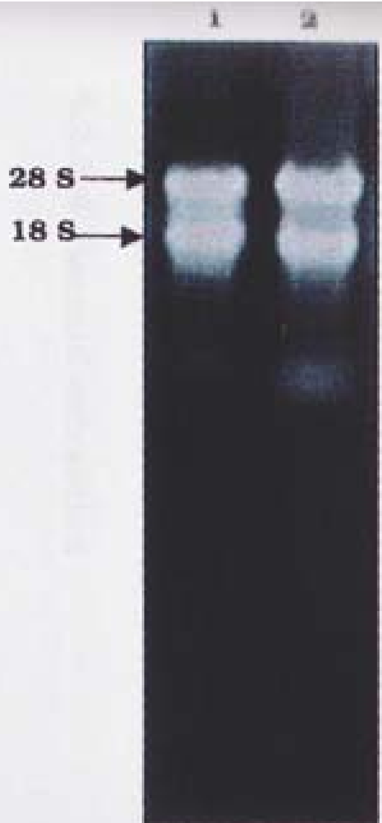



**Plate 3 : Formaldehyde agarose gel (1%) electrophoresis of total RNA extracted from RVD4 infected murine neuroblastoma cell lysate**

Lanes 1 & 2 - 28 S and 18 S rRNA bands

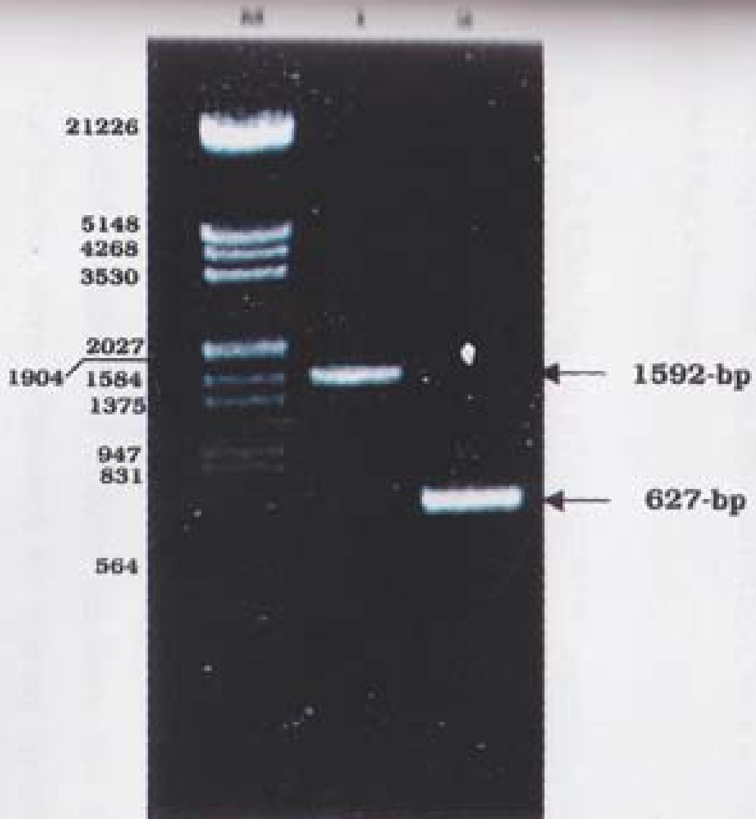



**Plate 4 : Agarose gel (1%) electrophoresis of PCR amplified Glycoprotein gene of local isolate**

Lane 1 - 1592-bp full length glycoprotein gene

Lane 2 - Nested PCR amplifying 627-bp

M - λ *EcoRI* / *HindIII* double digest marker

electrophoresis. The concentration was adjusted to 50 ng per µl and used for cloning.

#### 4.2.3.2 Cloning in pTARGET™ mammalian expression vector

The purified glycoprotein gene (1592 bp) was ligated with pTARGET™ mammalian expression 'T' vector and transformed into *Escherichia coli* DH5α cells. The transformants were spread on to LB ampicillin X-gal/IPTG plates for recombinant selection. Blue and white colonies were seen on the plates on overnight incubation (Plate 5). White colonies were supposed to harbour the recombinant plasmids while blue colony harbors the recircularized plasmids. The plates were processed for colony hybridization to confirm the presence of insert in the recombinant colonies.

#### 4.2.3.3 Colony hybridization

The nylon membrane containing the lifted colonies from the plates were hybridized with digoxigenin labeled 627 bp internal PCR product of the glycoprotein gene. Post hybridization, the membrane was processed for immunological detection using anti-digoxigenin antibody coupled with alkaline phosphate. Purple precipitate appeared on the membrane where the white colonies were lifted (Plate 6). The membrane was placed on the plate and aligned with the colonies. The colonies that aligned with the purple precipitate were picked and processed for plasmid extraction.

#### 4.2.3.4 Plasmid extraction

Plasmid was extracted from the white colonies that aligned with the purple colour on the nylon membrane. The plasmid sample were

**Plate 5 :** LB agar ampicillin/X-gal/IPTG plates showing wild type blue and recombinant white colonies

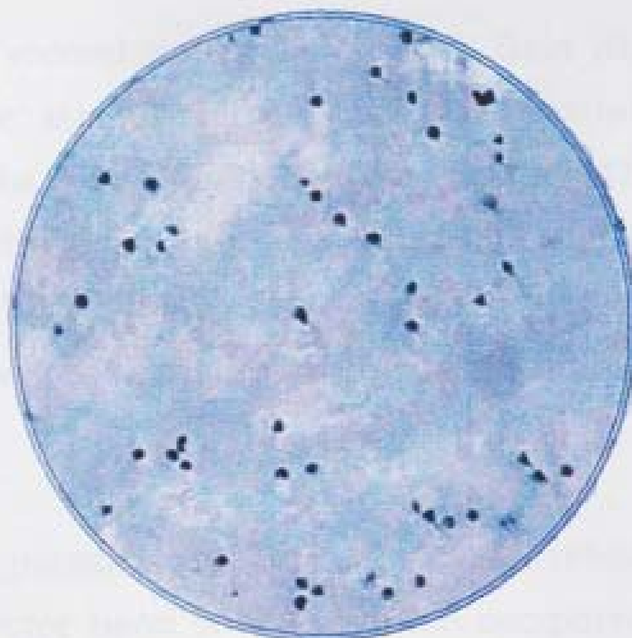


**Plate 6 :** Colony hybridization using 627-bp glycoprotein gene product as probe.(Purple colour signals indicate the location of recominant colonies)

run on a 1% agarose gel in TAE along with normal pTARGET™ mammalian expression vector and supercoiled DNA ladder as marker. The recombinant plasmids were around 7.2 kb in size while the normal pTARGET™ vector was around 5.6-kb when compared with the super coiled DNA ladder. The plasmids that were around 7.2-kb in size were subjected to restriction enzyme digestion for confirming the presence of the insert (Plate 7).

#### 4.2.3.5 Restriction enzyme digestion of the recombinant plasmid

The plasmids that were around 7.2 kb in size were subjected to digestion with Bam HI, Bam HI / Kpn I and Eco RI to confirm the presence of the insert and also to check the orientation of the insert.

Digestion of the recombinant plasmid with Bam HI, linearised the plasmid and the size of the plasmid was around 7.2 kb when compared with the 1 Kb plus DNA ladder marker. This linearization of the plasmid with *BamHI* gives an indication that the glycoprotein gene has been cloned upstream in the expression vector. However, final confirmation requires sequencing of the recombinant plasmid (Plate 7).

Digestion of the plasmid with Bam HI / Kpn I released an insert of 1.6 kb and a vector band of 5.6 kb when compared with the 1 Kb plus DNA ladder marker (Plate 7).

Digestion of the plasmid with *EcoRI* released an insert of 1.7 kb and a vector band of 5.5 when compared with the 1 Kb plus DNA ladder marker (Plate 7).

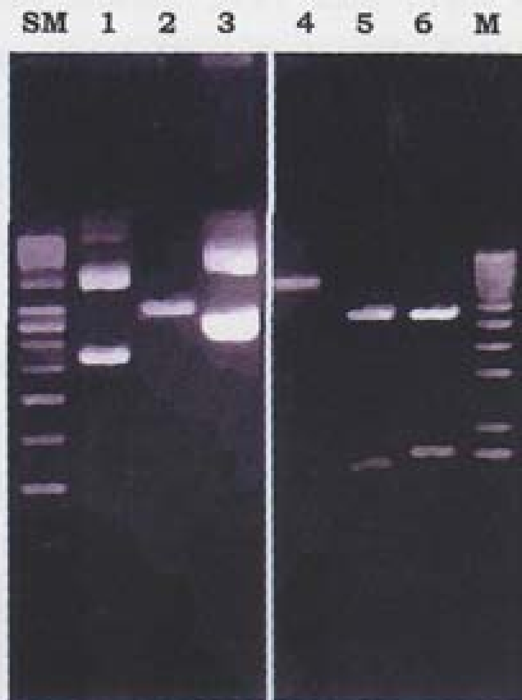



**Plate 7** : Agarose gel (1%) electrophoresis of pTarget vector and recombinant pTarget vector

SM - Supercoiled DNA ladder

Lane 1 - Uncut pTarget vector (5.6 kb)

Lane 2 - pTarget vector (linearized with *BamHI / KpnI*)

Lane 3 - Uncut recombinant plasmid (7.2 kb)

Lane 4 - Recombinant plasmid (linearized with *BamHI*)

Lane 5 - Recombinant plasmid digested with *BamHI*

Lane 6 - Recombinant plasmid digested with BamHI/KpnI (insert 1.6 kb released)

Lane 7 - Recombinant plasmid digested with *EcoRI* (increase in size of insert due to the vector sequence)

M - 1kb plus DNA ladder

Two recombinant colonies labeled pT17 and pT29 that had the insert in the upstream orientation were selected for southern hybridization and subsequent sequencing.

#### 4.2.3.6 Southern blotting and hybridization

The digested recombinant plasmids pT17 and pT29 were electrophoresed in a 1% agarose gel in TAE (Plate 8) and transferred to nylon membrane. Hybridization was performed with digoxigenin labeled 627 bp internal PCR product of the glycoprotein gene. Post hybridization, the membrane was processed for immunological detection.

Purple colour band appeared in the insert area both in pT17 and pT29, in the uncut recombinant plasmids (pT17 and pT29) and plasmids digested with Bam HI (plasmids linearised) (Plate 8). No purple colour band appeared in the uncut and digested control plasmid (pTarget vector uncut and digested with Bam HI/ *KpnI*). The recombinant plasmids pT17 and pT29 were chosen for sequencing.

### 4.3 Sequencing

#### 4.3.1 Preparation of the plasmids for sequencing

The recombinant plasmids pT17 and pT29 was prepared by inoculating the colony into LB broth. Plasmid was extracted using QIAprep spin column and finally the plasmid was eluted in 30 µl of nuclease free water. The plasmid was run on a 1% agarose gel to check the size and purity. The concentration of the plasmid was adjusted to 100ng per µl for sequencing.

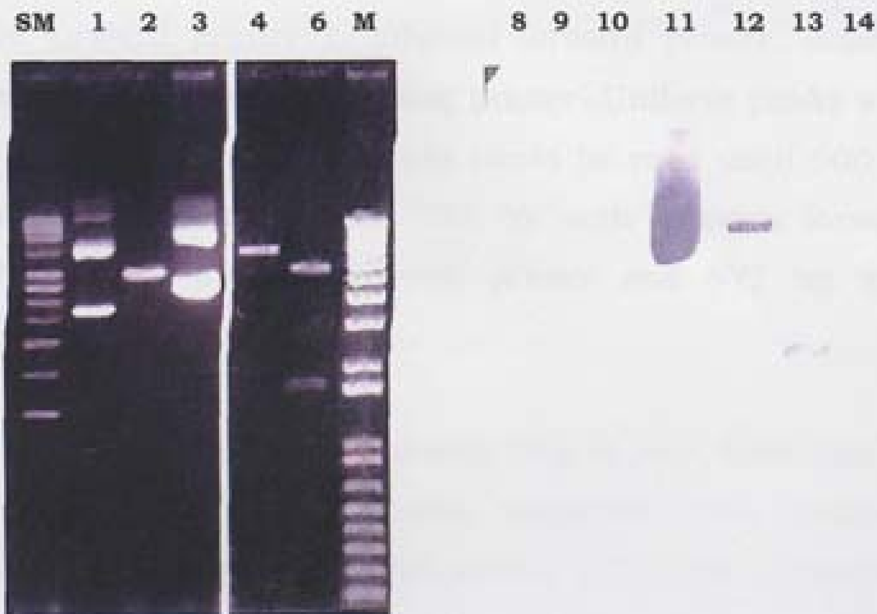



**Plate 8**: Southern blotting and Hybridization with digoxigenin labelled 627 bp product as probe

SM - Supercoiled DNA ladder
Lane 1 - Uncut pTarget vector (5.6 kb)
Lane 2 - pTarget vector (linearized with *BamHI / KpnI*)
Lane 3 - Uncut recombinant plasmid (7.2 kb)
Lane 4 - Recombinant plasmid (linearized with *BamHI*)
Lane 4 - Uncut recombinant plasmid (clone pT17)
Lane 5 - Recombinant plasmid digested with *BamHI*
M - 1kb plus DNA ladder
Lane 6 - Recombinant plasmid digested with *EcoR I*(insert 1.7 kb released)
Lane 11 - Recombinant vector (Purple colour band)
Lane 12 - Recombinant vector linearized by *Bam HI* digestion
Lane 13 - Purple colour band (1.6 kb Insert released)

### 4.3.2 Sequencing the recombinant plasmid

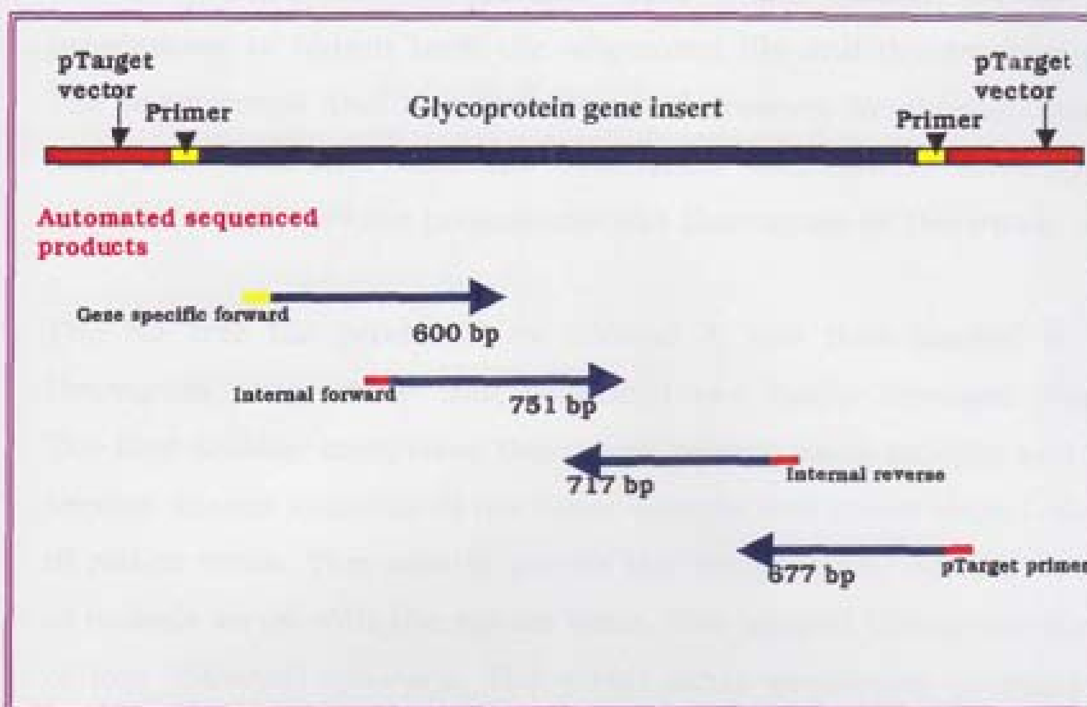
The recombinant plasmids pT17 and pT29 was sequenced with the gene specific forward primer 1, internal forward primer, internal reverse primer and pTarget sequencing primer. Uniform peaks were obtained in sequencing and sequences could be read until 600 bp with gene specific forward primer, 751 bp with internal forward primer, 717 bp with internal reverse primer and 677 bp with pTarget sequencing primer (Fig.1).

The gel file of the automated sequencing (Fig.1) was subjected to analysis of each sample lanes using sequence data analysis software to obtain sequence with each primer. The eight sequences obtained with the primers were aligned using the Autoassembler package in the automatic sequencer. The nucleotides (N) that could not be assigned to any specific base were corrected by aligning and checking the reverse primer generated sequence of the same sample. The analyzed and thus corrected sequence was stored on to separate text files. Using the auto assembler package the reverse primer generated sequences were reverse complemented and aligned with the forward primer sequence to generate the complete sequence of the glycoprotein gene of 1592 bp (Fig.1). The full-length sequence was stored as a text file in FASTA format and used for further analysis.

## 4.4 SEQUENCE ANALYSIS

### 4.4.1 Phylogenetic analysis of the obtained glycoprotein sequence with software CLUSTAL X

The nucleotide sequence of RVD4 was stored along with the nucleotide sequence of other vaccine and street virus isolates in




GEL FILE

Full length Glycoprotein gene sequence obtained after aligning

GCGGATCCATGGTTCCTCAGGCTCTCTTGTTTGTACCCCTTCTGGTTTTTTCATTGTGTT
TTGGGAAATTCCCTATTTACACGATACCAGACAAACTTGGTCCCTGGAGTCCGATTGATA
TACATCATCTCAGCTGCCCAAACAATTTGGTTGTGGAGGATGAAGGATGCACCAACCTAT
CAGGATTCTCCTACATGGAGCTCAAAGTAGGATATATCTCAGCAATAAAAGTGAACGGCT
TCACTTGTACAGGTGGGGTTACAGAGGCGGAGACCTACACTAACTTTGTTGGTTATGTCA
CCACCACGTTCAAAAGAAAGCATTTCCGCCCAACACCCGATGCGTGTAGGGCCGCGTACA
ACTGGAAACTGGCCGGCGACCCTAGATATGAAGAGTCTCTACACAATCCGTACCCTGACT
ACCATTGGCTTCGAACTGTAAAAACCACAAAGGAGTCTCTCGTTATCATATCTCCAAGTG
TGGCAGATTTGGACCCATATGACAAATCCCTTCACTCGAGAGTCTTCCCTAGCGGAAATT
GCTCAGGAGTAGCGGTGTCTTCTACCTACTGCTCCACTAACAACGATTACACCATCTGGA
TGCCCGAGAATCCGAGACTAGAGATGTCTTGTGACATTTTTACCAATAGTAGAGGAAAGA
GAGCATCTAAAGGGAGTAAGACCTGCGGCTTTGTAGATGAAAGAGGCCTGTATAAGTCGC
TAAAAGGGGCTTGCAAACTCAAATTGTGTGGGGTGCCCGGACTCAGACTCATGGACGGAA
CGTGGGTCGCAATACAGACGTCAGATGAGACCAAATGGTGCCCCCCTGATCAGTTAGTAA
ATCTACATGACTTTCGCTCAGATGAAATCGAACATCTCGTCGTGGAAGAACTAGTCAAGA
AAAGAGAAGAGTGTCTAGATGCACTGGAGTCTATCATGACCACCAAGTCCGTAAGTTTCA
GACGTCTCAGTCACCTGAGGAAACTCGTCCCTGGGTTCGGGAAAGCATATACTATATTCA
ACAAAACCTTGATGGAGGCTGATGCTCATTACAAGTCAGTCCGGACTTGGAACGAGATCA
TCCCCTCAAAAGGGTGTCTAAGAGTCGGAGGAAGGTGTCATCCTCACGTGAATGGGGTAT
TTTTTAATGGCATAATATTGGGCCCTGATGGCCATGTTCTGATCCCGGAGATGCAGTCAT
CCCTCCTCCAACAACACATGGAGTTGTTGGAGTCCTCAGTCATCCCCTTAATGCATCCAT
TAGCAGACCCGTCAACAGTTTTCAAGGACGGTGATGAGGCAGAAGATTTTGTTGAGGTCC
TCCTTCCGGATGTGCACAAACTGATCTCAGGGGTTGATCTCGGTCTTCCCAGCTGGGGGA
AGTATGTGTTAATGAGTGCAGGTGTCCTAACAGTACTGATGTTGATCATTTTCTTGATGA
CGTGTTGCAGGAAGAGTCAATCATCCAGCGTCACAAATAACAGTCCCAGGGGGATGGGGA
GGAAAGTGTCTGTCACCAGCCAGAACGGGAAGGTCATATCTTCCTGGGAGTCATATAAAA
ACGGGGGTGAGACCGGACAGTGA

**Fig.1 : Sequence data generated by automated sequencing of the cloned glycoprotein gene**

FASTA format. The sequences were then loaded on to the programme to obtain both the alignment file and dendrogram file. The programme also has the option of drawing Neighbour-Joining (NJ) tree from the distance estimation calculated. However, it requires a tree-viewing programme like Drawgram or Drawtree.

The NJ tree file produced by Clustal X was then loaded to the Drawgram programme that produced two major lineages (Fig.2). The first lineage comprises the rabies related virus mokola and the second lineage consists of the other vaccine and street virus isolates of rabies virus. This clearly proves the antigenically diverse nature of mokola virus with the rabies virus. The second lineage consisted of four different clusters. The street virus sequences reported by Benmansour et al., 1992 (namely RAVGPRC, RAVGPRA and RAVGPRB) formed one cluster. The second lineage also consisted of two different clusters namely the vaccine viruses; HEP, LEP and CVS forming one cluster and ERA, SAD and Vnukovo formed the other cluster. The RVD4 isolate and SHBRV remained as separate entities in the lineage

## 4.5 *IN-VITRO* EXPRESSION

### 4.5.1 Open reading frame (ORF) and Protein sequence determination

The complete nucleotide sequence of the messenger sense strand of the cloned glycoprotein RVD4 aligned with the amino acid is shown in (Fig.4). Computer software based analysis revealed only one long open reading frame extending from the first ATG codon at position 7 to the stop codon TGA at position 1581, which was able to code for

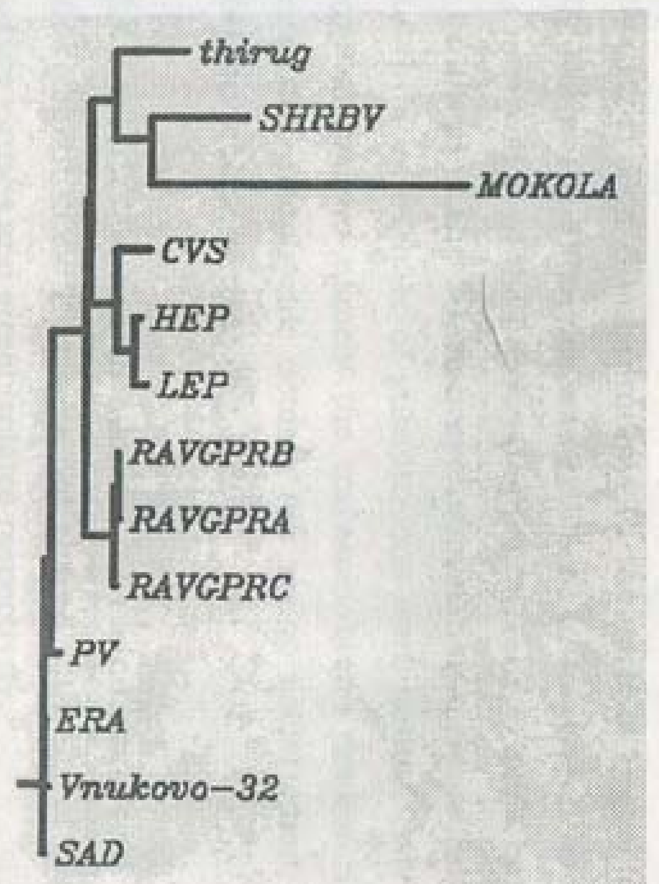



**Fig.2 : Phylogenetic grouping of RVD4 with other vaccine and street isolate sequences using CLUSTAL X software and NJ method**

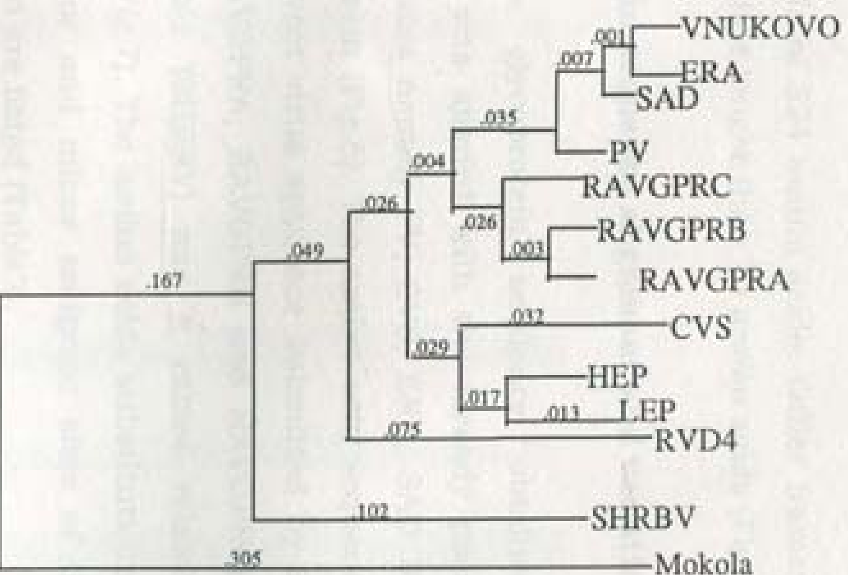



**Fig.3 : Phylogenetic grouping of RVD4 with other vaccine and street isolate sequences using MEGA - distance by jukes cantor method software and NJ method to draw tree**

a polypeptide of 524 amino acids. Other frames could not encode any polypeptide longer than 75 amino acids (Fig.5).

**4.5.2 Protein sequence alignment and variation estimation**

The rabies glycoprotein sequence obtained by conceptual translation was aligned with the already predicted sequence of vaccine viruses namely PV, CVS, ERA, SAD B19, HEP, LEP and Vnukovo strain (Fig.6). The amino acid sequence was also aligned with the street virus sequence submitted by Benmansour et al., (1992 - RAVGPRA, RAVGPRB and RAVGPRC), silver haired bat rabies isolate (SHBRV) and a rabies related virus (MOKOLA) sequence (Fig.7). The amino acids substitutions that had occurred in the major and minor antigenic sites of the ectodomain are minimal and are listed (Table2).

Other than the antigenic sites the substitutions were localized in 3 different areas of the ectodomain namely 8 substitutions in the regions between 161 to 191, 5 substitutions in the regions between 224 to 241 and 6 substitutions in the regions between 417 and 438. Not much difference was exhibited in the region implicated in the recognition of the neuronal receptors (amino acids between 189 to 214) and in the T-lymphocyte stimulating residues (amino acids 18-44). More number of substitutions were noticed only in the c-terminal regions namely the transmembrane domain (11 amino acids) and cytoplasmic domain (26 amino acids) when compared with the vaccine viruses (Fig. 8 & 9).

The isolate RVD4 had in addition 22 amino acids and 25 amino acids substitutions that were not shared with any of the strain of vaccine viruses and street isolates respectively.

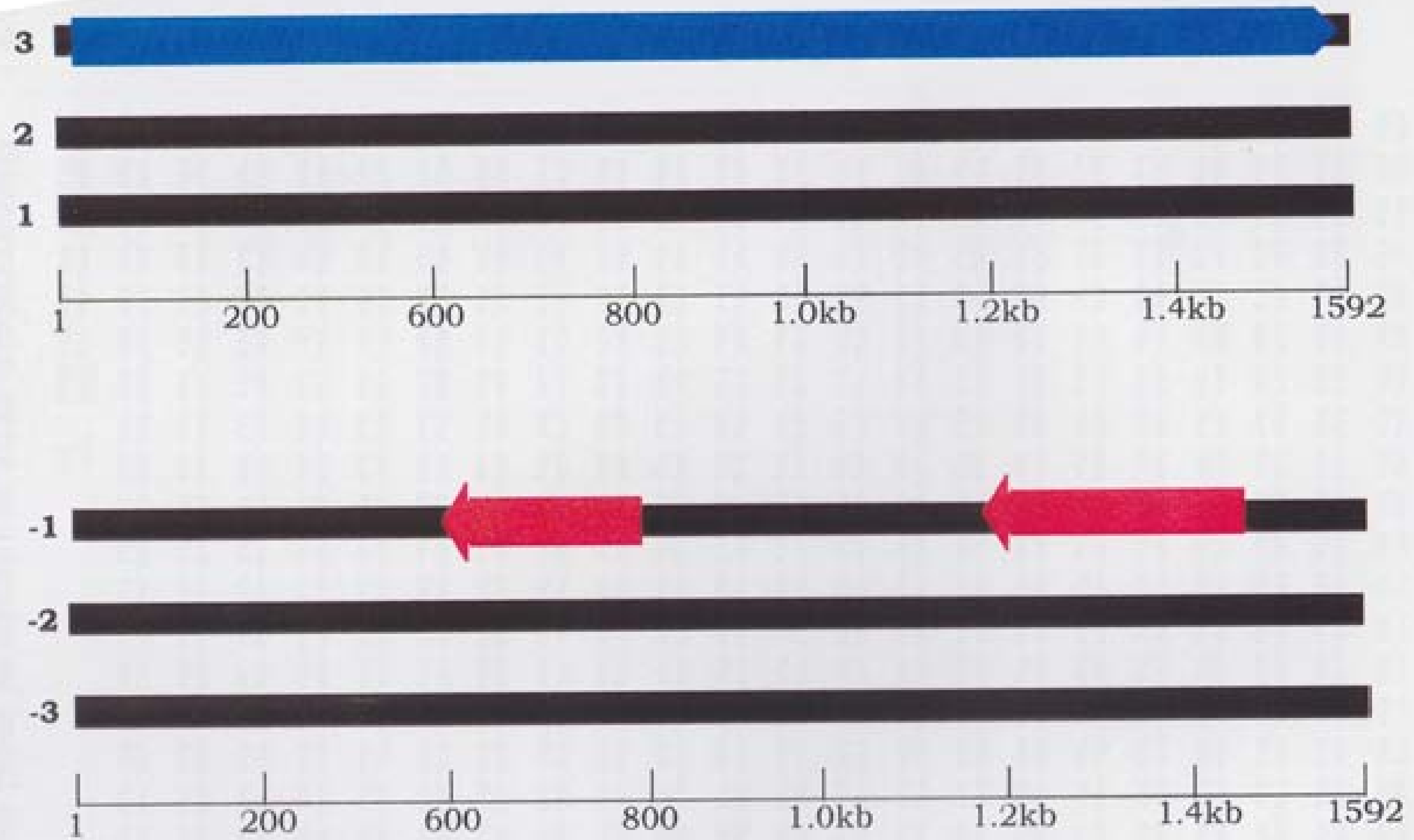



**Fig .5 : Different Open Reading Frames of RVD4 depicting the length of polypeptide coded. Only the third reading frame codes ( ■ ) for the full-length polypeptide of 524 amino acids**

```
gga.tcc atg.gtt.cct.cag.gct.ctc.ttg.ttt.gta.ccc.ctt.ctg.gtt.ttt.tca.ttg.tgt.ttt   60
Gly Ser Met Val Pro Gln Ala Leu Leu Phe Val Pro Leu Leu Val Phe Ser Leu Cys Phe   20

ggg.aaa.ttc.cct.att.tac.acg.ata.cca.gac.aaa.ctt.ggt.ccc.tgg.agt.ccg.att.gat.ata  120
Gly Lys Phe Pro Ile Tyr Thr Ile Pro Asp Lys Leu Gly Pro Trp Ser Pro Ile Asp Ile   40

cat.cat.ctc.agc.tgc.cca.aac.aat.ttg.gtt.gtg.gag.gat.gaa.gga.tgc.acc.aac.cta.tca  180
His His Leu Ser Cys Pro Asn Asn Leu Val Val Glu Asp Glu Gly Cys Thr Asn Leu Ser   60

gga.ttc.tcc.tac.atg.gag.ctc.aaa.gta.gga.tat.atc.tca.gca.ata.aaa.gtg.aac.ggc.ttc  240
Gly Phe Ser Tyr Met Glu Leu Lys Val Gly Tyr Ile Ser Ala Ile Lys Val Asn Gly Phe   80

act.tgt.aca.ggt.ggg.gtt.aca.gag.gcg.gag.acc.tac.act.aac.ttt.gtt.ggt.tat.gtc.acc  300
Thr Cys Thr Gly Gly Val Thr Glu Ala Glu Thr Tyr Thr Asn Phe Val Gly Tyr Val Thr  100

acc.acg.ttc.aaa.aga.aag.cat.ttc.cgc.cca.aca.ccc.gat.gcg.tgt.agg.gcc.gcg.tac.aac  360
Thr Thr Phe Lys Arg Lys His Phe Arg Pro Thr Pro Asp Ala Cys Arg Ala Ala Tyr Asn  120

tgg.aaa.ctg.gcc.ggc.gac.cct.aga.tat.gaa.gag.tct.cta.cac.aat.ccg.tac.cct.gac.tac  420
Trp Lys Leu Ala Gly Asp Pro Arg Tyr Glu Glu Ser Leu His Asn Pro Tyr Pro Asp Tyr  140

cat.tgg.ctt.cga.act.gta.aaa.acc.aca.aag.gag.tct.ctc.gtt.atc.ata.tct.cca.agt.gtg  480
His Trp Leu Arg Thr Val Lys Thr Thr Lys Glu Ser Leu Val Ile Ile Ser Pro Ser Val  160

gca.gat.ttg.gac.cca.tat.gac.aaa.tcc.ctt.cac.tcg.aga.gtc.ttc.cct.agc.gga.aat.tgc  540
Ala Asp Leu Asp Pro Tyr Asp Lys Ser Leu His Ser Arg Val Phe Pro Ser Gly Asn Cys  180

tca.gga.gta.gcg.gtg.tct.tct.acc.tac.tgc.tcc.act.aac.aac.gat.tac.acc.atc.tgg.atg  600
Ser Gly Val Ala Val Ser Ser Thr Tyr Cys Ser Thr Asn Asn Asp Tyr Thr Ile Trp Met  200

ccc.gag.aat.ccg.aga.cta.gag.atg.tct.tgt.gac.att.ttt.acc.aat.agt.aga.gga.aag.aga  660
Pro Glu Asn Pro Arg Leu Glu Met Ser Cys Asp Ile Phe Thr Asn Ser Arg Gly Lys Arg  220

gca.tct.aaa.ggg.agt.aag.acc.tgc.ggc.ttt.gta.gat.gaa.aga.ggc.ctg.tat.aag.tcg.cta  720
Ala Ser Lys Gly Ser Lys Thr Cys Gly Phe Val Asp Glu Arg Gly Leu Tyr Lys Ser Leu  240

aaa.ggg.gct.tgc.aaa.ctc.aaa.ttg.tgt.ggg.gtg.ccc.gga.ctc.aga.ctc.atg.gac.gga.acg  780
Lys Gly Ala Cys Lys Leu Lys Leu Cys Gly Val Pro Gly Leu Arg Leu Met Asp Gly Thr  260

tgg.gtc.gca.ata.cag.acg.tca.gat.gag.acc.aaa.tgg.tgc.ccc.cct.gat.cag.tta.gta.aat  840
Trp Val Ala Ile Gln Thr Ser Asp Glu Thr Lys Trp Cys Pro Pro Asp Gln Leu Val Asn  280

cta.cat.gac.ttt.cgc.tca.gat.gaa.atc.gaa.cat.ctc.gtc.gtg.gaa.gaa.cta.gtc.aag.aaa  900
Leu His Asp Phe Arg Ser Asp Glu Ile Glu His Leu Val Val Glu Glu Leu Val Lys Lys  300

aga.gaa.gag.tgt.cta.gat.gca.ctg.gag.tct.atc.atg.acc.acc.aag.tcc.gta.agt.ttc.aga  960
Arg Glu Glu Cys Leu Asp Ala Leu Glu Ser Ile Met Thr Thr Lys Ser Val Ser Phe Arg  320

cgt.ctc.agt.cac.ctg.agg.aaa.ctc.gtc.cct.ggg.ttc.ggg.aaa.gca.tat.act.ata.ttc.aac 1020
Arg Leu Ser His Leu Arg Lys Leu Val Pro Gly Phe Gly Lys Ala Tyr Thr Ile Phe Asn  340

aaa.acc.ttg.atg.gag.gct.gat.gct.cat.tac.aag.tca.gtc.cgg.act.tgg.aac.gag.atc.atc 1080
Lys Thr Leu Met Glu Ala Asp Ala His Tyr Lys Ser Val Arg Thr Trp Asn Glu Ile Ile  360

ccc.tca.aaa.ggg.tgt.cta.aga.gtc.gga.gga.agg.tgt.cat.cct.cac.gtg.aat.ggg.gta.ttt 1140
Pro Ser Lys Gly Cys Leu Arg Val Gly Gly Arg Cys His Pro His Val Asn Gly Val Phe  380

ttt.aat.ggc.ata.ata.ttg.ggc.cct.gat.ggc.cat.gtt.ctg.atc.ccg.gag.atg.cag.tca.tcc 1200
Phe Asn Gly Ile Ile Leu Gly Pro Asp Gly His Val Leu Ile Pro Glu Met Gln Ser Ser  400

ctc.ctc.caa.caa.cac.atg.gag.ttg.ttg.gag.tcc.tca.gtc.atc.ccc.tta.atg.cat.cca.tta 1260
Leu Leu Gln Gln His Met Glu Leu Leu Glu Ser Ser Val Ile Pro Leu Met His Pro Leu  420

gca.gac.ccg.tca.aca.gtt.ttc.aag.gac.ggt.gat.gag.gca.gaa.gat.ttt.gtt.gag.gtc.ctc 1320
Ala Asp Pro Ser Thr Val Phe Lys Asp Gly Asp Glu Ala Glu Asp Phe Val Glu Val Leu  440

ctt.ccg.gat.gtg.cac.aaa.ctg.atc.tca.ggg.gtt.gat.ctc.ggt.ctt.ccc.agc.tgg.ggg.aag 1380
Leu Pro Asp Val His Lys Leu Ile Ser Gly Val Asp Leu Gly Leu Pro Ser Trp Gly Lys  460

tat.gtg.tta.atg.agt.gca.ggt.gtc.cta.aca.gta.ctg.atg.ttg.atc.att.ttc.ttg.atg.acg 1440
Tyr Val Leu Met Ser Ala Gly Val Leu Thr Val Leu Met Leu Ile Ile Phe Leu Met Thr  480

tgt.tgc.agg.aag.agt.caa.tca.tcc.agc.gtc.aca.aat.aac.agt.ccc.agg.ggg.atg.ggg.agg 1500
Cys Cys Arg Lys Ser Gln Ser Ser Ser Val Thr Asn Asn Ser Pro Arg Gly Met Gly Arg  500

aaa.gtg.tct.gtc.acc.agc.cag.aac.ggg.aag.gtc.ata.tct.tcc.tgg.gag.tca.tat.aaa.aac 1560
Lys Val Ser Val Thr Ser Gln Asn Gly Lys Val Ile Ser Ser Trp Glu Ser Tyr Lys Asn  520

ggg.ggt.gag.acc.gga.cag tga   1581
Gly Gly Glu Thr Gly Gln Ter    526
```

**Fig.4 : Glycoprotein gene sequence of local isolate (RVD4) of rabies virus aligned with the amino acid coded**

(The start and stop codon are boxed, the signal peptide is underlined bold)

***Table2:* Comparison of Conceptually translated amino acid sequence of RVD4 with various field and vaccine strains**

| Sequence compared | Conceptually translated amino acid sequence of RVD4 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Major antigenic sites | | | | Minor sites | | | Arginine 333 | Toxic loop 189 to 214 |
| | Site II | | Site III 330-338 | Site a 231 | Site I 231 | Site VI 264 | Site V 294 | | |
| | 34 to 42 | 198 to 200 | | | | | | | |
| **PV** | - | - | - | - | 1 | - | - | - | 1 |
| **CVS** | 1 | - | - | - | 1 | - | - | - | 1 |
| **ERA** | - | - | - | - | 1 | - | - | - | 1 |
| **VNUKOVO** | - | - | - | - | 1 | - | - | - | 1 |
| **SAD B19** | - | - | - | - | 1 | - | - | - | 1 |
| **HEP FLURY** | - | - | 1 | - | 1 | - | - | 1 (Q) | 2 |
| **LEP FLURY** | - | - | 1 | - | 1 | - | - | 1 (G) | 2 |
| **RAVGPRC** | - | - | - | - | 1 | - | - | - | - |
| **RAVGPRA** | - | - | 1 | - | 1 | - | - | 1 (Q) | - |
| **RAVGPRC** | - | - | - | - | 1 | - | - | - | - |
| **SHBRV** | 1 | 1 | 1 | - | - | 1 | - | - | 5 |
| **MOKOLA** | 5 | 2 | 5 | 1 | - | - | 1 | 1 (D) | 9 |

```
RVC4     MVPQALLFVP LLVFSLCFGK FPIYTIPDKL GPWSPIDIHH LSCPNNLVVE DEGCTNLSGF   SYMELKVGYI SAIKVNGFTC TGGVTEAETY TNFVGYVTTT FKRKHFRPTP DACRAAYNWK 101
PV       .......... ....P..... .......... .......... .......... ..........   .......... ....M..... ..V....... .......... .......... ..........
CVS      ....V..... ..G....... .......... .......... .......... ........E.   .......... .......... ..V....... .......... .......... ..........
ERA      .......... ....P..... ......L... .......... .......... ..........   .......... L...M..... ..V.....N. .......... .......... ..........
Vnukovo  .......... ....P..... .......... .......... .......... ..........   .......... L...M..... ..V.....N. .......... .....L.... ..........
SADB19   .......... ....P..... .......... .......... .......... ..........   .......... L......... ..V....... .......... .......... ..........
HEP      ....V...A. ....P..... .......... .......L.. .......... ..........   .......... .......... ..V....... .......... .......... ..........
LEP      ....L...A. ....P..... .......... .......YT. .......... ..........   .......... .......... ..V....... .......... .......... ..........

RVC4     LAGDPRYEES LHNPYPDYHW LRTVKTTKES LVIISPSVAD LDPYDKSLHS RVFPSGNCSG   VAVSSTYCST NNDYTIWMPE NPRLEMSCDI FTNSRGKRAS KGSKTCGFVD ERGLYKSLKG 221
PV       M......... .......... .......... .......... .....R.... ....G.....   .......... .H........ ....G..... .......... ...E...... ..........
CVS      M......... .......... ....R..... .I......T. .......... ....G.K...   IT........ .H........ ...PRTP... .......... ..N....... ..........
ERA      M......... ........R. .......... .......... .....R.... ......K...   .......... .H........ ....G..... .......... ...E...... ..........
Vnukovo  M......... ........S. .......... .......... .....R.... ......K...   .......... .H........ ....GK.... .......... ...E...... ..........
SADB19   M......... ........R. .......... .......... .....R.... ......K...   .......... .H........ ....G..... .......... ...E...... ..........
HEP      M......... .......... .......... ........T. .......... ....G.....   IT........ .H........ .L..GT.... ..H....... ..D....... ..........
LEP      M.R....... .......... .......... ........T. .......... ....G.....   IT........ .H....A... .L..GT.R.. .......... ..G....... ..........

RVC4     ACKLKLCGVP GLRLMDGTWV AIQTSDETKW CPPDQLVNLH DFRSDEIEHL VVEELVKKRE   ECLDALESIM TTKSVSFRRL SHLRKLVPGF GKAYTIFNKT LMEADAHYKS VRTWNEIIPS 341
PV       .........L .......... .M...N.... ...G...... .......... ..........   .......... .......... .......... .......... .......... ..........
CVS      ..R......L .......... .M........ .......... .......... ..........   .......... .......... .......... .......... .......... ..........
ERA      .........L .......... .M...N.... .......... .......... ......R...   .......... .......... .......... .......... .......... .......L..
Vnukovo  .........L .......... .M...N.... .......... .......... ......R...   .......... .......... .......... .......... .......... .......L..
SADB19   .........L .......... SM...N.... ....K..... .......... ......R...   .......... .......... .......... .......... .......... .......L..
HEP      .........L .......... .M........ ...G...... .......... .E........   .......... .......... .......... .......... .......... .Q........
LEP      .........L .......... .M........ ...G...... .......... ..........   .......... .......... .......... ..G....... .......... .G........

RVC4     KGCLRVGGRC HPHVNGVFFN GIILGPDGHV LIPEMQSSLL QQHMELLESS VIPLMHPLAD   PSTVFKDGDE AEDFVEVLLP DVHKLISGVD LGLPSWGKYV LMSAGVLTVL MLIIFLMTCC 461
PV       .......... .......... ........N. .......... .......V.. ..........   .......N.. ........H. ...ER..... ....N..... .L...A..A. .........W
CVS      ....K..... .......... .......... .......... .......K.. ..........   .......E.. ........H. ..Y.Q..... ....N..... ..T..AMIG. V..FS...W.
ERA      .......... .......... ........N. .......... .......... ....V.....   .......... ........H. ...NQV.... ....N..... .L...A..A. ..........
Vnukovo  .......... .......... ........N. .......... .......... ....V.....   .......... ........H. ...NQV.... ....N..... .L...A..A. ..........
SADB19   .......... .......... ........N. .......... .......... ....V.....   .......... ........H. ...NQV.... ....N..... .L...A..A. ..........
HEP      .......E.. .......... .....S.... .......... .......... ..........   .......... V.......H. ....QV.... ....K..... ..I..A.IA. ..........
LEP      .......E.. .......... .....S.R.. .......... .......... ..........   .......... V.......H. ....QV.... ....N..... ..I..A.IA. ..........

RVC4     RKSQSSSVTN NSPRGMGRKV SVTSQNGKVI SSWESYKNGG ETGQ   504
PV       .RVNR.EP.Q HNL..T..E. ...P.S..I. .......S.. ...L
CVS      .RANRPESKQ R.FG.T..N. .....S.... P......S.. .IRL
ERA      .RVNR.EP.Q HNL..T..E. ...P.S..I. .....H.S.. ..RL
Vnukovo  .RVNR.EP.Q HNL..T..E. ...P.TW.I. .....H.S.. ..RL
SADB19   .RVNR.EP.Q HNL..T..E. ...P.S..I. .....H.S.. ..RL
HEP      .RVNRPES.Q SNLG.T..N. ..P..S.... .......S.. ..RL
LEP      .RVNRPESSQ S.LGET..N. .....S.... .......S.. ..RL
```

Note:

The references for the sequences are

| | | | | |
|---|---|---|---|---|
| PV | - Tordo et al., 1986 | ; | CVS | - Briggs et al., 1998 |
| ERA | - Anilionis et al., 1981 | ; | Vnukovo | - Fodor et al., 1994 |
| SADB 19 | - Conzelmann et al., 1990 | ; | HEP | - Morimoto et al., 1989 |
| LEP | - Chandrasekar et al., 1992 | | | |

**Fig. 6: Conceptually translated RVD4 glycoprotein sequence aligned with other vaccine virus glycoprotein sequences**

```
RVC4     MVPQALLFVP LLVFSLCFGK FPIYTIPDKL GPWSPIDIHH LSCPNNLVVE DEGCTNLSGF   SYMELKVGYI SAIKVNGFTC TGGVTEAKTY TNFVGYVTTT FKRKHFRPTP DACRAAYNWK 101
RAVGPRB  .......... ....P..... .......... .......... .R........ ..........   .......... .......... ..V....... .......... .......... ..........
RAVGPRA  .......... ....P..... .......... .......... .R........ ..........   .......... .......... ..V....... .......... .......... ..........
RAVGPRC  .......... .......... .......... .......... .......... ..........   .......... .......... ..V....... .......... .......... ..........
SHBRV    .I....Q... ..IP...... .......... .......... ........A. .....S....   .......... .......... ..V....... .......... ........M. ......HD..
MOKOLA   .NIPCFAVIL S.ATTHSL.E ..L....E.I EK.T...MI. ......MLS. E...NTE.P.   T.F...S..L AHQ..P.... ..V.N..... .......... ......K..V A...D.....

RVC4     LAGDPRYEES LHNPYPDYHW LRTVKTTKES LVIISPSVAD LDPYDKSLHS RVFPSGNCSG   VAVSSTYCST NNDYTIWMPE NPRLEMSCDI FTNSRGKRAS KGSKTCGFVD ERGLYKSLKG 221
RAVGPRB  M......... .......... .......... .......... .......... ..........   IT........ .H........ .....T.... .......... .......... ..........
RAVGPRA  M......... .......... .......... .......... .......... ..........   IT........ .H........ .....T.... .......... .......... ..........
RAVGPRC  M......... .......... .......... .......... .......... ..........   IT........ .H........ .....T.... .......... .......... ..........
SHBRV    M.......D. .Q........ .......... .......... .......... ......K.L.   IT........ .H.......V EA..GT.... ....K..K.. ..GR.W.... ..........
MOKOLA   VS........ ..T....SS. ....T....A .L.....IVE M.I.GRT... PM....K..K   LYP.VPS.T. .H...L.L.. DSS.SLI... ..S.S.QK.M N..RI...K. ...F.R....

RVC4     ACKLKLCGVP GLRLMDGTWV AIQTSDETKW CPPDQLVNLH DFRSDEIEHL VVEELVKKRE   ECLDALESIM TTKSVSFRRL SHLRKLVPGF GKAYTIFNKT LMEADAHYKS VRTWNEIIPS 341
RAVGPRB  .........L .......... .M........ .......... .......... ..........   .......... .......... .......... .......... .......... ..........
RAVGPRA  .........L .......... .M........ .......... .......... ..........   .......... ......L... .......... .......... ....E..... .Q........
RAVGPRC  .........L .......... .M........ .......... .......... ..........   .......... .......... .......... .......... .......... ..........
SHBRV    .......... .......... S.....DI.. .......... ..H....... ......I...   G......... .......... .......... ........N. .......... ......V...
MOKOLA   S...T...K. .I..F..... SFTKP.VHV. .T.N....I. ND.L..V... I.DDII....   ....T..T.L MSQ....... ..F......Y ......L.GS ...TNVY..R .DR.AD.L..

RVC4     KGCLRVGGRC HPHVNGVFFN GIILGPDGHV LIPEMQSSLL QQHMELLESS VIPLMHPLAD   PSTVFKDGDE AEDFVEVLLP DVHKLISGVD LGLPSWGKYV LMSAGVLTVL MLIIFLMTCC 461
RAVGPRB  .......... .......... .......... .......... .......... ..........   .......... .......H.. ....QV.... ....N..... .L...T.IA. ..........
RAVGPRA  .......... .......... .......... .......... .......... ..........   .......... .......H.. ....QV.... ....N..... .L...T.IA. ..........
RAVGPRC  .......... .......... .......... .......... .......... ..........   .......... G......H.. ....QV.... ....N..... .L...T.IA. ..........
SHBRV    ....K..... ..P....... ........N. .......... .......... ....T.....   .......... .......H.. ....QV.EI. .........L .......AT. I.A...I...
MOKOLA   R...K..QQ. MDP.K..L.. ...K....QI .......EQ. K...D..KAA MF..R...IN   REA...KDGN .D...DLHM. ..Q.SV.D.. ....H..FWL .VG.T.VAFV V.ACL.RV..

RVC4     RKSQSSSVTN NSPRGMGRKV SVTSQNGKVI SSWESYKNGG ETGQ      504
RAVGPRB  .RVNRPKS.E R.LGET.... .....S.... ........S. ..RR
RAVGPRA  .RVNRPKS.E R.LGET.... .....S.... ........S. ..RR
RAVGPRC  .RVNRPKS.E R.LGET..E. .....S..I. ........S. ..RR
SHBRV    .RANRTES.Q RGR.ES.G.. ..AP....I. ....L..S.S ..GL
MOKOLA   .RMRRRRSLR ATQDIPLSVA PAPVPRA..V .....S.GLP G.        502
```

**Note:**

Sequences **RAVGPRB, RAVGPRC & RAVGPRA** (Benmansour et al., 1992)
**SHBRV** (Silver haired bat rabies virus- Morimoto et al., 1996)
**Mokola** (Rabies-related virus – Tordo et al., 1993)

**Fig. 7: Conceptually translated RVD4 glycoprotein sequence aligned with other predicted street isolate and mokola glycoprotein sequences**

The RVD4 isolate exhibited much differences when compared with the rabies-related virus namely Mokola. The differences were distributed in all the three domains of the glycoprotein gene (Fig.8).

### 4.5.3 Predicted characteristics of the cloned glycoprotein gene product

The first 19 amino acids from the start codon ATG of the predicted sequence constitute the hydrophobic signal peptide. This signal peptide would be cleaved off post-translationally, so that the mature Glycoprotein would have 505 amino acids, making its molecular weight 56,999 Da. The 20th amino acid residue is Lysine, which becomes the $NH_2$-terminal amino acid of the mature glycoprotein. The mature glycoprotein has an ectodomain of 439 amino acids, a transmembrane domain of 22 amino acids, and a cytoplasmic domain of 44 amino acids (Fig.10).

The hydropathicity gene plot calculated from the deduced amino acid sequence of the cloned glycoprotein is shown (Fig.11). The mature glycoprotein has an uninterrupted hydrophobic domain of 22 amino acids, bounded by Glycine (438), Lysine (439) (numbered from the $NH_2$-terminal Lysine) and Arginine and Lysine at positions 462 and 463 near carboxy terminus, that corresponds to the presumptive anchor domain of transmembrane glycoprotein. The hydrophilic residues downstream of the transmembrane domain could be regarded as the cytoplasmic domain because the glycoprotein is supposed to interact with the M2 protein/nucleocapsid complex.

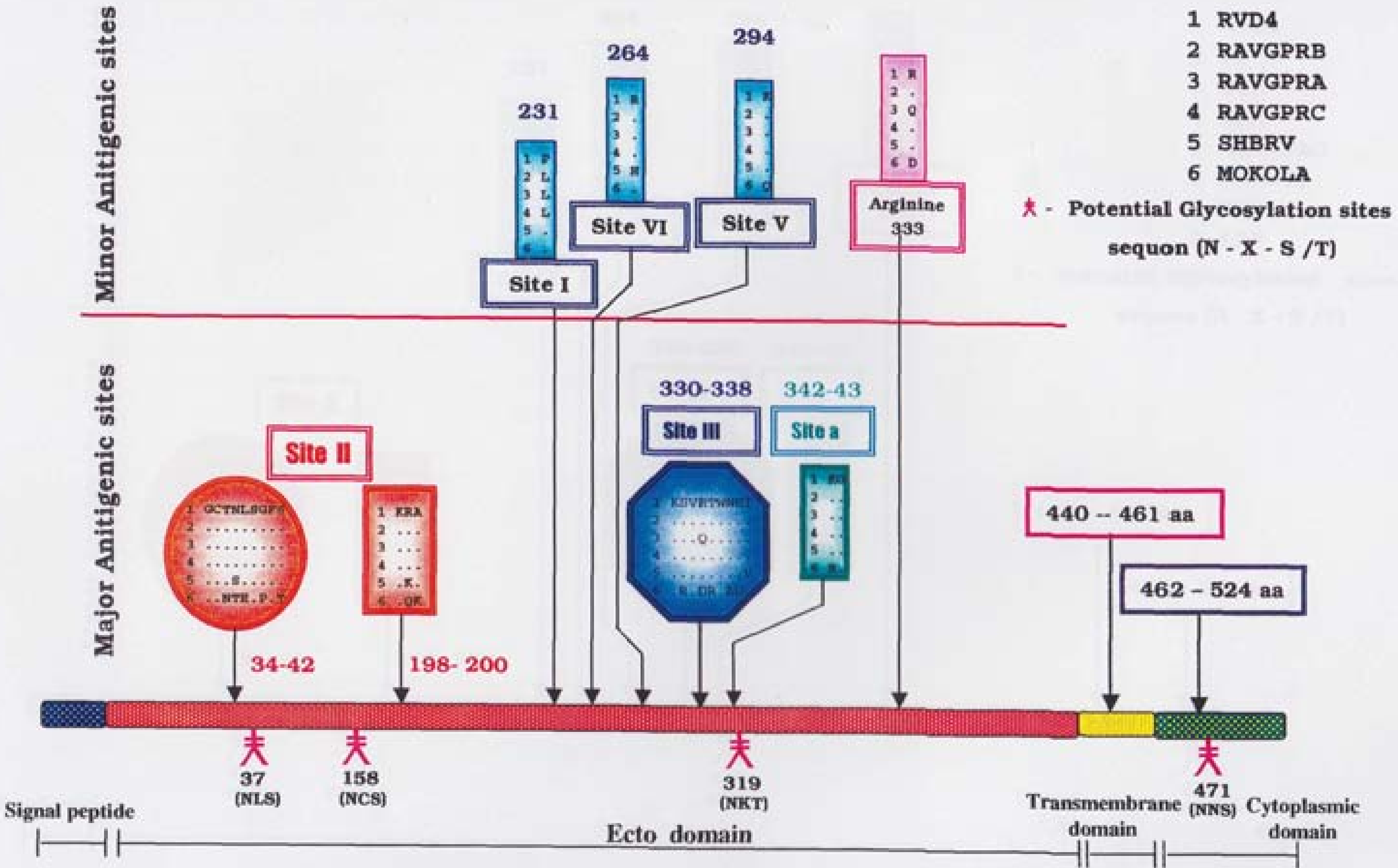



**Fig.8 : Feature map of RVD4 depicting the comparison of the amino acids constituting the antigenic domains of RVD4 with other field isolates**

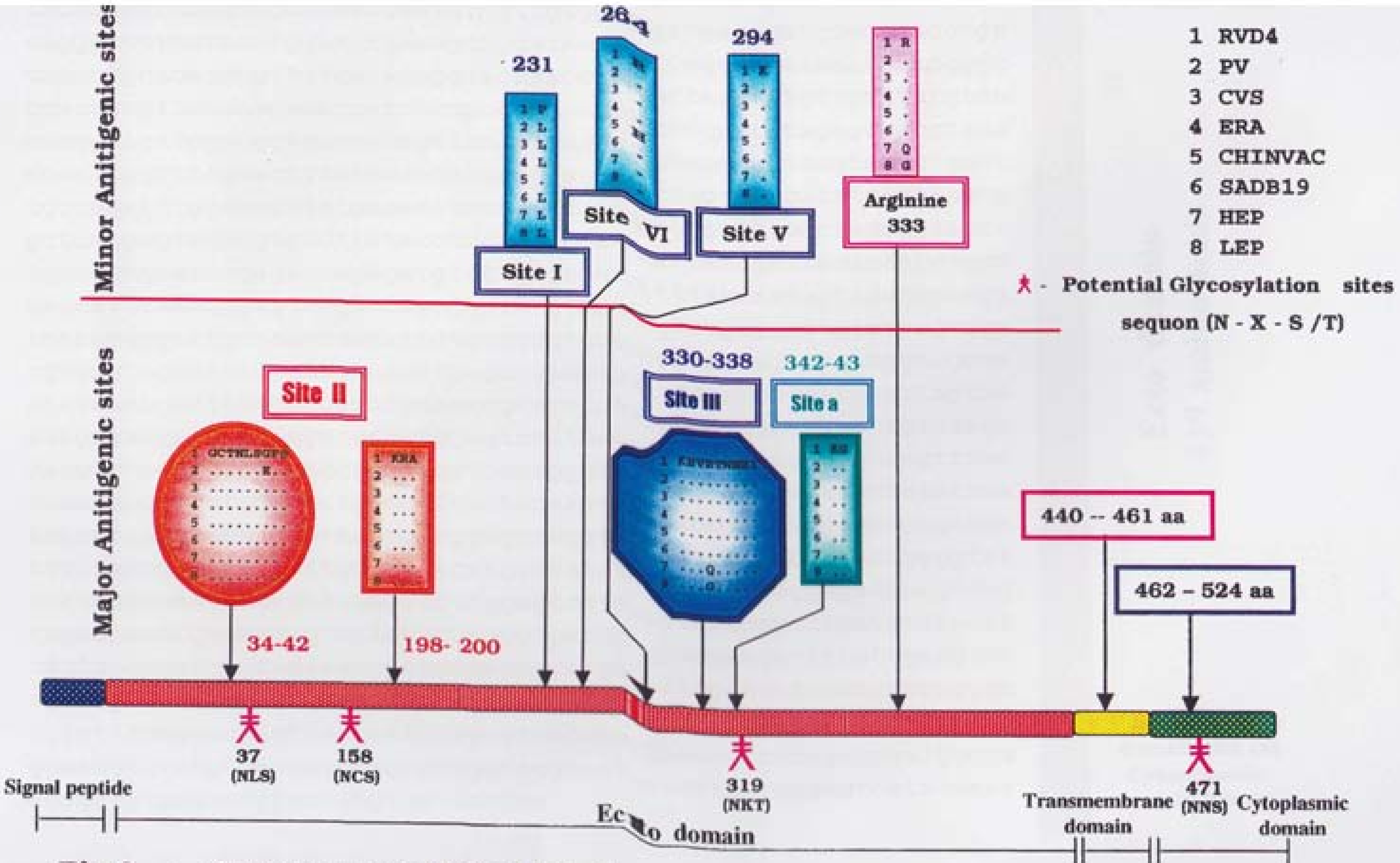



**Fig.9 : Feature map of RVD4 depicting the comparison of the amino acids constituting the antigenic domains of RVD4 with other vaccine strains**

```
gcggatccatggttcctcaggctctcttgtttgtacccctctggtttttcattgtgtt
ttgggaaattccctatttacacgataccagacaaacttggtccctggagtccgattgata
tacatcatctcagctgcccaaacaatttggttgtggaggacgaaggatgcaccaacctgt
caggattctcctacatggaacttaaagttggatacatctcagccataaaaatgaacgggt
tcacttgcacaggcgttgtgacagaggcagaaacctacactaactttgttggttatgtca
ccaccacgttcaaaagaaagcatttccgcccaacaccagacgcatgtagagccgcgtaca
actggaagatggccggtgaccccagatatgaagagtctctacacaatccgtaccctgact
accattggcttcgaactgtaaaaaccacaaaggagtctctcgttatcatatctccaagtg
tggcagatttggacccatatgacaaatcccttcactcgagggtcttccctagcggaaatt
gctcaggagtagcggtgtcttctacctactgctccactaacaacgattacaccatctgga
tgcccgagaatccgagactagagatgtcttgtgacatttttaccaatagtagaggaaaga
gagcatctaaagggagtaagacctgcggctttgtagatgaaagaggcctgtataagtcgc
taaaagggggcttgcaaactcaaattgtgtggggtgcccggactcagactcatggacggaa
cgtgggtcgcaatacagacgtcagatgagaccaaatggtgcccccctgatcagttagtaa
atctacatgactttcgctcagatgaaatcgaacatctcgtcgtggaagaactagtcaaga
aaagagaagagtgtctagatgcactggagtctatcatgaccaccaagtccgtaagtttca
gacgtctcagtcacctgaggaaactcgtccctgggttcgggaaagcatatactatattca
acaaaaccttgatggaggctgatgctcattacaagtcagtccggacttggaacgagatca
tcccctcaaaagggtgtctaagagtcggaggaaggtgtcatcctcacgtgaatggggtat
tttttaatggcataatattgggccctgatggccatgttctgatcccggagatgcagtcat
ccctcctccaacaacacatggagttgttggagtcctcagtcatccccttaatgcatccat
tagcagacccgtcaacagttttcaaggacggtgatgaggcagaagattttgttgaggtcc
tccttccggatgtgcacaaactgatctcaggggttgatctcggtcttcccagctgggga
agtatgtgttaatgagtgcaggtgtcctaacagtactgatgttgatcattttcttgatga
cgtgttgcaggaagagtcaatcatccagcgtcacaaataacagtcccaggggatgggga
ggaaagtgtctgtcaccagccagaacgggaaggtcatatcttcctgggagtcatataaaa
acgggggtgagaccggacagtgaggtaccgga
```

Signal peptide ( 19 amino acids)

Ecto domain 439 amino acids

Transmembrane domain (22 aa)

Cytoplasmic domain (44 aa)

Fig.10 : Feature map of RVD4 Glycoprotein gene Sequence

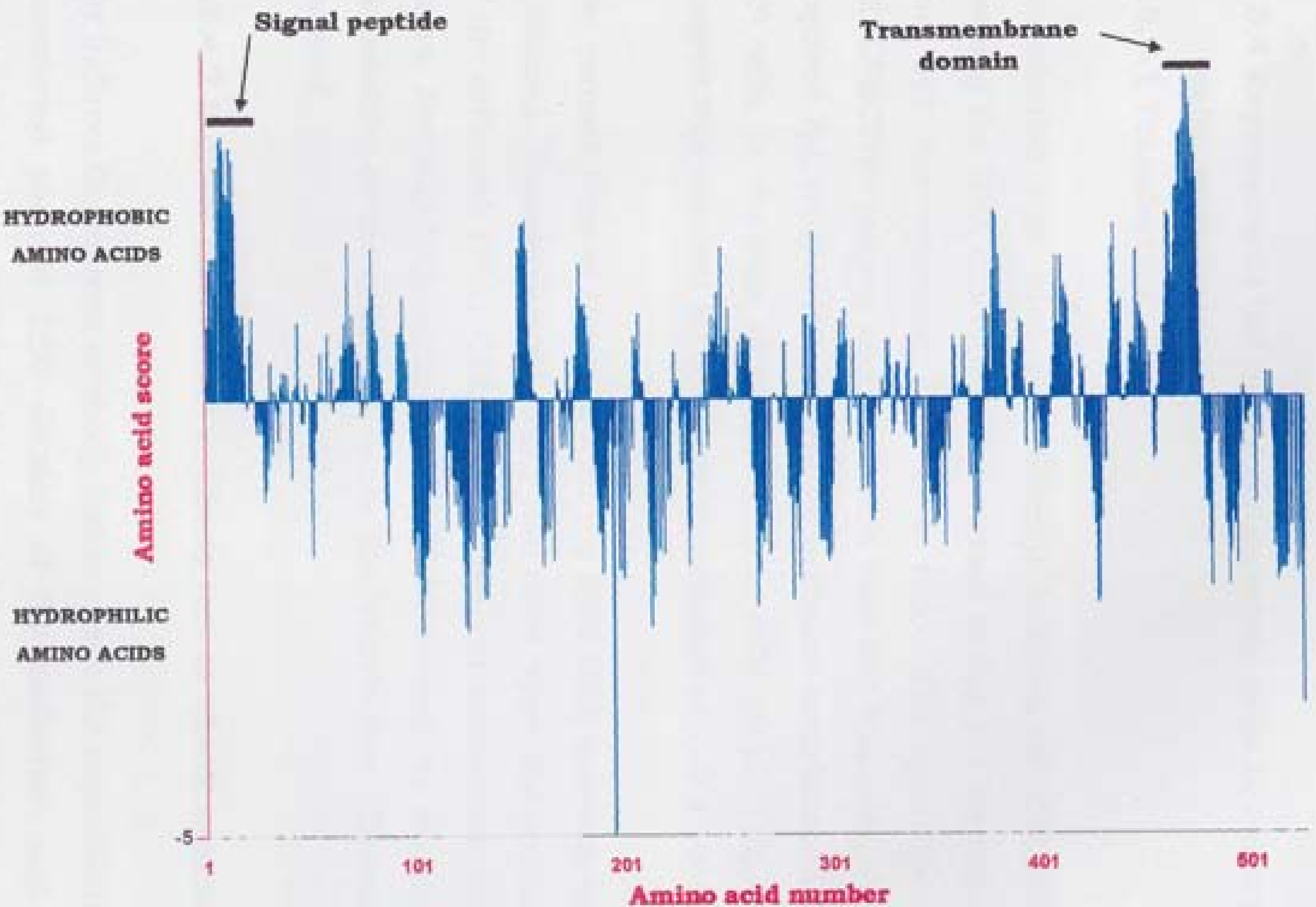



**Fig.11 : Hydropathicity profile of RVD4 glycoprotein (determined using SEQAIDII) depicting the stretch of hydrophobic amino acids constituting Signal Peptide and Transmembrane domain**

On the exoplasmic domain of the cloned Glycoprotein, four putative N-linked glycosylation sites (Asn-X-Thr/Ser sequence) at asparagine residues 37,158,319 and 471 were found. Sites 37 and 319 are common to all rabies strains so far sequenced. The amino acid at position 333 of the cloned Glycoprotein is Arginine, an important residue involved in pathogenicity of an isolate.

### 4.5.4 Expression of the rabies glycoprotein gene in eukaryotic cells

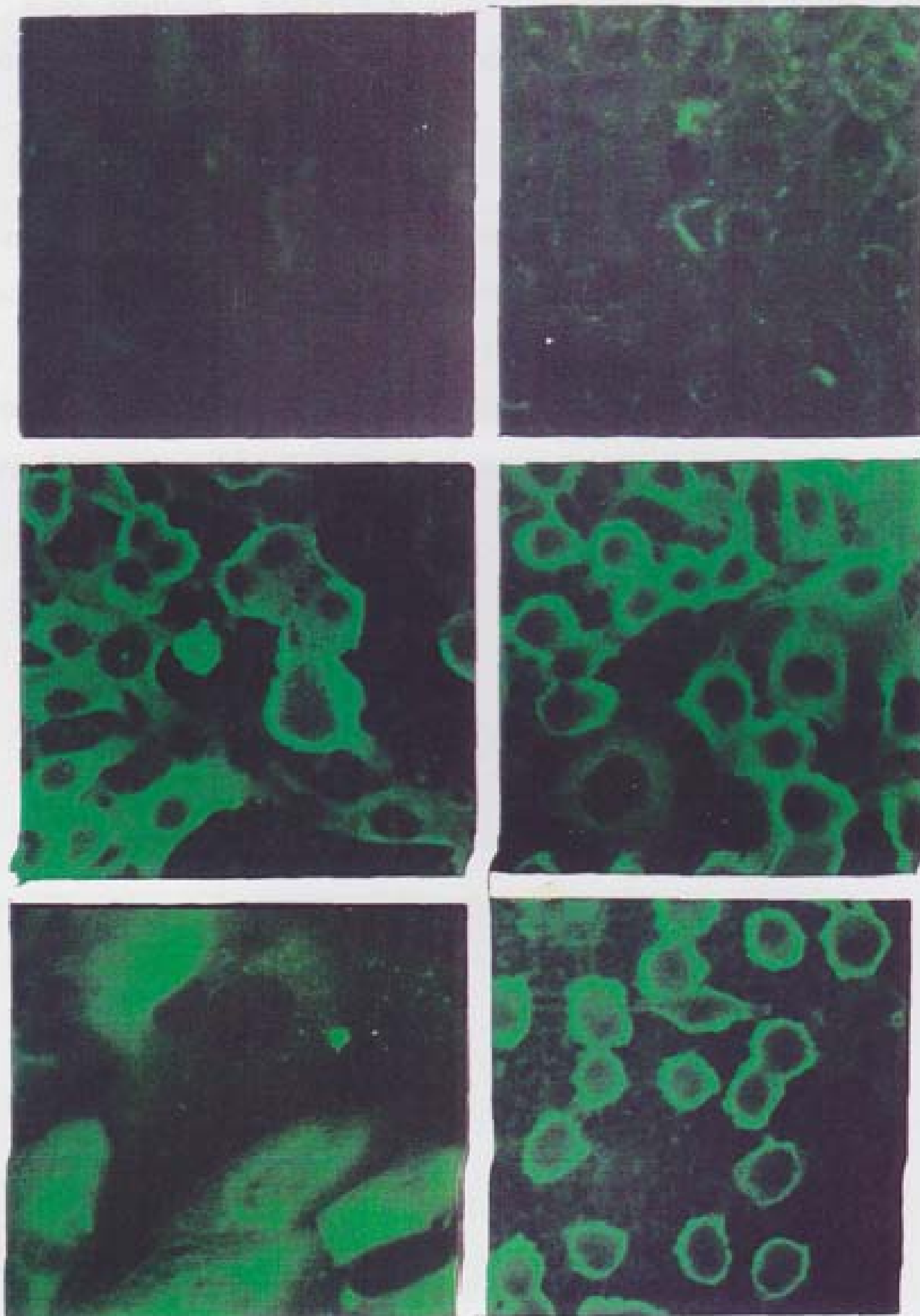
#### 4.5.4.1 Transfection

Transfection was tried out different volumes of CELLFECTIN$^R$ keeping the DNA concentration constant at 2µg per transfection to find out the optimum lipid DNA ratio. The quantity of the CELLFECTIN$^R$ reagent varied between Vero and N2a cells. Vero cells required 7µl (7µg) of the reagent for optimum transfection of 1- 2 x $10^5$ cells. In the case of Neuroblastoma cells 10 µl (10µg) of the reagent was required for optimum transfection of 1- 2 x $10^5$ cells.

The contact time of the cells with the lipid DNA complex was also optimized. Transfection efficiency was more when the contact time of the cells with DNA/ CELLFECTIN$^R$ reagent suspension was 8-10 hours. Prolonged exposure was found to be toxic as evidenced by vacuolation of the cells and hence the transfection efficiency also reduced.

#### 4.5.4.2 Indirect fluorescent antibody test to check expression

For indirect fluorescent antibody test to check the expression of the transfected plasmid 1:50 dilution of the antiserum and 1:150 dilution of the FITC-labeled secondary antibody was found



**te 9 : Immmofluorescence of Vero cells and Murine roblastoma cells transfected with recombinant vector (RVD4)**

A,C & E – Vero cells B,D & F – N2a cells

are negative controls ; *B,D,E- cytoplasmic fluorescence indicating* expression of the cloned ; F – surface fluorescence exhibited in IFAT on live cells

optimum. In the case of cells permeabilized with acetone 30-35 % of the cells were positive. The fluorescence pattern in this case was uniform and diffuse. Moderate fluorescence in the cytoplasmic membrane of the cells was observed 24 hours post transfection (Plate 9).

When live, intact cells (cells not permeabilized with acetone) were subjected to indirect FAT approximately 30 % of the cells were positive, and 5 % were highly positive. The positively stained transfected cells exhibited surface fluorescence that was uniform, strong and comparable to that observed for permeabilized cells (Plate.9) .

# DISCUSSION

# CHAPTER V
# DISCUSSION

Rabies continues to be a serious problem in both developed and developing countries due to the reservoir of rabies virus in wild life and domestic animals. More than 50,000 people and millions of animals die in developing countries and in India, approximately 30,000 people die of rabies annually and 7,00,000 to 1 million undergo rabies prophylaxis (Sehgal,S. 1997). Protection against this lethal infection can be achieved by pre-or, frequently, post-exposure vaccination. Limited access to high quality cell culture-based anti-rabies vaccines coupled with their high cost and lack of a cold chain are responsible for these deaths. The tools of molecular biology have broadened the ways in which the immune system has been investigated, providing new means for identification of antigenic determinants for many infectious diseases, and has also created new tools for the production of candidate vaccines. This study has been undertaken to isolate and identify a local isolate of rabies virus for vaccine production. Attempt had been made to clone the glycoprotein gene of the local isolate and express the glycoprotein *in-vitro* in eukaryotic cells

## 5.1 ISOLATION, IDENTIFICATION AND ADAPTATION OF LOCAL ISOLATE OF RABIES VIRUS

### 5.1.1 Sample collection and examination by fluorescent microscopy

Rabies virus is a typical neurotropic virus. Neurons in the brain are the major target of street rabies virus. Intensive replication of virus occurs in the central nervous system (Matsmoto and Kawai, 1969).

Since The virus is abundant in hippocampus major, cerebellum and cerebral cortex (Tierkel and Atanasiu, 1996) the brain samples were preferred as the source for virus isolation and identification for the present study.

Demonstration of Negri bodies in brain tissues is a simple and less expensive method for rabies diagnosis. But this technique has the disadvantage of producing a proportion (of up to 15 percent) of false negative results (Barnard and Voges, 1982). Presence of Negri bodies does confirm the diagnosis but its absence does not rule out the disease. Negri bodies cannot always be found in the brain of animals dying of rabies. In such cases tests with higher sensitivity, like Fluorescent Antibody Test (FAT), need to be carried out to confirm the result.

Direct FAT with antinucleocapsid antibody conjugate detects all the rabies virus strains. FAT could be carried out with high efficiency for all the samples. The test result could be obtained within an hour of collection of the specimen. The test was found to be rapid and very accurate method for rabies diagnosis. This agrees with the report of earlier workers (Wilsnack, 1960; Dean and Abelseth, 1973). FAT may occasionally yield positive results when the mouse inoculation is negative, since FAT detects inactivated as well as live antigen.

In the present study 28 samples were collected from rabies suspected animals and on screening with rabies antinucleocapsid antibody conjugate, 12 samples were found to be positive for rabies. Concurring with the earlier reports (Ganesh, 1997) the incidence is clearly higher in dogs proving the importance of dogs in the spread of the disease .

### 5.1.2 Detection of serotype variation by Monoclonal antibody (Mab) Screening

Differentiation of rabies and rabies related virus (Lagos bat, Mokola and Duvenhage) infection becomes necessary since it has been reported that vaccination failures could be attributed to serotype variations (Sureau et al., 1983).

The availability of panel of Mabs now allows identification of various Lyssa virus types and subtypes and the differentiation of the strains used for vaccine production from field virus isolates

The vaccine strains of rabies viruses used for prophylaxis share sufficient cross-reactivity with all field strains of rabies virus to give protection to exposed individuals. The identification of non-rabies Lyssa viruses such as Lagos bat, Mokola and Duvenhage, with antigenic characteristics quite different from the vaccine strains illustrates the antigenic diversity within Lyssa virus genus. Recognition of this diversity is essential to the interpretation of comparative tests of vaccine efficiency (Smith and King, 1996).

Monoclonal antibody W502-2 reacts with rabies and non-rabies Lyssa viruses, whereas C 15-2 reacts with only rabies virus. Monoclonal antibody W 422-5 reacts with Lagos bat, Mokola and Duvenhage viruses but do not react with rabies virus. In the present study all the 12 samples that were positive by direct FAT using antinucleocapsid antibody conjugate also gave positive reaction with both W 502-2 and C15-2 monoclonal antibodies. The results of the present study revealed that all the samples subjected to the screening test had been infected by the classical rabies virus

(serotype I) and not rabies related virus. Earlier reports (Jayakumar et al., 1993) also revealed the presence of rabies virus and not rabies-related viruses.

### 5.1.3 Adaptation of rabies virus in neuroblastoma cell line

Neuroblastoma cells of human or murine origin are most widely used in rabies virus investigations. These cells are highly suitable for primary isolation of rabies virus from field specimens. Murine neuroblastoma cells are more susceptible to rabies virus infection than any other cell lines tested (Webster et al., 1989). Virus isolation in neuroblastoma cell culture is as efficient as the fluorescent antibody test and mouse inoculation test for demonstrating small amounts of rabies virus (Rudd and Trimarchi, 1987).

In murine neuroblastoma cells, the isolate did not produce any characteristic cytopathic effect, as has been reported earlier (Crick and King, 1988). Rapid and intensive multiplication of rabies virus in the murine neuroblastoma cells was indicated by the presence of large number of fluorescent foci in the infected monolayer, which is in agreement with the observation of Jayakumar et al. (1994).

## 5.2 AMPLIFICATION OF FULL LENGTH GLYCOPROTEIN GENE AND CLONING IN EXPRESSION VECTOR

Amplification of the full glycoprotein gene requires RNA to be extracted from the suspected sample, which is then converted to cDNA. Using a set of primers the glycoprotein gene was amplified and the specificity was checked by a nested PCR before purification and cloning into expression vectors.

### 5.2.1 Preparation of total RNA

The guanidinium thiocyanate phenol chloroform extraction method of Chomczynski and Sacchi (1987) was a convenient method for isolation of RNA from infected cell cultures. Guanidinium thiocyanate is one of the most effective protein denaturants and is the denaturant of choice for preparation of RNA from sources enriched in RNase activity (Chirgwin et al., 1979). The addition of exogenous RNase inhibitors was not required. The use of phenol is a disadvantage of this method, as being a toxic chemical, it poses problems in handling and disposal.

Formation of the 28S and 18S ribosomal bands is a good indication that the RNA sample is intact and has not been degraded by RNase (Farrel, 1993).

### 5.2.2 Polymerase chain reaction for amplification of full length glycoprotein gene

The PCR for amplification of the full-length glycoprotein gene amplified a 1.6-kb product as already reported (Benmansour et al., 1992). Nested PCR is routinely done in any laboratory to check the specificity of the amplification if the PCR has been done from a crude sample. The specificity of the full-length amplification was checked in this study by a nested PCR with the inner primer pair that amplified 627-bp central region of the glycoprotein gene.

### 5.2.3 Cloning into pTARGET™ mammalian expression vector and Recombinant selection

The purified glycoprotein gene (1592 bp) was ligated with pTARGET™ mammalian expression 'T' vector and transformed into *Escherichia coli* DH5α cells. The transformants were spread on to LB ampicillin X-gal/IPTG plates for recombinant selection. The plates were processed for colony hybridization to confirm the presence of insert in the recombinant colonies. Purple precipitate appeared on the membrane where the white colonies were lifted. The plasmids that were around 7.2 kb in size were subjected to digestion with Bam HI, Bam HI / Kpn I and Eco RI to confirm the presence of the insert and also to check the orientation of the insert (Plate 7).

### 5.2.4 Restriction enzyme digestion of the recombinant plasmids

Digestion of the plasmid with Bam HI / Kpn I released an insert of 1.6 kb and a vector band of 5.6 kb. However, digestion of the plasmid with *EcoRI* released an insert of 1.7 kb and a vector band of 5.5. The increase in the size of the insert is due to the vector sequences added to the insert. The pTarget vector has *BamHI* restriction enzyme site upstream of the 'T' cloning site. The amplified full-length glycoprotein also has *BamHI* restriction enzyme site in the forward primer. Hence, digestion of the plasmid with *BamHI* linearises the plasmid and also indicates that the colonies had the insert in the upstream orientation (Plate 7).

## 5.3 SEQUENCE ANALYSIS

The target genes chosen to be indicators of relatedness for phylogeny should fulfil two requirements. One is that they should either be essential elements in all the microorganisms studied or

have a conserved function in order that relatedness could be assessed. The other is that, some segments of these genes should evolve rapidly between closely related strains in order to distinguish between variants.

Three different approaches are commonly employed for phylogeny estimation. The first one is based on a distance matrix generated from all pair-wise comparisons of DNA sequences (Felsenstein, 1984). The other method UPGMA (un-weighted pair-group mean) is the simplest method, but is of limited value. The NJ method (Saitou and Nei, 1987) is extremely fast and highly efficient at finding the correct tree. Hence the NJ method was used in getting the phylogenetic tree.

### 5.3.1 Phylogenetic analysis of the obtained glycoprotein sequence with software CLUSTAL X

To date, studies on the genetic variability of wild isolates of Lyssa viruses have been essentially focused on the N, G and Pseudogenes (Tordo et al., 1994). The use of glycoprotein region for phylogenetic analysis (Sacramento et al., 1992, Tordo et al., 1993) provided good balanced evaluation of the rabies virus isolates.

Alignment of the sequences by Clustal X produced two major lineages, with mokola forming one lineage. The isolate RVD4 and the other field isolates remained in the same cluster along with the vaccine viruses in the other lineage. SAD B19, ERA and Vnukovo-32 had evolved from SAD virus that had its common origin from a rabid dog. This relationship is clearly exhibited in the phylogeny as these sequences were grouped together in the cluster. The RVD4 isolate was differently grouped even-though it constituted the same

cluster. This clearly indicates that there is sufficient cross reactivity of the field isolates with vaccine viruses. Hence the vaccines available as on date would offer protection to individuals exposed to the field isolates (Ganesh, 1997). The separate lineage of Mokola clearly illustrates the antigenic diversity within Lyssa virus genus and thus vaccines prepared from the available fixed viruses might not protect against mokola infection. This antigenic diversity within the Lyssa virus genus requires further manipulations on the antigenic domains to improve upon the existing vaccines to the entire Lyssa genus. Jallet et al., (1999) had also reported that chimeric Lyssa virus glycoprotein using the capacity of site II part of PV to correctly present the site II part of Mokola or European bat Lyssa virus b1 can broaden the range of protection against Lyssa viruses.

The two distance estimation methods Viz., Jukes-Cantor and Kimura 2-Parameter distances and NJ tree had been used for comparison of Newcastle Disease virus fusion and matrix protein sequences (Seal et al., (1996). The two methods were found to be equally effective. Similar topography was obtained when the rabies nucleotide sequences were fed in to MEGA (Fig.3) and the distance estimated using the above two methods. This coincides with the grouping of the rabies virus done by CLUSATL X.

## 5.4 *IN-VITRO* EXPRESSION

The full-length glycoprotein gene sequence of RVD4 obtained had been compared with the available glycoprotein sequence of other vaccine and field isolates. The sequence was confirmed its identity and its phylogenetic relationship with the vaccine and field rabies viruses had been determined. The nucleotide sequence of RVD4 was conceptually translated and the protein characteristics were studied

in-silico. The recombinant plasmid was then used to transfect eukaryotic cells for *in-vitro* expression.

### 5.4.1 Open reading frame (ORF) and Protein sequence determination

This polypeptide is equivalent in size and organization to previously characterized rabies glycoprotein coding for 524 amino acids with the mature peptide having 505 amino acids (Anilionis et al., 1981; Tordo et al., 1986; Morimoto et al., 1989; Conzelman et al., 1990).

### 5.4.2 Predicted Characteristics of the conceptually translated glycoprotein

The first 19 amino acids from the start codon ATG of the predicted sequence constitute the hydrophobic signal peptide. This hydrophobic peptide was followed by the amino acid sequence Lysine–Phenylalanine as already reported (Lai et al., 1981). The hydropathicity plot calculated (Plate) clearly depicts the hydrophobic region of 19 amino acids at the N-terminus of the cloned Glycoprotein. The plot also shows another stretch of 21 hydrophobic amino acids from 440 to 461 that would constitute the transmembrane domain. The amino terminal hydrophobic signal peptide initiates the translocation of the nascent protein before being cleaved into the mature protein and thus plays a major role in processing of the protein. This is clearly evidenced when the cloned glycoprotein was subjected to *in-vitro* expression studies. Indirect FAT on The transfected cells exhibited surface fluorescence which indicated that in these cells the recombinant protein was processed,

transported and anchored into the plasma membrane confirming the role played by the signal peptide.

N-linked glycosylation plays a critical role in the expression of most cell-surface and secreted protein and is often required for protein stability, antigenicity, and biological action (Kornfield et al., 1985). The effects of N-linked glycosylation often depend on the number and position of N-linked oligosaccharides added to a protein chain (Rademacher et al., 1989). Four putative N- linked glycosylation sites (Asn-X-Thr/Ser sequence) at asparagine residues 37,158,319 and 471 were found in the sequenced glycoprotein. The amino acid 319, which is glycosylated, appears to be important, both because it is present in all Lyssa virus strains sequenced up to now, and also it is the only region that shows homology with Glycoprotein of Vesicular Stomatitis Virus. This confirms that the cloned glycoprotein protein would also behave similar to the glycoprotein of reported strains (Rose et al., 1982).

The amino acid at the position 333 in the predicted sequence of RVD4 is arginine which coincides with the report that neurovirulence seem to be directly related to the maintenance of Arginine or Lysine in the position 333 (site III). Mutants with other amino acids in this position cannot infect certain type of neurons presumably because they are unable to recognize the receptors (Tuffereau et al., 1989).

It has also been proposed that the region of the Glycoprotein from amino acid 189 to 214 might play an important role in recognizing the specific receptor molecule on the cell surface that would help the virus to invade the CNS. This region was found to resemble the toxic loop of the snake venom neurotoxin that recognizes and binds

to the nicotinic acetylcholine receptor molecule (Lentz., 1984). Not much difference is noticed in this region between the fixed and street virus (table2) confirming the neurovirulence property of the RVD4 isolate. The strains namely HEP and LEP, which have amino acid substitutions at this location, justifies this fact in that they are attenuated. This site could be exploited in developing site-directed mutants of local isolates and thereby developing an attenuated vaccine from local isolates.

Many substitutions have been noticed in the region from amino acids 189 to 214 in Mokola virus and also an aspartic acid at the position 333. Hence, the affinity to invade the CNS should be reduced. However, it has been found that mokola is highly neurotropic in mice and causes more severe encephalitis than rabies. Thus tissue specificity is complex and lot of residues would interact in determining the virulence of an isolate.

Overall, much of the substitutions were not noticed in the regions contributing to the antigenic sites. The isolate sequenced shares sufficient cross reactivity with the vaccine strains used for prophylaxis and vaccines available could offer protection to exposed individuals. However, the difference exhibited by Mokola clearly illustrates the antigenic diversity within Lyssa virus genus and thus vaccines prepared from the available fixed viruses would not protect against mokola infection.

The substitutions not shared with any vaccine and local isolates which is localized to 3 different areas of the ectodomain namely between161 to 191, 224 to 241 and between 417 and 438 requires to be investigated. Benmansour et al., (1992) reported that a short region delimited by amino acids 224-241 shows heterogenecity and

also postulating that it might be a major antigenic site in dogs. The derived amino acid sequences of RVD4 also show heterogenicity in this area, confirms that the isolate is from a dog.

### 5.4.3 Expression of the cloned rabies glycoprotein in eukaryotic cells

The study of eukaryotic gene regulation and expression has been advanced by technology which allows the introduction of nucleic acids into eukaryotic cells. An ideal method to transfer genes or macromolecules of interest into eukaryotic cells should exhibit the following features namely high efficiency of transfer, low toxicity, reproducibility and suitability for *in-vitro* and in-vivo applications. Cationic liposome-mediated transfection is one method in the chemical arsenal techniques designed for gene transfer, and was first reported by Felgner et al., (1987). Liposomes can deliver DNA to some "difficult" cell types more efficiently than other methods. In addition, liposomes are easier to use, require no special equipment and have been successfully used for in-vivo applications (Fahood et al., 1994). CELLFECTIN^R reagent used in this study was also found to be efficient in transfection and could transfect both Vero and neuroblastoma cell lines.

Using indirect immuno-fluorescence, the expressed protein was detected on the plasma membrane of the transfected cells in a manner similar to that found with virus-infected cells (Lodmell ad Ewalt, 1987; Wiktor et al., 1984). The pattern of fluorescence exhibited by the transfected Vero and neuroblastoma cells both with and without permeabilization was as already described by Burger et al., (1991). The surface fluorescence indicated that in

these cells the recombinant protein was processed, transported and anchored into the plasma membrane.

Eukaryotic cell lines, which express Glycoprotein, as described in this study and by previous authors like Lecocq et al., (1985) offer several advantages over other available means to study this protein. Although intact rabies virus infects a broad range of tissue culture cells *in-vitro* (Wunner, 1987) it is inconvenient to work with infected cells owing to the risk of human infection from live virus and because of the cytopathic effect of the virus on the host cells. Use of live recombinant vectors like vaccinia to study this protein also has a disadvantage that it also produces cytopathic effect in the cells. Use of mammalian expression vectors to express the glycoprotein would permit further study of this protein eliminating the disadvantages by other systems. Since antigenic epitopes on the G are preserved in transfected cells, this suggests that comparison of wild-type and mutant Glycoproteins in transfected mammalian cell lines will be useful for studying both humoral and cellular host immune responses to rabies virus (Morgeaux et al., 1989). This is an early attempt to transiently express the glycoprotein of rabies virus in eukaryotic cells. Attempts can be made in the future for stable expression in eukaryotic cells to purify the protein. This purified glycoprotein could be used in developing an ELISA kit for screening antibodies to rabies in animals and humans. In addition this attempt would also help in preparing a DNA vaccine from the local isolate.

### 5.5 Conclusion

We report here a primer pair with built in restriction sites for use in Polymerase Chain Reaction to amplify and for directional cloning of

the full-length glycoprotein gene of rabies virus. The glycoprotein gene of local isolate of rabies has been amplified and cloned in eukaryotic expression vector. The glycoprotein gene had been confirmed for its orientation and identity by sequencing. The sequenced glycoprotein has been compared with other sequences of vaccine and field isolates of rabies virus. Overall, much of the substitutions were not noticed in the regions contributing to the antigenic sites. The isolate sequenced shares sufficient cross reactivity with the vaccine strains used for prophylaxis and vaccines available could offer protection to exposed individuals. However, the difference exhibited by Mokola clearly illustrates the antigenic diversity within Lyssa virus genus and thus vaccines prepared from the available fixed viruses would not protect against mokola infection. The recombinant vector carrying the glycoprotein gene had been transfected in eukaryotic cells to express the protein *in-vitro*. High-level expression of the protein was obtained 48 hours post transfection. The surface fluorescence indicated that in these cells the recombinant protein was processed, transported and anchored into the plasma membrane. This study is an initial attempt on transient expression of the glycoprotein in eukaryotic cells. Attempts can be made in the future for stable expression in eukaryotic cells to purify the protein in large scale. In addition this attempt would help in preparing a DNA vaccine from the local isolate.

# SUMMARY

# CHAPTER VI

## SUMMARY

- In the present study, 28 brain samples from rabies suspected animals were examined by direct fluorescent antibody test with antinucleocapsid conjugate. 12 samples were found to be positive for rabies. Monoclonal antibody screening with W502-2, C15-2 and W422-5 revealed that all the 12 samples belonged to the classical rabies group.

- Of the 12 positive samples, a dog sample designated RVD4 adapted well to murine neuroblastoma cells and the virus infected cells revealed specific fluorescence in the direct fluorescent antibody test.

- Primers have been designed with built in restriction enzyme sites for amplification of the full-length glycoprotein gene of rabies virus. A nested PCR have also been developed to check the specificity of the amplification of the full-length glycoprotein gene. Use of the designed primers would amplify a 1592 bp glycoprotein gene and favour directional cloning of the amplified gene.

- The PCR amplified glycoprotein gene had been cloned in to pTargeT™ Vector Mammalian Expression T-Vector and transformed in to DH5α cell. Screening of the colonies yielded two clones pT17 and pT29 that had the glycoprotein gene in the upstream orientation. The orientation was checked by digestion with *BamHI*, *BamHI* / *KpnI* and *EcoRI* Restriction enzymes.

- The two recombinant colonies were sequenced with the gene specific outer forward primer, inner forward primer, inner reverse primer and pTarget sequencing primer to obtain the full-length glycoprotein gene sequence and also to check the orientation.

- The full-length glycoprotein gene sequence was compared with available vaccine virus and field isolate sequences using the programme CLUSTAL X 1.8. Not much difference was exhibited in the antigenic regions and also in the region implicated in the recognition of the neuronal receptors (amino acids between 189 to 214) and in the T-lymphocyte stimulating residues (amino acids 18-44). More number of substitutions were noticed only in the c-terminal regions namely the transmembrane domain and cytoplasmic domain when compared with the vaccine viruses.

- Phylogenetic analysis of the sequence of RVD4 by CLUSTAL X and Neighbour Joining method to draw the tree revealed two main lineages. The first lineage comprises the rabies related virus mokola and the second lineage consists of the other vaccine and street virus isolates of rabies virus. This clearly proves the antigenic diverse nature of mokola virus with the rabies virus. The second lineage consisted of four different clusters. The street virus sequences reported by Benmansour et al., 1992 (namely RAVGPRC, RAVGPRA and RAVGPRB) formed one cluster. The vaccine viruses, HEP, LEP and CVS forming one cluster and ERA, SAD and Vnukovo formed the other cluster. The RVD4 isolate and SHBRV remained as separate entities in the lineage.

- The conceptually translated amino acid sequence of RVD4 revealed only one long open reading frame extending from the first ATG codon at position 7 to the stop codon TGA at position 1581, which was able to code for a polypeptide of 524 amino acids. Other frames could not encode any polypeptide longer than 75 amino acids. Programmes predicted the other characteristics of the derived amino acid sequence, which coincided well with the earlier reports.

- The recombinant harbouring the full-length glycoprotein gene was used to transfect Vero and Murine neuroblastoma cell line using Cellfectin$^{R}$ reagent. The quantity of Cellfectin$^{R}$ and the contact of the DNA-liposome complex have been optimized.

- Indirect immunofluorescent test was done on both live non-permeabilized and cells fixed with acetone. In the case of cells permeabilized with acetone 30-35 % of the cells were positive 24 hrs post transfection and the fluorescence pattern was uniform and diffuse. In live, intact cells approximately 30 % of the cells were positive, and 5 % were highly positive. The positively stained transfected cells exhibited surface fluorescence that was uniform, strong and comparable to that observed for permeabilized cells. The surface fluorescence indicated that in these cells the recombinant protein was processed, transported and anchored into the plasma membrane.

# REFERENCES

# REFERENCES

Almeida, J.D., A.F. Howston, L. Pinteric and P. Fenje. (1962) Electron microscope observation of rabies virus by negative staining. Virology. 18:147.

Anilinonis,A., W.H. Wunner and P.J. Curtis. (1981). Structure of the glycoprotein gene in rabies virus. Nature. 294: 275-278.

Barnard,B.J.H. and S.F. Voges, (1982). A simple technique for the diagnosis of rabies in formalin-preserved brain. Onderstepoort. J. Vet. Res., 49: 193-194.

Bause,E. H. Herkamp and G. Legler. (1982). Conformational aspects of N-glycosylation of proteins. Studies with linear and cyclic peptides as probes. Biochem. J. 203. 761-768.

Benmansour. A., H. Leblois, P. Coulon, C. Tuffereau, Y. Gaudin, A. Flamand and F. Lafay. (1991). Antigenicity of Rabies Virus Glycoprotein. J. Virol. 65: 4198-4203.

Benmansour,A, M. Brahimi, Ç. Tuffereau, P. Coulon, F. Lafay and A. Flammand. (1992). Rapid sequence evolution of street rabies glycoprotein is related to the highly heterogenous nature of the viral population. Virology, 187: 33-45

Birnboin,H.C. (1983). A rapid alkaline extraction method for isolation of plasmid DNA, Meth. Enzymol. 100:243-255

Biswas,S., M.S. Ashok, G.S. Reddy, V.A. Srinivasan and P.N. Rangarajan. (1999).Evaluation of the protective efficacy of a rabies DNA vaccine in mice using an intracerebral challenge model. Current Science. 76: 1012-1016.

Biswas,S., G.S. Reddy, V.A. Srinivasan and P.N. Rangarajan. (2001). Pre-exposure efficacy of a novel combination DNA and Inactivated Rabies virus vaccine. Human Gene Therapy. 12: 1917-1922.

Bourhy.H, B. Kissi and N.Tordo.(1993) Molecular diversity of the Lyssavirus genus.Virology. 194:70-81.

Bunschoten. H, M.Gore, I.J. Claassen, F.G. Uytdehaag, B. Dietzschold, W.H. Wunner and A.D. Osterhaus. (1989) Characterization of a new virus-neutralizing epitope that denotes a

sequential determinant on the rabies virus glycoprotein. J Gen Virol. 70:291-8.

Burger,S.R., A.T. Remaley, J.M. Danley, J. Moore, R.J. Muschel, W.H. Wunner and S.L. Spitalnik. (1991). Stable expression of rabies virus glycoprotein in Chinese hamster ovary cells. J. Gen. Virol. 72: 359-367.

Celis,E., B. Dietschold and H. Koprowski. (1989). Rabies virus specific T-cell hybridomas : identification of class I MHC-restricted T cell epitopes using synthetic peptides. Hybridoma. 8:263-275.

Celis, E., C.E. Rupprecht and S.A. Plotkin. (1990). New and improved vaccines against rabies. In: Woodgrow G.C., Levine MM eds. New Generation vaccines. New York, Marcel Dekker, 1990:419-439.

Chirgwin,J.M., S.R.Przybyla, R.J. Mc Donald and W.J. Rutter. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry. 18: 5294.

Chomczynski,P. and P. Sacchi. (1987). Single-Step method of RNA isolation by Acid Guanidium Thiocyanate-Phenol Chloroform extraction. Anal. Biochem., 162:156-159

Christopher Prehaud, P. Coulon, F. Lafay, C. Thiers and A. Flamand. (1988). Antigenic site II of the Rabies Virus Glycoprotein: Structure and Role in Viral Virulence. J. Virol. 62: 1-7.

Clark,H.F. and T.J. Wiktor. (1972) Rabies virus. In: Strains of human viruses Eds., M. Majer and S.A. Plotkin. Karger, Basel. Pp177-182.

Conzelmann,K.K., J.H. Cox, L.G. Schneider and H.J.Thiel, (1990). Molecular cloning and complete Nucleotide sequence of the attenuated Rabies virus SAD B19. Virology, 175:485-489.

Coslett,G.D., B.P. Holloway and J.F. Obijeski. (1980) The structural proteins of rabies virus and evidence for their synthesis from separate monocistronic RNA species. J. Gen. Virol., 49: 161-180.

Crick,J. and A. King. (1988). Culture for rabies virus in-vitro. In: Rabies (Eds.) J.B. Campbell and K.M. Charlton. Kluwer Academic Publishers, Boston. Pp47-66.

Dean,D.J. and M.K. Abelseth. (1973). The fluorescent antibody test. In: Laboratory techniques in rabies (Eds.) F.X. Meslin, M.M. Kaplan and H. Koprowski. 3rd edn. World Health Organization, Geneva, pp. 73-84.

Dietzschold,B, T.J. Wiktor, W.H. Wunner and A. Varrichio. (1983). Chemical and immunological analysis of the rabies soluble glycoprotein. Virology. 124: 330-337.

Dietzschold. B, H.H. Wang, C.E. Rupprecht, E. Celis, M.Tollis, H.C.J.Ertl, E. Heber-Katz and H. Koprowski. (1987) Induction of protective immunity against rabies by immunization with rabies virus ribonucleoprotein. Proc. Natl. Acad. Sci. U S A. 84:9165-9.

Dietzschold. B, M. Gore, D. Marchadier, H.S. Niu, H.M. Bunschoten, L. Otvos Jr, W.H. Wunner , and H.C.Ertl. (1990) Structural and immunological characterization of a linear virus-neutralizing epitope of the rabies virus glycoprotein and its possible use in a synthetic vaccine. J Virol. 64:3804-9.

Farhood H, X.Gao, K. Son, Y.Y. Yang, J.S. Lazo, L. Huang, J.Barsoum , R. Bottega and R.M. Epand (1994). Cationic liposomes for direct gene transfer in therapy of cancer and other diseases. Ann N Y Acad Sci. 31:23-34.

Farrel, R.E. (1993). In: RNA methodologies . A laboratory guide for isolation and characterization. Academic press. Inc., New York, pp:133-135.

Felgner ,P,L., T.R. Gadek, M. Holm, R. Roman, H.W. Chan, M.Wenz, J.P. Northrop, G.M. Ringold and M.Danielsen.(1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci U S A. 84:7413-7

Felsenstein, J. (1984). Distance methods for inferring phylogenies: a justification. Evolution. 38: 16-24.

Ganesh,V. (1997). Molecular characterization of rabies virus. Thesis submitted to the Tamil Nadu Veterinary and Animal Sciences University, Chenai-7.

Gaudin. Y, C. Tuffereau , A. Benmansour, and A. Flamand . (1991). Fatty Acylation of rabies virus proteins. Virology.184:441-4.

Gaudin Yves., Rob W.H. Ruigrok, Christine Tuffereau, Marcel Knossow and Anne Flamand. (1992). Rabies Virus Glycoprotein is a Trimer. Virology. 187: 627-632.

Gaudin, Y., R.W.H. Ruigrok, M. Knossow and A. Flammand. (1993). Low-pH conformational changes of rabies virus glycoprotein and their role in membrane fusion. J. of Virol. 67: 1365-1372.

Grinnell.B.W. and R.R. Wagner. (1985). Inhibition of DNA-dependant transcription by the leader RNA of vesicular stomatitis virus: role of specific nucleotide sequences and cell protein binding. 5: 2502-2513

Habel,K., and H. Koprowski (1955) Laboratory data supporting the clinical trial of antirabies serum in persons bitten by a rabid wolf. Bull. WHO., 13: 773-779.

Hiramatsu. K, K.Mifune, K. Mannen, A. Nishizono, H. Kawano, Y.Ito and A.Kawai.(1992) Mapping of the antigenic determinants recognized by monoclonal antibodies against the M2 protein of rabies virus. Virology. 187:472-479.

Isabelle Seif, P. Coulaon, P.E. Rollin and A. Flamand. (1985). Rabies Virulence: Effect on Pathogenicity and Sequence Characterization of Rabies virus mutations affecting Antigenic site III of the glycoprotein. J. Virol. 53: 926-934.

Jallet,C., Y. Jacob, C. Bahloul, A. Drings, E. Desmezieres, N. Tordo and P. Perrin. (1999). Chimeric lyssavirus glycoproteins with increased immunological potential. J. Virol. 73: 225-233.

Jayakumar,R., K. Kumanan, A. Sundararaj and V.D. Padmanaban. (1993). Monoclonal antibody screening of rabies virus infection in and around Madras. Ind. J. Virol., 9: 62-64.

Jayakumar, R., K. Kumanan, K. Vijayarani and V.D. Padmanaban. (1994). Isolation and identification of rabies virus in murine neuroblastoma cells. Ind. J.Anim. Sciences. 64: 847-848.

Kawano.H, K. Mifune, M. Ohuchi, K. Mannen, S. Cho, K. Hiramatsu and A. Shichijo.(1990) Protection against rabies in mice by a cytotoxic T cell clone recognizing the glycoprotein of rabies virus. J Gen Virol. 71:281-7.

Kieny.M.P., R. Lathe, R. Drillien, D. Spehner, S. Skory, D. Schmitt, T. Wiktor, H. Koprowski and J.P. Lecocq. (1984). Expression of

rabies virus glycoprotein from a recombinant vaccinia virus. Nature. 321: 163-166.

Kissling, R.E. and D.K. Reese. (1953) Growth of rabies virus in tissue culture. J. Immunol., 91: 362.

Koprowski, H. (1966). In-vitro production of antirabies vaccine. Symposia series in immunological standardization. 1:357-266.

Kornfeld,R. and S. Kornfeld. (1985). Annu. Rev. Biochem. 54: 631-664.

Lafay. F, P. Coulon, L.Astic , D. Saucier, D. Riche , A. Holley and A. Flamand. (1991) Spread of the CVS strain of rabies virus and of the avirulent mutant AvO1 along the olfactory pathways of the mouse after intranasal inoculation. Virology. 183:320-30.

Lai, C.Y. and B. Dietzschold. (1981). Amino acid composition and terminal sequence analysis of the rabies virus glycoprotein : identification of the reading frame on the cDNA sequence. Biochem. Biophy. Res. Com. 103: 536-542.

Larson.J.K, W.H. Wunner, L. Otvos Jr and H.C. Ertl. (1991) Identification of an immunodominant epitope within the phosphoprotein of rabies virus that is recognized by both class I- and class II-restricted T cells. J Virol. 65:5673-9.

Lathe.F.R., M.P. Kieny, D. Schmitt, P. Curtis and J.P. Lecocq. (1984). M13 Bacteriophage vectors for the Expression of foreign protein in Escherichia coli: The Rabies Glycoprotein. J. Mol. App. Genetics. 2: 331-342.

Lecocq,J.P., M.P. Kieny, Y. Lemonie, R. Drillien, T. Wiktor, H. Koprowski and R. Lathe. (1985). New rabies vaccines : recombinant DNA approaches. In Worlds Debt to Pasteur, (Eds.) H. Koprowski and S.A. Plotkin, Alan R. Liss, New York. Pp. 259-271.

Lentz.T.L., T.P. Wilson, E.Hawrot and D. W.Speicher. (1984). Amino acid sequence similarity between rabies virus glycoprotein and snake venom curaremimetic neurotoxins. Science. 226:847-848.

Levaditi,M.C. (1913) Rabies and in-vitro cell culture. C.R. Soc. Biol., 75:505.

Lodmell,D.L. and L.C. Ewalt. (1987). Immune sera and antiglycoprotein monoclonal antibodies inhibit in-vitro cell-to-cell spread of pathogenic rabies viruses. J. Virol. 61:3314-3318.

Luckow ,V,A. and M.D. Summers (1988). Signals important for high-level expression of foreign genes in Autographa californica nuclear polyhedrosis virus expression vectors. Virology. 167:56-71.

Matsumoto,S. and A. Kawai, 1969. Rabies virus. Advances in Vir.Res., 16:257-301.

Mcdonald,A.S., Kevin Schifferli, Dina Anderson, Joel Hessee and Valentina Ciccarone. (2001). A simple method for cationic lipid reagent selection for transfection. Focus. 18: 6-9.

Mcfarlan,R.I., B. Dietzschold, T.J. Wiktor, M. Kiel, R. Houghten, R.A. Erner, J.G. Sutcliffe and H. Koprowski. (1984). T cell responses to cleaved rabies virus glycoprotein and to synthetic peptides. J. immunol., 1333: 2748-52.

Mcfarlan,R.I., B. Dietzschold and H. Koprowshi. (1986). Stimulation of cytotoxic T-lymphocyte responses by rabies virus glycoprotein and identification of an immunodominant domain. J. Mol. Immunol. 23: 733.

McGarvey,P.B., J. Hammond, Margaret M. Dienelt, D.C. Hooper, Zhen Fang Fu, B. Dietzschold, H. Koprowski and Frank H. Michaels. (1995). Expression of the Rabies virus Glycoprotein in Transgenic Tomatoes. Biotechnology., 13: 1484-1487.

Meslin,F.D., D.B. Fishbein and H.C. Matter. (1994). Rationale and prospects for rabies elimination in developing countries. In: Lyssaviruses (eds.). B.Dietzschold and H. Koprowski. Srpinger, Berlin. Pp1-26.

Modelska, A., B. Dietzschold, N. Sleysh, Z.F. Fu, K. Steplewski, D.C. Hooper, H. Koprowski and Vidadi Yusibov. (1998). Immunization against rabies with plant-derived antigen. Proc. Nat. Acad. Sciences., 95: 2481-2485.

Morgeaux,S. M.L. Joffret, C. Leclerc, P. Sureau and P. Perrin. (1989). Evaluation of the induction of specific cytotoxic T lymphocytes following immunization of F1 hybrid mice with rabies antigens. Research in Virology. 140: 193-206.

Morimoto,K., A. Ohkubo and K. Kawai. (1989) Structure and transcription of the glycoprotein gene of attenuated HEP-Flury strain of rabies virus. Virology, 173:465-477.

Perrin,P., Y.Jacob, A. Aguilar-Setein, E. Loza-Rubio, C. Jallet, E.Desmezieres, M. Aubert, F. Cliquet and N. Tordo. (1999). Immunization of dogs with a DNA vaccine induces protection against rabies virus. Vaccine.18:479-486.

Prehaud,C., P.Coulon, F. LaFay, C. Thiers and A. Flamand. (1988) Antigenic site II of the rabies virus glycoprotein: structure and role in viral virulence. J Virol. 62:1-7.

Prehaud,C., K.Takehara, A. Flamand and D.H. Bishop.(1989). Immunogenic and protective properties of rabies virus glycoprotein expressed by baculovirus vectors.Virology. 173:390-99

Prevec. L, J.B. Campbell, B.S.Christie, L.Belbeck and F.L.Graham. (1990). A recombinant human adenovirus vaccine against rabies. J Infect Dis. 161:27-30.

Rose.J.K, R.F. Doolittle, A. Anilionis, P.J. Curtis and W.H. Wunner. (1982). Homology between the glycoproteins of vesicular stomatitis virus and rabies virus. J Virol. 43:361-4.

Rudd,R.J. and C.V. Trimarchi. (1987). Comparison of sensitivity of BHK-21 and murine neuroblastoma cells in the isolation of a street strain of rabies virus. J.Clin. Microbiol. 25: 1456-1458.

Sacremento,D, H. Badrane, H. Bourhy and N. Tordo. (1992). Molecular epidemiology of rabies in France: comparison with vaccinal strains. J. Gen. Virol. 73: 1149-1158.

Saitou, N. and M.Nei. (1987). The Neighbour-Joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.

Sakamoto, S., Toshio Ide, S. Tokiyoshi, J. Nakao, F. Hamada, M. Yamamoto, J.A. Grosby, Yajin Ni and Akihiko Kawai. (1999). Studies on the structures and antigenic properties of rabies virus glycoprotein analogues produced in yeast cells. Vaccine. 17: 205-218.

Seal,B.S. (1996). Analysis of matrix protein gene nucleotide sequence diversity among Newcastle disease virus isolates

demonstrates that recent disease outbreaks are caused by viruses of psittacine origin. Virus Genes. 11: 217-224.

Sehgal,S., (1997). In : Rabies control in Asia (eds., Dodet, B and Meslin,F.X.), Meslin Elsevier, Paris, pp. 140-145.

Seif, I., P. Coulon, P.E. Rollin and A. Flammand. (1985). Rabies virulence: effect on pathogenecity and sequence characterization of rabies virus mutations affecting antigenic site III of the glycoprotein. J. virol. 53: 926-934.

Shakin-Eshleman,S.H., Steven L Spitalnik and Lakshmi Kasturi. (1996). The amino acid at the X position of an Asn-X-Ser sequon is an important determinant of N-linked core-glycosylation efficiency. J. Biol. Chemistry. 271:6363-6366.

Smith,J.S. and A.K. King. (1996). Monoclonal antibodies for identification of rabies and non-rabies lyssa viruses. In: Laboratory techniques in rabies (Eds). F.X.Meslin, M.M. Kaplan and H. Koprowski), 4th edn. World Health Organization, Geneva, pp.145-155.

Sureau,P., P. Rollin and T.J. Wiktor. (1983). Epidemiological analysis of antigenic variations of street rabies virus detection by monoclonal antibodies. Am. J. Epidemiol. 117: 605-609.

Taylor. J, R. Weinberg, B. Languet, P. Desmettre and E. Paoletti. (1988) Recombinant fowlpox virus inducing protective immunity in non-avian species. Vaccine. 6:497-503.

Tierkel,E.S. and P. Atanasiu. (1996). Rapid microscopic examination for Negri bodies and preparation of specimens for biological tests. In: Laboratory techniques in rabies (Eds.) F.X.Meslin, M.M. Kaplan and H. Koprowski), 4th edn. World Health Organization, Geneva, pp.55-58.

Tordo,,N., O. Poch, A. Ermine and G. Keith. (1986) Primary structure of leader RNA and nucleoprotein genes of the rabies genome: segmented homology with VSV. Nuc. Acid. Res., 14: 2671-2783.

Tordo,N. and O. Poch. (1988) Structure of rabies virus. In: Rabies (eds) J.K. Campbel and K.M. Charlton. Kluwer Academic Publishers, Boston. Pp. 25-45.

Tordo,N., H. Badrane, H. Bourhy and D. Sacramento. (1993). Molecular epidemiology of lyssa viruses: focus on the glycoprotein and pseudogenes. Ond. J. Vet. Res. 60: 315-323.

Tordo, N., H.Bourhy and D. Sacramento. (1994). PCR technology for lyssa virus diagnosis, in: The polymerase chain reaction for human diagnosis, edited by J.P. Clewley. London: CRC press.

Tuffereau. C, S. Fischer and A. Flamand. (1985) Phosphorylation of the N and M1 proteins of rabies virus. J Gen Virol. 66:2285-9.

Tuffereau,C., H. Leblois, J. Benejean, P. Coulon, F. Lafay and A. Flamand. (1989). Arginine or lysine in position 333 of ERA and CVS glycoprotein is necessary for rabies virulence in adult mice.Virology. 172:206-12.

Webster,W.A., K.M. Charlton and G.A. Casey. (1989). Persistent infections of f field strain rabies virus in murine neuorblastoma (NA-C1300) cell cultures. Can. J. Vet. Res. 53:445-448.

Whitt,M.A., L.Buonocore, C. Prehaud and J.K. Rose. (1991) Membrane fusion activity, oligomerization and assembly of the rabies virus glycoprotein. Virology. 185: 681-688.

Wiktor.T.J, E. Gyorgy, D. Schlumberger, F. Sokol and H. Koprowski.(1973) Antigenic properties of rabies virus components. J Immunol. 110:269-76.

Wiktor,T.J. (1977). Cell-mediated immunity and post-exposure protection from rabies by inactivated vaccines of tissue culture origin. Dev. Biol. Stand. 40: 225.

Wiktor,T.J. and H. Koprowski. (1980) Antigenic variants of Rabies virus. J. Exp. Med., 152:99-112.

Wiktor,T., R. Macfarlan, K. Reagan, B. Dietzschold, P.J. Curtis, W.H.Wunner, M.P. Kieny, R. Lathe, J.P. Lecocq, M. Mackett, B. Moss and H. Koprowski. (1984). Protection from rabies by a vaccinia virus recombinant containing the rabies virus glycoprotein gene. Proc. Natl. Acad. Sciences. 81: 7194-7198.

Wilsnack,R.E. (1960). The Fluorescent Antibody Diagnosis of rabies. J.Am. Vet. Med. Assoc., 137: 319-320.

Wojczyk. B, S.H. Shakin-Eshleman, R.W. Doms, Z.Q. Xiang, H.C.Ertl, W.H. Wunner and S.L. Spitalnik. (1995) Stable secretion

of a soluble, oligomeric form of rabies virus glycoprotein: influence of N-glycan processing on secretion. Biochemistry. 34:2599-609.

Wunner, W.H., B. Dietzschold, P.J. Curtis. and T.J. Wiktor. (1983). Rabies subunit vaccines. J. Gen. Virol., 64: 1649-56

Wuner,W.H. (1987). Rabies viruses pathogenesis and immunity. In: The Rhabdoviruses, Eds. R.R. Wagner, Plenum Press, New York. Pp. 361-426.

Wunner,W.H., J.K. Larsen, B. Dietzschold and C.L. Smith. (1988) The molecular biology of rabies virus. J. Infec. Dis., 10:571-584.

Xing,Z.H., S. Spitalnik, M. Tran, W.H. Wunner, H. Cheng and H.C.J. Ertl. (1994). Vaccination with a plasmid vector carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus. Virology. 199:132-140.

Xuan, X., K. Tuchiya, I. Sato, Y. Nishikawa, Y. Onoderaz, Y.Takashima, A. Yamamoto, A. Katsumata, A. Iwata, S. Ueda, T. Mikami and H. Otsuka. (1998). Biological and immunogenic properties of rabies virus glycoprotein expressed by canine herpesvirus vector. Vaccine. 9: 969-976.

Yelverton.E., Shirley Norton, John F. Objeski and David V. Goeddel. (1983). Rabies virus Glycoprotein Analogs: Biosynthesis in Escherichia coli .Science. 219: 614-621.