CHAPTER II

LITERATURE REVIEW

Classification and Morphology of Rabbies virus

Rabies virus is a rhabdovirus belonging to the family of Rhabdoviridae. Members of the family Rhabdoviridae infect vertebrates, insectes and plants. Five genera have been established in the Rhabdoviridae: three genera of animal rhabdoviruses, namely, *Vesiculovirus, Lyssavirus, Ephemerovirus*, and two genera of plant rhabdoviruses, namely, *Cytorhabdovirus and Nucleorhabdoviruses* (Wunner *et al.*, 1994). The Vesiculoviruses comprises of four principal serotypes which is classified as vescular stomatis virus (VSV) Indiana, VSV New Jersy, chandipura virus and piry virus. The ephemeroviruses are arthropod-borne rhabdoviruses which cause acute febrile infection in cattle and water buffalo. These include kimberley virus, berrimah virus, Adelaide river virus (ARV) and bovine ephemeral fever virus (BEFV), the type member of ephemeroviruses. The plant rhabdoviruses are primarily distinguised on the basis of the site of virus maturation. Nucleorhabdoviruses which include sonchus yellow net virus (SYNV) and the type member, potato yellow dwarf virus, mature in the nucleus of infected cells. Lettuce necrotic yellows virus (LNYV) which is the type member of the genus *Cytorhabdovirus*, accumulates in the cytoplasm of infected cells (Welzel *et al.*, 1994).

The neuropathogenic lyssaviruses were subdivided in four serotypes on the basis of serological neutralization and monoclonal antibody studies (WHO Expert Committee on rabies, 1984): the type member rabies virus (serotype1) and "rabies-related" viruses. The rabies-related viruses is classified into three serotypes: Lagos bat (serotype2), Mokola (serotype3) and Duvenhage (serotype4). European bat lyssaviruses (EBL) subtype 1 and 2 were proposed to constitue serotype5 (Bouhry *et* al., 1992). Recently, the *Lyssavirus* was classified into six clearly distinct genotypes according to their amino acid similarity (Bouhry *et al.*, 1993). Genotype 2 (Lagos bat virus) and 3 (Mokola virus) are the most phylogenetically distant from the classical and vaccinal rabies virus of genotype 1. Genotype 4 (Duvenhage virus) and 5 (EBL1) are closely related to each other. Genotype 6 represented by EBL2 is the most proximal to genotype 1.

Rabies virion particles have a bullet-shaped structure with an average length of 180 nm and a diameter of 75 nm (Hummeler *et al.*, 1967). The particle, illustrated in figure 1, is hemispherical at one end and usually planar at the other end. Each particle contains a helical nucleocapsid which is surrounded by a lipid bilayer envelope, derived from the plasma membrane of an infected cell. The external surface is covered with 10 nm spike-like projection of 67-kDa glycoprotein (G) anchored in a lipid bilayer. The envelope sheath, which consists of lipids derived from the host cell plasma membrane, is closely associated with a second 26-kDa matrix protein (M), located on the inner side of the virus envelope. The space within the lipoprotein shell is occupied by the nucleocapsid core. The core of virus particle is in the form of helix structure with 30-35 coils extending from end to end within the virion. The nucleocapsid consists of an RNA molecule associated with three internal proteins; the 190-kDa transcriptase (L), the 55-kDa nucleoprotein (N) and the 38-kDa nonstructural phosphorylated protein (NS).

Propagation and Assay in Cell Culture

Rabies virus has been successfully cultured. This was initially accomplished with fixed strains and later with street rabies strains (Kissling, 1958). Although numerous cell culture lines have been used to propagate rabies virus, baby hamster kidney (BHK) cells have been favorite host for virus growth. These cells are highly susceptible to viral infection, making it easy to obtain purified virus in milligram quantities. This has permitted a systematic approach to virus characterization, study of its growth *in vivo* and development of new assay methods. In addition, BHK-21 cells have been widely used for production of animal rabies virus vaccines. Recently human diploid cells, Vero cells, and purified chicken embryo cells have been used to produce human rabies vaccines (Wiktor *et al.*, 1964; Montagnon *et al.*, 1985; Barth *et al.*, 1984). The chemical composition, particularly the carbohydrate and lipid content can be modified by the host cell. The strains of rabies virus have been isolated from street rabid animal and human and adapted by many passages in cell culture to obtain the fixed strains. The standard fixed strains have been known as the Louis Pasteur virus (PAS), Pastuer virus (PV), Pitman-Moore (PM), Challenge Virus Standard (CVS), Avirulent Orsay (AvO-1), Street Alabama Dufferin (SAD), Evelyn-Rockitnicki-Abelseth (ERA), High Egg Passage (HEP-Flury). Most vaccines still derived from these isolates obtained between 50 and 100 years ago. Despite the technical progress in improving the efficiency and safety of classical or subunit vaccines, very

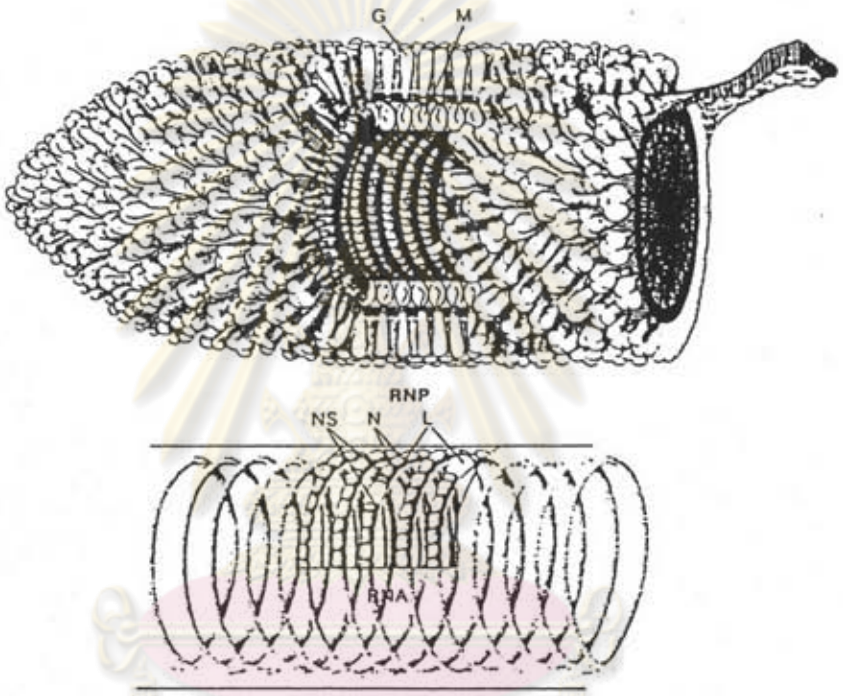

Figure 1. Drawing of rabies virus (above) and helical nucleocapsid core (below). Rabies viral glycoprotein (G) and matrix protein (M) are associated with the membrane envelope. The helical single-stranded RNA genome is tightly bounded with the ribonucleoprotein (RNP), comprise of nucleoprotein (N), phosphoprotein (NS) and polymerase (L).

(From : Wunner *et al.*, 1988)

little has been done to renew the strains used as seed.

Rabies Virus Genome

A. Organization of the Genome

The genome of rabies virus is a single-stranded RNA and is of negative-sense polarity. RNA length is equivalent to 11,932 nucleotides agree with the molecular weight estimation of 4.6×10^6 (Tordo *et al.*, 1986; Tordo *et al.*, 1988; Sokol *et al.*, 1969). Figure 2 shows the total length of genome which contains the 3' leader sequence, N, NS, M, G, and L genes, the intergenic sequences and a 5' noncoding region. The gene order, 3'-N,NS,M,G,L-5', defined by transcriptional mapping studies is the same as that in the other rhabdoviruses (Flamand and Delagneau, 1978). The genetic informations encoded in the single-stranded RNA genome of rabies virus is transcribed into five monocistronic messenger RNAs, each contains a 3' poly(A) tail at its end (Coslett *et al.*, 1980; Hollowey *et al.*, 1980). The 3' and 5' ends of the rhabdoviruses genomic RNA has a high degree of complementary allowing the formation of a putative "panhandle" structure (Conzelmann *et* al., 1990; Wetzel *et al.*, 1994).

B. Leader Sequence

At the extreme 3' end of rabies virus genome lies a small stretch of 58 nucleotides which precedes the tetranucleotide sequence, UUGU- which denotes at the beginning of the N gene sequence. The 3' trinucleotideUCG at position 1-3 followed by a U-rich region is found in the sequence of the other known rhabdoviruses (Walker *et al.*, 1992; Wetzel *et al.*, 1994). The leader RNA of rabies virus is similar to that of VSV in number of nucleotide and high content of A-residue (50%, Tordo *et al.*, 1986; Conzelmann *et al.*, 1990). The leader sequence is transcribed into a complementary plus-strand leader RNA whose function has not been established. From study with VSV, it appears that transcription of plus-strand leader RNA is necessary for the polymerase complex to reach the start of the first gene. The presence of 3' leader region implies that events such as the initiation of transcription and replication, the switching between these two function and the initiation of encapsidation may take place here (Blumberg *et al.*, 1983; Blumberg *et al.*, 1981).

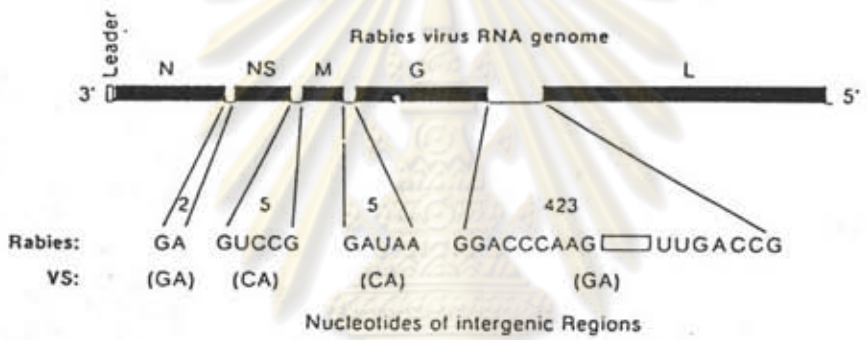

Figure 2. Organization of rabies virus genome. The schematic shows leader RNA, five structural genes and intergenic regions. The intergenic sequences are compared to that of vesicular stomatitis (VS) virus.

(From : Wunner *et al.*, 1988)

C. Structural Genes

Recently, the genomic information of Pasteur virus (PV) rabies strain has been confirmed by cloning and sequencing of complete viral genome (Tordo *et al.*, 1986; Tordo *et al.*, 1988). The nucleotide length of mRNA templates is measured from the tetranucleotide UUGU at the 3' end to the stretch of $(U)_7$ at the end of each gene. Location and nucleotide length of rabies mRNA sequences is shown in Table 1. The sequence closet to the 5' end of the mRNA after the tetranucleotide AACA up to the AUG start codon varies in both length and composition in each mRNA. The 5' noncoding regions of the N, NS, M, G, and L mRNAs are 12, 29, 15, 27 and 30 nucleotides long, respectively. The 3' noncoding regions of the N, NS, M, G, and L mRNAs are 62, 71, 184, 75 and 19 nucleotides in length, respectively. Each mRNA contains a single long open reading frame starting at first AUG and terminating with the stop codon UAA (for N, NS, and M) or UGA (for G and L). The mRNAs of the N, NS, M, G, and L encode the structural proteins of 450, 297, 202, 524, and 2142 amino acids in length, respectively. The coding capacity of mRNAs for five structural proteins is derived from 90% of the total nucleotides in the rabies virus genome. The nucleotide lengths for N, NS, M, and G genes of the ERA and CVS-11 strain are identical to that of PV strain (Wunner *et al.*, 1988). An exception has been found in mRNA encoding the G protein of HEP-Flury strain. The G-mRNA contains a 3' noncoding region which is 423 nucleotides longer and homologous to the G-L intergenic sequence in the genome of other strains, suggesting that the HEP-Flury genome lacks the polyadenylating signal prior to the G-L intergenic region (Morimoto *et al.*, 1989).

D. Intergenic Sequence

The rabies virus genome differs in many features from that of VSV. The most notable differences of the two RNAs is the variable length and composition of the intergenic sequences. The intergenic sequences of rabies virus: the N-NS, NS-M, M-G and G-L are as long as 2, 5, 5, and 423 nucleotides, respectively. Three short intergenic regions (N-NS, NS-M, and M-G) are identical in the ERA, CVS-11 and PV strains of rabies virus. However, 5% in the G-L intergenic sequences of the ERA and 18% in the same region of the CVS-11 genome are different from the corresponding genome of the PV strain (Wunner *et al.*,1988). There are two sequences at either end of the G-L intergenic regions, resemble to the consensus mRNA start and stop signals. Neverthless this region cannot

Table 1. Location and Nucleotide length of rabies mRNA sequences in rabies virus genome. Information is based on nucleotide sequence of PV strain (Tordo *et al.*, 1986; Tordo *et al.*, 1988)

mRNA	Nucleotide position	Length of mRNA
N	59-1482	1424
NS	1485-2475	991
M	2481-3285	805
G	3291-4964	1674
L	5388-11862	6475

encode a peptide larger than 18 amino acids and the corresponding transcript has never been reported. In VSV, each of the intergenic regions contains dinucleotides, either GA or CA (Rose, 1980). A stretch of nucleotides in the G-L intergenic region, a sixth gene encoding of a 1.2 kDa nonviral protein, has been report existing in the genome of fish rhabdovirus (Kurath *et al.*, 1985).

Structure and Function of Rabies Virus

A. RNA-dependent RNA Polymerase

As in other rhabdoviruses, the RNA-dependent RNA polymerase in rabies virus is the largest polypeptide with a molecular size, deduced from the coding sequence of L gene, of 244,206 Da (Tordo *et al.*, 1988). The number of L protein molecules per virion is extremely small in comparison with that of other structural proteins. The calculated number of L protein molecules is 33 per virion (Madore and England, 1977). The number of L polypeptide molecules per virion appears to vary among different rabies virus strains (Dietzshold *et al.*, 1979). This number of L protein molecules per rabies virion is approximately half the number of that found in the VSV (Bishop and Roy, 1972).

Because of the large size and the requirement in RNA synthesis both *in vitro* and *in vivo*, the L protein is thought to be responsible for a variety of enzyme activities; i.e. RNA synthesis, mRNA capping, methylation and polyadenylation of viral mRNAs (Banerjee, 1987). In addition the L protein of VSV has been claimed to phosphorylate the NS protein whereas the rabies L protein may lack this activity (Sanchez *et al.*, 1985; Tordo *et al.*, 1988). However, the kinase activity has been found associated with the purified virions of VSV and rabies virus (Tuffereau *et al.*, 1985). The molecule contains 2142 amino acids. Two strongly hydrophobic domains include residues at 851 to 869 and 1962 to 1980. A relative long hydrophillic stretch of amino acids spans from residue 1552 to 1634 and is rich in asparagine and glutamine (Tordo *et al.*, 1988). It shares an overall 33% amino acids similarity to the VSV L protein. The conserved amino acid stretch at the central part of the molecule is considered to be the possible location of functional domains responsible for the catalytic activities of the L protein (Poch *et al.*, 1990).

B. Nucleoprotein

Molecular size of N protein has been estimated by SDS-PAGE to be 54,000 to 62,000 Da. The stoichiometric amount in a virion is approximately as 1800 molecules (Madore and England,1977). The amino acid sequence deduced from the N gene shows a high degree of similarity among fixed strains: ERA, CVS and PV as illustrated in figure 3 (Wunner *et al.*, 1988). The N protein of rabies virus is phosphorylated, as are those of serveral rabies-related viruses and some fish rhabdovirus, but not the nucleoprotien of VSV (Sokol and Clark, 1973; Sokol *et al.*, 1974). The site of phosphorylation is located near the carboxy terminus (Dietzschold *et al.*, 1988). The N protein as well as NS protein are tightly associated with the RNA genome in the nucleocapsid core structure. Asssociation in the ribonucleoprotein (RNP) complex appears to be in a molar ratio of 2:1. The close packing of N protein protects the RNA from ribonucleases and keeps the RNA genome in a suitable configuration for transcription. Evidence from the studies with VSV indicates that as N protein accumulates in infected cell, it plays a key role in regulating the switching from monocistronic RNA transcription to genome-length plus-sense RNA intermediate and, then, replication of minus-strand progeny RNA genome (Blumberg *et al.*, 1981; Blumberg *et al.*, 1983). Once the nascent RNA becomes encapsidated by N protein, the polymerase is prevented from recognition the mRNA termination sites or transcription signal on the genome RNA. Experiments have not been carried out to determine the role of N protein in rabies virus replication. There is only one invesigation, employing anti-rabies N protein monoclonal antibody, which inhibits the viral releasing as measured by intracytoplasmic nucleocapsid accumulation (Lafon and Lafage, 1987).

C. Non-structural Protein

Molecular weight of NS protein determined by SDS-PAGE is assumed to be 37,000-40,000 Da. The number of protein molecules per virion is suggested as 950 (Madore and England, 1977). The deduced amino acid sequence of the NS phosphoprotein reveals that the molecule contains 297 residues. As shown in figure 3, comparison of the deduced amino acid sequences of the three fixed strains shows a closer relationship between the ERA and PV strains (98% homology) than between the ERA and CVS strains (92% homology). Analysis of the NS protein sequence also indicates extensive hydrophillic portions at the central region and at the NH_2-terminus (Wunner *et al.*, 1988). The phosphorylation sites in the NS protein of rabies virus have not been determined. There are 16 out of

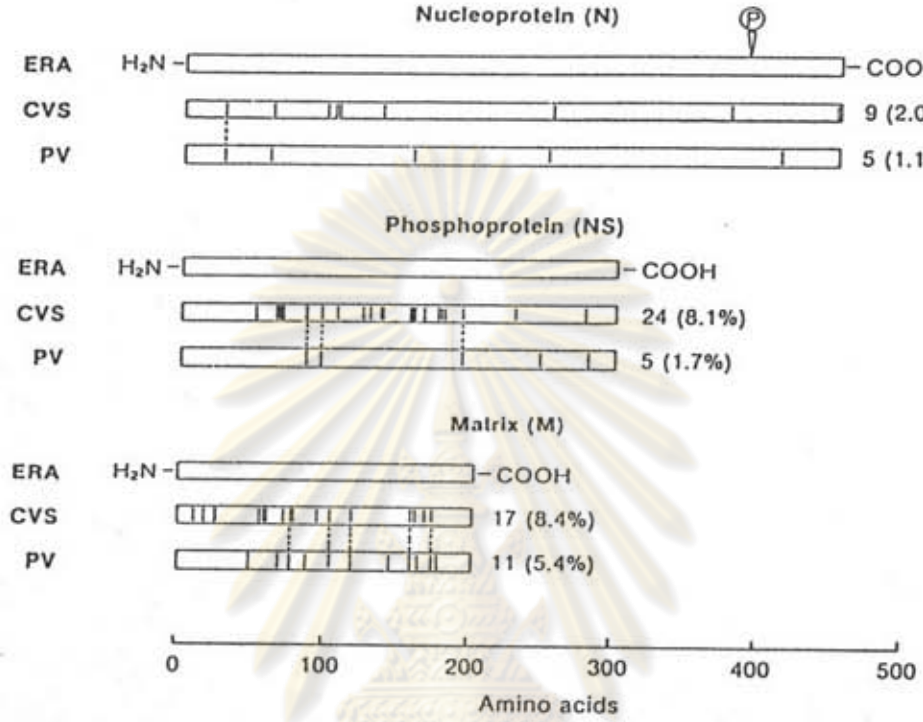

Figure 3. Comparison of deduced amino acid sequences of rabies virus proteins from the ERA, CVS-11 and PV strains of rabies virus. Vertical lines indicates the positions of amino acid differences. The percentage of amino acid substitutions is given for the CVS-11 and PV sequences.

(From : Wunner *et al*., 1988)

40 serine plus threonine residues that may provide potential phosphorylation sites within the NH_2-terminus of the molecule, where the sites of phosphate addition have been mapped in NS protein of the VSV (Bell and Prevec, 1985). NS protein contains one epitope localized at the NH_2-terminus that is recognized by both B and T cells (Dietzschold *et al.*, 1987) In rabies virus, the nonstructural phosphorylated protein interacts with trascriptase and RNP to form the active complex that is responsible for the transcription as well as replication of the genome RNA. Analysis of the interaction between rabies virus N and NS protein mutants with either C- or N-terminal deletions suggested that both terminal domains of NS protein are involved in binding to the N protein (Fu *et al.*, 1994). Little is known about the function of the NS protein. The binding of NS protein to the helical RNP structure, the nature of N and NS protein complexes in the rabies virion, interaction of NS protein and L protein in active polymerase complex in infected cells remain unclear. However, there are some structural and stoichiometric parallels among the NS proteins of viral strains to be noted. Two forms of NS protein, have been detected in CVS, PM, and HEP strains; suggests that it exists in at least two distinct phosphorylation states in these strains (Dietzschold *et al.*, 1979). The studies of transcription, replication and encapsidation of VSV-infected cells indicate that the active RNP complexes consists of N and NS proteins in a 1:1 molar ratio (Peluso, 1988; La Fera and Peluso, 1988)

C. Matrix Protein

Matrix (M) protein is the smallest structural proteins of rabies virus, 202 amino acids in length. Molecular weight is estimated by SDS-PAGE to be 21,000 to 26,000 Da (Tordo *et al.*, 1986; Wunner*et al.*, 1988). The central segment of 19 amino acids is rich in hydrophobic residues suggesting a high probability of being membrane bound. The sequence similarity between M protein of the ERA, PV and CVS-11 strains is less than those of the other internal proteins of the corresponding strains (figure 3). This suggests that a structural homology in M protein molecules exists without sequence homology. The deduced amino acid sequence of the rabies M protein indicates no detectable similarity with that of VSV, although they appear to share funtional homology (Rayssiguier *et al.*, 1986). The M protein of VSV plays a negative role in regulating endogenous viral trascription (Clinton *et al.*, 1978). Transcription inhibition activity of M protein is located within the first 31 N-terminal amino acid residues (Li *et* al., 1989). A major function of M protein may involve with the interaction between the cytoplasmic portion of membrane-anchored viral glycoprotein and the nucleocapsid, since it locates on the inner

surface of the viral envelope. Little information has been determined so far about its funtions in viral replication or morphogenesis.

D. Glycoprotein

The most extensively analyzed protein of rabies virus has been the surface glycoprotein owing to its immunogenic and antigenic properties. The polypeptide sequence deduced from nucleotide sequences reveals that the nascent protein consists of 524 residues with molecular weight of 58,000 Da (Tordo *et al.*, 1986; Anilionis *et al.*, 1981; Yelverton *et al.*, 1983). Comparison of the primary structures of rabies G proteins from four different fixed strains in Figure 4 illustrates following features: (1) the antigenic domain; (2) the transmembrane (TM) domain; (3) the cytoplasmic domain. The G protein of rabies virus is an integral membrane protein. Insertion of such proteins into cellular membrane is accomplished by two types of sequence: the insertion signal sequence at the amino terminus and the stop-transfer sequence near the carboxy terminus. The first 19 amino acids predicted from rabies G-mRNA constitutes the hydrophobic signal peptide. The 20th residue, lysine, becomes the NH_2-terminal amino acid of mature glycoprotein (Lai and Dietzschold, 1981). This hydrophobic domain at NH_2-terminus functions as the leader translocating the nascent protein across the RER membrane into the lumen where glycosylation of the protein begins. At some point during translocation, the signal sequence is cleaved from the G protein molecule and presumbly remains in the infected cells. The deduced sequence also has a hydrophobic domain of 22 amino acids at the carboxy terminus locate between residue 439 and 462 that corresponds to TM domain or stop-transfer sequence. Extending from the TM to the COOH terminus is a long stretch of 44 charged and uncharged residues. This hydrophillic internal segment possibly provides a site for interaction with the viral M and nucleocapsid proteins.

Since the laboratory strains of rabies have been propagated in cell culture many times with different passage histories, high proportion of amino acid substitutions is present in the TM and cytoplasmic portions. However, molecular function is still retained. The divergence in amino acids in ectodomain alters the pathogenic, antigenic or immunogenic properties of rabies virus. One of these evidences is a single amino acid change in avirulent (AvO) strain altering the characteristic virulence (Coulon *et al.*, 1983; Tuffereau *et al.*, 1989). Replacement of arginine at position 333 of G protein with isoleucine, glutamine or glutamic acid does not disturb the molecular conformation by creating neither an alpha helix or

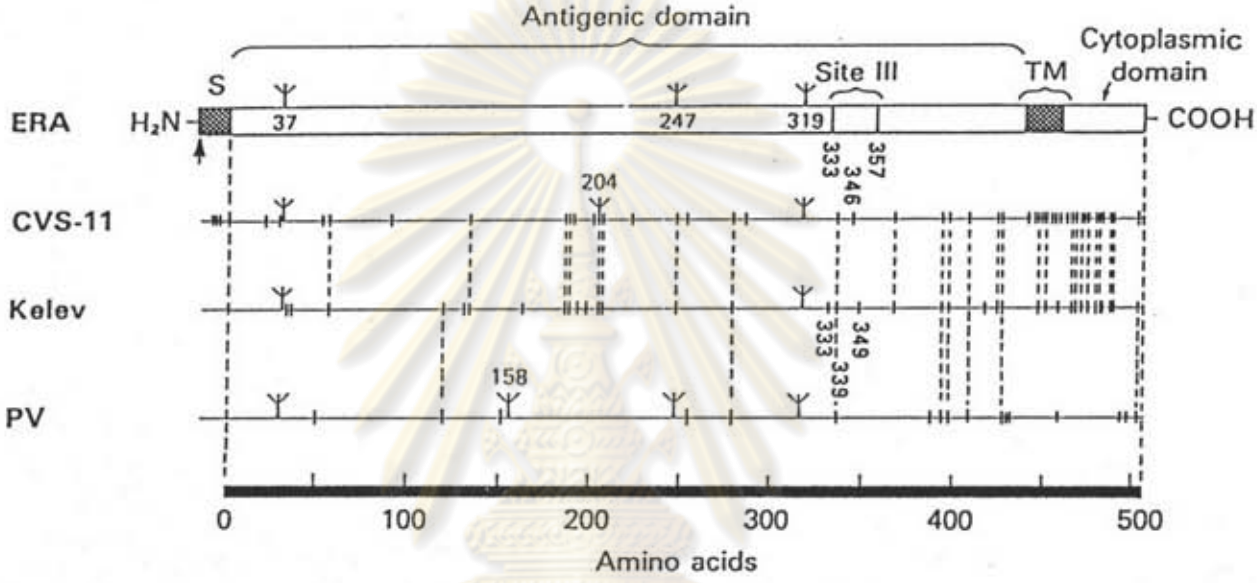

Figure 4. Comparison of rabies viral glycoprotein from fixed laboratory strain (ERA, CVS-11, Kelev and PV). Schematic shows signal peptide (S), antigenic domain, transmembrane portion (TM) and cytoplasmic domain. Glycosylation sites are indicated by branched symbols. Amino acid substitutions is identified by vertical lines. 'Site III' refers to the major antigenic site.

(From : Wunner *et al.*, 1988)

a beta turn. However, the ability to kill adult mice by intracerebral inoculation is lost. Thus the replacement of arginine by a non-charged amino acid at position 333 usually leads to an attenuated phenotype and vice versa. A number of field isolates (street rabies virus) has been reported their virulence for adult mice with substitution of arginine 333 (Benmansour *et al.*, 1992). This discrepency may be compensated by the effects from one or both of the two accompanying substitutions at position 298 and 326 on street viruses G protein. The antigenic domain of the G protein is responsible for both the antigenic and the immunogenic activity. The entire region of 439 amino acids is exposed to the external surface of virus, including host cell surface receptors, and elements such as viral neutralizing antibody. The deduced amino acid sequence has three possible sites for sequence-specific oligosaccharide attachment as illustrated in figure 4 (Wunner *et al.*, 1988). The potential of N-linked sites defined by the sequences, is asparagine-X-serine and asparagine-X-threonine (X refer to any amino acids except proline). The N-linked of asparagine residue at position 37 and 319 are conserved in the ERA, CVS, Kelev, and PV strains, though the potential site at 37 is not glycosylated both in ERA and CVS. The middle glycosylation site at residue 247 is also conserved in ERA and PV strains. An amino acid substitution at position 247 abolishes the middle glycosylation site of Kelev strains. The middle glycosylation site of the CVS shifts to position 204 due to mutations that correspond the residue 204 for residue 247. It is interesting that asparagine 204 in CVS G protein sometimes appears to be glycosylated which are separated into two bands, GI and GII, in SDS-PAGE (Wunner *et al.*, 1985). An extra carbohydrate attachment site of PV appears at asparagine 158 in the polypeptide chain. The percentage of amino acid sequence similarity between the ectodomain portion of the ERA, CVS and PV indicates that these viruses are closely related. The ERA is more closely related to the PV strain (96.4% similarity) than it is to the CVS strain (91.1% similarity). In contrast, the amino acid sequence of G protein of rabies (ERA), compared to that of the VSV, only contains 20% homology. It must be noted that the glycosylation site at asparagine 319 in rabies virus G protein is perfectly registerd in the second of the two glycosylation sites within the VSV G protein (Rose *et al.*, 1982).

The antigenic determinants of many neutralizing-resistant rabies virus varaints have been described on the basis of their reactivity with the panel of anti-G monoclonal antibodies (Flamand *et al.*, 1980; Wiktor *et al.*, 1980; Lafon *et al.*, 1983). Three major antigenic sites (I, II, and III) have been described on the glycoprotein of the ERA and CVS-11 strains and two additional sites (IV and V) on the ERA glycoprotein. It is clear that a

single amino acid change in physical mapping is correlated to the resistance of virus to neutralization by antibody binding (Dietzschold *et al.*, 1988; Prehaud *et al.*, 1988). The antigenic structure of site III is the largest representation of virus-neutralizing epitopes, and therefore has the potential for being a major binding site. Antigenic site III is represented by at least three epitopes that consist of amino acids in close proximity within the linear sequence and are thereby most likely continuous determinants. One epitope locates some distance from the others in the linear sequence and may be linked to antigenic site III by folding in native protein molecule.

Rabies virus-infected cells secrete a truncated form of the rabies glycoprotein called "soluble" antigen (Gs protein). The Gs protein (61,000 Da) lacks 58 amino acids from the carboxy terminus, resulting from proteolytic cleavage of G protein in the portion of TM region. Lack of two-thirds of hydrophobic TM segment prevents the Gs protein from aggregation in the presence of immunogenic rosette-forming agent *n*-octylglucoside. The rosette structure of G protein induces high levels of virus neutralizing antibodies. Although the Gs protein is antigenically identical to the G protein, it fails to protect against lethal challenge with rabies virus (Dietzschold *et al.*, 1983). The difference in immunization efficacy can be explained by the lack of protein-protein interaction as well as protein-lipid interacton in Gs protein.

Rabies Virus Membrane

The rabies virus particle envelope is a bilayer lipid structure derived from cell membrane. The lipid which form the membrane matrix are phospholipids, neutral lipids and glycolipids (Blough *et al.*,1977; Schlesinger *et al.*, 1973). Phospholipids are the major lipids constituent (42-66% of total lipid) of rabies virus membrane. Phosphatidylethanol-amine, phosphatidylcholine, and sphingomyelin are the most abundant phospholipids. Triglyceride and cholesterol are the major neutral lipids. The ratio of cholesterol to phospholipid ranges from 0.48 to 0.92.

Replicative Cycle of Rabies Virus

Life cycle of rabies virus has been studied on a limit scale due to the virulence of the infectious particles. The knowledge of replicative cycle of rabies virus is presumbly similar to those of VSV and others minus-strand RNA virus such as orthomyxoviruses and paramyxoviruses. Rabies virus particle attaches to the cell membrane mediated by the interaction of

viral glycoprotein with receptors on the host-cell surface. Immunofluorescence studies indicate that the rabies virus antigen accumulates at the myotubes which is abundant with clusters of acetylcholine receptors (Lentz *et al.*, 1982). After the intial attachment, virus enters the cell by receptor-mediated endocytosis as described in BHK and CER cells (Iwasaki *et al.*, 1973; Superti *et al.*, 1974). The viral genome is extruded into the cytoplasm, and replication begins. The transcription and the replication mechanisms of rabies virus genome are illustrated in figure 5. Upon entry into the host cell, the rabies nucleocapsid acts as a template for the primary transcription of the viral mRNAs, producing a small uncapped, non-polyadenylated leader RNA and monocistronic transcripts of 5' capped polyadenylated mRNAs corresponding to the five known structural genes (Coslett *et al.*, 1980). Upon translation of the primary transcripts, the nucleocapsid can now act as a template for synthesis of a full-length positive sense strand, an intermediate which subsequently serves as template for the synthesis of a large number of the genome nucleocapsid. The newly synthesized genome nucleocapsid can either be encapsidated into progeny virions or serve as templates for further mRNA synthesis, the so-called secondary transcription.

The transacriptional-replicative model, proposed for the regulation of VSV replication (Blumberg *et al.*, 1981), suggests that viral transcription and genome replication are carried out by the same viral polymerase. Whether the viral L protein transcribes the minus strand nucleocapsid genome or replicates the viral genome is determined according to this model by concentration of the viral N protein. The N protein would modulate transcription and replication by its ability to suppress a termination signal on the minus-strand genome template which terminates the primary transcription and leads to the synthesis of plus-strand. Since stoichiometric amounts of the viral N protein are required for nucleocapsid assembly, this replication mode of viral RNA synthesis would be dependent on the continuation of protein synthesis. The leader RNA termination signal located on the plus-strand full length RNA template would prevent minus-strand RNA synthesis under condition where there is insufficient N protein to assembly the plus-strand RNA into nucleocapsid.

Transcription of Rabies Virus

Five distinct mRNA species have been synthesized *in vitro* by detergent-disrupted rabies virion and each can be identified by *in vitro*

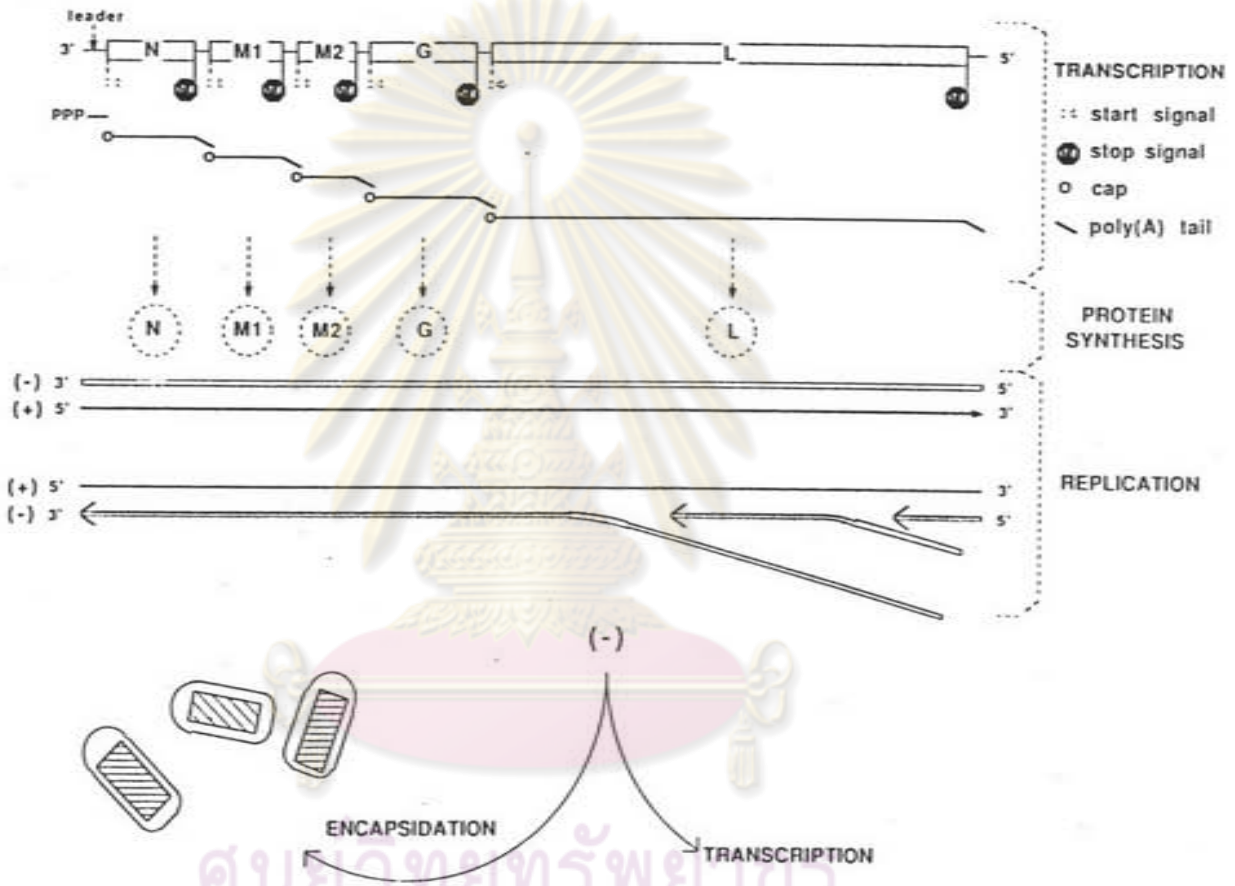

Figure 5. Transcriptional and replicative mechanisms of the rabies virus genome. (From: Tordo and Poch, 1988)[21]

translation (Coslett *et al.*, 1980; Pennica *et al.*, 1980). The *in vitro* synthesized mRNAs contain features common to the typical eucaryotic mRNA, such as a 5'-terminal cap structure (GpppA) and a 3'-polyadenylic acid tract. The *in vitro* mRNA synthesis of VSV has been shown to decrease in the order of leader RNA, N, NS, M, G and L. The unique polar effect on transcription suggests that the polymerase most likely initiates at a single site at the 3' end of the viral genome. Cleavage model has been proposed to occur at specific sites on the growing nascent chain (Banerjee *et al.*, 1977). Five mRNA species are produced, followed by capping and polyadenylation at the 5' ends and 3' ends of the chains, respectively. This model lacks support mainly due to insufficient data demonstrating the cleavage reaction or the existence of uncleavage precursor RNA *in vitro*. The second model, named the stop-start model, has been proposed that the initiation of RNA synthesis starts at the 3' end of the genome RNA and synthesizes the leader RNA first. The same enzyme then reinitiates on the N gene to continue the synthesis and polyadenylates by slippaged on the U residues present at the intergenic junction. The same enzyme continues the synthesis of NS, M, G, and L mRNAs in the manner as described above (Emerson, 1982). This process will generate a concentraion gradient of the RNA species in the order of leader RNA> N> NS> M> G> L.

The multiple (independent) initiation model has been proposed that the the transcription initiation occurs at multiple internal sites (Testa *et al.*, 1980). As illustrated in figure 6, RNA synthesis begins at multiple promotor sites but the elongation of each RNA oligonucleotides depends on the prior transcription of the 3' proximal gene. The secondary structure of the transcribing RNP is proposed to play a role in this cascade mode of transcription. Although this model basically agrees with the stop-start model in having transcripts synthesized from a separate initiation event, the initiations are proposed to be independent.

Detection of Rabies Virus

There are a vareity of laboratory methods available for detection of rabies virus in a biological sample.

A. Histological Technique

The inflammatory lesions found in rabies are common to all viral infections of the nervous system. Specific inclusions can be observed in the cytoplasm of neurons and identified by histological and immunological

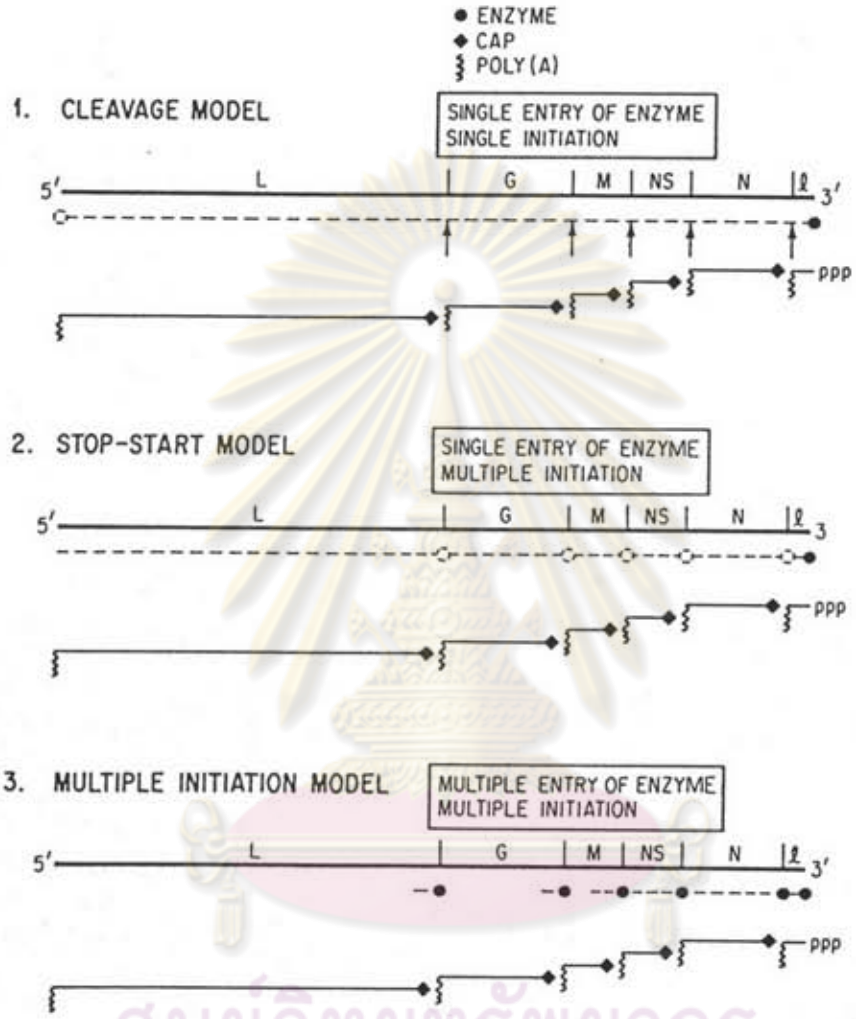

Figure 6. Models for Rhabdovirus mRNA synthesis. Arrows in the cleavage model indicate the putative cleavage sites.

(From: Banerjee, 1987)

technique. The Negri bodies is a compacted mass of viral nucleocapsid, surrounded by numerous bullet-shaped viral particles budding from intracytoplasmic membranes. Smears or impressions of fresh specimens should be examined for Negri bodies either by Seller's stain or FA test. The Seller's technique should only be used when FA is not available.

1. Seller's Staining

The Negri body is stained pink with hematoxylin-eosin and red with Mann's stain. The size of these inclusions varies from 0.24 to 27 μm and are frequently seen in the Ammon's horn. The viral inclusions of rabies, Lagos bat and Mokola viruses are indistinguishable. In addition, non-specific inclusion could sometimes be found in healthy animals.

2. Fluorescence-Antibody Test (FA)

An immunofluorescene method for the demonstration of rabies antigen in infecetd brain tissue was reported by Goldwasser and Kissling (1958), and adapted for use as a diagnostic procedure by others. In principal, antibodies to rabies nucleocapsid in the γ-globulin fraction can be labeled with fluorescent marker to produce a fluorescent conjugate. When this labeled rabies antibody is covered over a smear or section containing antigen, the antigen-antibody complex occurs, fixing the fluorescent compound to the homologous antigen sites. All the conjugate constituents except reacted labeled antibody can then be washed away. the smear is viewed on a microscope fitted with an illuminator and filters appropriate for fluorochrome excitation. The FAT demonstrated a sensitivity that approached 100% compared with the isolation of virus by the intracerebral inoculation of mice. Since rabies FA is rapid, simple and extremely sensitive, it has been recognized as the best single test available for the rapid diagnosis of rabies (Kaplan, 1973). Although potentials exist for superior accuracy when the FA is applied by experienced technologists using high quality reagents, this level of performance is not always achieved in routine diagnostic programs. The immunofluorescene procedure has numerous applications in rabies laboratory besides direct diagnosis on specimens of CNS. It has also been instrumental in the evaluation of rabies pathogenesis, for example, distribution of virus in neural tissues, and the mechanism of infection in cell culture as well as in tissue culture isolation techniques for rabies virus. Moreover, FA is also employed to identify and quantify rabies serum antibody and screen for monoclonal antibody.

B. Animal Inoculation

Intracerebral inoculation of mice with suspect brain material is one of the most widely used tests in the laborytory diagnosis of rabies. According to the WHO guidelines, this test should be performed whenever humans have been bitten by unvaccinated animals and the direct FA test gave a negative result. The method has been proposed in 1930 by Hoyt and Jungeblut (Kaplan, 1973). The mice for inoculation must be carefully selected. The animals must be obtained from a healthy breeding colony, free of latent virus infection. The white swiss mouse is the animal of choice for rabies diagnosis, but other strains can be used, since all are susceptible to the intracerebral inoculation of street rabies virus. Incubation period varies according to the isolate, usually occurs between 8-15 days. Paralyzed mice may be sacrificed and their brains removed for confirmation of the diagnosis by FA. Although MI is recommended for rabies diagnosis, other laboratory animal species (rabbit, guinea pig and hamster) may also be used when mice are not available.

C. Cell Culture Inoculation

Rabies tissue culture test (RTCIT) is presently the best technique for street rabies virus isolation. This technique is performed instead of MI, with the same reliability, for rapid confirmation, the direct FA that is always performed as the first step with diagnostic specimens. Several cell lines may be used such as the BHK-21 cell line (Rudd *et al.*, 1980), the CER cell line (Smith *et al.*, 1977) and mouse neuroblastoma cell line (Smith *et al.*, 1978). The supernatant of virus and cell suspension are incubated in chamber slides overnight. The rabies viral inclusions are then stained with an anti-nucleocapsid rabies FITC conjugate and viewed under a fluorescent microscope.

D. Rapid Rabies Enzyme Immuno-Diagnosis (RREID)

This technique was designed to provide a rapid, sensitive, and specific test for rabies diagnosis in laboratories which have difficulties in using the direct FA test. Rabies virus antigen is incubated in the wells of sensitized microtiter plate, resulting in the binding of rabies nucleocapsid to the antinucleocapsid antibody which is bound to the solid phase. The bound viral antigen is then quantified with the same antinucleocapsid antibody conjugated to peroxidase; the color appears when the substrate is added. The RREID has been shown to have a specificity virtually comparable to

that of the FA test, however, their sensitivities were slightly lower that the use of RREID instead of FA is not recommended (Bouhry *et al.*, 1989). The ease of technique and the possibility of reading the results with naked eye, is particularly useful in laboratories which do not have FA and in large-scale epidemiological surveys.

E. Detection of Rabies Genetic Materials

1. Dot Hybridization Assay

Nucleic acid hybridization technique for the detection of specific nucleic acid sequences have been applied to the diagnosis of a variety of infectious disease. The method of dot hybridization for diagnosis of rabies infection was first introduced by Ermine *et al.*(1988). RNA was extracted from brain of suspected animals. RNA was fixed on membranes and hybridized with cDNA probes complementary to rabies gene sequence. Hybridized, labelled probes were detected by autoradiograph. The sensitivity of the method allows the detection of rabies viral RNA as little as 80 ng of brain RNA. Dot hybridization can be used for diagnostic purposes since they correlated well with direct FA and cell culture isolation methods. Non radio-labelled probes are prefered, since ^{32}P-labelled probes cannot be easily used for routine diagnostic procedures as serveral days are required for autoradiography. Improvement of the dot hybridization technique is still required.

2. Polymerase Chain Reaction (PCR)

PCR is an *in vitro* method of nucleic acid synthesis by which a particular segment of DNA can be specifically replicated. It involves two oligonucleotide primers that flank the DNA fragment to be amplified and repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers with DNA polymerase. These primers hybridize to opposite strand of the target sequence and are oriented so that the DNA synthesis by the polymeraase proceeds across the region between the primers. Since the products synthesized in one cycle can serve as a template in the next, the number of target DNA copies approximately doubles at every cycle. The result is an exponential accumulation of the specific target fragment. PCR amplification of rabies virus nucleic acid was firstly proposed in by Ermine (Ermine *et al.*, 1990). The PCR amplification of the rabies RNA for

diagnosis of brain specimen coupled with dot-blot analysis of the amplified products with a non-radioactive digoxigenin labelled probe performed a perfect concordance (100%) with the three methods, FA, RTCIT and RREID, in rabies diagnosis (Sacramento *et al.*, 1991). In addition, the PCR is proven to be a powerful tool for molecular epidemiology studies, allowing analysis of highly variable genomic area of the street virus strain without prior cell culture adaptation (Sacramento *et al.*, 1991; Smith *et al.*, 1991).

During the last 30 years, significant advances have been made in the diagnosis of rabies virus. The histological diagnosis of Negri bodies in infected brain has been replaced by direct detection of viral nucleocapsid inclusions on brain smears with a fluorescent polyclonal antibody. The FA has become a basic method for routine rabies diagnosis. In addition, viral isolation by either MI or RTCIT is also performed to allow further analysis of etiological agent. RTCIT has reduced viral isolation from 5-11 days to only 1 day, particularly when highly susceptible murine neuroblastoma cell cultures are used. Such rapidity promotes the RTCIT from a simple isolation technique to a comfirmatory test for diagnosis. A method based on the ELISA technique, the RREID has been developed. Each of these techniques has its merits and disadvantages. FA is rapid (2-3h) and inexpensive but requires availability and maintenance of UV microscopes and suitably trained personnel. RREID is simple, rapid (4h) and cost effectiveness. MI is neither rapid nor ethical, whereas the RTCIT is fast but expensive and requires technical expertise in cell culture. Dot hybridization has great potential for the diagnosis of rabies. The method does not need sophisticated equipment but requires the use of radioactive-labelled probes and time consuming. Dot hybridization with non radio-labelled probes is less sensitivity but can be coupled with PCR technique for rabies diagnosis within 2 days. However, the laboratory techniques require expensive equipment and well-trained workers.