1.0 Introduction and review of literature:

1.1 History:

Chandipura Virus (CHPV), a member of the family *Rhabdoviridae* genus vesiculovirus, (Fig. 1) is an important arbovirus in India as it causes encephalitis with high mortality in children. CHPV was first isolated in 1965 from the serum of a patient with febrile illness from Chandipura village near Nagpur in India during the investigation of dengue/Chikungunya outbreak *(Bhatt and Rodrigues, 1967)*.

CHPV was subsequently isolated in 1967 from sandflies collected from Aurangabad district Maharshtra state during a routine entomological investigation in 1967 *(Dhanda et al, 1970)* and later from a child suffering from encephalopathy in Jabalpur, Madhya Pradesh *(Rodrigues et al, 1983)*.

1.2 Clinical importance of CHPV:

CHPV gained substantial attention in 2003, when an explosive outbreak of CHPV was reported in several districts of Andhra Pradesh, India. This epidemic was comprehensively studied at National institute of Virology (NIV), Pune. In this epidemic, IgM antibodies were negative for the common encephalitis causing viruses such as Japanese encephalitis, West Nile, dengue, and measles. Attempt to detect RNA of coronavirus, paramyxovirus, enterovirus, and influenza viruses yielded negative results. Importantly virus isolation was achieved with six samples. These were identified as Rhabdovirus by electron microscopy (EM) visualizing typical bullet shaped 150-165 nm long, 50-60 nm wide particles (Fig. 2). PCR for rabies virus was negative but CHPV RNA was detected in clinical samples from nine patients. Sequencing of five of these RNA samples showed 96.7–97.5% identity with the reference strain of 1965. Immunofluorescent antibody test and PCR confirmed the presence of CHPV. Thus, this was the first evidence of the epidemic potential of CHPV *(Rao et al, 2004)*.
(a) Taxonomy of CHPV:

Group: GroupV\([-\)ssRNA]\nOrder: Mononegavirales
Family: Rhabdoviridae
Genus: Vesiculovirus
Species: Chandipura virus

(b) Schematic presentation of CHPV

(c) Genome organization of CHPV:

![Genome organization of CHPV](image)

**Fig. 1** (a) Taxonomy of Chandipura virus, (b) Schematic presentation of CHPV and (c) Genome organization of CHPV, showing all the proteins and bullet shaped morphology (*Courtesy-Fields Virology, Ed IV Vol II*).

Next year, i.e. in year 2004 another epidemic of CHPV was reported in the eastern districts of Gujarat with a case fatality rate of 78.3% (*Chadha et al., 2005*). Rapid onset of disease, high case fatality rate and death within 24-48 hr of onset of symptoms were found to be the hallmark of CHPV encephalitis.

Scientists at NIV not only did detailed analysis of all the recent epidemics but also conducted a hospital-based surveillance of cases with acute encephalitis in children between May 2005 and April 2006, to determine the contribution of CHPV to sporadic acute viral encephalitis cases in children, from an affected district of Andhra Pradesh, India, seroconversion in recovered cases and to compare the seroprevalences of anti-CHPV IgM and N antibodies in areas reporting cases with those without any cases of acute viral encephalitis (*Tandale et al., 2008*). CHPV etiology was detected in 25 cases (48.1%, n=52; RNA in 20, IgM in 3 and N antibody seroconversion in 2). Anti-CHPV N antibody seroprevalence in <15 years contacts (66/90, 73.3%) and noncontacts (77/109, 70.6%) was significantly lower than in contacts (75/77, 97.4%) and non-contacts (302/321, 94.1%) more than 15 years respectively. Thus CHPV appears to be the major cause of acute viral
encephalitis in children in endemic areas during early monsoon months with acute onset of fever and central nervous system involvement in the form of one or more symptoms such as altered sensorium, unconsciousness, coma and convulsions (Rao et al, 2004; Chadha et al, 2005; Tandale et al, 2008). The place where CHPV is in active circulation is depicted in Fig. 3 (Mishra AC, 2004)

![Fig. 2: Transmission electron micrographs of primary Chandipura virus isolates from culture. Bar=100 nm in both micrographs. (A) Two negative stained virus particles showing the stain filled canals and basal attachments. (B) Negative stained CHPV particle showing typical vesiculovirus morphology, including the internal ribonucleoprotein coil. Inset shows a virus particle with a released helical ribonucleoprotein coil (Rao et al, 2004).](image-url)
Epidemics of the disease were also recognized in the several districts of Maharashtra including Bhandara, Nagpur, Gadchiroli and adjoining border of Andra Pradesh (NIV, Unpublished observation). In year 2009 same area has been affected and the epidemic is still on.
1.3 Seroprevalance studies conducted in India:

Sero-prevalance studies were done by NIV, Pune in different animals. Out of 180 animal sera from highly affected areas of the Karimnagar and Warangal districts of Andhra Pradesh, 33 (18.3%) had virus-neutralizing antibodies to CHPV. The positive animals consisted of pigs (30.6%), buffalos (17.9%), cattle (14.3%), goats (9.3%) and sheep (7.7%). However there is no evidence of viremia present in domestic animals (Joshi et al, 2005). Thus the question of reservoir still remains unanswered.

1.4 Diagnostics:

Following the crucial role of CHPV in year 2003 and 2004, diagnosis becomes an important issue. While investigating the outbreak of Andhra Pradesh, 2003, an assay for the detection of IgM anti-CHPV was quickly developed (Rao et al, 2004). However, considering rapid course of diseases and early mortality, detection of viral RNA emerged as a method of choice. Accordingly heminested RT-PCR (Rao et al, 2004) and nested RT-PCR (Chadha et al, 2005) were developed and used. Usually titers of the CHPV in blood are low and therefore single PCR does not yield any positivity necessitating use of nested RT-PCR. But, in view of the problems of cross contamination with nested RT-PCR an attempt was made to develop a real time one step RT-PCR (Kumar et al, 2008). This would be useful for diagnosis as well as quantitation of the virus in vitro and in vivo.

1.4.1 Real time one step RT-PCR:

Real time RT-PCR is a technical advancement over conventional RT-PCR, this method combines PCR chemistry with fluorescent probe based detection of amplified product in the same reaction tube. In comparison with conventional RT-PCR, the fluorogenic assay present many advantages. In addition to its greater/equal sensitivity, this technique is rapid, allowing several samples to be processed in few hr. The assay is a closed system in which the tube is never opened post amplification, thus eliminates the possibility of cross-contamination. Therefore real time RT-PCR provides advantage of quantifying RNA amount with a high degree of reproducibility and precision without contamination (Reviewed by Mackey et al, 2002; Leutenegger CM,
Moreover, measurement of viral load by real time PCR has become a widely used approach to studying the effect of antiviral therapies or the emergence of drug-resistant variants (Clarke JR, 2002; Pas et al, 2005). Well known examples include the hepatitis viruses C and B (HCV; HBV), or human immune deficiency virus (HIV) infections, in which clinical management is based on the monitoring of viral load in peripheral blood (Najioullah et al, 2001; Garson et al, 2005; Palmisano et al, 2005). The importance of quantitative virus analysis is further underscored by the fact that different viruses may persist in a latent state after primary infection in healthy immunocompetent individuals as well as in asymptomatic patients, and cause universally positive results in PCR assays. Mere detection of these viral pathogens by PCR may not be relevant for the clinical outcome in these individuals. By contrast, consecutive assessment of the virus load seems to play an important role for the diagnosis and prognosis in patients with viral reactivation, by providing a basis for timely initiation of appropriate treatment (Yoshikawa T, 2003; Zhong et al, 2004; Cesaro et al, 2005).

1.5 Prevention/Treatment:

Keeping unavailability of any antiviral or therapeutics for CHPV infection, we at NIV, developed a recombinant Glycoprotein (rGp) based vaccine (Venkateshwarlu and Arankalle, 2009). Current rGp based CHPV vaccine has achieved great success in mice. Assessment of this candidate vaccine in higher animals is need of hour keeping recent outbreaks in mind.

1.6 Genome organization:

It is important to mention in the beginning itself that although, CHPV closely resembles with the prototype vesiculovirus, Vesicular Stomatitis Virus (VSV), it could be readily distinguished by its ability to infect humans and based on serology. Therefore research on otherwise extensively studied family member VSV has been addressed in detail where no information specific for CHPV is available in this review.

The CHPV genome consists of a linear, single stranded negative sense RNA molecule of approximately 11,120 Kb (Marriot AC, 2005). Encoding five
genes, the nucleocapsid protein (N), phosphoprotein (P), matrix protein (M),
glycoprotein (G) and polymerase large protein (L). A 49-nucleotide leader (Le)
RNA is transcribed from the 3’ genomic terminus, which is non-translated,
uncapped, and non-polyadenylated and a 46-nucleotide trailer (Tr) sequence
at the 5’ end remain untranscribed (Fig 1C). Transcription of viral genes
occurs in sequential manner from a single promoter at 3’-end of the genome
resulting in the decrease amount of each transcript in order 3’- Le-N-P-M-G-L-
5’ similar to VSV (Reviewed by Basak et al, 2007).

Despite its growth in high titer in various cell lines and other laboratory
animals, no attempt was made to sequence the full genome or other related
studies primarily because of its low pathogenicity to humans or domestic
animals until the 2003 outbreak. However, quite a number of studies have
been carried out on the virus genome leading to fragmented information about
all the genes of CHPV (Giorgi et al, 1983; Masters and Banerjee, 1987;

The full genome map of 1965 isolates needs to be further analyzed
with full genome sequences of 2003, 2004 and 2007 CHPV isolates for the
better understanding CHPV (NIV, unpublished data). Interestingly,
comparative sequence analysis revealed that CHPV is evolutionary
equidistant from the new world vesiculoviruses, i.e. VSV Indiana (VSVind) and
VSV New Jersey (VSVnj) and is closely related to the Asian vesiculovirus
Isfahan (Marriott AC, 2005).

1.7 CHPV proteins and its function:

1.7. 1 N protein:

N protein is the most abundant viral protein in the infected cells. CHPV
N gene codes for a 422 amino acid polypeptide. Expression and purification of
the N protein led to its biochemical and biophysical characterization
(Majumder et al, 2001). Sequence analysis of CHPV N gene revealed almost
50% sequence homology to N protein of VSVind or VSVnj serotypes
(Masters and Banerjee, 1987). The biological function of N protein is to bind
with nascent leader RNA to initiate encapsidation of replication product

simultaneously to synthesis (Blumberg et al, 1983; Wertz GW 1983; Patton et al, 1984; Banerjee AK, 1987a).

Aggregation prone nature of N protein poses a major obstacle towards biochemical characterizations. However, Majumder et al, (2001) were able to express a soluble form of CHPV-N protein in bacteria without the need for co-expressed P protein. Transmission electron microscopy of homogenously purified CHPV N protein removed from detergent showed disc-like appearance as obtained for VSV N–P complex. Subsequent studies revealed a role for osmolytes in preventing further aggregation of this oligomer N protein (Majumder et al, 2001). P protein was identified as N protein specific chaperone that prevented N protein aggregation in vitro and in vivo independent of its phosphorylation status (Majumdar et al, 2004).

1.7.2 P protein:

P protein, a 32.5-kDa protein, plays an important role in transcription and replication of CHPV (Chattopadhyay and Chattopadhyay, 1994). CHPV P gene codes for a 293 amino acid polypeptide. In VSV it is well documented that RNA genome is transcribed and replicated by RNA dependent RNA polymerase. It is composed of two polypeptides; the large protein L which has the catalytic activity and P protein acts as cofactor. The P protein of CHPV is known to be phosphorylated by casein kinase II, at Ser62 similar to VSVind, VSVnj (Chattopadhyay et al, 1997; Basak et al, 2003). Recombinant expressed P protein has shown that N-terminal 46 amino acid is responsible for phosphorylation-mediated dimerisation (Raha et al, 2000).

To gain the insight for the mechanism of transcription performed by the P protein of CHPV, in vitro reconstitution of transcription with purified components was done by Chattopadhyay et al (1997). This study concluded that when purified L protein was incubated with N-RNA complex in a reaction mixture for in vitro transcription, it was unable to synthesize viral mRNA. However, addition of viral P protein along with L, and not P alone, allowed for viral mRNA synthesis. This confirmed the role of P protein as an activator of viral transcription (Chattopadhyay et al, 1997) similar to VSV (Banerjee AK, 1987b).
Based on cloning and expression of P protein of CHPV revealed the molecular weight of the protein was calculated to be 32.5 KDa (Chattopadhyay and Chattopadhyay, 1994), however further studies revealed about the serine-62 at the N-terminal acidic domain of the P protein which is Casein Kinase II (CKII) phosphorylated and essential for transcription (Chattopadhyay et al, 1997) as seen by mutation studies. Mutated version of P protein with alanine substituted for serine62, when tested in vivo was unable to activate transcription and rather inhibited viral mRNA synthesis in a trans-dominant manner (Basak et al, 2003). While the unphosphorylated form of P protein (P0) is essential for the replication in VSV (Pattnaik et al, 1997), the phosphorylation of P protein at Ser-62 (P1) is indispensable for transcription. However, P1 undergoes further round of modification by L-associated kinase (LAC) at its C-terminal domain to form P2. This sequential phosphorylation event was proposed to modulate transcriptional activation property of VSV-P (Barik and Banerjee, 1992a, b; Banerjee AK, 1987b; Chattopadhyay and Banerjee, 1987).

Overall, it seems logical to target the P protein, as L protein works in close association of P protein. Its small size and important function make it an attractive target to modulate CHPV replication with less effort.

1.7.3 M protein:

CHPV matrix protein (M protein) is a 229 amino acid polypeptide of 26.3 kDa molecular weight. It lies in the inner surface of the virion to join core nucleocapsid to the membrane and has a highly basic N-terminal domain, with eight lysine residues, which enables membrane binding (Ogden et al, 1986) and is separated from the rest of the polypeptide by a triple proline sequence (Rose and Gallione, 1981). M protein plays an important role in virus life cycles, for example, it is important for viral assembly and budding (De et al, 1982). However, M protein in VSV is observed to be the most lethal protein for the hosts mainly because it induces the cytopathic effect in the infected cells (Licata and Harty, 2003). It was shown to shut off host transcription by RNA polymerase I and II (Ahmed and Lyles, 1998) and it is also found responsible for the inhibition of host gene expression in VSV (Enninga et al, 2002). Efforts of Marriot and co-workers lead to identification
of the CHPV M protein specific shut off of transcription by cytomegalovirus immediate early promoter in vivo (Taylor et al, 1999). As this protein is most lethal among the different proteins encoded by CHPV, we decided to target this gene also by siRNA.

1.7.4 G protein:

Glycoprotein (G protein) a 59.5 kDa protein, is the spike protein of CHPV that enables virus absorption, assembly and budding. In rhabdoviruses G protein elicits antibody response and therefore it is considered as a major antigenic determinant (Neumann et al, 2002). Cloning and expression studies on CHPV-G protein revealed presence of a N-terminal cleavable signal peptide, two N-liked glycosylation sites at the N-terminal ectodomain, a membrane anchor domain and a cytosolic domain at the C-terminus (Masters et al, 1989). It was proposed that low pH induced conformational change in the G protein within endosome subsequent to viral entry enables membrane fusion to release core particle in two sequential steps into the host cytoplasm (Le Blanc et al, 2005). However, recently solved crystal structure of the VSV-G protein ectodomain in its postfusion state revealed classic hairpin conformations, large numbers of protonated residues stabilized at low pH state and also a novel fold combined features of class I and class II fusogenic peptides (Roche et al, 2006). G protein of CHPV was found to be ideal candidate for the vaccine development (Venkateshwarlu and Arankalle, 2009). In this study CHPV G protein was expressed in baculovirus expression system and the purified protein was further evaluated for its immunogenicity in mice. The encouraging result obtained in this study is a testimonial for its potential as candidate vaccine. This protein was also exploited for the development of recombinant ELISA for the detection of IgG antibodies (NIV unpublished data).

1.7.5 L protein:

L protein is largest viral protein of 2092 amino acid polypeptide of approximately 238.5 kDa in size (Marroit AC, 2005). Viral transcriptase is composed of L and P proteins. L protein retains the catalytic activity of RNA polymerization, capping and polyadenylation. Comparison of deduced amino
The acid sequence of L protein of CHPV with that of different rhabdoviruses exhibited a high degree of homology. Four conserved motifs present in VSV (Poch et al, 1989) are also present within CHPV L protein in a central block that is thought to mediate RNA polymerization (Marriott AC, 2005). A unique feature of VSV mRNA synthesis is the involvement of L protein in 5’-end modification of the nascent mRNA that includes methylation and capping. However, unlike cellular guanylyltransferase, L protein incorporates GDP rather than GMP in the capped structure as Gp(α)p(β)-p(α)A. The 5’ end modification events were proposed to be successive to transcription initiation, whereby, nascent mRNA termini maintains contact with transcribing polymerase until modified (Stillman and Whitt, 1999). It is important to note here that leader RNA lacks 5’ cap structure. Addition of poly(A) tail to the viral mRNA is also because of the L protein, where, polymerase slippage during transcription termination at U7 tract is believed to add A residues at the 3’ end of mRNA (Barr et al, 1997). L protein has shown to have the protein kinase activity as mentioned elsewhere and it is also known to associate with cellular translation elongation factors (Banerjee AK, 1987a, b, Das et al. 1998).

1.8 Life cycle of CHPV:

Here we will discuss the general steps involved in the replication of VSV. Although many of these events involved in the life cycle of VSV occur simultaneously in an infected cell, it is convenient to consider the process of infection as a linear series of events that proceeds in the following order: adsorption, entry and uncoating, transcription, replication, assembly, and budding.
Fig. 4 Diagram of Rhabdovirus life-cycle. The steps illustrated are virus adsorption and penetration by endocytosis, envelope fusion with endosome membranes, release of nucleocapsids containing parental genomes into the cytoplasm, primary (1°) transcription, genome replication to produce nucleocapsids containing antigenomes and progeny genomes, secondary (2°) transcription, and assembly by budding from host plasma membrane. 

(Courtesy-http://www.bioweb.uncc.edu/Faculty/Grdzelishvili/index_files/image002.jpg).

1.8.1 Adsorption:

Rhabdoviral infection is initiated by attachment of virus to a receptor on the host cell surface. The receptors for the attachment of rhabdoviruses have been difficult to identify because of the generally broad host range and the binding properties of rhabdovirus particles. Virus adsorption is inefficient and difficult to quantitate for viruses in this family. For example, VSV binding is pH dependent, and maximal binding occurs between pH 6.5 and 6.0 (Fredericksen and Whitt, 1998); yet even at the optimal pH, binding fails to reach equilibrium (Matlin et al, 1982).
Furthermore, when virus is prebound at 4°C, only a limited amount of virus is internalized when cells are warmed to 37°C (Matlin et al, 1982). However, virus infectivity can be increased more than 20-fold when virus is adsorbed at pH 6.3 versus when virus is bound at neutral pH (Fredericksen and Whitt, 1998). The pH dependence of binding correlates well with pH-induced conformational changes in the G protein (Fredericksen and Whitt, 1998), suggesting that the receptor-binding site in the envelope protein becomes exposed during the transition from the neutral to the acid conformation. When analyzed in a competitive binding assay, high-affinity binding of $^{35}$S-methionine–labeled VSV to Vero cells (Schlegel et al, 1983). The binding inhibitor was resistant to protease and neuraminidase but was inactivated by phospholipase C, suggesting it was a phospholipid. Of all phospholipids tested, only phosphatidylserine totally inhibited the high-affinity binding of VSV to Vero cells and also inhibited VSV plaque formation by 80% to 90% but did not block herpesvirus plaque formation (Schlegel et al, 1983). Based on these results, it is thought that phosphatidylserine may be one of the VSV receptors, at least on Vero cells. The identification of phospholipid binding domains in other rhabdovirus glycoproteins supports this idea and suggests that phosphatidylserine binding is a common feature of rhabdovirus envelope proteins (Estepa and Coll, 1996).

1.8.2 Entry and uncoating:

After binding, the virions are endocytosed through a clathrin-dependent pathway typical of receptor-mediated endocytosis (Matlin et al, 1982). Subsequent reductions in the pH of the endocytic compartment eventually trigger a membrane fusion reaction between the envelope of the endocytosed virion and the endosomal membrane. This fusion event is catalyzed by the G protein and results in the release of the ribonucleoprotein (RNP) core into the host cell cytoplasm (Matlin et al, 1982). Either concomitant with membrane fusion or immediately after, M protein dissociates from the RNP core (Rigaut et al, 1991). The combined processes of membrane fusion and M protein dissociation constitute the uncoating event for rhabdoviruses. The trigger for M protein dissociation from RNPs is not known.
1.8.3 Transcription:

The first synthetic event that occurs after uncoating of RNPs is transcription of viral-specific mRNAs by the L–P3 (trimer of P protein) polymerase complex brought into the cell by the virion. Primary transcription occurs in the absence of protein synthesis, unlike genome replication, which requires newly synthesized N and P proteins and ongoing translation (Davis and Wertz, 1982). The template for VSV transcription is the genome RNA complexed with the N protein in a ribonuclease-resistant form.

Using UV-radiation for in vitro transcriptional mapping analysis the order of transcription of VSV genes was determined. These studies revealed that VSV mRNAs are synthesized in an obligatory sequential manner after polymerase entry at single 3' end of the genome termini i.e. at the beginning of leader gene (Ball and White, 1976; Abraham and Banerjee, 1976; Testa et al, 1980). Determination of relative molar ratios of different viral mRNAs within infected cells revealed that their abundance decreased with increasing distance from the 3' promoter in an order N>P>M>G>L, thus indicating a mechanism that also ensures polar transcription (Villarreal et al, 1976; Iverson and Rose, 1981).

An engineered version of VSV was utilized to further confirm these findings where an additional transcriptional unit (I) was inserted at N–P, P–M, M–G or G–L gene junction. Quantitation of I mRNA relative to the 3’ proximal VSV N mRNA expressed within cells infected with recombinant virus, consistently, revealed a gradual decrease in I mRNA synthesis with increase in distance from 3' termini of the genome (Wertz et al, 2002). Stop and start model of Emerson et al (1982) remains most widely accepted, till date. In this model, subsequent to single site entry at the beginning of leader gene, viral polymerase sequentially transcribes the genome with progressive attenuation at each gene boundary to result in decreasing amount of transcripts for genes that are distant from the entry site. Each termination event may lead to the polymerase to fall off from the template or may allow for re-initiation at the downstream promoter. Therefore, transcription from downstream genes is dependent on termination of upstream genes and reinitiation.
1.8.4 Replication:

Replication of CHPV is characterized by read-through of the gene boundaries by viral polymerase to synthesize an exact complement of (−) sense genome RNA. As discussed earlier transcriptase is mainly composed of an L–P3 complex, whereas the replicase consist of an L–(N–P) complex in which P protein is not phosphorylated.

During this process, the polymerase switches to replicative mode to copy entire genomic template into an exact polycistronic complement that acts as replication intermediate to produce many more copies of (−)ve sense genomes upon further rounds of replication. Progeny (−)ve sense genomes are also subjected to transcription, referred to as secondary transcription.

It is important to note here that virus specific genomic analogues, and not mRNAs, always remain encapsidated by N, while, it is believed that progressive encapsidation of nascent genome RNA during its synthesis is necessary for replication and/or protecting replication product from cellular RNases (Banerjee AK, 1987a; Barr et al., 2002).

The molecular mechanism that allowed for a switch in polymerase function from transcription to replication has remained obscure. Studies in recent years on both CHPV and VSV led to a different proposal to explain vesiculovirus transcription-replication switch.

According to “two separate promoters for transcription and replication proposal”, initiation of viral transcription occurs at the beginning of N gene, while polymerase initiates replication at the beginning of leader gene. Both the processes utilize same nucleocapsid template, whereas, modifications of the viral polymerase allow for binding at separate sites during replication (Whelan and Wertz, 2002; Barr et al, 2002).

Replication activity was seen in a complex from VSV-infected cells composed of L, P and N protein, in vitro (Gupta et al, 2003). Phophorylation of P was shown to be dispensable for the activity of this proposed tripartite replicase complex, thus, separating it from the classical L-P1 transcriptase complex. Accordingly, it was postulated that the integration of de novo synthesized N protein into a replicase complex resulted in differential promoter recognition by polymerase to initiate replication phase.
However, such model could not satisfactorily explain generation of leader RNA during viral RNA synthesis (Blumberg and Kolakofsky, 1981). But may be the abortive replication attempts by polymerase resulted in accumulation of leader RNA within cells. However, *in vitro* transcription with Chandipura virus nucleocapsid resulted in synthesis of leader RNA (Basak et al, 2003). Even as replication byproduct, presence of discrete leader RNA species within virus-infected cells implies a strong attenuation signal for polymerase at the leader-N boundary, that needs to be suppressed for productive replication. Also this model was proposed based on studies on an engineered version of VSV that can substantially differ from wild type virus in nucleocapsid conformation, thus, may allow for observed internal entry of the polymerase.

But according to another theory which considers the involvement of N protein and an auxiliary function for P protein, which maintains the N protein in its soluble form is important for swiching from transcription to genome replication.

**1.8.5 Assembly and budding:**

Assembly and budding of enveloped viruses is a well-orchestrated process, which occurs at defined membrane locations within the cell. For viruses that bud from intracytoplasmic organelles, key viral components, such as the viral glycoproteins, typically contain specific signals that result in targeting, or retention, at that site.

For rhabdoviruses, virus budding takes place primarily at the plasma membrane, which was initially thought to be the default site for virus budding. However, the recent identification of cellular proteins and lipids, which constitute components of the cellular budding machinery and that localize to sites of virus budding, have altered our view of virus budding such that the concept of a default budding site is no longer viable (Pornillos et al, 2002).

Thus, one of the major questions related to the mechanism of rhabdovirus budding is how different components of the assembling virion are recruited to sites of virus assembly at the plasma membrane and are specifically incorporated into the budding virion. Due to lack of data on CHPV and availability of plethora of data on VSV assembly and budding; here we
will use VSV as the prototype member and discuss literature available on other rhabdoviruses in comparison to VSV, wherever possible.

The early models of rhabdovirus assembly were primarily based on data from biochemical and genetic studies of temperature sensitive mutants and pseudotyped viruses produced from mixed infections. These models suggested that virus assembly occurs at the cell surface as the result of specific interactions between G protein delivered to the plasma membrane and M protein in association with nucleocapsids. The site of virus budding was thought to be determined by the location of the spike glycoprotein. This hypothesis was supported by the observations that VSV buds exclusively from the basolateral surface of polarized epithelial cells similar to the localization of the VSV G protein (Boulan and Pendergast, 1980; Fuller et al, 1984), but these results were difficult to reconcile with the observation that VSV readily formed pseudotypes with many different types of viral glycoproteins. Therefore it appeared that there was a lack of specificity in the assembly process (Zavada J, 1982). Interactions between the G and M proteins were thought to cause conformational changes in M monomers, which were converted from a soluble form into a two-dimensional network underlying G protein in the plasma membrane. Polymerization of M protein was proposed to occur concomitantly with its binding to the nucleocapsid and formation of bullet-shaped particles. These models suggested that the outward bulging of the membrane was caused by the spike proteins “pulling” the membrane and concomitantly anchoring the M protein layer to the membrane (Simons and Garoff, 1980). Host proteins were excluded from the budding sites by the closely apposed layer of M protein located just beneath the plasma membrane. Thus, it appeared that G protein was the initiator of the assembly process and that M protein aided in the process to mediate virus budding.

In a scholarly review, Jayakara et al (2004) proposed a simplified model of assembly and budding of rhabdoviruses, which involved different steps (Fig. 5).

(i) The first step is formation of the nucleocapsid core through binding of N protein to genomic (or antigenomic) RNA while the RNA is being synthesized. N protein polymerization on the nascent RNA occurs in the cytoplasm through
an exchange reaction in which P protein is released from N:P dimers as N associates with the sugarphosphate backbone of the RNA. Some cytoplasmic M protein may associate with the newly formed RNPs, but this is not sufficient for complete condensation into skeletons.

(ii) While RNPs are forming in the cytoplasm, G protein in the plasma membrane localizes to, or forms, sites (i.e., microdomains) that are favorable for the initiation of budding and RNP condensation by M protein (Fig. 5, steps I and II). Such sites favorable for budding must be formed soon after delivery of G to the cell surface since virus budding commences as soon as 2–3 h post-infection.

(iii) When sufficient amounts of M protein have accumulated in the cytoplasm and a sub-population has localized to the inner leaflet of the plasma membrane, nucleocapsids become localized to the plasma membrane and are condensed into tightly coiled structures (skeletons) via interaction with M protein. The condensation of RNPs occurs at regions of the plasma membrane containing locally high concentrations of G protein, which results in formation of the bud site (Fig. 5, steps II and III). These G-enriched microdomains may favor membrane curvature and virion extrusion.

(iv) Interaction of M–RNPs with the bud site and the progressive condensation of the RNP core results in evagination of the membrane in which envelopment of the underlying condensed RNP core occurs via recruitment of both soluble and membrane-associated M protein into the condensing skeleton. Thus, condensation of RNPs by M protein results in formation of bullet-shaped protrusions extending from the plasma membrane (Fig. 5, steps III and IV).

(v) Cellular components, such as Nedd-4 or a related ubiquitin ligase, or other as of yet undefined proteins, associate with the PY motif of M which either directly or indirectly result in fission and release of mature virions (Fig. 5, step V).

While this model was just an attempt to incorporate all the findings done till that time, there are several key unanswered questions that still needed to be addressed experimentally. For example, despite high concentrations of both RNPs and M protein in the cytoplasm of infected cell, why does RNP condensation apparently occur primarily at the plasma
membrane? What components in or near the plasma membrane initiate the budding process? Is M protein located inside or outside of the RNP coil, or both? What determines the site of budding at the plasma membrane? What is the contribution of host factors to rhabdovirus budding?

**Fig 5** Model for rhabdovirus assembly and budding. (I) Primed nucleocapsid-Mi pre-assembly complexes associate with the inner leaflet of the plasma membrane via interaction with membrane-associated M protein. (II) Soluble M protein polymerization and additional membrane-associated M –RNP-M interactions initiate RNP condensation, which results in formation of bud sites. The bud sites form preferentially at regions of the plasma membrane that contain locally high concentrations of G protein (G microdomains). (III) Continued RNP condensation and interaction between membrane-associated M protein results in extrusion of the forming virion. (IV) Cellular proteins, brought to the bud site via interaction with the PY-motif in M protein complete the final step in budding and facilitate the release of virions (V) via a membrane fission event. The inset shows a model of an assembled virion in cross-section with the central M protein core scaffold, the membrane-associated M proteins bound to the outside of the RNP core, and the glycoprotein spikes protruding from the viral envelope.
1.9 Immunology of CHPV:

Tripathy et al (2005) reported significant change in INF-γ, IL-2 in patients compared to controls, whereas IL-6 and TNF-α were higher in patients with >2 days of illness compared to controls. However, in another study, the authors have shown that there is blood brain barrier (BBB) breach early in infection as measured by Evans blue dye exclusion test. Infant mice are susceptible to infection intravenous (i.v.), intraperitonial (i.p.) and intracerebral (i.c.) mode whereas adults are susceptible only via intracerebral route. Raise in proinflammatory cytokines like TNF- α, INF-γ, IL2, IL6 with 24 hr of infection can aid leak in BBB. Authors also shown that IgM antibody for virus appears as early as 72 hr post infection (PI) which seems to decrease blood viral load but could not decrease CNS load. CD4+, CD8+ and CD19+ cells were also less in infected mice and antigen specific suppression of T cell proliferation was witnessed at 72 hr. Authors also report that passive immunization was able to prevent infection before viral infection and not at 24 hr PI. Thus this paper is a significant step in understanding the etiopathogenesis of this viral infection (Balakrishnan and Mishra, 2008).

1.10 RNAi:

RNA interference (RNAi) was originally described as a natural antiviral mechanism in plants. In RNAi long double– stranded RNA (dsRNA) is processed by the enzyme dicer into small, 21- to 25-nt dsRNA molecules called short interfering RNAs (siRNAs), which mediate the sequence-specific degradation of the target mRNA (reviewed by Dixon RA, 2001; Tijsterman et al, 2002; Plasterk RH, 2002; Bernstein et al, 2001; Ahlquist P, 2002; Mello and Conte, 2004; Meister and Tuschl, 2004; Hannon and Rossi, 2004). RNAi has emerged as a powerful tool for gene silencing with a potential for therapeutic use in viral infections (Novina et al, 2002; Bennink and Palmore, 2004; Bitko et al, 2005).
1.11 History and mechanism of action:

Post transcription gene silencing (PTGS) was initially described in plants, when attempts to increase the expression of chalcone synthase in petunias resulted in the effective suppression of both the endogenous gene as well as the introduced gene; the phenomenon was coined “cosuppression” (Napoli et al., 1990). Later, the use of antisense RNA [single-stranded (ss) RNA] sequence that is complementary to a particular mRNA to suppress the expression of the par-1 gene in Caenorhabditis elegans yielded interesting results, injecting sense strand RNA (essentially duplicating the par-1 gene transcript) also resulted in gene silencing (Guo and Kempheus, 1995). It was finally determined that dsRNA was even more effective than ssRNA in gene silencing; the phenomenon was called "RNA interference" (Fire et al., 1998).
During the last 10 years, tremendous progress has been made in revealing the underlying mechanism of RNAi and related silencing responses. The molecular bases of the silencing process have emerged from combination of biochemical and genetic studies in several model systems.

1.12 Mechanistic studies:

Although the basic mechanisms of RNA silencing were first described in plants as mentioned earlier, it was the work of Fire and co-workers (1998) in *C. elegans* which demonstrated that dsRNA could trigger the silencing. In both plants and *C. elegans*, exposure to dsRNA results the occurrence of sequence-specific silencing mainly at posttranscription level (*Fire et al 1998; Waterhouse et al, 1998*). Loss of gene expression in both cases was due to degradation of the target mRNA (*Fire et al, 1998; Vaucheret et al, 1998*). These observations suggested that dsRNA destabilize homologous mRNA, probably through a process that acted in the cytoplasm. But the mechanism remained elusive until the development of cell free systems that recapitulate RNAi.

1.13 Models for study the mechanism of RNAi:

The key breakthrough was Kennerdell and Carthew’s demonstration that dsRNA could induce sequence specific silencing upon introduction to *Drosophila* embryos (*Kennerdell and Carthew, 1998*). After that the broad outlines of a biochemical pathway for RNAi have emerged from experiments using cell free lysates of *Drosophila* syncytial blastoderm embryos or extracts from cultured S2 cells.

The pathway has been deciphered by studying the ATP dependence at each step, by using intermediates in the pathway to initiate RNAi, and by classical biochemical fractionation of the protein enzymes and protein RNA complexes required for RNAi. These studies have revealed a number of key steps. First, experiments using *in vitro* systems demonstrated that dsRNA target the corresponding mRNA for destruction (*Tuschi et al, 1999*). Second, *in vitro* studies established that cleavage of long dsRNA produces approximately 22 nucleotide of short interfering RNA (siRNA) by the action of Dicer (*Zamore et al, 2000*). Third, *in vitro* experiments demonstrated that
siRNA are intermediate in the RNAi pathway (Nykanen et al., 2001; Elbashir et al., 2001a) and siRNA directs an RNA protein complex called RNA induced silencing complex (RISC), to destroy the target mRNA (Hammond et al., 2000). Fourth in vitro studies showed that the siRNA programmed RISC directs the endonucleolytic cleavage of the target mRNA at a single phosphodiester bond, whose position on the target is determined by its distance from the 5’-end of the siRNA (Zamore et al., 2000; Elbashir et al., 2001a). Finally, studies in cell free extracts demonstrated that RISC-associated activities can completely destroy the target mRNA (Hammond et al., 2000).

The most important breakthrough from in vitro studies was the identification of the structure and function of the siRNAs. In a series of remarkable studies, Tuschl and co-workers sequenced the small RNA produced upon incubation of long dsRNA in a Drosophila embryo lysate, deduced their architecture, and then demonstrated that synthetic siRNA duplexes direct target cleavage in vitro (Elbashir et al., 2001a). This further lead to the discovery that synthetic siRNA can trigger RNAi ex vivo in cultured mammalian cells (Caplen et al., 2000; Elbashir et al., 2001b) and in vivo in mice (Lewis et al., 2002; McCaffrey et al., 2003; Song et al., 2003) as well as plants (Klahre et al., 2002).

1.14 Production of siRNAs by Dicer:

Biochemical studies on Dicer are still in their infancy, as a lot of intriguing enzymological questions need to be answered. Dicer is similar to bacterial RNase III in that it can cleave dsRNA in an apparent sequence-nonspecific manner. The siRNA products possess two nucleotide 3’ overhangs, with 5’ phosphate, 3’-hydroxyl termini. On the basis of common protein domain features, the type of product termini, and the divalent metal ion requirement, it is expected that the chemistry of phosphodiester hydrolysis is the same as that used by bacterial RNase III. The sequence nonspecificity is an asset because it allows recognition and cleavage of virtually any dsRNA. The structural features of siRNA important for function have been analyzed. siRNA with two-nucleotide 3’ overhangs were most efficient in Drosophila embryo lysates (Elbashir et al., 2001c). There is not a strict ribose
requirement, however, as substitution of terminal residues with 2'-deoxyribose or 2'-O-methylribose residues does not affect function (Elbashir et al, 2001c).

The biochemical properties of purified recombinant human Dicer have been described. Human Dicer requires a divalent metal ion (Mg$^{2+}$, Mn$^{2+}$ or Co$^{2+}$) for activity, with cleavage of dsRNA directly providing the mature approximately 21-23 bp siRNA species. However, human Dicer does not require ATP to cleave dsRNA (Provost et al, 2002; Zhang et al, 2002), which is consistent with the ATP independence of E. coli RNase III, which can bind ATP. Immunofluorescence analysis has localized Dicer to the endoplasmic reticulum (ER), with no evident of nuclear staining (Provost et al, 2002). The ER location would place Dicer in proximity to the RISC complex, which is associated with the translation machinery (Hammond et al, 2000). Thus apparent lack of nuclear localization suggests that cleavage takes place in the cytoplasm to produce mature siRNA/miRNA.

1.15 Dicer protein in different organism:

Dicers are large multidomain proteins found in eukaryotes (e.g., animals, plants and Schizosaccharomyces pombe, but not in Saccharomyces cerevisiae). Metazoan and plant Dicer proteins generally contain ATPase/helicase, DUF283, PAZ, Two RNase III, and a dsRBD, but Dicer of lower eukaryotes frequently has less complex organization (Fig. 4). The PAZ, dsRBD, and RNase III domains are involved in dsRNA binding and cleavage. The PAZ domain is also found in PPD (PAZ and Piwi Domain) or argonaute proteins that are involved in RNAi pathways (Jaskiewicz and Filipowicz, 2008).

Mammalian genome encodes only one Dicer protein. On the other hand, plants, such as Arabidopsis thaliana, poplar and rice express four Dicer like proteins (Dcl). Fungi, such as Neurospora crassa, and insects (Drosophila and mosquito) contain two Dicer genes.
A new classification for RNase III enzymes. Class I contains bacterial and fungal RNase III orthologs, class II contains Dicer and Drosha proteins. Drosha enzymes are found only in animals. Schematic organization of selected proteins of the RNase III family with different domains are shown (Courtesy- Current topics in microbiology and immunology, RNA interference, vol 320, Springer)

1.16 Proteins interacting with Dicer:

Although recombinant Dicer is active as a dsRNA-specific endonuclease in vitro, in cells it generally functions in association with other proteins as a component of multiprotein complexes.

1.16.1 dsRBD-Containing Cofactors of Dicer in mammals:

Cleavage of pre-miRNA and dsRNA substrates seems to be invariably catalyzed by Dicer in association with double stranded RNA binding domain (dsRBD)-protein cofactors. The first such dsRBD protein, Rde-4 (RNAi deficient-4), was identified in a genetic screen in C. elegans (Tabara et al,
But in human cells, TRBP [Human immunodeficiency virus-1 (HIV-1) transactivating response (TAR) RNA binding protein] was found to be a dsRBD protein partner of Dicer (Chendrimada et al., 2005; Haase et al., 2005). TRBP is required for the optimum function for gene silencing by siRNAs and miRNAs. Another mammalian dsRBD protein, PKR activator (PACT), which is 42% identical to TRBP, has also been recently found to interact with Dicer. Its depletion strongly affected the accumulation of mature miRNAs in human cells (Lee et al., 2006). TRBP may be important for Dicer function in vivo since its titration by overexpression of TAR RNA in human cells leads to the inhibition of Dicer activity (Bennasser et al., 2006).

1.16.2 Argonautes/PPD:

Another group of well-characterized Dicer partners is represented by PPD or Argonaute proteins. Members of the PPD proteins family contain two signature domains: a PAZ domain in the center and a PIWI domain at the carboxyl terminus. Genetic and biochemical studies have indicated that PPD proteins can be divided into two subgroups: those that are homologous to the Arabidopsis Argonoute-1 and are ubiquitously expressed, and those that are most similar to the Drosophila Piwi, expressed in germline stem cells (Carmell et al., 2002; Tolia and Joshua-Tor, 2007). These sub-groups are referred to as Argonaute (Ago) and Piwi proteins. The interaction between human Dicer and two PPD proteins, Ago-2 and Hiwi, has been investigated in detail (Tahbaz et al., 2004), revealing that a subregion of the PIWI domain, the PIWI-box, binds directly to the Dicer RNase III domain. Ago-2, Hiwi, and Dicer are present in soluble and membrane-associated fractions, including that interactions between these two types of proteins may occur in multiple cellular compartment.

1.16.3 Other proteins interacting with Dicer:

Several other proteins have been found to interact with Dicer. In C. elegans, the RNA-helicase-related protein DRH-1, which is required for RNAi, was found to interact with Rde-4 and Dicer (Tabara et al., 2002). FMRP, an mRNA-binding protein involved in the pathogenesis of fragile X syndrome, has been shown to interact with Dicer and Ago-1 in mammalian cells (Jin et
al, 2004) and Drosophila dFXR, a fly ortholog of FMRP, interacts with Dicer-1 and Ago-2 (Caudy et al, 2002; Ishizuka et al, 2002). In mammalian male germ cells, Dicer was shown to interact with mouse vasa homolog (MVH), with both proteins localizing to the P body-related structure known as chromatoid body (Kotaja et al, 2006).

1.17 Role of Dicer in vivo:

Knockout experiments indicated that Dicer is essential for vertebrate development. Disruption of the Dicer gene in mice arrests embryogenesis at day 8.5 (Bernstein et al, 2003), while mice with a strong hypomorphic mutation, resulting from the deletion of the first two Dicer exons, die between 12.5 and 14.5 days of gestation and display defects in angiogenesis (Yang et al, 2005). Very recently a Dicer deficient mouse line was established and functional analysis showed them to be hypersusceptible to VSV infection due to impaired function of miR-93 and miR-24 (Otsuka et al, 2007).

1.18 RISC: the catalytic machinery of RNAi:

The development of in vitro assays for RISC from Drosophila embryo lysate and S2 cells extracts opened the way for the isolation of this enzyme complex and identification of its component. RISC is a multiprotein complex reported to be in the range of 200 to 500 kDa. Several in vitro experiments have been done using minimal RISC containing only the AGO “slicer” and the guide strand of siRNA; these complexes are in the range of 150-200 kDa. It is postulated that the various protein components found in vivo in the RISC might play an important role in assembly, target cleavage, formation of a distinct effector complex, or all the above. RISC has been found to have following components upon purification: (1) Ago2, (2) dFXR (Drosophila ortholog of fragile X mental retardation protein), (3) VIG (vasa intronic gene), (4) Tudor-SN (a nuclease with tudor domain and bearing five nuclease domain homologous to the Staphylococcus nuclease domain), (5) R2D2 (a dsRNA binding protein with two dsRNA binding domains), (6) Aubergine (an Ago family protein), (7) Armitage-RNA helicase, and potentially other unidentified factors (Reviewed by Kavi et al, 2008).
RISC is an endonuclease as revealed by the biochemical characterization of its 5'-PO$_4$ and 3'-OH cleavage products, which are of equal length. Thus RISC is a 5'-phosphomonoester producing RNA endonuclease (Meister et al., 2004). The cleavage site on the target RNA lies between the 11$^{th}$ and 12$^{th}$ nucleotide, where the 1$^{st}$ nucleotide on the target base pairs with the 21$^{st}$ nucleotide on the siRNA guide strand. RISC requires Mg$^{2+}$ for its catalytic activity. As addition of EDTA (ethylenediaminetetraacetate) reduces the target RNA cleavage (Schwarz et al., 2004).

It is believed that the two nonbridging oxygens of the scissile phosphodiester bond between the 11$^{th}$ and 12$^{th}$ nucleotide on the target RNA may be a ligand for Mg$^{2+}$. The substitution of the ribose sugar of the target (substrate) with bulky moieties such as 2'-O-methyl groups severely decrease target cleavage; however, substitution of the ribose with 2'-deoxy modification did not affect the target cleavage significantly. This result highlights the fact that the limiting step in the case of RISC is not the rate of chemical cleavage but rather steric hinderance, conformational transitions associated with active site residues, or both (Revised by Kavi et al., 2008).

### 1.19 RISC assembly and target mRNA cleavage:

Recent studies utilizing Drosophila embryo extract has indicated that the assembly of active siRNA loaded RISC takes place in a step-wise manner. During these steps many different proteins together with the thermodynamic properties of the siRNA duplex play a critical role in the selection of bona fide siRNA “guide strand,” which enters the RISC. The siRNA duplex, which has been synthesized chemically, has different thermodynamic stabilities at either end. The combined action of dcr2-R2D2 ensures that the strand whose 5'-end is near the relatively thermodynamically unstable end of the siRNA duplex enters the RISC (Tomari et al., 2004b). R2D2 binds to the 5'-end of the strand, which lies near the more thermodynamically stable end; this strand is referred to as “passenger strand.” This strand is then cleaved by Ago2 and finally discarded.

First step in the RISC assembly is the binding of the R2D2-Dicer-2 heterodimer to the siRNA duplex. This complex is now known as RDI (R2D2-Dicer-2 initiator) complex (also known as R1). Both proteins are required for
loading siRNA into RISC and both dsRNA binding domains of R2D2 are required (Liu et al., 2006).

Recently, it was found that RDI complex itself forms holo-RISC (active RISC) (Pham and Sontheimer, 2005). The formation of this complex was ATP independent. Recent evidence showed the formation of another complex known as RLC (RISC loading complex). In the RLC, siRNA is unwound with help of AGO2, which cleaves the passenger strand bound by R2D2 and is thus discarded. This paves the way for the guide strand entry into the holo-RISC. The catalytic activity of the RNAi machinery lies in this 80S holo-RISC. In-depth analysis revealed the importance of 5'-PO$_4$ group, which needs to be present on both strand of the siRNA duplex. Absence of both 5'-PO$_4$ resulted in failure of the duplex to initiate RNAi, while presence of one of the groups can initiate the formation of early complexes such as RDI and R2 but not holo-RISC. It is believed that 5'-PO$_4$ groups are recognized at multiple steps in the RISC assembly. The 2'-hydroxyl group (of the ribose sugar) of the 5'-terminal nucleotide, when substituted with 2'-deoxyribose, results in selective entry of this modified strand into RISC. Studies performed with mammalian and HeLa cell lines indicated that P bodies/GW bodies are the sites of target mRNA degradation (Coller and Parker, 2004). This observation is also consistent with earlier reports showing that P bodies (GW bodies) are enriched with decapping enzyme, protein involved with degradation of mRNA and translation inhibitory proteins (Lui et al., 2005a; b).

1.20 Types of small silencing RNA:

Basis of classification:

siRNAs can be classified based on the following properties: (1) Proteins involved in biogenesis of small RNA, (2) Mode of regulation of these small RNA and (3) Size of these small RNA.

In a recent review, the major types of siRNAs were classified according to the molecules that trigger their production — a classification scheme that best captures the biological distinctions among small silencing RNAs (Reviewed by Ghildiyal and Zamore, 2009) (Fig. 8). The proteins involved in
the biogenesis of these small RNA and their functions has been mentioned in Table 1.

Fig. 8 Classification of small RNA based on the molecules involved in their biogenesis.
<table>
<thead>
<tr>
<th>Name</th>
<th>Organism</th>
<th>Length (nt)</th>
<th>Proteins</th>
<th>Sources of Trigger</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>miRNA</td>
<td>Plants, algae, animals, viruses, protists</td>
<td>20-25</td>
<td>Drosha (animals only) and Dicer</td>
<td>Pol II transcription (pri-miRNAs)</td>
<td>Regulation of mRNA stability, translation</td>
</tr>
<tr>
<td>casiRNA</td>
<td>Plants</td>
<td>24</td>
<td>DCL3</td>
<td>Transposons, repeats</td>
<td>Chromatin Modification</td>
</tr>
<tr>
<td>tasiRNA</td>
<td>Plants</td>
<td>21</td>
<td>DCL4</td>
<td>miRNA-cleaved RNAs from the TAS loci</td>
<td>Post-transcriptional Regulation</td>
</tr>
<tr>
<td>natsiRNA</td>
<td>Plants</td>
<td>22-24</td>
<td>DCL1, DCL2</td>
<td>Bidirectional transcripts induced by stress</td>
<td>Regulation of stress-response genes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exo-siRNA</td>
<td>Animals, fungi, protists</td>
<td>~21, 21, 24</td>
<td>Dicer</td>
<td>Transgenic, viral or other exogenous dsRNA</td>
<td>Post-transcriptional regulation, antiviral defense</td>
</tr>
<tr>
<td></td>
<td>Plants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endo-siRNA</td>
<td>Plants, algae, animals, fungi, protists</td>
<td>~21</td>
<td>Dicer (except secondary siRNAs in C. elegans)</td>
<td>Structured loci, convergent and bidirectional transcription, mRNAs paired to antisense pseudogene transcripts</td>
<td>Post-transcriptional regulation of transcripts and transposons; transcriptional gene silencing</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>piRNA</td>
<td>Metazoans excluding Trichoplax adhaerens</td>
<td>24-30</td>
<td>Dicer-independent</td>
<td>Long, primary transcripts</td>
<td>Transposon regulation, unknown functions</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>piRNA-like (soma)</td>
<td>Drosophila melanogaster</td>
<td>24–30</td>
<td>Dicer-independent</td>
<td>In ago2 mutants in Drosophila</td>
<td>Unknown</td>
</tr>
<tr>
<td>21U-RNA piRNAs</td>
<td>Caenorhabditis elegans</td>
<td>21</td>
<td>Dicer-independent</td>
<td>Individual transcription of each piRNA</td>
<td>Transposon regulation, unknown functions</td>
</tr>
<tr>
<td>26G RNA</td>
<td>Caenorhabditis elegans</td>
<td>26</td>
<td>RdRP</td>
<td>Enriched in sperm</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
1.21 Class of small RNA used for the gene silencing exogenously:

![Small RNA expression using Vector backbones](image)

**Fig. 9** Showing different strategies to express small RNA using vector backbone for the exogenous gene silencing.

1.21.1 Short hairpin RNA (shRNA):

At present, a variety of workable strategies have been developed that effect stable silencing in mammalian cells. In each case, the expression of the interfering RNA is driven by mammalian promoters from DNA vectors that are introduced into the cell by transfection or infection method and either propagated episomally or integrated into the genome. Most expressed dsRNA are very short, simple hairpin with 19-29 nucleotide double stranded stems. Because RNA polymerase III initiates and terminates small highly structured RNA transcripts precisely (Goomer and Kunkel, 1992), many groups have employed polymerase-III-dependent promoters to derive the expression of these molecules.

Mouse (Yu et al, 2002) and human U6 snRNA promoters (Paddison et al, 2002b; Paul et al, 2002; Sui et al, 2002), the human RNase P (H1) promoter (Brummelkamp et al, 2002; McManus et al, 2002a) and human Val-tRNA promoter (Kawasaki and Taira, 2003) have been used successfully. These promoters were found to be active in most if not all the embryonal and somatic cell types and offer similar level of constitutive expression. However, expression of miRNA-like structure from the RNA polymerase II Cytomegalovirus (CMV) immediate early promoters could also serve as substrate for Dicer (Zeng et al, 2002). Dicer recognizes the double-stranded region of this transcript and produces functional siRNAs. In another version, separate U6 promoters have been used to derive the transcription of single-stranded 21-nucleotide RNAs (Miyagishi and Taira, 2002). These transcripts presumably anneal within the cells to form structure identical to siRNAs.
In practice, the production of encoded hairpin constructs is relatively straightforward. In the simplest form, a hairpin target site sequence is chosen. Although this can be done “by hand,” a number of design tools are available on the Internet that simplify this mind-bending process (http://katahdin.cshl.org:9331/RNAi/, http://jura.wi.mit.edu/bioc/siRNA/, http://www.dharmacon.com, http://www.ambion.com/techlib/misc/siRNA_finder.html).

Chemically synthesized DNA oligonucleotides that encode the chosen sequence are annealed to form a double-stranded fragment, which is then cloned into a hairpin expression vector downstream from a eukaryotic promoter. The introduction of shRNA constructs to cells can be accomplished using any of the various gene transfer techniques. Although stable silencing of genes using expressed shRNA has already been put to work in a variety of applications, improvement and more ambitious applications of this technology are sure to occur. Improved shRNA-based gene silencing strategies may allow delivery of lower doses, thus reducing the likelihood of RNAi-related side effects.

1.2.1.2 Artificial miRNA (amiRNA):

Recent advances in gene silencing methodology are to modify the stem sequence of miRNA hairpin to achieve knockdown of artificially targeted genes (Zeng et al, 2002; McManus et al, 2002a; Boden et al, 2004; Zhou et al, 2005; Stegmeier et al, 2005; Silva et al, 2005; Dickins et al, 2005). A number of natural miRNA hairpins exist in clusters of multiple identical or different copies (Lagos-Quintana et al, 2001; Lau et al, 2001). This finding suggests that polycistronic transcripts might be naturally used to enhance the efficiencies of target gene repression or to achieve linked multigene repression. Thus many groups have tested these polycistronic transcripts to generate miRNA artificially for better knockdown and linked multi-gene knockdown by modified miRNAs (Zhu et al, 2007).

A flexible cloning platform for generation of plasmid vectors and lentivirus based for miR-shRNAs expression against multiple target genes was used by Zhu et al (2007). To date, shRNA and artificial miRNA-based strategies have been compared with conflicting results (Boden et al, 2004; Silva et al, 2005; Li et al, 2007). In certain comparisons, the shRNAs tested
had suboptimal 5’ overhangs due to variable arrangements of transcription start and stop sequences, some caused inadvertently by the use of restriction enzyme sites during vector production (Boden et al, 2004; Silva et al, 2005). This raises concerns since recent reports have demonstrated that 2-nucleotide (nt) 3’ overhangs, often observed in natural pre-miRNAs, are optimal substrates for Exportin-5 and Dicer (Zeng and Cullen, 2004; Vermeulen et al, 2005).

Furthermore, none of the prior hairpin-based comparisons assessed the equivalency of strand biasing (Boden et al, 2004; Silva et al, 2005; Li et al, 2007). Strand biasing refers to which strand of the siRNA duplex enters the RISC and mediates gene silencing. In general, the strand with the weakest base-pairing near the 5’ end will be incorporated into the RISC (Khvorova et al, 2003; Schwarz et al, 2003). Hence, shRNA and artificial miRNA comparisons may be confounded if the vectors, following processing, release siRNAs that exhibit differential strand biasing (i.e., one loading the intended antisense strand and the other loading the unintended sense strand). Indeed, a single base-pair shift during RNAi substrate processing may alter which strand of the resulting siRNA preferentially enters the RISC, thus highlighting the importance of evaluating strand biasing in RNAi vector comparison studies.

Inevitably, the selection of which hairpin-based RNAi expression system is most suitable for a given application relies on several factors: project goals, delivery options, target expression levels, and desired silencing efficiency. Thus, empirical testing of these shRNA expression systems may be required for each target and tissue on a per study basis.

1.22 shRNA/artificial miRNA Vs siRNA:

In an attempt to compare between siRNA and shRNA Li et al (2007) have carried out a very systematic study, where they compared 190 shRNAs and 360 siRNAs against 19 and four targets respectively and observed that the functional shRNAs and siRNAs displayed similar but not identical nucleotide preferences (Table 2). They, therefore, recommended for development of more accurate algorithm to facilitate the selection of the functional shRNAs.
Advantages of using shRNA:
1. Low doses of administration.
2. shRNA allows long-term down-regulation of a gene.
3. Stable transfection can be carried out
4. Economical alternative to siRNA.

Table 2. Nucleotide preferences in functional siRNA and shRNA

<table>
<thead>
<tr>
<th>Functional siRNA</th>
<th>Functional shRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong preference for GC at positions 1–2</td>
<td>Starting with G or GG</td>
</tr>
<tr>
<td>Strong preference for AU at positions 3–7</td>
<td>Strong preference for AU at positions 3–7</td>
</tr>
<tr>
<td>Relatively GC-rich at positions 14–16</td>
<td>Relatively GC-rich at positions 14–16</td>
</tr>
<tr>
<td>Strong preference for AU at positions 17–19</td>
<td>Strong preference for AU at positions 17–19</td>
</tr>
<tr>
<td>Strong preference for AU at positions 3, 6, 13, and 18</td>
<td>Strong preference for AU at positions 3, 6, 13, and 18</td>
</tr>
<tr>
<td>Preference for GC at position 9</td>
<td>Preference for AU at position 9</td>
</tr>
<tr>
<td>Weak preference for GC at position 11</td>
<td>Preference for GC at position 11</td>
</tr>
</tbody>
</table>

1.23 RNA Viruses and RNAi:

Soon after the recognition of potentials of RNAi several studies were reported for different viruses. For the sake of clarity and relative importance these have been divided into in vitro and in vivo.

1.23.1 RNAi studies in vitro:

Adelman et al (2002) showed that C6/36 (Aedes albopictus) cells could stably be transformed with the plasmid containing dsRNA that was designed to transcribe an inverted-repeat RNA (irRNA) derived from the genome of dengue virus type 2 (DEN-2). Results demonstrated that 44% cell lines designed to express irRNA were resistant to DEN-2 infection, with more than 95% of the cells showing no DEN-2 antigen accumulation.
In a review by Barik S (2004) control of nonsegmented negative strand RNA viruses replication by siRNA was mentioned *in vitro*.

Dash *et al* (2008) used two siRNA against nsP3 and E1 genes of Chikungunya virus. Their results indicated a reduction of virus titer up to 99.6% in siRNA transfected Vero cells compared to control. The viral inhibition was most significant at 24 h (99%), followed by 48 h (65%) post infection.

Dector *et al* (2002) showed that a small interfering RNA corresponding to the *VP4* gene efficiently inhibited the synthesis of this protein in virus infected MA104 (monkey kidney) cells. A large proportion of infected cells had no detectable VP4 protein and the yield of viral progeny was reduced. Most of the virus particles purified from these cells were not infectious. The *VP4* gene silencing was specific because the synthesis of *VP4* gene from rotavirus strains with different target sequence was not affected. These findings offer a novel therapeutic approach for the assembly of viral particles.

Gitlin *et al* (2002) used two siRNAs against capsid and polymerase protein of polio virus and both of these siRNA inhibited the plaque formation in Hela cells. It is an early paper that showed in vitro inhibition of poliovirus and pointed out the potential problem of viral escape.

Kapadia *et al* (2003) reported that *NS3* and *NS5B* siRNAs could specifically inhibit HCV RNA replication and protein expression in Huh-7 cells. In their experiments, HCV RNA replication was inhibited within 2 days of siRNA transfection, and the effect lasted at least 6 days.

McCown *et al* (2003) showed that transfection of siRNAs specific for WNV capsid or NS5 ORF in 293T cells resulted in decreased WNV protein expression, genomic RNA synthesis, and infectious virus production.

Novina *et al* (2002) used five siRNAs targeted to the HIV-1 cellular receptor CD4 and the viral structural Gag protein. They found that infection with HIV-1 could be efficiently inhibited in mammalian cells. This is one of the initial papers to use RNAi *in vitro* to inhibit HIV and introduced the concept of inhibiting both viral and host genes to suppress viral entry and replication.
Zhang et al (2004) showed that the DNA vector-driven siRNA against the spike protein of SARS-CoV could selectively and specifically silence gene expression of the Spike protein in SARS-infected 293T cells.

1.23.2 RNAi studies in vivo:

Bitko et al (2005) showed that intranasal administration of siRNA can be used for the treatment of respiratory syncytial virus (RSV) and parainfluenza virus (PIV) in mice. In this study three P gene siRNA of RSV and two siRNA for hemagglutinin-neuraminidase with fusion protein of PIV were used. Thus this study showed efficient silencing and in vivo effectiveness of nasally administered uncomplexed siRNAs for the clinically important respiratory syncytial virus.

Huang et al (2009) showed that A549 cells transfected with Hepatitis E Virus (HEV) specific siRNA-RdRp-1 were capable of preventing infection of host cells by HEV. Further by using HEV-specific shRNA expression plasmids they showed suppression of HEV infection in piglets. In Group of piglets treated with shRNA-RdRp-1, no HEV RNA was detected throughout the study. In addition, HEV antigens were reduced in the liver, spleen, and kidney of these piglets, as were activities of ALT, AST and the level of TBIL. Thus, indicated the potential of the RNAi approach for the treatment of HEV infection.

Kumar et al (2007) for the first time showed that rabies virus glycoprotein (RVG) could be used for the delivery of siRNA by intravenous injection for the treatment of Japanese encephalitis (JE) and West Nile virus (WNV) infection in mice.

Li et al (2005) reported a screen of 48 siRNA candidate targeting t the genome of SARS Coronavirus in fetal Rhesus monkey kidney cells, however, only two were selected for further studies; first in mice with a reporter system and second in a Rhesus macaque SARS model. This study had provided strong evidence that siRNA could be used as a potential prophylactic and therapeutic agent in the treatment of SARS infection without any adverse effects. This is the first study to show therapeutic efficacy of siRNAs in a nonhuman primate to treat respiratory infection by the SARS coronavirus.
Palliser et al (2006) for the first time showed the potential for vaginal application of siRNAs to prevent or treat sexually transmitted infections. In total, seven siRNAs were used in NIH3T3 and Vero cells, and three were found very effective in both the cell lines. In further studies, the most effective siRNA was used in mice for the treatment of Herpes Simplex virus-2 infection intravaginally.

Song et al (2003) studied the application of RNAi in fulminant hepatitis. By designing six siRNAs targeted to Fas, a gene related to apoptosis in a broad spectrum of liver diseases, the researchers found that the longevity of 82% experimental mice could be significantly enhanced (Song et al, 2003).

Zhang et al (2005) showed that Respiratory syncytial virus (RSV) infection can be treated with intranasal administration of siRNA using nanoparticles targeting the viral NS1 gene.

Apart from the above landmark studies, there have been several key publications in the area. Mentioning each and every publication in the field is out of the scope of this review. However important and pioneering research papers with special reference to route and agent used for delivery of the siRNA against viral infections has been tabulated in Table 3.
Table: 3. Selected examples of in vivo efficacy using siRNA in viral disease

<table>
<thead>
<tr>
<th>Target</th>
<th>Formulation</th>
<th>Route</th>
<th>Model</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulmonary</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSV-P, PIV-P</td>
<td>Saline or Lipoplex</td>
<td>Intranasal</td>
<td>RSV infection, PIV infection</td>
<td>Bitko et al, 2005</td>
</tr>
<tr>
<td>SARS</td>
<td>D5W or Surfactant</td>
<td>Intranasal</td>
<td>SARS infection</td>
<td>Li et al, 2005</td>
</tr>
<tr>
<td>Influenza A-NP, PA</td>
<td>PEI</td>
<td>Intravenous</td>
<td>Influenza virus infection</td>
<td>Ge et al, 2004</td>
</tr>
<tr>
<td>Nervous System</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JEV and WNV envelop</td>
<td>Lipoplex</td>
<td>Intracranial</td>
<td>JEV and WNV infection</td>
<td>Kumar et al, 2006</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBV</td>
<td>Liposome</td>
<td>Intravenous</td>
<td>Plasmid-based HBV replication</td>
<td>Morrissey et al, 2005</td>
</tr>
<tr>
<td>Ebola L gene</td>
<td>PEI or Liposome</td>
<td>Intraperitoneal</td>
<td>Ebola virus infection</td>
<td>Geisbert et al, 2006</td>
</tr>
<tr>
<td>Viral induced Cancer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV-E6</td>
<td>Lipoplex</td>
<td>Intratumoral or intraperitoneal</td>
<td>Subcutaneous tumour xenograft</td>
<td>Niu et al, 2006</td>
</tr>
<tr>
<td>Other organs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSV2-UL27, UL29</td>
<td>Lipoplex</td>
<td>Intravaginal</td>
<td>Herpes simplex 2 virus infection</td>
<td>Palliser et al, 2006</td>
</tr>
</tbody>
</table>

1.24 RNA based Therapeutics:

Because RNA-based therapeutics can tremendously expand the number of ‘druggable’ targets by overcoming the major limitation of existing medicines, which are able to target only a limited number of proteins involved in disease pathways. Over the past 20 years, scientific and technical breakthroughs have significantly advanced the field of RNA-based therapeutics. Several classes of molecules and approaches have been investigated as RNA therapeutics, including antisense RNA, ribozymes, aptamers, small interfering RNA (siRNA) and microRNA (miRNA) (Dorsett
and Tuschi, 2004). However, applications of RNA drugs in the treatment of human diseases remain in their infancy (Table 4). Nevertheless, this area presents a major opportunity, as indicated by the increasing number of high-profile partnerships and merger and acquisition deals between pioneering biotechnology companies and traditional large pharmaceutical companies (reviewed by Melnikova I, 2007).

1.25 Drugs on the market:

The only approved antisense RNA drug so far is fomivirsen (Vitravene; Isis Pharmaceuticals/Novartis) for the treatment of cytomegalovirus retinitis in patients with AIDS. However, No ribozyme was ever successfully developed as drug. Although, the only marketed aptamer is pegaptanib (Macugen; OSI Pharmaceuticals/Pfizer) for wet age-related macular degeneration (AMD). Neither drug can be called commercially successful, as fomivirsen is offered only on a limited basis, while sales of pegaptanib eroded owing to competition from ranibizumab (Lucentis; Genentech), which contributed to the decision by OSI Pharmaceuticals to divest its ophthalmology assets.

From the first in vivo evidence of RNAi-based therapeutic efficacy in an animal disease model in 2003 (Song et al, 2003), the pace of drug development has been rapid because of the entrance of several siRNA molecules in different phases of clinical trials (Table 4).
<table>
<thead>
<tr>
<th>Company</th>
<th>Programme</th>
<th>Indication</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antisense</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isis</td>
<td>ISIS301012</td>
<td>High cholesterol</td>
<td>Phase II</td>
</tr>
<tr>
<td></td>
<td>ISIS113715</td>
<td>Diabetes</td>
<td>Phase II</td>
</tr>
<tr>
<td>OncoGenex, Isis</td>
<td>OGX-011</td>
<td>Cancer</td>
<td>Phase II</td>
</tr>
<tr>
<td>Eli Lilly, Isis</td>
<td>LY2181308</td>
<td>Cancer</td>
<td>Phase I</td>
</tr>
<tr>
<td>AVI BioPharma</td>
<td>Resten AVI-5126</td>
<td>Restenosis</td>
<td>Phase II</td>
</tr>
<tr>
<td></td>
<td>Resten AVI-4065</td>
<td>CABG</td>
<td>Phase I/II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HCV</td>
<td>Phase II</td>
</tr>
<tr>
<td>Topigen</td>
<td>TPI-ASM8</td>
<td>Asthma</td>
<td>Phase I</td>
</tr>
<tr>
<td>Lorus Therapeutics</td>
<td>GTI-2040</td>
<td>Renal cell carcinoma</td>
<td>Phase II</td>
</tr>
<tr>
<td><strong>Aptamers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Archemix</td>
<td>ARC1779</td>
<td>Acute coronary syndrome, Percutaneous coronary</td>
<td>Phase I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>intervention</td>
<td></td>
</tr>
<tr>
<td>Antisoma, Archemix</td>
<td>AS1411</td>
<td>Renal cancer, acute myeloid Leukaemia</td>
<td>Phase II</td>
</tr>
<tr>
<td><strong>siRNA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Opko Health</td>
<td>Bevasiranib (C &amp; S)</td>
<td>Wet AMD</td>
<td>Phase III</td>
</tr>
<tr>
<td>Allergan</td>
<td>AGN211745 (Sirna-027)</td>
<td>Wet AMD</td>
<td>Phase II</td>
</tr>
<tr>
<td>Silence Therapeutics, Biotech,</td>
<td>RTP801i</td>
<td>Wet AMD</td>
<td>Phase I</td>
</tr>
<tr>
<td>Pfizer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alnylam</td>
<td>ALN-RSV01</td>
<td>RSV infection</td>
<td>Phase II</td>
</tr>
</tbody>
</table>

Table: 4. Selected RNA-based therapy in development
1.26 Problems associated with use of siRNA in clinics:

In reality using siRNA in the clinics will not be possible in the near future, mainly because of inadequately addressed issues like delivery, distribution, metabolism and excretion of these synthetic siRNA. The intravenous administration of siRNA is inefficient mainly because of unstability of naked/unmodified siRNA and non-specific biodistribution. Variety of particles were used by several groups to deliver siRNA for example, positively charged iron oxidenanoparticles (Medarova et al, 2007), liposomes (Bisanz et al, 2005; Kim et al, 2007) or copolymer-based nanoparticles (Bartlett et al, 2007; Rozema et al, 2007).

In many cases, the incorporation of siRNA into particles and ribonucleoprotein complexes involves the use of chemical modification of 3'-end of the sense siRNA strand with membrane-tropic moieties including cholesterol (Soutschek et al, 2004) or fatty and bile acids (Wolfrum et al, 2007). Hydrophobic moieties result in recombination of modified siRNA with HDL and LDL particles and increased liver tropism. Alternatively, special liver-targeted ApoB siRNA-binding polymer delivery systems were designed to release the contents of nanoparticles in the endosomes (Rozema et al, 2007).

In a recent study, use of 29 amino acid residue of rabies virus glycoprotein (RVG) was found to be safe and non-invasive approach for the delivery of siRNA in the brain. Thus, these molecules not only increase the stability of siRNA but also selectively deliver the siRNA in the target organs (Kumar et al, 2007).

Determining biodistribution of siRNA is extremely important for target and nontarget organs of potential RNAi therapies and for predicting potential toxicities to these organs. These experiments require covalent “tagging” of oligoribonucleotides. For example, oligoribonucleotides can be labeled by tritium at C8 positions of purines using heat-exchange method (Mook et al, 2007).

In the recent detailed study, chemically modified ApoB siRNA which, utilized 5’-phosphorylation of siRNA with γ-AT($^{32}$P) was used for optimization of delivery and distribution (Wolfrum et al, 2007). Tracking of $^{32}$P-siRNA or
$^3$H-siRNA and its metabolites requires scintillation counting of tissues removed from sacrificed animals or, alternatively, relies on experiments with perfused isolated organs. The alternative to $^{32}$P-labeling is noninvasive imaging which is ideally suited for determining biodistribution of constructs used for gene delivery and transfer in vivo (reviewed in Bogdanov and Weissleder, 1998; Bogdanov and Weissleder, 2002). For example, both major radionuclide-based tomographic imaging modalities, positron emission tomography (PET) and single-photoncomputedtomography (SPECT), can potentially be used for imaging siRNA biodistribution in live animals.

To study the metabolism as well as excretion of these small molecules from the systems it essential to utilize the emerging molecular imaging tools (Reviewed by Bogdanov, 2008). The fast kidney filtration is typical for all siRNA since the duplexes are small, and retention in the liver is probably due to scavenging of phosphorothioates by endothelial cells of the liver as in the case of oligodeoxyphosphorothioates (Bijsterbosch et al, 1997).

Several studies have shown the effectiveness of siRNA in the central nervous system (CNS) (Zhang et al, 2003; Xia et al, 2004; Shankar et al, 2005; Ralph et al, 2005). However, for synthetic siRNA to be used as potential antiviral to suppress viral encephalitis it is of paramount importance that siRNA should be delivered directly to the brain without invasion. Use of 29 amino acid residue of rabies virus glycoprotein (RVG) was found to be safe and non-invasive approach for the delivery of siRNA in the brain for the treatment of Japanese encephalitis virus (JEV) infection in mice (Kumar et al, 2007).