1. Introduction and review of literature:

Encephalitis literally means inflammation of the brain. Encephalitis may be caused by a variety of afflictions i.e. Bacterial, parasitic, protozoal and viral. Viral encephalitis refers to encephalitis caused by a virus and remains a significant source of neurologic morbidity and mortality worldwide. It is one of the major public health concerns in many developing countries (Chatterjee P. 2005; Lam et al. 2005; Mackenzie et al. 2005; Mudur et al. 2005).

Several outbreaks of encephalitis with high mortality remained undiagnosed in India, the first report being as early as 1954 in Jamshedpur in central India, (Khan N. 1954) followed by those in Nagpur in 1958 and in Raipur in 1965, (Rodrigues et al.1972). During last couple of years, at least 4 outbreaks of encephalitis in India have remained undiagnosed despite extensive investigations (NIV, unpublished data). Table 1 provides the list of viruses responsible for causing encephalitis.

Table 1: Causes of viral encephalitis in humans

- Arbovirus encephalitis
  - La Crosse encephalitis
  - California encephalitis virus
  - Japanese encephalitis
  - St. Louis encephalitis
  - Equine encephalitis
  - Murray Valley encephalitis virus
  - Tick-borne meningoencephalitis
  - Powassan encephalitis
  - West Nile virus
- Herpes simplex
- Herpes zoster
- RSSE (Russian spring-summer encephalitis) viruses.
- Rabies virus
- Chandipura virus
- HIV
1.1. History:

CHPV was first discovered by National Institute of Virology (NIV), Pune from a serum sample of a patient, collected during an outbreak of febrile illness in Nagpur area of Maharashtra state, India in 1965. During investigations of this outbreak, serum samples collected from two febrile cases negative for Degue and Chikungunya viruses were inoculated in BS-C-1 cells and produced cytopathic effect (CPE). The filterable agent recovered was identified as a new virus and named as “Chandipura virus” after the locality from where it was isolated (Bhatt et al. 1967).

Later CHPV was also isolated from sandflies during a routine entomological investigation in Aurangabad in 1967 (Dandha et al. 1970) and subsequently, from serum sample of a child suffering from encephalopathy in Jabalpur, Madhya Pradesh (Rodrigues et al. 1983).

The virus did not gain any public health importance until 2003, when investigations by NIV associated CHPV with large encephalitis outbreak in children in many districts of Andhra Pradesh and Maharashtra, India (Rao et al. 2004).

Various viral encephalitis outbreaks has been associated with CHPV; 2003 (Maharashtra, and Andhra Pradesh), 2004 (Gujarat), 2005 and 2007 (Maharashtra, and Andhra Pradesh) (Rao et al 2004, Chadha et al. 2005 and NIV unpublished data). There are enough evidences suggesting existence of CHPV for more than 50 years. However, CHPV has attained the status of an important emerging pathogen of public health significance only recently.

1.2. Classification:

Chandipura virus is a member of Order: Mononegaloviridae; Family Rhabdoviridae and belongs to genus vesiculovirus. (Figure-1). Viruses belonging to this family owe their name rhabdo, meaning rod-shaped in Greek, to the typical bullet shaped morphology and include human pathogens such as rabies and CHPV (Figure-2).

CHPV was found to be similar in many aspects to VSV for example, genetic makeup, polypeptide composition, life cycle and single stranded RNA
genome of negative sense. Comparison of protein and nucleic acid composition also revealed marked similarity to VSV. Thus, CHPV was included within Vesiculovirus genus and is placed between Vesicular stomatitis virus (Newjersey) (VSVNJ) and Vesicular stomatitis virus (Indiana) (VSVI) (Rose and Whitt, 2001).

Figure-1: Classification of CHPV.

(Picture courtesy: Indian academy of sciences, 15th Mid-year meeting, http://www.ias.ac.in/meetings/myrmeet/15mym_talks/dchattopadhyay/)

1.3. Morphology:

Morphologically all rhabdoviruses are enveloped, rod or cone-shaped particles approximately 100 to 430nm long and 45 to 100nm in diameter. CHPV is 150-165nm long, 50-65nm wide showing distinct surface projections of 9-11nm in size and stain filled canal at the base of the virus particle (Figure-3) (Rao et al. 2004).
Figure-2: Schematic representation of a typical bullet shaped CHPV (http://education.expasy.org/images):

Figure-3: Transmission electron micrographs of primary CHPV isolates from tissue culture (Rao et al. 2004):

Bar=100 nm in both micrographs.
A: Two negative stained virus particles showing the stain filled canals and basal attachments.
B: Negative stained Chandipura virus particle showing typical vesiculovirus morphology, including the internal ribonucleoprotein coil. Inset shows a virus particle with a released helical ribonucleoprotein coil.
1.4. Viral genomic organization:

Chandipura virus is a negative stranded RNA virus. The genome consists of approximately 11,119nt and codes for five polypeptides (Figure-4), namely, Nucleocapsid protein N, Phosphoprotein P, Matrix protein M, Glycoprotein G and Large protein L (Marriot A.C, 2005)

Though morphologically/genetically very close to VSV, serology can distinguish CHPV and VSV. VSV remains one of the best-studied models among rhabdoviruses. Therefore VSV has been taken as an example in the present review, where no or little information specific for CHPV is available

Figure-4: The genomic organization of a Chandipura virus

1.5. CHPV Proteins:

1.5.1. The Nucleoprotein (N):

CHPV N gene was sequenced almost two decades ago to reveal ~50% sequence homology with N gene of VSVI and VSVNJ (Masters and Banerjee 1988b). Subsequently, cloning and recombinant expression of N gene led to its biochemical and biophysical characterizations (Majumder et al. 2001).

CHPV N gene codes for a 422 amino acid polypeptide (~49 kDa) with no reports of post-translational modifications. N protein encapsidates genomic RNA in a precise structure that can be compared with histone-mediated enwrapping of a DNA molecule into a nucleosome structure (Figure-5). This encapsidated form of the genome alone can be recognized by viral polymerase as its template during both transcription and replication (Banerjee. 1987a,b).
The N protein of Chandipura virus not only protects the viral genome from RNases, but is also thought to play some vital regulatory roles in the transition from transcription to replication in the viral life cycle, referred to as the transcription-replication switch (Majumdar et al. 2004, Bhattacharya et al. 2006). The major biological property of N protein is to bind with nascent leader RNA to initiate encapsidation of replication product concurrent to synthesis (Blumberg et al. 1983, Wertz 1983; Patton et al. 1984; Banerjee 1987a). However, aggregation prone nature of N protein poses a major obstacle towards biochemical characterization. Encapsidation competent N protein synthesized in a cell-free system was not sufficient for extensive structure-function analysis (Patton et al. 1983, 1984).

**Figure-5:** A proposed view of Chandipura virus genome RNA encapsidated with Nucleocapsid protein. Nucleocapsid protein binds to viral RNA to enclose it in a disc like structure. This disc like structures stack on each other to generate a helical assembly, as depicted, to form core nucleocapsid. Phosphoprotein P and Large Protein L remain associated with N-RNA (Basak et al. 2007):

1.5.2. The Phospoprotein P:

CHPV P gene codes for a 294 amino acids polypeptide with molecular weight of ~29 kDa. P and L proteins constitute the viral RNA dependent RNA polymerase (RdRp), where L is the catalytic subunit and P is the cofactor. Bacterially expressed non-phosporylated form of P protein (P0) of CHPV was found to be efficiently phosphorylated in vitro by Casine Kinase II (CKII). The phosphorylated form (P1) of the P protein supported the transcription in vitro but the non-phosphorylated form could not (Chattopadhyay et al. 1997).
Studies with recombinant CHPV P revealed CKII, indeed similarly phosphorylates P protein \textit{in vitro} (Chattopadhyay and Chattopadhyay 1994), although, CHPV-P exhibited less than 20% homology with P protein from other vesiculoviruses (Masters and Banerjee 1987).

Mutational analysis identified serine (S-62) in CHPV P protein as single phosphorylation site \textit{in vivo} (Basak et al. 2003). Kinetic data suggested that CKII incorporates one molecule of phosphate on serine (S62) and if this site gets mutated the P protein does not support \textit{in vitro} transcription. The phosphorylated P protein is eluted by gel filtration at the position of its dimer in contrast to the non-phosphorylated protein eluting as a monomer (Chattopadhyay et al. 1997). So the dimer formation of phosphorylated P protein indicates that it is involved in transcriptional activities.

Mutated P protein with alanine substituted for serine, when tested \textit{in vivo} was unable to activate transcription and rather inhibited viral mRNA synthesis in a trans-dominant manner (Basak et al. 2003). Therefore, CKII mediated phosphorylation appeared to be indispensable for P protein to act as a transcriptional activator.

The role of P protein in transcription–replication switch using VSV and CHPV as model systems was studied. Based on these studies it was proposed that viral transcription-replication switch where P protein acts as a modulator of genome transcription and replication by its ability to bind to the nascent leader RNA (sequence at the 3’ end of the viral genome (Giorgi et al 1983)) in its unphosphorylated (P0) form, promotes read-through of the transcription termination signals (intergenic sequences) and initiates nucleocapsid assembly on the nascent RNA chain (Basak et al. 2003).

Incubation of L protein with N-RNA in a reaction mixture did not synthesize viral mRNA. However, addition of viral P protein along with L, and not P alone, allowed viral mRNA synthesis. These studies confirmed a role of Phosphoprotein as an activator of viral transcription (Chattopadhyay et al. 1997) and this proposed function was consistent to the observations made earlier in VSV and CHPV (Banerjee 1987b).

1.5.3. The Matrix protein M:
The matrix protein, M consists of 229 amino acids and is ~27kDa in size (Taylor et al. 1999). It lies on the inner surface of the virion to tether core nucleocapsid to the membrane. Highly basic N-terminal domain, with eight lysine residues, 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).

Marriot and co-workers cloned CHPV M gene in 1999 to subsequently show its detrimental effect on transcription from cytomegalovirus immediate early promoter in vivo. Despite only 28% amino acid sequence identity with VSV, CHPV M protein preserved the ability to inhibit host nucleo-cytoplasmic transport (Petersen et al. 2001).

Research in the last few years, however, recognized M protein as a deadly weapon in rhabdoviral arsenal that perhaps alone may account for the observed cytotoxicity in virus infected cells (Licata and Harty 2003). VSV M protein was shown to shut off host transcription by RNA polymerase I and II (Ahmed and Lyles 1998). It was capable of inhibiting nuclear export of host mRNA and snRNA (Petersen et al. 2001) by targeting nucleoporin Nup98 present at the nuclear rim (von Kobbe et al. 2000). M protein mediated inhibition of host gene expression constitute an example of viral mechanism to suppress cellular interferon response (Enninga et al. 2002). Additional work on CHPV M protein is required to completely understand the functions of this protein.

1.5.4. The Glycoprotein G:

The Glycoprotein, G, is the sole spike protein of CHPV that enables virus absorption, assembly and budding. It also elicits antibody response thus acting as a major antigenic determinant (Banerjee AK 1987a, 1987b, Barr et al 2002, Neumann et al. 2002). CHPV G protein expressed from a cloned DNA 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).

Recently, crystal structure of the VSV G protein ectodomain has been shown in its post-fusion state to reveal classic hairpin conformations, large numbers of protonated residues stabilizing low pH state and a novel fold.
combining features of class I and class II fusogenic peptides (Roche et al. 2006). In light of the prediction that other rhabdoviral G proteins display similar folds (Roche et al. 2006), it is interesting to note that almost all of the amino acid substitutions in G protein sequences of epidemic associated virus strains were observed within the ectodomain, a viral tool for host invasion (Arankalle et al. 2005). Homology modeling of G-gene sequences derived from various epidemic associated strains may allow us to gain insight into viral pathogenesis. However, it needs to be studied if the changes occurring at ectodomain region of CHPV G protein play any role in epidemiology of the virus.

1.5.5. The Large protein L:

L protein encodes for a polypeptide of 2092 amino acids with molecular weight of 238.5 kDa. Viral transcriptase is composed of L and P-protein. L protein retains the catalytic activity of RNA polymerization, capping and polyadenylation. Comparison of deduced amino acid sequence of L protein of CHPV with that of different rhabdoviruses exhibited a high degree of homology along the length of the protein. 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 2005).

Nevertheless, L protein remains associated with translation elongation factor, EF1; while, EF1–bc complex was proposed to constitute L-associated kinase (LAK) activity, EF1–a may participate in the capping by virtue of GTP/GDP binding activity (Das et al. 1998).

Therefore, it seems L protein in conjunction with P protein and other cellular components, synthesize viral mRNA within infected cells and offers mechanisms that could be potential drug targets, such as its unique capping or RNA dependent RNA polymerase Factivity. Nevertheless, a recombinant expression system to study structure and function of CHPV L protein is yet not available and is required for additional analyses.

1.6. Biology:
Life cycle of viruses belonging to Rhabdovirus family involves different stages e.g. Adsorption, Penetration, Uncoating, Transcription, Replication, Assembly and progeny virus budding (Figure 6).

**Figure-6:** Diagram of rhabdovirus replication 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. *(Figure courtesy: Field’s Virology fifth edition)*.

### 1.6.1. Adsorption

The viral infection is initiated by attachment 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, Matlin et al, 1982); yet even at the optimal pH, binding fails to reach equilibrium (Matlin et al, 1982).

Competitive binding assay revealed 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 that 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 proposed 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).

1.6.2. 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.

1.6.3. Transcription

The first 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 (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. 1980a,b). 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 and Holland, 1976, Iverson and Rose 1981).

1.6.4. 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 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 (Whelan and Wertz, 2002, Barr et al. 2002).
1.6.5 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).

1.7. Growth of CHPV In-vitro and In-ovo systems:

CHPV can be grown in high titer in chicken eggs as it causes lethal infection in this system. Stocks of three Indian CHPV isolates; one isolate from a febrile case in 1965, and two isolates from two pediatric encephalitis cases from Andhra Pradesh, 2003 were inoculated in 10-day-old chick embryos by allantoic route. All three virus isolates replicated in chick embryos showing titer of log 10(12) to log 10(13) EID\textsubscript{50}. It was demonstrated that chick embryos are susceptible to CHPV and virus grows to high titers in this system and chick embryos can be used as an alternative host system for cultivation and isolation of CHPV as they are less expensive than laboratory animals and have several other advantages over cell cultures (Pawar et al. 2005). CHPV is moderately adapted to Drosophila (Busscereau F, 1975).

Though CHPV was found to be infectious for Vero cells and rat cells, Vero cells were more susceptible to the virus (Zavada et al. 1979). Chinese hamster lung (CHL) cells and human peripheral blood (HPB) leukocytes were susceptible to CHPV and induced chromosomal aberrations in these cell lines (Paranjape and Wagh, 2003). Aedes krombeini cell line (NIVI-AK-453) is susceptible to CHPV and the virus grows in high titers (Pant et al, 1992). The virus also grows in recently established Aedes aegypti cell line established at National Institute of virology (Sudeep et al. 2009).
Isolation of the virus was attempted by inoculation of clinical specimens into Vero, Madin-Darby canine kidney (MDCK), rhabdomyosarcoma (RD), Vero E6 and porcine kidney cells (PS cell) (Rao et al. 2004). Among these cell lines the Vero E6 cells and RD cells are widely used for CHPV propagation.

1.8. Host Range:

1.8.1. Potential animal reservoirs:

In order to determine the possible role of domestic animals in the outbreak of acute encephalitis associated with CHPV, a serological survey of domestic animals was carried out during an epidemic in July 2003. Out of 180 animal sera from highly affected areas of the Karimnagar and Warangal districts of Andhra Pradesh, 33 (18.3%) had virus-neutralizing (VN) antibodies. The antibody positive animals consisted of pigs (30.6%), buffalos (17.9%), cattle (14.3%), goats (9.3%) and sheep (7.7%). However, evidence of viremia was not documented in domestic animals (Joshi et al. 2005). Thus the question of reservoir still remains unanswered.

1.8.2. Laboratory animal models and *in-vivo* propagation of CHPV:

Unlike other Rhabdoviruses, CHPV enjoys the wide host range including vertebrates and insects. From available evidence it is fairly clear that mammals may represent the “Dead-end hosts” (Raha et al. 2000).

CHPV is lethal to young mice by peripheral as well as central routes of infection but adult mice are susceptible only through central route of infection (Bhatt et al. 1967). Antibody response to CHPV was studied in different laboratory animals, viz., mice, guinea pigs, rabbits, monkeys and chickens by complement fixation (CF), neutralization (N) and agar gel diffusion (AGD) tests. The CF antibody response was good in guinea pigs and monkeys, poor in rabbits and negligible in chickens. The mice, rabbits, guinea pigs and monkeys appeared to be the animals of choice to prepare high titer immune sera by single inoculation of CHPV (Kelkar SD, 1976). Low levels of neutralizing activity were detected in serum sample of CHPV infected steers, pigs, contact sheep, and one contact goat (Wilks and House, 1986).
1.9. The Disease:

1.9.1. CHPV encephalitis in Human:

Although the virus was isolated in 1965 from a febrile case and in the mid 70s from child with acute encephalitis in Raipur in central India (Rodrigues et al. 1983), the epidemic potentiality of the CHPV and public health importance was recognized only in 2003. CHPV was the cause of a large outbreak of encephalitis in children aged 2 Months to 15 years, with high case fatality rate (183 of 329, 55% cases) from Andhra Pradesh state in 2003 (Rao et al. 2004) and a small outbreak with 75% mortality in Gujarat, in 2004 (Chadha et al. 2005).

The absence of neutralizing antibodies to CHPV in most (17 of 20) of the patients indicates a primary infection with CHPV (Chadha et al. 2005). Significantly lower frequency of neutralizing antibodies among younger children suggests that a significant proportion of the pediatric population is susceptible to CHPV and is consistent with the fact that encephalitis cases occurred only among children (Chadha et al. 2005).

1.9.2. The patients and clinical manifestations of the disease:

Although the clinical spectrum is not very clear, patients infected with CHPV were observed to be suffering from high-grade fever of short duration, vomiting, altered sensorium, generalized convulsions, decerebrate posture, leading to Grade IV coma, acute encephalitis / encephalopathy and death within a few hours to 48 hrs of hospitalization (Rao et al. 2004).

1.9.3. The treatment:

No specific antivirals are available. Therefore, a child infected with CHPV is treated only symptomatically. Once the patient shows the symptoms of Glasgow coma score of <7 the treatment is highly impossible (Narasimha et al. 2008, Rao PN et al. 2004; Hussain et al. 2004; John TJ. 2004).

1.10. Epidemiology:

Though CHPV can infect many mammalian species in different places, human cases have only been reported from India. Retrospective analysis revealed that this virus is highly prevalent at least since 1955 in India (Bhatt et
al. 1967), Srilanka (Peiris et al. 1993), and Africa (Nigeria, Senegal) (Fontenilla et al. 1994, Traore-Lamizana et al. 2001). Figures 7-11 illustrates geographical distribution of the Virus, world wide (Figure 7) and India (8-11)

Figure 7: Worldwide prevalence of CHPV

![Worldwide prevalence of CHPV](image1)

Figure-8: The Geographic distribution of CHPV in India.

![Geographic distribution of CHPV in India](image2)
Figure-9: The geographical distribution of CHPV in Andhra Pradesh (Rao et al. 2004).

Figure-10: Map of Gujarat, India showing areas with cases of encephalitis. • = Cases; gray lines=district borders; black lines=state borders (Chadha et al. 2005).
**Figure-11:** Geographic distribution of acute viral encephalitis cases among children in North Telangana, Andhra Pradesh, India (May 9, 2005 to March 22, 2006, N=52). The affected districts with the number of reported cases showed in brackets.
1.11. Transmission:

Since CHPV is emerging as an important encephalitis causing pathogen in India, need for better understanding of the transmission mechanisms including the role of vectors and other ecologic factors was felt.

1.11.1. Potential vectors:

For the first time, Dhanda et al described that the CHPV is transmitted to humans by sandflies (Dhanda et al. 1970). It was observed that the virus multiplied in sandflies (Phlebotomus papatasi) following intrathoracic inoculation. Within 24 hours, mean virus titers in infected flies increased approximately 4 logs. Experimentally infected P. papatasi transmitted the virus by bite to newborn mice and by transovarial transmission to their progeny. Eight percent of the F1 offspring of experimentally infected female parents were infected with CHPV (Tesh and Modi, 1983).

However, mosquitoes also supported the virus growth and transmission in experimentally infected 2 days old suckling Swiss albino mice. Ae. aegypti was found the most susceptible mosquito species for CHPV virus (Ilkal et al, 1991).

Experiments in the laboratory documented vertical and venereal transmission of CHPV in Aedes aegypti (L). The minimum filial infection rate among the progeny of infected females was 1.2%, the rate among male and female progeny was 0.9 and 1.4%, respectively. The venereal infection rate of CHPV among inseminated females was 32.7%. This study indicates the possible occurrence of vertical and venereal transmission of CHPV in insect vectors (Mavale et al, 2005). Another study described that infected males are capable of passing on the virus to female sand flies while mating. The infection rate was found to be 12.5% in uninfected females when mated with infected males. The occurrence of venereal transmission of this virus may have epidemiologic importance in the natural cycle of CHPV (Mavale et al, 2006).

In laboratory studies it was shown that P. argentipes is susceptible to CHPV infection and the virus is disseminated to salivary gland by overcoming the midgut barrier and midgut escape barrier within 4–5 days and is then
transmitted to other vertebrate hosts by crossing salivary gland barrier in the next 24 hours. This indicates that the extrinsic incubation period of CHPV in *P. argentipes* is approximately 5–6 days. In *P. papatasi* and some mosquito species, it was shown that CHPV has an extremely short extrinsic incubation period (Mavale et al. 2007).

On the basis of above studies it is clear that Sandflies are potential vectors for CHPV. But the role of mosquitoes in the CHPV life cycles needs to be explored.

1.12. Diagnosis:

Several molecular, tissue culture based and serological tools are available for diagnosis of CHPV infection. These includes:

- **Virus Isolation:** Cell culture, infant mice and embryonated eggs.
- **Antigen detection:** ELISA, IFA.
- **Genome detection:** RT-PCR, real time PCR.
- **Serological tests:** IgM and IgG ELISA, complement fixation (CF) and Neutralization tests.

1.13. Vaccine:

A vaccine is generally referred to as a medical preparation given to a person to provide immunity from a disease. Table 2 provides major achievements in the field of vaccines.

1.13.1. History of vaccines:

Table 2: Major achievements in the field of vaccine.

<table>
<thead>
<tr>
<th>Year</th>
<th>Achievement</th>
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<tbody>
<tr>
<td>1789</td>
<td>Edward Jenner developed <strong>smallpox vaccine</strong>. In the year 1980, WHO declared that the disease had been eradicated.</td>
</tr>
<tr>
<td>1885</td>
<td>Louis Pasteur injected a <strong>rabies vaccine</strong> to two individuals.</td>
</tr>
<tr>
<td>1896</td>
<td>Vaccine for <strong>cholera</strong> and <strong>typhoid</strong> were developed using killed bacteria.</td>
</tr>
<tr>
<td>1897</td>
<td>A killed vaccine for <strong>plague</strong> was developed.</td>
</tr>
</tbody>
</table>
| 1923 | A powerful toxin from **diphtheria** bacteria was chemically inactivated and used as a “toxoid”.
| 1926 | A killed vaccine for **pertussis** (“whooping cough”) was |
developed, using the whole pertussis organism.

1927 Tetanus "toxoid" was developed. By the late 1940s tetanus was combined with diphtheria and pertussis as the children's vaccine "DPT."

1954 Jonas Salk developed **killed polio vaccine**.

1961 Alfred Sabin developed **oral polio vaccine (OPV)** using live attenuated virus.

1963 A safe and effective **measles** vaccine was developed.

1964 **A killed rabies vaccine** was developed, but required up to 30 painful shots in the abdomen. By 1980, a newer version required only five shots in the arm to protect against this deadly disease.

1967 A vaccine for **mumps** was licensed.

1970 Several strains of **rubella** were weakened to make a vaccine. By 1971, measles, mumps and rubella vaccines were combined into a single shot known as MMR.

1970s & 80s **Meningococcal**, pneumococcal and haemophilus influenza type b (Hib) vaccines were developed.

1982 Plasma derived **Hepatitis B** vaccine was licensed.

1986 A recombinant protein based vaccine for **hepatitis B** was licensed.

1990 A killed vaccine for **hepatitis A** was developed.

1995 **A varicella (chicken pox)** vaccine was licensed for use in children.

1996 The first "DPTa" vaccine was approved, using only part of the pertussis organism, combined with diphtheria and tetanus.

2005-06 **RotaTeq (live attenuated virus)** vaccine was licensed for the prevention of life-threatening rotavirus gastroenteritis.

Types of vaccines:

- **Killed whole virus vaccines**
- **Subunit vaccines** – purified or recombinant viral antigen
- **Recombinant viral vector based vaccines**
- **Anti-idiotypic vaccines**
- **DNA vaccines**
A. Live virus vaccines:

✓ Prepared from attenuated strains.
✓ Use of related virus from another animal, e.g. cowpox to prevent smallpox.
✓ Multiply inside human host and provide continuous antigenic stimulation over a period of time.
✓ Prepared by passage of virus in an “unnatural host” or host cells. After repeated passages the virus is administered in natural host

Example:
1. The 17D strain of yellow fever (Live attenuated)
2. Poliovirus (Live attenuated)
3. Measles virus (Live attenuated)

Potential safety problems:

1. Under attenuation
2. Mutation(s) leading to reversion to virulence
3. Preparation instability
4. Contaminating viruses in cultured cells
5. Heat labiality
6. Should not be given to immunocompromized or pregnant patients
B. Inactivated whole virus:

- Inactivated by heat or chemicals.
- The outer virion coat should be left intact but the replicative function should be destroyed.
- Chemicals used include formalin, β-Propiolactone (BPL), Binary ethylenimine (BEI)

Formalin, is 37% formaldehyde by weight (40% by volume), 6-13% methanol, and the rest water. It reportedly reacts with many functional groups of proteins that lead to conformational change of the outer protein shell of the virus.

BPL, an alkylating agent, reacts with nucleophilic reagents including nucleic acids and proteins. It induces nicks in DNA, cross-linking between DNA and proteins as well as between the DNA strands in the double helix. It also alters the capability of residual/contaminating cell DNA to be used as template by various polymerases.

BEI, is an inactivant used in the inactivation of the foot-and-mouth disease virus.

Examples:

1. Formaline inactivated polio vaccine.
2. BPL inactivated Hepatitis A vaccine.
3. BPL inactivated Cholera vaccine.

Table 3: Attenuated v/s inactivated vaccines

<table>
<thead>
<tr>
<th>Features</th>
<th>Attenuated Vaccine</th>
<th>Inactivated vaccine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Number of doses</td>
<td>Single</td>
<td>Multiple</td>
</tr>
<tr>
<td>Need for adjuvent</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Duration of immunity</td>
<td>Many years</td>
<td>Less</td>
</tr>
<tr>
<td>Ab response</td>
<td>IgG</td>
<td>IgA, IgG</td>
</tr>
<tr>
<td>CMI</td>
<td>Good</td>
<td>Poor</td>
</tr>
<tr>
<td>Reversion to virulence</td>
<td>Possible</td>
<td>Not-possible</td>
</tr>
</tbody>
</table>
Potential safety problems:

1. Incomplete inactivation
2. Increased risk of allergic reactions due to large amounts of antigen involved

C. Subunit vaccine:

- A type of vaccine that consists of immunogenic viral proteins stripped free from whole virus particles, then purified from other irrelevant components, thereby reducing the risk of adverse reactions and residual infectious virus.
- Highly purified.
- Increasing purification may lead to loss of immunogenicity, and this may necessitate coupling to an adjuvant, such as an aluminum salt.

Examples:

1. HA vaccines for influenza A and B.
2. HBV (HBsAg) vaccine derived from the plasma of carriers.

Adjuvants:

1. Used to potentiate the immune response.
2. Functions to localize and slowly release antigen at or near the site of administration.
3. Functions to activate APCs to achieve effective antigen processing or presentation.

D. Recombinant viral proteins:

- Viral proteins have been expressed in bacteria, yeast and mammalian cells.
- Recombinant hepatitis B vaccine is the only recombinant vaccine licensed at present.
- An alternative application of rDNA technology is the production of hybrid virus vaccines. For example, Vaccinia; foreign gene is inserted into the plasmid vector along with a vaccinia virus promoter and vaccinia thymidine kinase sequences. The resultant recombination vector is
then introduced into cells infected with vaccinia virus to generate a virus that expresses the foreign gene.

☑ Excellent potential for producing **polyvalent** live vaccines.

☑ At present, efforts are being made to attenuate vaccinia virus further.

**Advantages:**

1. Production and quality control simpler.
2. No other viral or external proteins, therefore less toxic.
3. Safer in cases where viruses are oncogenic or establish a persistent infection.
4. Feasible even if virus cannot be cultured.

**E. Synthetic peptides:**

☑ Development of synthetic peptides depends on the identification of immunogenic sites/epitopes.

☑ Synthetic peptides can be highly immunogenic in their free form provided they contain, in addition to the B cell epitope, T-cell epitopes recognized by T-helper cells. Such T-cell epitopes can be provided by carrier protein molecules, foreign antigens or self adjuvanted peptide molecule
F. **Anti-idiotype vaccines:**

- **Idiotype:** An antigen-binding site in an antibody is a reflection of the 3-D structure of part of the antigen, which is of a particular epitope.
- Can be thought of as a mirror of the epitope in the antigen.
- Antibodies (anti-ids) are raised against the idiotype antibody by injecting the antibody into another animal.

**Examples:**
1. Idiotypic vaccination for B cell malignancies.
2. Anti-ids raised against antibodies to HBsAg elicit anti-viral antibodies.

G. **DNA vaccines:**

- DNA coding for the foreign antigen is directly injected into the host so that the host cells produce the antigen.
- These are often called DNA vaccines but would better be called DNA-mediated or DNA-based immunization since it is not the purpose to raise antibodies against the DNA molecules themselves but to get the protein expressed by host cells.
- It should be noted that sometimes the plasmid does not replicate in the cells of the vaccinee, but only protein is produced.
- The plasmid DNA is taken up by muscle cells after injection.
- One microgram of DNA could potentially introduce a thousand different genes into the vaccinee.

1.14. **Development of a candidate vaccine for Chandipura infection:**

At present there is no vaccine available to prevent CHPV infection. Based on various previous studies, documenting immunological efficacy of Glycoprotein of other closely related rhabdo viruses such as Rabies and VSV, we decided to use a recombinant Glycoprotein of CHPV for vaccine development.
1.14.1. **G protein as candidate vaccine**

The G protein in the family *Rhabdoviridae* is involved in virus attachment to the receptors of host cells and initiates infection. G protein has neutralizing epitopes for most of the members of the family Rhabdoviridae. For example the rabies G protein molecule (Figure 12) was found to have immunodominant epitopes involved in inducing virus neutralizing antibodies (VNAb) and a strong specific humoral immune response. It also induced a specific cellular immune response (*Sureau and Perrin. 1989*).

**Figure-12:** The G protein protruding external spikes on the surface of CHPV (*Basak et al. 2007*)

1.14.2. **G protein of VSV and its Immunogenicity:**

The VSV G protein is the major structural protein showing external spikes, arranged in a tightly packed coat on virion membrane. It has been studied in great details not only as a vaccine candidate but also to understand other important functions such as membrane protein and biogenesis in rhabdoviruses. (*Crise et al. 1989*).

Careful examination of VSV G protein revealed its three major domains including (1) 29 amino acids from the c-terminus comprise the cytoplasmic domain, (2) 20 hydrophobic residues form the transmembrane domain and (3) the major segment of the G-protein, extending from the transmembrane to the N-terminus, is the external antigenic domain (*Coll JM. 1995*). The single transmembrane VSV G protein is involved in virus entry and cell fusion (*White JM. 1990; Florkiewicz and Rose. 1984; Riedel et al. 1984*).
The G protein plays an essential role in virus infection. It mediates both virus attachment to the cell membrane and subsequent pH-dependent fusion between the viral envelope and the endosomal membrane, leading to the release of the ribonucleoprotein in the cytoplasm (Jeetendra et al. 2002; Matlin et al. 1982). It is involved in the differential pathogenesis and the G protein is a determinant of VSV virulence in VSV Indiana (VSVI) and VSV Newjersy (VSVNJ) (Martinez et al. 2003).

The VSV G protein was shown to induce virus specific neutralizing antibodies (Kelley et al. 1972; Dietzschold et al. 1974) and these anti-G antibodies are highly effective in preventing reinfection (Roberts et al. 1999).

Two major serotypes of VSV, New Jersey (VSVNJ) and Indiana (VSVI) have been described based on neutralizing antibodies to the surface G protein (Cartwright and Brown. 1972; Kelley et al. 1972). The selective removal of G protein from the surface of the virion by proteolytic enzymes resulted in loss of infectivity (Cartwright et al 1969, 1970). All monoclonal antibodies secreted by mouse hybridomas were neutralized with VSV G protein and serotype-specific neutralizing antibodies against the VSV G protein are important in protecting mice (Gobet et al. 1988; Lefrancois et al. 1984).

One epitope on the VSVI G protein was defined by a monoclonal antibody that could bind to the G proteins of both serotypes, but it could neutralize the infectivity of only VSVI (Lefrancois and Lyles, 1982). In VSV G protein many non-neutralizing antibodies, both cross-reactive and serotype specific, have also been described (Bricker et al. 1987; Lefrancois and Lyles, 1982).


The G protein exchange vectors of VSV have the same N, P, M, and L genes but express a G protein from either the New Jersey (NJ) or CHPV and VSVI and they do not generate cross-neutralizing antibodies and allow reinfection and effective boosting of antibody responses to foreign proteins encoded by the vector (Rose et al. 2000).
Laboratory mice have been used extensively to study the innate and acquired immune response to VSV G protein (Rose et al. 2001).

Recombinant viruses are promising candidates for developing safe and efficacious vaccines that allow unambiguous differentiation of vaccinated from naturally infected animals by introducing, for example, foreign genes or epitopes to which a serological immune response can be detected. A VSVI-GNJGI recombinant virus (VSV G protein of new jersey & Indiana) containing G protein gene to induced cross-reactive neutralizing antibody response in swine provided protection against a high dose challenge with VSVI or VSVNJ. (Kim et al. 1998; Roberts et al. 1998; 1999; Zinkernagel et al. 1978).

1.14.3. G protein of Rabies virus and its Immunogenicity:

As in the case of VSV G protein, the rabies virus G protein has also been evaluated as a potential vaccine (Rosenthal et al. 1980, Crise et al. 1989). Rabies virus G protein is a trans membrane protein that forms the viral envelope and induces the production of antibodies that neutralize the virus. It can be potentially formulated as a subunit vaccine useful in animal/human immunization protocols against hydrophobia and it can also be used in diagnostic kits for rabies detection (Fabiana et al. 2008). The pseudorabies virus expressing G protein of rabies was very effective in eliciting both humoral as well as cellular immune responses in mice (Patial et al. 2007), dogs (Patial et al. 2007, Donald et al. 2006), Horse (Fischer et al. 2003) and in foxes (Yuan et al. 2008).

The G protein expressed in vaccinia virus vectors was highly immunogenic and induced both humoral and cell-mediated responses in mice, foxes and raccoons by intradermal, intramuscular, and oral routes and protected from challenge with street rabies virus (Rupprecht et al. 1988, Lambot et al. 2001). This approach is widely used even in human vaccination.

In rabies infection, the neutralization of viral infectivity is due to antibodies to the envelope G protein (Cox et al.1977, Wunner et al. 1985). Although virus-neutralizing antibodies are not the only factor responsible for protection against rabies, the presence of neutralising antibodies in serum of
vaccines is taken as a reliable indicator for the success of active immunization (Moore et al. 2005; Perrin et al. 1986; Sureau et al. 1982).

Rabies immunosomes (G protein anchored on pre-formed liposomes) have been shown to be efficient in post-exposure treatment of laboratory animals that had been experimentally infected with lethal dose of rabies wild strain (Perrin et al. 1985). Protection induced in mice was ten to twenty fold higher with rabies immunosomes than with purified G protein or virosomes (Perrin et al. 1985).

A chimeric peptide containing antigenic determinants from rabies virus G protein (amino acids 253-275) was tried as edible vaccine and good humoral immune response was observed in human (Yusibov et al. 2002).

In one study, monkeys that had been vaccinated one-time i.m., or via gene gun, with different concentrations of G gene were challenged 1 year after vaccination. Overall, 60% (3/5) of the gene gun vaccinated monkeys and 87% (5/6) of the i.m. Vaccinated monkeys survived lethal viral challenge (Donald et al. 2002).

The oral rabies vaccine (ORV) is genetically engineered (modified-live vaccinia vectored vaccine derived from an attenuated strain of the vaccinia virus). As the first recombinant ORV, vaccinia-rabies G protein (V-RG) was developed by inserting the ERA (Evelyn-Rokitnicki-Abelseth) rabies strain G protein gene into the thymidine-kinase region of the Copenhagen strain of vaccinia virus genome (Kieny et al. 1984; Wiktor et al. 1984). RABORAL V-RG is one of two ORV products recommended by the WHO to immunize wildlife against rabies. It was endorsed by WHO based on results of target and non-target wild animal safety testing, as well as the demonstration of efficacy in several rabies reservoir species (WHO expert consultation on rabies, 2005; Brochier et al. 1989; Pastoret et al. 1992; Hammami et al. 1999). Pseudorabies virus (PRV) expressing the G protein has been licensed for use as a live vaccine in pigs and possesses an excellent safety and efficacy record (Qian et al. 2004).

Efficacy of the RABORAL V-RG vaccine was proved under laboratory conditions in the red fox (Vulpes vulpes) (Blancou et al. 1986), dog (Canis familiaris) (Chappuis et al., 1993), raccoon (Procyon lotor) (Rupprecht et al., 1982).
1988) and striped skunk (*Mephitis mephitis*) (Tolson et al., 1987). Efficacy in field was demonstrated by distribution of millions of RABORAL V-RG doses into wildlife habitat in Western Europe to vaccinate red foxes and in the United States of America (US) to vaccinate raccoons, coyotes (*Canis latrans*), and under experimental conditions, gray foxes (*Vulpes cinereoargenteus*) (Brochier et al. 1996, Roscoe et al. 1998, Fearneyhough et al. 1998; Mackowiak et al. 1999, Sidwa et al. 2005). RABORAL V-RG has also been used in Israel to eliminate rabies in red foxes and golden jackals (*Canis aureus*) (Yakobson et al., 2006). Large-scale vaccination campaigns using RABORAL V-RG have contributed to the elimination of red fox rabies in Belgium, France, and Luxembourg (Brochier et al. 1991, Brochier et al. 1995, Masson et al.1996, Aubert et al. 2004, Cliquet et al. 2004).

In a study two, rabies post-exposure therapies were comparatively evaluated: BALB/c mice were challenged at day 0 with rabies virus and then received either a single dose of rabies G-gene based DNA vaccine administered at day 0, or five doses of cell culture-derived rabies vaccine administered at days 0, 3, 7, 15 and 28. Both regimens rapidly triggered protective levels of neutralizing antibodies against rabies virus in vaccinated mice. In addition, one injection of DNA vaccine protected 53% of the challenged mice, compared to 40% of mice protected after five injections of cell culture-derived vaccine (Bahloul et al.2003).

Post-exposure vaccination in non-human primates with DNA of G protein gene, in combination with a one-time treatment with human rabies immune globulin (HRIG), protected 50 and 75% of the monkeys, respectively, as compared to 75% mortality of the controls (Lodmell et al. 2002).

Vaccine trials targeting dogs living in field conditions in Tunisia further established that rabies DNA-based vaccination induced a stronger induction of virus neutralizing antibodies compared to Rabisin® (Bahloul et al. 2006). It has been shown that the expression of two copies of the rabies virus (RV) G-gene from a replication-competent RV vector significantly increases anti-RV-specific immune responses (Faber et al. 2002).

In dogs, administration of 100mg DNA by the i.m. route resulted in stronger and more durable rabies virus neutralizing antibody (RVNA) titers
than those obtained by i.d. inoculation. In contrast, i.m. vaccination of cats with a similar dose was less effective in terms of mean titer and seroconversion frequency. However, efficacy was improved by increasing the dosage to 300mg of DNA per immunization. Interestingly, i.d. inoculation of cats appeared to be a superior route of delivery in this species, resulting in higher seroconversion frequency than i.m. administration. In addition, geometric mean RVNA titers in i.d. inoculated cats increased over four-fold during a seven month period following a second and final immunization. It was reported that non-facilitated, naked DNA vaccines could elicit strong, antigen-specific immune responses in dogs and cats (Osorio et al. 1999).

The red fox, dog, and raccoon dog were given Oral rabies vaccines (ORV) through a bait containing a vaccinia-rabies G protein (V-RG) vaccine and induction of rabies virus neutralizing antibodies and protection after a virulent rabies challenge were observed (Cliquet et al. 2008).

A self-replicating RNA vaccine encoding rabies virus G protein gene was developed and mice were immunized with 10ug of Sin-Rab-G RNA and immune responses were compared with mice immunized with rabies DNA vaccine and commercial cell culture vaccine (Rabipur). The self-replicating rabies RNA vaccine generated cellular and humoral IgG responses similar to rabies DNA vaccine. On challenge with rabies virus CVS strain, rabies RNA vaccine conferred protection similar to rabies DNA vaccine. These results demonstrated that replicon-based self-replicating rabies RNA vaccine with 10ug dose was effective in inducing immune responses and protection similar to rabies DNA vaccine (Saxena et al. 2008).
1.15. Combination vaccines:

A combination vaccine is a formulation containing several individual vaccines. This method of vaccine development is preferred in order to reduce dose schedules and multiple needle pricks.

Multi-disease combination:
Vaccines include individual vaccines for different diseases.
Mixed at one of three stages:
(i) During the manufacturing process such that the combination is filled into vials or syringes;
(ii) At the time of administration by mixing different vaccines in a vial then taking the mixture up into a syringe for injection or
(iii) At the moment of administration from the 2 chambers of a dual-chambered syringe.
Example: Polio vaccine, DPT vaccine, *Haemophilus influenzae* type b (Hib), MMR (measles-mumps-rubella)

Multivalent combination vaccines:
Are directed against serotypes or serogroups of the same viral or bacterial pathogen.
Invariably are mixed during the manufacturing process.
Developed as such from the outset rather than developing and licensing each individual serotype vaccine separately.
Example: Pneumococcal vaccine

1.15.1. DPT:

The first combination vaccines available were mixtures based on the Diphtheria, Tetanus and wild type pertussis (DPTw) vaccines. Along with DPTw the combinations included; DPTw-Inactivated polio (IPV), DPTw-Hib (the capsular P serotype of Haemophilus influenza type B conjugated to D or T
toxoids), DPTw-HB (Hepatitis B), and DPTw-Hib-IPV (Vidor et al.1999). The ‘ultimate’ combination being developed, was DPT-Hib-IPV-HB, covers 6 different diseases and has up to 12 different vaccine antigens. Given the compatibility in their schedules for immunization, it would enable a reduction of needle-sticks in infants and children.

The available literature cited above strongly suggests utility of recombinant G protein as a vaccine candidate though DNA based immunizations of animals did induce high neutralizing antibody titers and are being successfully used by different countries for wild life immunization against rabies.

As our aim was to attempt to develop a vaccine for predominant pediatric age group, we opted for G protein of CHPV for all our experiments and fortunately, we were successful.