CHAPTER 2

REVIEW OF LITERATURE
Historical Perspectives

Rabies is an ancient disease, perhaps as old as humankind. 3000 years BC, the word rabies originated from "rabhas" which in Sanskrit means "to do violence". The Greek word for rabies, "lyssa" is derived from "lud" which means "violent". Thus, the family of viruses to which rabies belongs is lyssa. The first description of the disease dates from the 23rd century BC in the Eshuma Code of Babylon.

50,000 years ago, various pathogens could have migrated during the exchanges of fauna and human populations over the Bering Strait. Reports of rabies-like disease among native people of the entire region of Pacific Northwest support this concept [Winkler, 1975]. Rabies as well as the link between human disease and animals, especially dogs was well known. Girolamo Fracastoro a famous Italian scholar, observed the disease in many patients following which he described the disease and its routes of contamination in 1530, i.e. 350 years before Louis Pasteur.

Records at the time of the Spanish conquest in Middle America associate vampire bats with human illness [Baer, 1994]. Terrestrial rabies surfaced for the first time in California in the 1703 [Baer, 1994]. Throughout the late 1700s, dog and fox rabies outbreaks were reported in the mid-Atlantic colonies [Winkler, 1975]; this probably aggravated by the introduction of dogs and red foxes, imported for hunting, throughout New England in the 1800s. Skunk rabies was also reported frequently throughout the western states by the 19th century [Baer, 1994]. Canine or street rabies was a scourge everywhere in the 19th century, especially in Europe. Fear of rabies, improper knowledge about mode of contamination, absence of any efficacious treatment, was almost irrational. Patients killed themselves or were killed when bitten by a dog, believed to be rabid. In this world of irrational terror, the first post-exposure treatment in 1885 gave Louis Pasteur an international aura that his previous major scientific works had not been able to provide.

The causative organism

Rabies virus belongs to the order Mononegavirales, viruses with non-segmented, negative-stranded RNA genomes. Within this group, viruses with a distinct "bullet" shape are classified in the Rhabdoviridae family, which includes following genera of animal viruses:
i) **Lyssa viruses:** They are categorized into seven major genotypes as tabulated in Table 1. In addition, four genotypes have been recently discovered, namely West Caucasian Bat virus, Aravan Virus, Khujand virus, and Irkut virus. Based on their biological properties, these genotypes are further subdivided into phylogroups 1 and 2. Phylogroup 1 includes genotypes 1, 4, 5, 6 and 7, while phylogroup 2 includes genotypes 2 and 3. The nucleocapsid region of lyssavirus is highly conserved from genotype to genotype across both the phylogroups; however, experimental data has shown that lyssavirus strains used in vaccinations are only from the first genotype of the first phylogroup. The type species is Rabies virus.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Virus</th>
<th>Source</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rabies virus</td>
<td>Dog, fox, raccoon, bat and others</td>
<td>Widespread</td>
</tr>
<tr>
<td>2</td>
<td>Lagos bat virus</td>
<td>Bats, cats; has not been detected in human beings</td>
<td>Africa (rare)</td>
</tr>
<tr>
<td>3</td>
<td>Mokola</td>
<td>Shrews, cats</td>
<td>Africa</td>
</tr>
<tr>
<td>4</td>
<td>Duvenhage</td>
<td>Insectivorous bat</td>
<td>Africa (rare)</td>
</tr>
<tr>
<td>5</td>
<td>EBLV Type 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Type 1a</td>
<td>Insectivorous bat</td>
<td>Netherlands, Denmark, Germany, Poland, Russia, Hungary, France</td>
</tr>
<tr>
<td></td>
<td>Type 1b</td>
<td>Insectivorous bat</td>
<td>Netherlands, France, Spain</td>
</tr>
<tr>
<td>6</td>
<td>EBLV type 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Type 2a</td>
<td>Insectivorous bat</td>
<td>Netherlands, UK, Germany, Ukraine</td>
</tr>
<tr>
<td></td>
<td>Type 2b</td>
<td>Insectivorous bat</td>
<td>Switzerland</td>
</tr>
<tr>
<td>7</td>
<td>Australian bat lyssavirus</td>
<td>Frugivorous bat (or flying fox); insectivorous bat</td>
<td>Australia, Philippines</td>
</tr>
</tbody>
</table>

**Table 1:** Lyssavirus genus of rhabdovirus family. Adapted from The Lancet, 2004.

ii) **Ephemero viruses:** They cross-react strongly in complement fixation and indirect immunofluorescence tests. They may show low level cross-reactions by indirect immunofluorescence with viruses of the genus Lyssavirus. No serogroups
within the genus have been established. Sequence comparison with other Rhabdovirus indicate that in evolutionary terms the Ephemeroviruses are closer to members of the genus Vesiculovirus than to those of other defined genera in the family. The type species is Bovine ephemeral fever virus, which causes Bovine ephemeral fever or the “Three Day Sickness”. It is an arthropod vector-borne disease of cattle. The characteristics of the disease are the sudden onset of fever, stiffness, lameness, nasal and ocular discharges, depression, cessation of rumination and constipation. Although the pathogenesis of the disease is complex, it seems clear that the host inflammatory responses mediated by the release of cytokines, are involved in the expression of the disease.

iii) Vesiculo viruses: They infect a wide range of vertebrates and invertebrates. The type species is vesicular stomatitis Indiana virus. It affects horse, cattle, swine, donkey, camelids and humans; sheeps and goats are resistant to the disease. The pathogen is transmitted by black fly, house fly and sand fly.

The Rabies virus

Rabies virus has RNA genome, which encodes for five proteins. These include nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the large virion associated transcriptase (L). The order and relative size of the genes in the genome are shown in the figure below:

![Fig. 1 Rabies Genome](image)

The arrangement of these proteins and the RNA genome determine the structure of the rabies virus. All rhabdoviruses have two major structural components: a helical ribonucleoprotein core (RNP) and a surrounding envelope. In the RNP, genomic RNA is tightly encased by the nucleoprotein. Two other viral proteins, P and L are associated with the RNP. The glycoprotein forms approximately 400
trimeric spikes which are tightly arranged on the surface of the virus. The M protein may be the central protein of rhabdovirus assembly as it is associated with both envelope and RNP. The basic structure and composition of rabies virus is depicted in following diagrams:

![Diagram of Rabies virion: longitudinal view](image)

![Diagram of Rabies virion: cross sectional view](image)

**Invasion and replication**

Rabies virus can infect a great variety of neuronal and non-neuronal cells *in vitro*. Specific binding occurs at neuromuscular junctions, where virus co-localizes with the nicotinic acetylcholine receptor [Lentz *et al.*, 1982; Lewis *et al.*, 2000]. Binding at this postsynaptic site is competitive with cholinergic ligands, including the snake venom neurotoxin α-bungarotoxin, which shows sequence homology with the envelope glycoprotein of rabies virus.
Rabies virus attaches specifically to two other receptors on neuronal cell membranes: the neural cell adhesion molecule [Thoulouze et al., 1998] and the p75 neurotropin receptor (p75NTR) [Langevin et al., 2002]. Two neurotransmitter receptors in the central nervous system, N-methyl-D-aspartate subtype R1 and Gamma-Aminobutyric Acid have also been suggested as possible receptors for rabies virus [Gosztonyi & Ludwig, 2001]. Non-specific viral attachment to several types of cell-surface receptors including carbohydrates, phospholipids, and sialylated gangliosides has been demonstrated [Warrell & Warrell, 2004].

Rabies virus enters cells by adsorptive endocytosis into endosomes. Soon after infection, virus may be associated with synaptic vesicles, since it co-localizes with synapsin I, or with early acidic endosomes [Lewis et al., 2000] whereby, viral glycoprotein could fuse with the endosomal membrane releasing the core ribonucleoprotein complex into the cytosol. The acidic pH of endosome is important because the glycoprotein needs to be exposed to acidic pH for it to facilitate fusion. Viruses move up towards the neuron's nucleus by retrograde axoplasmic flow [Fig. 3]. The retrograde transport is a natural function of nerve

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**Fig. 3: Retrograde transport.** The neuroinvasiveness of rabies virus results from its ability to migrate to CNS through retrograde axonal transport and transynaptic spread. Adapted from Current Topics in Microbiology and Immunology, 2005.
Review of Literature

cells to transport messenger molecules from axon to nucleus, where dynein molecules move along the microtubules of the axon. Here, the ribonucleoprotein complexes of the virus are carried by direct attachment to a dynein motor or by encapsulation in vesicles attached to a dynein motor [Dietzschold et al. 2005]. Rabies virus capitalizes on this mechanism to reach the spinal cord; moving at 8 to 20 mm per day.

Replication of virus can occur locally in striated muscle at the bite site, before any contact with nervous tissue; this could account for long incubation periods [Warrell & Warrell, 2004]. Virus is soon detectable experimentally at local motor or sensory nerve endings and, after superficial inoculation, in epithelial layers [Murphy, 1977]. It can directly enter the peripheral nerves as well.

The virion RNA polymerase then copies virion RNA. Single monocistronic mRNA encodes all viral proteins. Messenger RNAs are translated on host ribosomes and all the proteins are produced simultaneously. RNA replication occurs in the cytoplasm and is carried out by the viral RNA polymerase. The full length plus strand is coated with nucleocapsid protein as it is made. The new positive strand is copied into full length minus strand, which is also coated with nucleocapsid protein as it is made. The new negative strands may be used as templates for the synthesis of full length plus strands, mRNAs, or be packaged into virions. The virus consists of two "modules" - envelope and nucleocapsid. Transmembrane proteins are made on ribosomes bound to the endoplasmic reticulum. They are inserted into the endoplasmic reticulum membrane as they are made; glycosylated in the endoplasmic reticulum and pass through the Golgi body where substantial modification of the carbohydrate chains occurs. They are then transported, in vesicles, to the appropriate cell membrane. This constitutes the envelope. After synthesis of the nucleocapsid, the viral RNA polymerase complex associates with them as they are formed. Nucleocapsids bud out through modified areas of membrane that contain G and M proteins. The M protein interacts with patches of G in the membrane and with nucleocapsids and is thus, involved in assembly. Finally, the rabies virion buds from the host cell. Productive viral replication with budding from plasma membranes takes place predominantly in the salivary glands, excreting virus that is transmissible to other mammals. The sequence of infection and replication of rabies virion is depicted in Fig. 4.
Fig. 4: Sequence of infection and replication of rabies virion. The virion adsorbs to host membrane protein (1), the cell engulfs it in an endosome (2). A protein in the endosome membrane pumps protons from the cytosol into the endosome interior. The resulting decrease in endosomal pH induces a conformational change in the viral glycoprotein, leading to fusion of the viral envelope with the endosomal lipid bilayer membrane and release of the nucleocapsid into the cytosol (3&4). Viral RNA polymerase uses ribonucleoside triphosphates in the cytosol to replicate the viral RNA genome (5) and synthesize viral mRNAs (6). One of the viral mRNAs encodes the viral transmembrane glycoprotein (shown in blue), which is inserted into the lumen of the endoplasmic reticulum (ER) as it is synthesized on ER-bound ribosomes (7). Carbohydrate is added to the large folded domain inside the ER lumen and is modified as the membrane and the associated glycoproteins pass through the Golgi apparatus (8). Vesicles with mature glycoprotein fuse with the plasma membrane, depositing viral glycoprotein on the cell surface with the large folded domain outside the cell, the transmembrane α-helix spanning the plasma membrane, and the small cytoplasmic domain within the cell (9). Meanwhile, other viral mRNAs are translated on host-cell ribosomes into nucleocapsid protein, matrix protein, and viral RNA polymerase (10). These proteins are assembled with replicated viral genomic RNA (shown in dark red) into progeny nucleocapsids (11), which then associate with the viral transmembrane glycoprotein in the plasma membrane (12). As additional copies of the matrix protein on a single nucleocapsid associate with the cytoplasmic domain of additional copies of the viral transmembrane glycoprotein, the plasma membrane is folded around the nucleocapsid, forming a "bud" that eventually is released (13). Adapted from www.http://compbio.mcs.anl.gov/sulakhe/virus/interest.htm
**Pathogenesis**

Rabies virus (RV) pathogenicity has been studied for a considerably long duration (> 100 years); the data indicates that RV consists of a wide array of variants. These can range from highly pathogenic strains, such as silver-haired bat virus, to extremely attenuated RV vaccine strains, such as SAG-2, which are not pathogenic even in severe combined immunodeficiency (SCID) mice after oral application [Morimoto et al., 2000; 2001].

Infection usually begins with the bite of a rabid animal, which breaks the skin. The saliva containing virus first encounters muscle cells and viral glycoprotein attaches to the nicotinic acetylcholine receptor. Rabies virus replicates in these cells until enough concentration is built up after which some viral particles come in contact with sensory or motor nerve cells. The virus can also enter nervous system directly without local replication. It then multiplies in the brain and may then spread through peripheral nerves to the salivary glands and other tissues. Rabies virus is also found in pancreas, kidney, heart, retina and cornea.

Susceptibility to infection and the incubation period may depend on the host's age, genetic background and immune status; the viral strain involved, the amount of inoculum, the severity of lacerations and the distance the virus has to travel from its point of entry to the central nervous system. The clinical spectrum is divided into three phases: Prodromal, Acute neurological or excitation and Coma or terminal phase. Their symptoms are as follows:

**Prodromal phase:** this is a short phase, which usually lasts for 2-10 days. The symptoms are mild and nonspecific. They include: slight fever (100 F to 102 F), chills, malaise, headache, anorexia, nausea, sore throat (the beginnings of hydrophobia), photophobia, musculoskeletal pain and a persistent loose cough. Usually there is an abnormal sensation around the site of infection, including local or radiating pain, burning, or itching, a sensation of cold and/or tingling.

**Acute neurological or excitation phase:** this stage usually lasts for 2 to 7 days. Patients experience nervousness, anxiety, agitation, marked restlessness, apprehension, irritability, hyperesthesia, sensitivity to loud noises, hydrophobia, excessive salivation (1 to 1.5 liters in 24 hours), lacrimation (secretion of tears) and perspiration. As the virus begins replicating in the brain, impairment of the cranial nerve occurs. This causes eye conditions: palsies, lack of parallelism of the
visual axes of the eyes, asymmetrical dilation of constriction of the pupils and an absence of corneal reflexes. At the same time, there is weakness of facial muscles and hoarseness. Systemic symptoms are severe, and they include: tachycardia or bradycardia, cyclic respirations, urinary retention and a temperature of 103 F. The patient is often lucid between excitation and hydrophobic episodes.

**Coma or terminal phase:** This phase usually begins 2-7 days after onset of neurologic phase. It is characterized by generalized flaccid paralysis. The patient experiences peripheral vascular collapse, coma, eventually leading to death. The figure below shows some of the symptoms observed for rabies infection.

![Fig. 5: Symptoms of rabies infection. A-C: Facial bite wounds inflicted by a rabid dog and carrying a high risk of rabies D-F: Rabies encephalomyelitis: hydrophobic spasm. Adapted from http://www.cdc.gov/rabies/virus.html; and Rabies and Envenomings, WHO, 2007.](image)

In the critical stage, rabies manifests in two forms:

- **Furious rabies:** it is characterized by agitation, thrashing, biting, viciousness, choking, gagging, hyperventilation, cardiac arrhythmias, paralysis and death.
- **Paralytic or dumb rabies:** it is characterized more by paralytic symptoms, which may include apathy, apparent depression, increased blood pressure, tachycardia, confusion, hallucinations and disorientation. These symptoms are
followed by increased periods of hyperactivity, stiffness in the back of the neck, and an increase in the number of cells in the cerebrospinal fluid. Dumb or paralytic rabies ends with coma and death by respiratory failure. Paralytic rabies is most often experienced by patients who have been exposed to virus via bats.

**Molecular basis of pathogenesis**

RV pathogenesis has been associated with phosphoprotein and glycoprotein.

**Rabies virus Phosphoprotein (P):** studies indicate that specific interaction of a conserved domain within P and the cytoplasmic dynein light chain (LC8) is responsible for retrograde transport to the central nervous system and, therefore, P is partly responsible for RV pathogenesis [Jacob et al., 2000; Raux et al., 2000; Poisson et al., 2001]. Mebatsion showed that the deletion of 11 amino acids within P abolishes the P-LC8 interaction and reduces the efficiency of the peripheral spread of RV [Mebatsion, 2001].

**Rabies virus Glycoprotein (G):** A large body of evidence shows that G is the prime pathogenicity determinant. It is also major antigen responsible for the protective immune response as it is the primary target of the host humoral [Wiktor et al., 1973; Cox et al., 1977] and cellular immune responses [Macfarlan et al., 1986; Celis et al., 1988]. G is the viral attachment protein responsible for host cell receptor recognition [Tuffereau et al., 1998], rapid virus uptake [Dietzschold et al., 1983; Seif et al., 1985; Morimoto et al., 1999] and for low pH-induced fusion of the viral envelope with endosomal membranes [Gaudin et al., 1993]. RV-G is a 505-amino-acid membrane glycoprotein, containing a 44-amino-acid cytoplasmic tail and a 22-amino-acid transmembrane domain [Wunner et al., 1988]. An important pathogenicity marker of RV is the arginine residue at position 333 (R333) of G, and a change to glutamic acid (E333) or aspartic acid (D333) not only abolishes RV pathogenesis but also hampers the virus ability to enter motor neurons [Coulon et al., 1998]. E333 exchange can lead to complete attenuation of some of the partially attenuated vaccine strains; however some pathogenic RV strains cannot be fully attenuated with this mutation, indicating that other mutations in the RV-G may be necessary [Morimoto et al., 2001].
Laboratory Diagnosis

The diagnosis of animal and human rabies can be made by the following methods [Fig. 6]:

**Histopathology:** Histological examination of biopsy or autopsy tissues, particularly fixed archive material, is occasionally useful in diagnosing rabies. However, conventional tests based upon the detection of Negri bodies and histological tests such as Sellers’s or Mann’s tests are no longer considered reliable; as for example, the absence of Negri bodies doesn’t rule out rabies because they are not observed in at least 20% cases. Thus, their use has been largely discontinued.

**In-situ hybridization:** is based on the specific binding of complementary riboprobes labeled with digoxigenin, to the viral genome and messenger RNA. The ability to detect messenger RNA is indicative of the presence of replicating virus.

**Molecular methods:** techniques like polymerase chain reaction (PCR), Reverse transcription (RT)-PCR are employed for diagnosis of rabies virus RNA from a range of biological fluids and samples (e.g. saliva and skin biopsies of hair follicles).

**Virus cultivation:** the most definitive means of diagnosis involving virus cultivation from infected tissue. Tissue culture lines regularly employed for the same include WI-38, BHK-21 and CER. Immunofluorescence (IF) is routinely used to detect the presence of rabies virus antigen in the tissue culture. The more commonly used method for virus isolation is by the inoculation of saliva, salivary gland tissue and brain tissue intracerebrally into infant mice. The mice should develop paralysis and die within 28 days. Upon death, brain is examined for the presence of the virus by IF.

**Fluorescent antibody test (FAT):** also referred as the direct fluorescent antibody test (dFA), is the mainstay of all rabies diagnostic laboratories. This test requires post mortem brain tissue (hippocampus, cerebellum or medulla oblongata) from animals suspected of having rabies. The presence of rabies virus in a brain smear is detected using a fluorescent marker tagged anti-rabies antibody.

**Serology:** circulating antibodies appear slowly in the course of infection but they are usually present on onset of clinical symptoms. Earlier employed mouse
infection neutralization test (MNT) or the rapid fluorescent focus inhibition test (RFFIT) have now been largely superseded by enzyme immunoassays (EIAs). Serology had been reported to be the most useful method for the diagnosis of rabies.

**Fig. 6: Methods of diagnosing rabies.** 1: Histopathology showing Negri bodies; 2: In situ hybridization; 3: Molecular methods; 4: Virus cultivation; 5: Fluorescent antibody test (FAT); 6: Serology. Adapted from www.defra.gov.uk/vla/science/sci_rabies_diag.htm.

### Transmission

Worldwide, canid species are the main vector in the transmission of rabies to humans, particularly in developing countries where canine rabies is endemic [Krebs et al., 1995]. Cats are the second most important carrier of rabies for humans. In developed countries, rabies is mainly found in wild animals. The disease is transmitted to domestic animals and humans through exposure to infected saliva. In the past few years, bat rabies has emerged as a public health problem in America and Europe. For the first time in 2003, more people in South America died from rabies following exposure to wildlife, particularly bats, than from dogs. Human rabies deaths following exposure to wild foxes, raccoons, skunks, jackals and wolves are very rare. Livestock, horses and deer can become infected with rabies, but although they could transmit the virus to other animals or people, this rarely occurs.
Epidemiology of human rabies

Globally, rabies is the tenth leading cause of death due to infection in humans. The threat of rabies exists in most parts of the world [Fig. 7]. Predominantly, rabies affects poor people in developing countries and its true incidence may be underestimated. In the year 2005, there were reports estimating that nearly 60,000 human fatalities occur each year mostly in Asia and Africa [Knobel et al., 2005]. Since 1985, India has reported an estimated 25,000–30,000 human death from rabies annually [Sudarshan, 2005; Sudarshan et al., 2007]. The majority of people who die of rabies are people of low-socioeconomic status [Deshmukh et al., 2004]. In India, about 15 million people are bitten by animals, mostly dogs, every year and need post exposure prophylaxis. Since May 2006, rabies has become the leading cause of infectious disease mortality in China, killing 3,293 people in 2006, 27% more than in 2005. More than 99% of all human deaths from rabies occur in the developing world [WHO, 1998] and about 30% to 60% of the victims of dog bites (the primary mode of virus transmission) are children less than 15 years of age. The lack of supplies of rabies immunoglobulin and training

Fig. 7: Global distribution of lyssaviruses. Red: rabies virus; Green: rabies-related, not classic virus (rabies-free; as per WHO); White: rabies-free. Adapted from The Lancet, 2004.
in its correct use condemns people worldwide to die an agonizing death from rabies. Crowded urban centers with inadequate public health infrastructures are prone to transmission of rabies virus. In developed countries where canine rabies has retreated, the transmission of rabies by wild mammals accounts for 90% of human exposures [Hankins, 2004]. Rabid bats, especially silver-haired bats, are the most prevalent source of human rabies in the United States. 14 of the 18 cases acquired in the United States since 1980 involved rabies variants associated with insectivorous bats [CDC, 1994]. Between 25,000 and 40,000 people in the US are treated annually for exposure to rabid or potentially rabid animals [Hankins, 2004]. Based on available epidemiological data, approximately 9 million vials of rabies vaccine are required annually.

**Prevention and Management**

More than 99% of infections in humans and mammals that develop symptoms end fatally. Recovery and survival is extremely rare. It is thus essential that individuals at high risk receive preventive immunization and individuals be given immediate post-exposure prophylaxis after any case of exposure. No rabies deaths have been reported in people who have had pre-exposure treatment followed by a booster dose after exposure. Such a treatment should be initiated promptly ensuring symptoms have not been initiated. Prophylaxis is recommended for people at occupational risk and for travelers to areas where dog rabies is endemic. The most cost-effective strategy for preventing rabies in people is by eliminating rabies in domestic animals (mostly dogs) through mass vaccinations programmes. Vaccination of dogs has led to elimination of transmissions to people and thus, reduced disease burden in several developed and developing countries such as Japan and Malaysia. Prevention of human rabies must be a community effort involving both veterinary and public health services; and it is a realistic goal if proper management strategies are executed. When humans are exposed to suspect animals, attempts to identify, capture or humanely sacrifice the animal involved should be undertaken immediately. Animals that are sacrificed or have died should be tested for the virus, with results sent to concerned veterinary services and public health officials so that the situation in the area is well documented.
The Cost of Prevention

Underreporting is a characteristic of almost every infectious disease in developing countries, therefore the number of post exposure prophylaxis (PEP) cases reported worldwide are largely underestimated. An important consideration for rabies is that human infection is incidental to the reservoir of disease in wild and domestic animals; therefore, a more accurate projection of the impact of rabies on public health should include an estimate of the extent to which the animal population is affected and the expenses involved in preventing transmission of rabies from animals to humans. Despite evidence that control of dog rabies through programs of animal vaccination and elimination of stray dogs can reduce the incidence of human rabies, exposure to rabid dogs is still the cause of over 90% of human exposures to rabies and of over 99% of human deaths worldwide. The cost of these programs prohibits their full implementation in much of the developing world, and in even the most prosperous countries the cost of an effective dog rabies control program is a drain on public health resources. The annual cost of rabies is estimated USD 583.5 million in Asia and Africa alone [Knobel et al., 2005] and up to USD 300 million in the United States. These costs include the vaccination of companion animals, animal control programs, maintenance of rabies laboratories and medical costs, such as those incurred for PEP. The average cost of rabies immunizations after a suspicious animal bite is US$ 40 in Africa, and US$ 49 in Asia. This post-exposure treatment is a major financial burden for most households in these countries, where the average wage is about US$ 1 to US$ 2 per day, per person.

Prophylaxis:

The prophylaxis is aimed at pre and post exposure management [Warrell & Warrell, 2004]. The most successful form of rabies prevention is pre-exposure vaccination. No rabies deaths have been reported in people who have had pre-exposure treatment followed by a booster dose after exposure. In addition, rabies is entirely preventable even after severe exposure, provided post-exposure prophylaxis is given timely.
A. Pre-exposure treatment:
Prophylaxis is recommended for people at occupational risk such as vets, laboratory workers, animal handlers and wildlife officers and for travelers to areas where dog rabies is endemic. The standard pre-exposure regimen includes tissue-culture vaccines. Booster doses increase and prolong the antibody response. Thus, they should be offered to persons at continuing risk every one to three years.

B. Post exposure treatment:
Wound cleansing and immunizations, done as soon as possible after suspect contact with an animal and following WHO recommendations, can prevent the onset of rabies in virtually 100% of exposures. Recommended treatment varies depending upon the severity of the contact, according to which following categories are listed:

- **Category I** : touching or feeding suspect animals, but skin is intact; no treatment is required
- **Category II** : minor scratches without bleeding from contact, or licks on broken skin; immediate vaccination recommended
- **Category III** : one or more bites, scratches, licks on broken skin, or other contact that breaks the skin; or exposure to bats; immediate vaccination and administration of rabies immunoglobulin recommended.

**Immunization**
Active immunization employing anti-rabies vaccine is provided for Category II and III exposures. Currently, tissue culture derived vaccines are used, which include: Human diploid cell vaccine (HDCV), Rabies vaccine adsorbed (RVA), Live attenuated vaccine (PCEC). They are usually administered intramuscularly into the deltoid region, or, in small children, into the anterolateral area of the thigh muscle. The vaccine is given as 5 doses regimen, comprising of one dose of rabies vaccine administered on each of days 0, 3, 7, 14 and 28.

Passive immunization utilizing human or equine immunoglobulins is provided for Category III contact and immunosuppressed people. There is convincing evidence that combined treatment with rabies immunoglobulin and active
immunization is much more effective than active immunization alone. It is mediated by:

a) Human rabies immunoglobulin (HRIG) – refers to gamma globulin prepared by cold ethanol fractionation from the plasma of hyperimmunized humans. These are expensive and generally in short supply or non-existent in most developing countries.

b) Anti-rabies equine serum (ERIG) – refers to concentrated serum preparation of hyperimmunized horses. These provide at least a partial solution to current problems of insufficient quantities and the high cost of human immunoglobulin in developing countries.

Rabies Vaccines

The essential goal for rabies vaccine advancement is the development of safe, effective, economic and portable vaccines. Although vaccine strategies have greatly reduced the disease burden in developed countries, programs to vaccinate wild and domestic animals are the key to continue these trends. Such programs also need to be undertaken in developing countries to check the spread of rabies.

1. First generation of rabies vaccines

Nerve tissue vaccines (NTVs) – Over 124 years ago, in 1885, Louis Pasteur developed a crude nerve tissue vaccine for the post-exposure treatment of rabies. This form of vaccination used desiccated infected tissue and was found to prevent rabies infection in a 9 year old boy named Joseph Meister. These days, NTV preparation consists of inactivated suspension (5 %) of infected animal nervous tissue.

Nerve-tissue vaccines for animals

Inactivated nerve-tissue vaccines may be produced from the brains of lambs or newborn mice. Such vaccines have been shown to be effective in mass canine immunization programmes in North Africa as well as Latin America and the Caribbean using lamb brain based and suckling mouse brain based vaccines, respectively.
**Nerve-tissue vaccine for humans**

Semple nerve-tissue vaccine is employed for humans. It is derived from phenol-inactivated infected rabbit brain. These preparations are associated with the rare complication of demyelinating allergic encephalitis, probably brought about by the myelin basic protein in the vaccine. This complication was shown to occur in 4.6 cases for 1000 persons vaccinated by the Semple vaccine. The case-fatality proportion is 3.13%. Due to these side effects, its usage was largely discontinued. In order to reduce the level of encephalitogenic substances in the final product, vaccines are also prepared by inoculation in one day old mice. Such suckling mouse brain vaccine is used in some Central and South American countries.

**2. Second generation of rabies vaccines**

These modern cell culture vaccines were developed to replace unpurified reactogenic nerve tissue vaccines. Although they are safer and more efficacious than NTVs, they are expensive to produce and therefore not affordable for many developing countries with limited budgets for healthcare [Beran et al., 2005].

**Modified vaccines for animals**

Modified live-virus (MLV) and inactivated vaccines can be produced in cell culture, using either primary cells or continuous cell lines. The seed virus cell systems vary considerably between different manufacturers. Recent improvements in vaccine production techniques have enhanced usage of inactivated vaccines for animal immunization. Vaccines that provide stable and long-lasting immunity are recommended, because these represent the most effective method of controlling and eliminating the disease in animals and circumventing the transmission to humans. Several types of modified live-virus vaccines have been proposed for the oral immunization of animals in the past 20 years; however, only five have proved suitable for use in the field for vaccination of foxes (Canada and Europe) and raccoon dogs (Finland). All these vaccines are derivatives of the original Street Alabama Dufferin (SAD) vaccine strain. Live attenuated Purified Chick Embryo Cell (PCEC) vaccine has comparatively wider usage. Here, the live attenuated virus of Flury LEP-25 strain is adapted to growth in the chick embryo cell culture and then purified.
Modified vaccines for humans

They can be produced in embryonated eggs or cell lines. In the purified Duck Embryo Vaccine, fixed virus strain is grown in embryonated duck eggs and then inactivated by β-propiolactone. This vaccine is considerably less immunogenic. It also has side effects, which range from local reactions to constitutional symptoms such as fever, malaise, myalgia and generalized lymphadenopathy, however, with lower risk of allergic encephalitis. Vaccines prepared in cell culture are now widely available, and have been shown to combine safety with high immunogenicity. Several types of cell cultures are used for human rabies vaccine production:

(i) Primary cells like hamster kidney, dog kidney or chick embryo fibroblasts;
(ii) Diploid cell lines like human or rhesus monkey origin; and
(iii) Continuous cell lines like vero cells.

Advances in biotechnology, such as the culture of continuous cell lines on microcarriers in fermenters, have made possible the production of rabies vaccines on an industrial scale and at reduced costs. As high yields of rabies virus are produced, concentration of the virus is not required. Such vaccines include: Human Diploid Cell Vaccine (HDCV) and Rabies Vaccine Adsorbed (RVA).

HDCV: was introduced in 1978. In this vaccine, rabies virus Pitman-Moore L503 strain is adapted to growth in the WI-38 (US) human normal fibroblast or MRC-5 (Europe) cell line; followed by concentration by ultrafiltration and inactivation by β-propionlactone. Several studies have demonstrated its efficacy, wherein, antibodies were generated in 100% of all recipients. Adverse reactions to HDCV are extremely rare. However, high cost limits its wide usage. As human cell cultures are more difficult to handle than other animal cell culture systems and 5-6 doses of the vaccine are usually administered intramuscularly, the cost of immunization becomes very high (USD$ 100 for 6 doses). However, several studies suggest than smaller intradermal doses of HDCV may be as effective and thus it may be considered for use in poor developing countries.

RVA: this vaccine is made in fetal rhesus monkey lung cell line, followed by inactivation by β-propiolactone and concentration by aluminium phosphate.
3. Third generation of rabies vaccines

Several recombinant live vaccines of rabies have been constructed and tested for their immunological potential. The rabies virus glycoprotein-adenovirus type-5 recombinant vaccine has been tested experimentally in Canadian wildlife. It provided extensive protection in wild animals including raccoons, red foxes and ferrets [Prevec et al., 1990; Charlton et al., 1992]. However, this vaccine was not licensed by the Canadian government, largely due to the safety concerns of first-generation E1-replication-deficient Ad-5 in human gene therapy protocols [Marshall, 1999; Raper et al., 2002; Meyerholz et al., 2004]. A vaccinia virus expressing the rabies virus glycoprotein has been successfully used for the elimination of rabies in parts of Western Europe and North America [Wiktor et al., 1984; Brochier et al., 1991]. A single report of its adverse effect was reported; however, the patient was immuno-suppressed through pregnancy [Rupprecht et al., 2001]. In another study, the recombinant canine adenovirus type-2 (CAV-2) expressing the rabies virus glycoprotein was found to not only provide full protection against rabies virus but also stimulate effective immune response against canine adenovirus infection [Hua et al., 2006]. Thus, the third generation of recombinant viral vaccines were found to be largely safe and effective.

4. Fourth generation of rabies vaccines

"DNA VACCINES: TOWARDS A RADICAL CHANGE"

Following the first demonstration of vaccination by Jenner over 200 years ago, numerous attempts have been made to develop safer and more efficacious vaccines. Approximately 100 years after Jenner described immunization with a live vaccine; Pasteur introduced the concept of using killed vaccines to control infectious diseases. For the next 100 years, the great debate continued as to the advantages or disadvantages of one conventional vaccination strategy over the other. For example, killed vaccines are more potent inducers of antibody responses than cell-mediated immune responses. On the other hand, live vaccines generally induce a more balanced response; however, they may not be safe due to the potential risk of reversion to virulence [Hooke et al., 1985].

The advancement in molecular biology in the 1970’s heralded the era of recombinant DNA technology to produce a new generation of vaccines.
Vaccination is one of the major achievements of modern medicine. As a result of vaccination, diseases such as polio and measles have been controlled and smallpox has been eliminated. However, despite these successes there are still many diseases of microbial origin that cause tremendous suffering because either there are no vaccines or the available vaccines are inadequate. An ideal vaccine would be safe, effective, confer sustained protection from any subsequent exposure and account for practical considerations like low cost per dose, biological stability, ease of administration, fewer side-effects. The development of DNA based vaccines offers possibilities for developing the next generation of such vaccines.

The astonishing observation that intramuscular injections of "naked" DNA recombinant for β-galactosidase (β-Gal) resulted in high levels of expression of β-Gal in myocytes at the site of injection [Wolff et al., 1990], and that direct inoculation of an expression plasmid encoding a foreign protein into the skin of mice resulted in the induction of antibody responses [Tang et al., 1992], established the potential of DNA vaccination. Subsequently, it was demonstrated that intramuscular injections of plasmid DNA encoding influenza nucleoprotein could protect mice against a challenge with live influenza virus [Ulmer et al., 1993; Fynan et al., 1993]. In addition, investigators also showed the vaccine, based on one strain of influenza, could protect against a different strain. These observations have opened up new avenues for vaccine development.

The introduction of the DNA can be accomplished by intramuscular or intradermal injections using needles, as well as by propelling DNA-coated gold particles various tissues [Fynan et al., 1993], preferentially the dermis. Although very few cells can be transfected using these methods, the amount of protein produced as well as other parameters of the method lead to surprisingly Th2 immune responses [Davis et al., 1995], in the absence of any further adjuvant apart perhaps from the DNA itself. Subsequently, it was shown that DNA vaccines induce persistent, cell-mediated and humoral immune responses to antigens isolated from a variety of viral, bacterial and parasitic pathogens.

**Advantages of DNA vaccines**

DNA vaccines have an edge over traditional vaccines including protein subunit, live attenuated or inactivated viral vaccines; pertaining to several aspects. These include:
DNA vaccines are inherently safer than live attenuated vaccines or inactivated viral vaccines, which pose threat of reversion to virulence, making safety a concern.

DNA plasmids are relatively simple and inexpensive to design and create.

Their high stability and relatively temperature insensitivity, circumventing the need of cold chain, makes them highly suitable for mass production and distribution in both industrialized and unindustrialized nations.

DNA plasmid is amenable to the introduction of several open reading frames from one or more genes from a target infectious agent.

More effective in preventing viral diseases, resembling actual infection and developing life long immunity.

Protein produced is likely to be folded in its native conformation, which favours production of neutralizing antibodies.

Peptides synthesized from plasmid-DNA can be brought to cell surface and displayed by HLA Class I molecules - an essential step in evocation of cytotoxic T cells.

DNA plasmids themselves are not immunogenic. Thus, it is possible to successfully boost after DNA vaccination without producing a heterologous immune response to the chosen vector, in contrast to the case with viral or bacterial vectors [Mascola et al., 2005].

They may possess an inherent adjuvant capacity due to the presence of cytosine-phosphate-guanine oligonucleotide (CpG) sequences [Abdulhaqq & Weiner, 2008].

Design of DNA vaccines
Aiming at development of an ideal vaccine, which would be safe, effective, inexpensive, would rapidly elicit lifelong immunity, be delivered non-invasively in a single administration and impart protection against the said pathogen; following are the key to the construction of DNA vaccine:

Antigen considerations
Harmonization of the DNA sequence coding for the antigen, enables more efficient translation of non-mammalian DNA sequences by mammalian host cells
[Bergmann-Leitner & Leitner, 2004]. The basis for this approach is the preferential use of different codons for the same amino acid by bacteria, protozoan parasites, plants and mammals [Deml et al., 2001]. Through harmonization, rare codons, for which limiting amounts of tRNAs are available, will be removed, thus yielding more antigen without the risk of truncated translation products. Also, since the genes of many viral and bacterial antigens have codon profiles that are sub-optimal for mammalian expression, modifications in the codon usage of the plasmid DNA immunogens can be engineered to increase their expression significantly [Grosjean & Fiers, 1982]. Codon-optimization of HIV env led to significantly enhanced expression and immune responses [Andre et al., 1998] and modifications in HIV gag plasmid sequence allowed a 100-fold reduction in the amount of DNA required to induce CTL and antibody responses [zur Megede et al., 2000].

**Vector considerations**

⇒ **Vector Backbone**

DNA vaccines consist of bacterial plasmids into which target antigenic sequences are incorporated. Although many vectors are available, a consensus optimized vector is emerging as potentially the most acceptable for human use [Norman et al. 1997]. However, further modifications, such as the minimalistic, immunogenically defined gene expression (MIDGE) vectors, are being explored, both to increase performance and to remove non-essential backbone sequences [Moreno et al. 2004]. The basic attributes of a DNA vaccine vector are:

a. An origin of replication (ori) suitable for producing high yields of plasmid in *E. coli*. The ColE1 plasmid-derived bacterial ori is well known to provide high yields of plasmid DNA, up to 15–30 mg/l during culture in shake flasks using a rich nutrient medium.

b. An antibiotic resistance gene to confer antibiotic-selected growth in *E. coli*. The antibiotic marker ensures selective pressure to ensure growth of only plasmid harboring bacteria.

c. A strong enhancer/promoter and an mRNA transcript termination/polyadenylation sequence for directing expression in mammalian cells. Gene expression is commonly driven by the SV40 early promoter [Moreau et al., 1981],
the Rous sarcoma virus (RSV) LTR [Gorman et al., 1982], or the cytomegalovirus immediate early promoter (pCMVIE) [Boshart et al., 1985], used in conjunction with the SV40 or bovine growth hormone 30-untranslated region (BGH 30-UTR) transcript termination/ polyadenylation sequences [Pfarr et al., 1986]. An adjacent intron sequence may be included on which expression of many mammalian genes depends or for ensuring high transcription efficiency. Intron A is the most commonly employed sequence [Chapman et al., 1991].

Signal sequences

Intracellular targeting and intercellular spreading strategies for altering the cellular location of an antigen expressed by a DNA-based vaccine can modulate immune responses [Xiang et al., 1995; Boyle et al., 1997; Drew et al., 2000]. Thus, addition of signal sequences can retain the protein in cytosol or some sub-cellular compartments or direct it to cell membrane or secretion into extracellular milieu [Alves et al., 1998; Lu et al., 2003; Donnelly et al., 2005]. Therefore, the effectiveness of these vaccines can be distinct from each other since a different DNA construction strategy adopted may affect antigen presentation to the host immune system and consequently influence the elicited immune response [Alves et al., 1999; Donnelly et al., 2005]. Successful targeting was demonstrated for several pathogens including Human papillomavirus based on HPV-16 E6 antigen fused to both intracellular and intercellular targeting sequences [Peng et al., 2005], Influenza A based on nucleoprotein fused to a tissue plasminogen activator signal sequence [Luo et al., 2008]; Vaccinia based on TPA tagged vaccinia virus L1 antigen (tPA-L1R) [Golden et al., 2008]. In all these studies, targeted DNA vaccine induced more potent immune response and provided superior protection in vaccinated mice than the unmodified antigen based DNA vaccine.

DNA Delivery considerations

The quality of the immune response elicited by a DNA vaccine is also dependent by the procedure of DNA delivery that influences the mechanisms of DNA uptake, transgene expression and transgene product processing [Kirman & Seder, 2003]. The understanding of the immune mechanisms associated to the success of the method of DNA vaccination could provide useful information to determine the requirements of an optimal DNA vaccine [Smorlesi et al., 2006].
Intramuscular

Plasmid DNA-based vaccines conventionally have been delivered by direct intramuscular (IM) injection. This requires a large amount (~100 µg/mouse) of purified plasmid, because IM delivery of DNA vaccine is based on the transfection of a limited number of myocytes. The isotypes of the antibodies and the type of cytokines induced by the method of delivery greatly differ. Therefore, in majority of the cases, IM inoculation of the plasmid induces Th1 immune responses, whereas gene gun-based plasmid delivery induces Th2 immune responses [Gurunathan et al., 2000]. These differences in the immune responses elicited by the two modes of DNA delivery may have an important bearing on the vaccine efficacy of the plasmid.

Intradermal

In recent years, intradermal (ID) delivery of DNA vaccine has gained considerable popularity. The gene gun system permits simpler and rapid delivery with minute concentration of DNA (~1 µg/mouse), as compared to a typical needle injection. More importantly, the force of the particle bombardment propulsion is thought to propel the DNA-coated beads through the plasma membrane of cells [Lodmell et al., 1998]. Thus, any cells that are in the path of the beads are transfected, eliminating the need for a cellular internalization mechanism [Ulmer et al., 1996]. After bombardment, within the epidermal tissue, the key professional antigen-presenting cells (APCs), the Langerhans cells, receive the DNA and express the encoded antigen [Condon et al., 1996]. These antigen-expressing cells mature, and are then able to migrate to draining lymph nodes, where they interact with specific naïve T cells, resulting in their activation to effector T cells specific for the antigen encoded by the DNA vaccine [Porgador et al., 1998].

Electroporation

Presently electroporation appears to be the most effective delivery method for plasmid DNA and has been reported to increase gene expression 10–100-folds in tissues such as skin and muscle [Mir et al., 2005]. Here, a brief, high voltage electric pulse creates nanometer sized pores in the cell membrane. DNA may be taken directly into the cell cytoplasm either through these pores, or as a consequence of the redistribution of membrane components that accompanies closure of the pores. Electroporation may also enhance the immunogenicity of
DNA vaccines through the induction of an acute, localized infiltration of immune cells [McMahon et al., 2001; Babiuk et al., 2004] and the up-regulation of cytokines, heat shock proteins, and other co-stimulatory molecules [Pazmany et al., 1995]. Electroporation has been successfully employed to enhance immune response upon DNA immunization in mice [Widera et al., 2000], pigs [Babiuk et al., 2002], sheep [Scheerlinck et al., 2002] and lamb [Tsang et al., 2007].

Immunology of DNA vaccines

Preliminary and rational step towards vaccine design is the understanding towards immune mechanism of protection. Humoral (antibody mediated) immune response is protective for most of viral and bacterial diseases like rabies; while cell mediated immunity plays a dominant protective role in most of intracellular pathogens and parasites like Mycobacterium. Both arms of immunity are required for combating some pathogens like HIV, Herpes virus. Immunization with plasmid DNA has been shown to activate both humoral and cellular immune responses, including the generation of antigen-specific CD8+ cytotoxic T cells as well as CD4+ T helper cells [Donnelly et al., 1997]. Their mechanism of action is shown in Fig. 8.

![Fig. 8: Mechanism of action of DNA vaccines. Adapted from www.mogam.re.kr/eng/page/im01.asp](www.mogam.re.kr/eng/page/im01.asp)
Upon intramuscular immunization, when the DNA vaccine encoded gene is expressed intracellularly, epitope peptides are presented on MHC class I molecules, and are therefore capable of stimulating CD8+ T cells. These gene products expressed by muscle cells are also phagocytosed by antigen presenting cells and are either cross-presented on MHC class I molecules or presented by MHC class II molecules (Fig. 9), inducing CD4+ T cell responses. The plasmids may induce the expression of co-stimulatory molecules on antigen presenting cells, probably due to the presence of CpG motifs. This combination of co-stimulation with MHC Classes I and II presentation of antigen leads to the initiation of immune responses. However, the specific immunological events which lead to this effective immune response remain unclear.

**Fig. 9: Presentation of antigens to the immune system.** The MHC class I system mediates presentation of endogenous or intracellularly-produced proteins. The MHC class II system mediates presentation of exogenous or externally-produced antigens. The MHC I pathway is used predominantly for immune presentation of endogenously synthesized proteins such as would occur following DNA immunization. (1) Intracellular or endogenously produced proteins are broken down into peptides (8 to 12 amino acids long) by spliceosomes and then directed into the endoplasmic reticulum (ER) through transport associated protein (TAP) molecules; (2) Once in the ER, the peptide antigens bind to activated MHC class I molecules; (3) which subsequently transport the complexes through the Golgi toward the cell surface; (4) This pathway generates an immune response biased toward cell-mediated/cytotoxic CD8+ lymphocyte activation. II. Following endocytosis, the MHC II pathway presents peptide antigens which originated outside of the cell. Extracellular or exogenous antigens are taken up into specialized antigen presenting cells (APCs) by endocytosis; (5) The antigen is degraded in a lysosome; (6) into immunogenic peptides which bind to MHC class II molecules; (7) To produce an antibody biased immune response, the MHC class II-peptide antigen complex is transported to the cell surface; (8) where it binds to CD4+ helper T cells. Adapted from Medscape General Medicine 1999.
There are at least 3 mechanisms by which the antigen encoded by plasmid DNA is processed and presented to elicit an immune response:

- **Direct transfection of bone-marrow derived APCs.** Bone-marrow derived APCs have been shown to be absolutely required for the induction of MHC class I-restricted CTLs after im DNA vaccination [Corr et al., 1996; Doe et al., 1996; Fu et al., 1997; Iwasaki et al., 1997]. Dendritic cells (DCs) are the key antigen-presenting cells (APCs) required for priming of an immune response following DNA vaccination; however they constitute <1 % of nucleated cells in any tissue and only about 0.4 % of DCs are normally transfected following DNA vaccination [Nussenzweig et al., 1982; Casares et al., 1997].

- **Direct transfection of somatic cells.** Somatic cells such as myocytes or keratinocytes constitute the predominant cell populations transfected after DNA inoculation via muscle or skin injection, respectively, these cells may serve as a reservoir for antigen [Agadjanyan et al., 1999]. However, the DNA encoded antigen is poorly expressed and released from the transfected somatic cells for uptake by DCs [Boyle et al., 1997; Deliyannis et al., 2000]. Thus, somatic cells can be important in the induction of immune responses via cross-priming and may play a role in augmenting and/or maintaining the response [Gurunathan et al., 2000]. They can also up-regulate expression of MHC class I and co-stimulatory molecules, with production of cytokines and chemokines [Rice et al., 2008].

- **Cross-priming.** Activation by cross priming appears to be the most probable immune mechanism which occurs following DNA vaccination [Ulmer et al., 1996]. Cross priming may occur via exit of exogenous antigens from the endocytic compartments and its processing in the cytosol, recycling of MHC-I molecules through endosomal/lysosomal pathway and transfer of processed peptides to the endosomal compartments. It is well known that CD4+ T-cell stimulation can result from endocytosis of exogenous peptides or proteins followed by antigenic processing via MHC class II pathways [Watts, 1997].
Enhancement of DNA vaccine potency

DNA vaccines represent an attractive approach for generating antigen-specific immunity because of their stability and simplicity of delivery. One of the most significant hurdles of DNA vaccine development has been transferring the success of inducing protective immunity in small animal models to larger animal models, often due to their low potency. Optimal immune responses require their efficient delivery and presentation to the immune system. This may be achieved by:

Targeting antigen to sites of immune induction

Among different routes of administration of DNA vaccines, intradermal administration by gene gun has emerged as one of the most efficient routes of delivery of DNA into professional APCs, which express the encoded antigen. These APCs interact with specific naïve T cells, resulting in their activation to effector T cells specific for the antigen encoded by the DNA vaccine. Boyle et al. showed that by targeting antigen to lymph nodes or APCs by using L-selectin or cytotoxic T-lymphocyte antigen 4 (CTLA4) not only enhances specific antibody levels but also the T-cell proliferative response [Boyle et al., 1998]. Such targeting to sites of immune induction increases the effective dose of antigen, thereby enhancing the magnitude of immune response elicited.

Prolonging the life of dendritic cells (DCs)

Prolonging the life of DCs would enable the presentation of target antigen to T cells for longer duration, which may enhance the immune response elicited against it. Also, administration of DNA encoding anti-apoptotic agents resulted in prolonged DC survival and, therefore, an increased number of antigen-expressing DCs in the draining lymph nodes and enhanced activation of antigen-specific CD8+ T cells [Kim et al., 2003]. Among these antiapoptotic factors, BcL-xL and Bcl-2 DNA generated the greatest enhancement of antigen-specific immune responses and antitumor effects. It has also been demonstrated that this approach results in higher CD8+ T-cell avidity, contributing to the strong anti-tumor effect [Kim et al., 2004]. Prolonging the life of DCs along with intracellular targeting strategies led to significant enhancement of E7-specific CD8+ effector and memory T cells as well as significantly improved therapeutic effects against
established E7-expressing tumors in tumor-challenged mice; thus enhancing the potency of DNA vaccine [Kim et al., 2007].

**Linkage of heat shock proteins (HSPs) to target antigen**

DNA vaccines encoding antigen can also be chimerically linked to HSPs in order to increase the number of antigen-expressing professional APCs. HSPs, such as calreticulin (CRT) and *Mycobacterium tuberculosis* heat shock protein 70 (HSP70), are molecules capable of binding with scavenger receptors such as CD91. These receptors are highly expressed on the surface of DCs and may allow antigenic peptides linked to HSPs in the context of a DNA vaccine to be chaperoned into DCs [Basu et al., 2001; Srivastava, 2002]. Chen et al. showed that vaccines containing E7-HSP70 fusion genes increased the frequency of E7-specific CD8+ T cells by at least 30-fold relative to vaccines containing the wild-type E7 gene. More importantly, this fusion converted a less effective vaccine into one with significant potency against established E7-expressing tumors. Surprisingly, E7-HSP70 fusion vaccines exclusively targeted CD8+ T cells; immunological and antitumor effects were completely CD4 - independent. These results indicate that fusion of HSP70 to an antigen gene may greatly enhance the potency of DNA vaccines via CD8 - dependent pathways [Chen et al., 2000].

**Targeting antigens for rapid intracellular degradation**

Endogenous antigens synthesized within the APC are first polyubiquitinated and then degraded into their peptide components by the ubiquitin-proteasome system (UPS). UPS is responsible for rapid turnover of most of the cellular proteins and for generation of the bulk of peptides to be presented by MHC Class I molecules. To exploit this system, Shen et al., employed a chimeric DNA encoding mHSP65 fused with ubiquitin at its N terminus. The chimera effectively induced CD8+ T cells and made the melanoma more immunogenic [Shen et al., 2008]. Several other researchers have also reported such findings [Tobery & Siliciano, 1997; Rodriguez et al., 1998].
Intercellular spreading as an innovative strategy for enhancing DNA vaccine potency

DNA vaccines lack the intrinsic ability to amplify and spread in vivo. This severely limits their potency. Hung et al. showed that the linkage of VP22 (a herpes simplex virus type 1 tegument protein) to E7 (human papilloma virus oncogenic protein) led to a dramatic increase in the number of E7-expressing DCs in lymph nodes, enhancement of E7-specific CD8+ T-cell precursors in vaccinated mice, thereby significantly enhancing potency against E7-expressing tumors [Hung et al., 2001]. Thus, a strategy that facilitates the spread of an antigen to additional DCs should appreciably improve the potency of naked DNA vaccines [Hung et al., 2006].

Employment of cytokines

Several cytokines have been established for their immune enhancement potential of DNA vaccines, pertaining to T cell proliferation. IL-2 and IL-12 are primarily employed for the same. IL-2 is produced by activated T-cells and is responsible for clonal T-cell proliferation [Waldmann et al., 2006]. Several studies suggest that DNA vaccine encoding IL-2 in conjunction with antigen may potentially enhance the antigen-specific immune responses resulting in improved vaccine potency. DNA vaccines employing IL-2 co-expressed with the Hepatitis B surface antigen led to significant enhancement of the HBsAg-specific immune responses [Chow et al., 1997]. Furthermore, another study has shown that DNA vaccines encoding a fusion of HER2/Neu to IL-2 significantly enhanced the therapeutic efficacy of the DNA vaccine against HER2/Neu-expressing tumors [Lin et al., 2004]. In addition, co-expression of IL-2 has also been shown to enhance the immune response to the HSV1 glycoprotein D antigen in DNA vaccines [Li et al., 2006]. The linkage of IL-2 to HPV-16 E7 antigen significantly enhances the DNA vaccine potency against E7-expressing tumors [Lina et al., 2007]. IL-12, a pro-inflammatory cytokine mainly secreted by DCs; also secreted by macrophages and B cells; is a potent inducer of Th1 responses [Hsieh et al., 1993]. Kim et al. showed that coimmunization of mice with a HIV-1 DNA vaccine and IL-12 gene resulted in increased Th1 responses while Th2 responses were correspondingly reduced [Kim et al., 1997]. Sin et al. also reported that coimmunization of mice with HSV DNA vaccine and IL-12 genes inhibited antibody responses while
promoting cellular proliferation and significantly increasing the secretion of chemokines (CCL3 and CCL5) and Th1 cytokines [Sin et al. 1999]. More recently, Hirao et al. [Hirao et al., 2008] found that macaques receiving the IL-12 plasmid in addition to a HIV DNA vaccine had at least double the cellular responses of those macaques receiving DNA vaccine alone. Additionally, co-immunization with IL-12 resulted in nearly 10 times higher memory responses. Co-injecting GM-CSF was also found to enhance the protection conferred by malaria DNA vaccine [Weiss et al, 1998].

Co-stimulatory molecules
Naïve T cells can be efficiently activated by administration of plasmid DNA encoding co-stimulatory, that is, B7 family molecules. Many studies have demonstrated that this approach can improve DNA vaccine potency [Pasquini et al., 1997; Corr et al., 1997].

Co-administration with CpG oligonucleotides
CpG oligonucleotides or motifs, the unmethylated cytidine-phosphate-guanosine; represent regions of DNA commonly present in bacterial genomes and may be found in the antibacterial resistance genes placed within DNA plasmids sequences. They activate B cells and DCs and induce pro-inflammatory cytokines [Klinman et al., 1997]. The activation has been shown to through Toll-like receptor 9 (TLR9) - a receptor found on APCs [Hemmi et al., 2000]. The TLRs are pattern recognition receptors like macrophage mannose receptor (MMR), which enable macrophages and DCs to recognize bacteria, thus ensuring that an appropriate immune response is generated to defend against the particular pathogen causing infection [Medzhitov & Janeway, 1998]. Such interaction leads to the production of Th1 cytokines and promotes priming and differentiation of Th1 T cells [Sato et al., 1996; Tascon et al., 2000; Ahmad-Nejad et al., 2002]. Although CpG motifs are not essential for the induction of immune response, they are likely play a role, especially in mice, in the adjuvanting of immune response to DNA vaccines [Tudor et al., 2005]. Their role as adjuvants in primates including humans appears more complex. This is in part due to the differential expression of TLR9 in mice and primate immune cells [Abdulhaqq & Weiner, 2008].