1.1 RABIES

1.1.1 A historical perspective of rabies

Rabies in ‘Latin’ is *rabere*, which means rage or madness (Prescott, 2002). It is a disease of antiquity, one of the oldest sequelae of mankind and perhaps the most gruesome and dreadful of all communicable diseases afflicting human beings, dating back to the earliest recorded history (WHO, 2007).

The disease appears in ancient Egyptian writings and later in writings by Democritus (500 B.C.) and Aristotle (322 B.C.) in ancient Greece. During 2300 A.D., the Eshuma Codex, at Babylon, described policies for animal control recognizing that the bite of a rabid dog could cause death in humans. In the Vedic times, rabies was known in Sanskrit as ‘Rabbahs’, which means ‘to do violence’. In the 1st century A.D., Cornelius Celsus invented the term ‘hydrophobia’ or the fear of water and proved saliva as the source of transmission of rabies. He used the word "virus" in this connection for the first time. Hippocrates has made a mention of rabies encephalitis relating to animals and men. But an Italian, Girolamo Fracastoro put the true nature of rabies on record in his work entitled ‘The Incurable wound’ in 1584. The first recorded evidence of rabies is found in dogs of Virginia, 1753 (Ayres et al. 2005., MSMR, 2005).

Since time immemorial, rabies has been the object of human fascination, torment, always been associated with fearful connotations. The awful symptoms of rabies raise emotions of fear and helplessness very similar to that from AIDS.

Development of rabies vaccine by Louis Pasteur in 1880s marked the beginning of the scientific approach to treat post exposure cases. The pioneering work done by von Behring, Kitasato Roux and Calmette, led to the use of therapeutic antiserum at the end of 19th century, in 1894 (Bon, 1996). In the latter half of the twentieth century, the treatment became more focused with the use of interferons, ribavirin, cytosine arabinoside, and intravenous, intraventricular and intrathecal administration of human and equine rabies immunoglobulin (Wilde et al. 2008).

In the beginning of the immunological era, it was observed that animals immunized with specific antigens developed an antibody response, beneficial to
the treatment of many diseases, including rabies. Hence, passive immunization or “serotherapy” became a powerful tool. However, the unpurified serum, used to cause adverse effects. During the forth-coming years, the development of various purification methods (chromatographic, non-chromatographic and a combination of both) and different fractionation procedures yielded either intact IgG, or antibody fragments, F(ab)_2 / F(ab). Purification of antiserum not only ensured high potency, but also minimized the risk of hypersensitivity reactions (Hong et al. 1994., Meslin et al. 1996). Purified antibody preparations of therapeutic importance are available not only of equine origin but also of ovine and bovine origin (John et al. 1967., Peres et al. 2006). But the most standardized and widely manufactured is the preparation of equine origin. The recommendations of the WHO expert committee on Biological Standardization, regarding the final protein content in antirabies titer serve as important guidelines for various antirabies serum manufacturers worldwide (WHO, 1994., WHO, 2009). The antiserum should fulfill two important criteria “Safety”, in terms of causing nil or least allergic reactions and “Efficacy”, in terms of neutralizing the specific antigen using least dose of antiserum.

Since the rabies disease spreads more largely, due to bites of canines, skunks, raccoons and bats, the social implication of rabies shall never diminish, as long as there shall continue to persist frequent contacts between domestic dogs and humans (CDC, 1991., Sharp, 2008). Rabies is a major public health problem because it is widespread among animal reservoirs.

It is extremely important to understand that rabies is the only communicable disease of man that results in 100% fatality and 0% survival, despite the best of treatment. Our knowledge of rabies has advanced rapidly owing to the growth of molecular biology and to the magnitude of on-going research by dedicated teams of medical and veterinary scientists. Surveillance and control measures adopted & propagated by the global agencies like Centre for Disease Control (CDC) and World Health Organization (WHO) have led to significant control of rabies. Today, we have enough measures to control rabies
100%, if not eliminate rabies worldwide, at least the canine rabies (Wilde et al. 2009).

1.1.2 Epidemiology of rabies

1.1.2.1 Global threat of rabies

Rabies is endemic in most parts of the world except New Zealand and Antarctica and is the tenth leading cause of death due to infection in humans, predominantly affecting the poor people in the developing countries and remains a serious health problem (Fig.1.1). Even those countries declared rabies free like United Kingdom, Ireland, areas of Scandinavia, Japan, Australia, New Zealand, Singapore, Brunei, most parts of Malaysia, some islands of Indonesia, Papua New Guinea bear a risk due to the expanding prevalence of the disease (OIE, 2009., WHO, 2007., Wilde et al., 2008., Wilde et al., 2009).

About 65,000 people die due to rabies annually. This may be an underestimated figure because many cases go unreported and misdiagnosed (Wilde et al., 2008). Most cases are observed in Africa (24,000) and Asia (31,000); majority being in the developing countries; 99% of all the cases are due to dog bites (Sugiyama et al., 2007., WHO, 2010). Every 10 to 15 min. someone dies due to rabies, of which majority of the cases are children under 15 years of age. In children due to their short stature, dog-bites occur on the upper parts of the body, face, neck and hence are dangerous (WHO, 2007).

More than 3.3 billion people from over 85 countries are at risk of rabies and prevalence of rabies is in more than 150 countries and territories. Annually more than 15 million people receive post-exposure preventive treatment. Of 1.5 million dog bite cases, 24,000 to 80,000 are from rabid dogs and require post exposure prophylaxis (WHO, 2010).
Fig. 1.1 The worldwide distribution of rabies virus. The distribution of classical rabies virus (genotype 1 (GT1)) in 2007 is shown in orange. The distribution of rabies virus and other members of the lyssavirus genus are shown in yellow. The only known lyssavirus-free country is New Zealand (green).

1.1.2.2 Rabies in Asia

Annually, in Asia 32,000 cases of deaths due to rabies are observed. Countries like Japan, Singapore and Taiwan are rabies free nations; a trend has been observed towards spreading of the disease from infected areas to rabies free areas like South Korea, Flores island of Indonesia, signaling the potential re-emerging of this disease in rabies free countries (Sugiyama et al., 1997., Sugiyama and Ito, 2007). Pet boom is one of the sources of recent flourishing of rabies.

1.1.2.3 Indian scenario of rabies

Rabies is endemic in India, except for probably the islands of Lakshadweep and Andaman & Nicobar. In India at least 20,000 human deaths due to rabies occur per year. India is [second (only to Ethiopia) in the world], the country with the highest rabies fatalities in Asia. Seventy percent of the victims are children younger than 15 years. The main cities along with four metros contribute to 30 per cent of the total deaths caused by rabies in India (Cliquet et al. 2007., WHO SEA report 2009., WHO, 2010). The statistics of animal bites is 17.4 / 1000
population and accounts to one bite / two seconds and a death every 30 minutes. Bites are mostly associated with dogs (96%), domestic as well as stray, ownerless dogs (60%). The dog population of India is around 25 to 30 million with 3-10% being rabid (dog to human ratio, 1:40), and they constitute the main reservoirs of the rabies virus. Each year approximately seven million people undergo post exposure rabies treatment after a dog bite, of which ~96% of cases are due to bites from stray dogs. In India, mainly ownerless and stray dogs are responsible for the maintenance of the epizootic and the transmission of rabies to humans (Cliquet et al. 2007., Menezes, 2008., WHO, 2010).

Fig 1.2 A rabid dog.
1.1.3 The disease: Rabies

1.1.3.1 Clinical features in humans

Rabies is a viral disease of the central nervous system leading to acute meningoencephalitis in warm-blooded animals. The rabies virus infects the central nervous system, ultimately causing disease in the brain and death. It is almost invariably fatal if post-exposure prophylaxis is not administered prior to the onset of severe symptoms. It is a zoonotic disease, spread most commonly by a bite from an infected animal (Fig. 1.2), (Carroll et al. 2007., Prescott, 2002., Talaro and Talaro, 2002).

The disease is slow and progressive. The average incubation period of rabies is 1 to 2 months, but may vary from one week to greater than one year, depending upon the site of dog bite, virulence of the virus, and the inoculation dose. The incubation period is short if the bite is on face, scalp, or neck because of the proximity to the brain. The prodormal phase comprises fever, nausea, vomiting, headache, fatigue, and other nonspecific symptoms. Some patients experience symptoms like pain, burning, prickling, or tingling sensations at the wound site.

Rabies can be present in different clinical forms:

- **Prodormal Rabies** – The onset of clinical rabies in man includes 2 to 4 days of prodormal manifestations, most of which are non-specific. Elevated temperature, malaise, headache, sore throat are commonly observed symptoms. The most significant symptoms are discomfort or pain at the site of bite, numbness and tingling in limb. There may also be increasing nervousness, anxiety, irritability, depression and melancholia, with or without a sense of impending death.

- **Classical rabies or Furious rabies** - This is accompanied by a profound dysfunction of brain stem centers that produces symptoms of rabies. There is violent involuntary contraction of the diaphragm and accessory respiratory, pharyngeal and laryngeal muscles initiated by an extremely painful swallowing of liquids. The patient lapses into coma and subsequent failure of respiratory centre
in brain produces a prolonged painful apneic death. The symptoms are characterized by hallucinations, combativeness, agitation, seizures, twitching, disorientation, violent muscle spasms, anxiety, irritability, depression, fatigue, loss of appetite, fever and sensitivity to light and sound.

- **Paralytic Rabies** — or *rage tranquille* is also known as **dumb rabies**. It is characterized by Guillain Barre’ like ascending paralysis. In this clinical picture, animal is not ‘mad’; paralysis usually of the lower jaw, drooping head are initial signs of dumb rabies. The paralysis then spreads to limbs and vital organs leading to death. According to WHO, this form constitutes about 30% of the cases, but is often misdiagnosed and largely goes unreported (Talaro & Talaro, 2002).

### 1.1.3.2 Transmission of rabies

Rabies virus can cause disease in all warm blooded animals, especially mammals including humans. Rabies virus is predominantly neurotropic, killing the host after its growth in neurons. However before death, virus reaches salivary glands and is excreted in saliva. Mere licking of preexisting scratch in skin is adequate for transmission of virus. The major reservoirs and vectors of rabies virus are members of:

- **Mustelidae:** Skunks, Weasels, Stoat and Martens;
- **Canidae:** Dogs, Wolves, Foxes, Coyotes and Jackals;
- **Procyonidae:** Raccoons;
- **Viverridae:** Mongooses and Meerkats;
- **Order Chiroptera:** Bats

Humans are infected primarily through a bite or scratch by an infected animal. A few reports of transmission of virus through transplantation of infected corneas and other organs are reported. The rabies is rarely transmitted by aerosols; however caves populated by rabies-infected bats do exist. Dogs are the main host in transmission of rabies, in 99% of all the human cases the virus is transmitted by dog bites.
In the United States of America and Canada, bats are the main source of human rabies deaths. Human deaths following exposure to foxes, raccoons, skunks, jackals, mongooses and other wild carnivore host species are very rare. Human-to-human transmission by bite is the only theoretical possibility and has never been confirmed (OIE, 2009., WHO, 2010). Amongst the important reservoirs, dogs are the major cause of human deaths (99%) along with cats or wild animals (bats, monkeys & foxes). Transmission of the disease to animals of great ecological interest like cattle and horses have been reported (Frazatti-Gallina et al., 2004).

1.1.4 Etiology of rabies

1.1.4.1 Rabies virus

The rabies is caused by neurotropic virus belonging to the genus Lyssavirus of the Rhabdoviridae family. It is characteristically enveloped and bullet shaped with a single stranded and nonsegmented RNA genome with negative sense. The Rhabdoviridae family includes at least three genera of animal viruses, Lyssavirus, Ephemerovirus, and Vesiculovirus and contains around 60 different viruses, but only rabies Lyssavirus infects humans and hence, of medical importance. These viruses are very widely distributed in nature, infecting vertebrates, invertebrates, and plants. Rabies virus does not infect insects, unlike many other animal rhabdoviruses.

Six unique genetic groups, of the rabies virus have been identified based on the sequencing of N genes; out of these, four groups have been identified in Asia. The other members of the genus ‘Lyssavirus’ (Greek lyssa, rage or rabies) such as Lagos bat virus, Mokola virus, Duvenhage virus, the European bat virus and the Australian bat virus can cause symptoms very similar to the classical rabies (OIE, 2009).

1.1.4.2 Structure of the rabies virus

Rabies viruses are bullet shaped with a helical symmetry, having length of 100-300 nm and a cross-sectional diameter of about 75 nm. The structure of the
rabies virus is as shown in the Fig. 1.3. The virions consist of a central, dense cylinder formed by the helical ribonucleocapsid and a surrounding envelope (8 nm wide) covered with a 10 nm spike-like envelope containing glycoprotein peplomers, placed at a distance of 5 nm. These glycoprotein forms approximately 400 trimeric spikes which are tightly arranged on the surface of the virus. The virus has a single-stranded RNA (~12 kb) genome with negative-sense, packaged as a phosphorylated ribonucleoprotein (RNP) complex in which RNA is tightly bound to the viral nucleoproteins.

As seen in Fig. 1.4 a. the RNA genome of the virus encodes five genes preceded by a ~ 50 nucleotide leader sequence (lRNA). The proteins encoded by these five genes are:

- **Nucleoprotein (N)** -- There are 1750 molecules of nucleoprotein (N) per virion. It is a 450 amino acid polypeptide with a molecular weight of 57 kd. One of its functions is to encapsidate and protect the genome. Three antigenic sites have been characterized on this protein. The protein is phosphorylated.

- **Phosphoprotein (P)** -- Each virion has 950 molecules of phosphoprotein (P). There are two forms of phosphoprotein, P1 (65 kd) and P2 (65 kd). The protein is hydrophilic and 297 amino acids long bearing an overall negative charge, due to aspartate, glutamate and phosphorylation at some amino acid residues.

- **Matrix protein (M)** -- There are 1650 molecules of matrix protein (M) per virion (Fig. 1.3). It is a 202 amino acid long polypeptide. The protein has two palmitoylated forms M1 (25 kd) and M2 (24 kd). The protein is located within and between the viral envelope & the ribonucleocapsid. The protein M plays a crucial intermediate and catalytic role.

- **Glycoprotein (G)** -- Each virion has 1800 molecules of glycoprotein (G). This protein also exists in two forms G1 (70 kd) and G2 (65 kd) with different extents of glycosylation, both of which are acylated with palmitic acid. Trypsin selectively affects this protein. It is a 524 amino acid long polypeptide and contains two hydrophobic segments typical to its transmembrane nature. At
least eight antigenic sites have been mapped in different rabies strains and these different G protein regions are of importance in stimulating the B and T cells. This protein plays a key role in the pathogenesis of the disease.

- **RNA polymerase (L)** -- There are 20-150 molecules of RNA polymerase (L) per virion. This is a giant protein of ~2140 amino acids and occupies 54% of the rabies genome. Its nucleotide sequence is homologous with the L protein of other members of the *Mononegavirales* order (Meslin et al. 1996., Schnell et al. 2010). This protein functions as RNA-dependent RNA polymerase (160 kd).

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**Fig. 1.3 Structure of rabies virus.** The negative-stranded RNA genome is tightly encapsidated into the RNP, (yellow). Two other viral proteins are associated with the RNP, the viral polymerase (blue) and the phosphoprotein (orange), which make up the internal core or capsid. The capsid is engulfed by the host cell-derived membrane. The matrix protein (green) is located between the capsid and the virion membrane. The transmembrane glycoprotein, is organized as a trimer (purple).
1.1.4.3 Replication of the rabies virus

The stages involving the entry till the replication of the virus are shown in the Fig. 1.4.b.

• Adsorption, Penetration and Uncoating

Adsorption is the attachment of rabies virus spiked envelope to the host cell membrane through the interaction of G protein of virus with the cellular nicotinic acetylcholine receptor. This initiates the infection process. After adsorption, the virus penetrates the host cell and enters the cytoplasm by pinocytosis through clathrin – coated pits. Clathrin is a protein that plays a major role in formation of coated vesicles. The main function of these vesicles is to transport molecules between the cells. Within the cytoplasm, the virus particles aggregate in the endosomes. The fusion of the viral membranes with the endosomal membranes release rabies nucleoproteins (RNP) in the cytoplasm of the infected cell. Low endosomal pH induces this fusion process enabling the viral genome to reach the cytosol. The fusion is catalyzed by G protein. Mutant viruses without G protein fail to propagate and are therefore crucial for pathogenesis.

• Transcription and Translation

Once in the cytoplasm, linear single-negative-stranded ribonucleic acid (RNA) genome serves as a template for transcription by the polymerase complex and is transcribed into five mRNAs coding for nucleocapsid (N), polymerase proteins (L, P), matrix protein (M), and glycoprotein (G) (Fig.1.4.a). Transcription starts with a short uncapped leader RNA (leRNA) from the 3’ end, followed by 5’ end-capped (C) and polyadenylated (A) mRNAs, which encode the viral proteins. The polymerase complex stops at each signal sequence (U/ACUUUUUUU) and after the intergenic region of 2–24 nucleotides, restarts transcription at the start signal sequence (UUGURRNGA). Successful reinitiation of transcription at each gene-junction does not always occur resulting into a transcription gradient i.e. N gene transcribed maximum and L gene transcribed minimum.

The viral proteins N, P, M, G and L, are synthesized on free ribosomes in the cytoplasm. Continuous translation of proteins, especially that of N and P is
required for replication. The G proteins made on the free ribosomes, are glycosylated in the endoplasmic reticulum and golgi apparatus.

- **Replication**
The single polymerase serves as RNA replicase as well as transcriptase. The switching from transcription to replication depends upon the intracellular ratio of leader RNA to protein N. On activation of this switch, replication of the viral genome begins. The transcription becomes "non-stop" i.e. stop codons are ignored, the viral polymerase aligns on the 3’ end of the genome, and proceeds to synthesize full-length copies of the positive strands. These positive strands serve as templates for synthesis of multiple negative strands of RNA, to be further incorporated in the individual virion particle.

- **Assembly**
The new genomic RNAs associate with the viral transcriptase (L) and nucleoprotein (N) to form RNP cores in the cytoplasm; the M protein forms a capsule, or matrix, around the RNP. The RNP-M complex migrates to the plasma membrane which by now has a lot of glycoproteins inserted in it. The M-protein initiates coiling of glycoprotein studded membrane around virion.

- **Budding**
The M-RNP complex associates with the glycoprotein and the particles acquire an envelope by budding through the plasma membrane. The viral protein is on the inner side of the envelope, whereas the viral glycoprotein is on the outer layer in form of spikes.

The central nervous system (CNS), exhibits preferential viral budding from plasma membranes, but virus in the salivary glands buds primarily from the cell membrane into the acinar lumen. Viral budding into the salivary gland and virus-induced aggressive biting-behavior in the host animal maximize chances of viral infection of a new host (Carroll et al. 2007, Schnell et al. 2010).
Fig. 1.4 Life cycle of rabies virus (a) Transcription of the rabies virus genome. (b) Life cycle of rabies virus in an infected cell.
1.1.4.4 Pathogenesis of the rabies virus

As shown in Fig. 1.5, initially after entry into the host through the bite on the skin or mucous membrane, virus replicates in the striated muscle cells. It multiplies here for a week and then spreads to the peripheral nervous system (PNS) at the exposed neuromuscular junction sites and nerve endings at unmyelinated sites such as sensory and motor terminals.

Fig. 1.5 Pathogenesis of rabies.

The nicotinic acetylcholine receptor (nAchR) and the neural cell adhesion molecule (NCAM). nAchR enriches rabies virus at the neuromuscular junction or synaptic cleft, enabling more efficient infection of the connected motor neurons (Fig.1.6). It then spreads via peripheral nerve axoplasm to the central nervous
system (CNS) at the rate of ~3 mm/hour. In the brain neurons, the virus produces characteristic Negri bodies, which are masses of viruses or unassembled viral subunits that are visible in the light microscope (Prescott, 2002). Upon reaching the CNS, viral spread occurs to autonomic nervous innervated tissues like the salivary glands, kidneys, lungs, liver and heart. Passage of virus into the salivary gland followed by the viral replication facilitates further transmission via infected saliva.

Fig. 1.6 Entry of rabies virus into neurons.
1.1.5 Diagnosis of rabies disease

Various testing methods are available for diagnosis of rabies disease based on the detection of the rabies virus, most of which are standardized and used widely (Meslin et al. 1996., OIE, 2008., Shankar, 2009).

1.1.5.1 Immunochemical detection of rabies virus

**Fluorescent antibody (FA) test**

The method is based on microscopic examination (under ultraviolet light) of Negri bodies in brain tissue sample. The serum containing antirabies antibodies conjugated with fluorescein isothiocyanate are used to stain the virus. The results of the test can be obtained within 30 minutes of the receipt of the sample, although for routine purposes a period of two to four hours is desirable for fixation in cold acetone. The results obtained are accurate. This test is in 99% agreement with mouse inoculation test. This test is recommended both by Office of International Epizootic (OIE) and World Health Organization (WHO). Rabies virus can also be detected in the peripheral nerves, salivary glands, saliva, cornea and skin, apart from brain and spinal cord. The only condition for performing fluorescent antibody test is that the specimens should be of good quality and be refrigerated immediately after collection until the test is carried out.

**Enzyme-linked immunosorbent assay (ELISA)**

Rabies virus can also be detected by enzyme-linked immunosorbent assay. This test should be used after validation against numerous samples in different laboratories. The specificity and sensitivity of these antirabies enzyme conjugates for locally predominant virus variants should be ascertained before performing the test. Usually it is used in combination with confirmatory tests like fluorescent antibody test or virus isolation. Perrin et al. developed an ELISA called “Rapid Rabies Enzyme Immunodiagnosis” (RREID), based on the detection of rabies virus nucleocapsid antigen in the brain tissue (Perrin et al. 1986). Rapid Rabies Enzyme Immunodiagnosis test showed a good correlation (96%) with the
fluorescent antibody test. Being a simple, and relatively cheap technique, ELISA is used especially for epidemiological surveys, but should not replace fluorescent antibody test wherever it is already established.

1.1.5.2 Detection based on replication of rabies virus

Mouse inoculation test

Ten mice, 3–4 weeks old (12–14 g) are inoculated intracerebrally. The inoculum is the clarified supernatant of a 20% (w/v) homogenate of brain material in an isotonic buffered solution containing antibiotics. To reduce animal pain, mice should be anaesthetised when inoculated. The mice are observed daily for 21 days, and every dead mouse is examined for rabies using the fluorescent antibody test.

This *in-vivo* test is quite expensive. It does not give rapid results as compared with *in-vitro* inoculation tests, but when the test is positive, a large amount of virus can be isolated from a single mouse brain for strain identification purposes. Another advantage of this test is that it can be easily applied in situations where skills and facilities for other tests (e.g. cell culture) are not available.

Cell-culture techniques

This test is based on the detection of infectivity of a sample using cell lines viz: Baby hamster kidney cell lines (BHK-21), Chick embryo related (CER), and Neuroblastoma cells. Infectivity testing using BHK-21 cells is comparable in sensitivity to mouse inoculation test, whereas infectivity testing using neurablastoma cells is more sensitive than mice test, to infection by street rabies virus. In cases of known human exposure, where fluorescent antibody test has yielded uncertain or negative results, these tests are employed. Most researchers feels this test should replace the mouse inoculation test wherever possible (Meslin et al. 1996., OIE, 2008., Shankar, 2009).
1.1.5.3 Detection of rabies nucleic acid by polymerase chain reaction (PCR) technique

The polymerase chain reaction (PCR) technique along with its modifications has been used for typing of various strains of rabies (Dai et al. 2008., Zhang et al. 2008) when the diagnostic effectiveness of, the mouse intracerebral inoculation test (MICIT), the rabies tissue culture infection test (RTCIT), the rapid rabies enzyme immune diagnosis test (RREID) and the nested reverse transcription polymerase chain reaction (nRT-PCR) was compared, Nested RT-PCR proved to be a markedly more sensitive diagnostic procedure than RREID, MIT and RTCIT (Franka et al. 2004).

1.1.6 Control of rabies

1.1.6.1 Prevention and treatment of rabies in humans

The two main approaches to control rabies are ‘Pre exposure prophylaxis’ and ‘Post exposure prophylaxis’.

Pre exposure prophylaxis is the administration of anti-rabies vaccine prior to bite. Prophylaxis before exposure constitutes three doses of vaccine administered into the deltoid muscle on 0, 3rd and 28th days and boosters between six to 24 months. Vaccination before exposure simplifies the treatment and imparts protection from possible infection after a bite (Pounder, 2005). The types of vaccines presently used are cell culture based vaccines; the old neural tissue vaccines are phased out. The cell culture based vaccines are safe and effective but costly. The vaccination is primarily restricted to the people with high risk of exposure to rabies virus, e.g: veterinarians, dog breeders, dog trainers, laboratory staff in close contact with animals and also the people directly dealing with virus in research laboratories or production units.

Post exposure prophylaxis is the administration of anti-rabies vaccine with or without antirabies serum to persons afflicted with dog bite, depending on the nature of exposure to rabies virus. Administration of immunoglobulin is obligatory in the cases where the dog involved in biting is suspected or diagnosed as rabid dog. Usually if the biting dog is itself vaccinated or is under observation, the administration of serum can be avoided. This is especially because commonly
used antirabies immunoglobulins are of equine origin and human recipient may show allergy to horse proteins (Dutta and Dutta, 1994., Pounder, 2005).

### 1.1.6.2 Prevention of rabies in dogs

Canine rabies control is the most cost-effective way to reduce human rabies cases. It is a matter of public health and should therefore receive high priority in the public veterinary services and also in human health care sector. However canine rabies control is often only associated with voluntary animal welfare organizations or groups. In India, Jaipur is an example of a city where rabies control through mass vaccination and neutering of dogs has resulted in eradication of human rabies (Reece and Chawla, 2006). Moreover, a zero-garbage city or even better the Zero-Garbage-India, would be an excellent benefit for controlling stray dog population.

Other measures to control rabies include mass vaccination of stray dogs population by oral vaccine (SAG2) including bait casing, humane removal of stray animals, promotion of responsible pet ownership through education (Leung et al. 2007., Pradhan et al. 2008., Sudarshan et al. 2006)

### 1.1.6.3 Other measures to control rabies

To raise public awareness of the severity of the disease, Annual World Rabies Day in the month of September is initiated globally. In India, measures are being taken to make Rabies-Free, India; by 2020 (Pradhan et al. 2008).

Progress has been made in the control and elimination of wildlife rabies using oral immunization of wild carnivores as well as mass parenteral vaccination in domestic dogs in developed countries. But developing countries and more specifically, India carries the greatest burden of the disease. In India rabies by law is not a notifiable disease. There is the lack of sustainable centralized effort, no organized surveillance system of human and animal cases, and lack of awareness and proper education about the disease (Meltzer and Rupprecht, 1998., Haider, 2008).

Unfortunately, many conventional treatments still persist among rural communities. Many do not know that even washing their wounds after being
bitten greatly reduces the risk of infection. All this is compounded by poverty, lack of understanding of the need to start the vaccinations on the very same day and lack of availability of the rabies anti-serum. (Cleaveland et al., 2003., Lembo et al., 2010).

1.2 ANTIRABIES SERUM

1.2.1 Importance of antirabies serum
In the absence of confirmed information about ‘health status’ of biting dog, each dog bite case is treated as exposure to rabies virus (Fig. 1.7). In such case post-bite treatment involves both post-bite vaccination using rabies tissue cultured antigen (active immunization) as well as administration of antirabies immunoglobulin (passive immunization). Post-bite vaccination would result in development of immunoglobulins, but not earlier than 2-3 weeks and therefore simultaneous administration of rabies immunoglobulin (antirabies serum) constitutes the best approach (Parikh, 2000., Park, 2000). Antirabies serum is injected locally in the biting wound area, immediately after dog-bite or preferably within eight days (Corey, 2001).

1.2.2 Types of antirabies serum
Two types of antirabies serum products are available for post exposure prophylaxis: Human Rabies Immunoglobulin (HRIG) and Equine Rabies Immunoglobulin (ERIG). HRIG is prepared from the blood of human volunteers immunized with purified cell culture based rabies vaccine while ERIG is prepared from the blood of horses immunized with rabies vaccine.
Fig. 1.7 Post exposure rabies prophylaxis.
*RIG – Rabies immunoglobulin.
1.2.2.1 Human rabies immunoglobulin (HRIG)

Human Rabies Immunoglobulin (HRIG) is an homologous preparation of refined, purified rabies immunoglobulin, i.e. immunoglobulins are human-proteins. To produce this immunoglobulin, human volunteers are immunized with purified cell culture based vaccines. HRIG is expensive (five times that of ERIG) and available in only limited quantities, because of involvement of human volunteers. Being homologous in nature, they do not cause any sensitization. The use of homologous immunoglobulins for human post-exposure treatment virtually eliminates the risk of anaphylaxis and serum sickness associated with heterologous serum products. To avoid such reactions, human rabies immunoglobulin (HRIG) preparations have been developed and used for post-exposure treatment in most industrialized countries. Also HRIG being of human origin, are eliminated from the human body at much reduced rate.

Equine rabies immunoglobulin (ERIG) becomes the immunoglobulin of choice, especially for the developing countries, where the animal bite cases are very much higher leading to greater requirement of immunoglobulins (Hong et al. 1994., Folbe and Cooke, 2006).

1.2.2.2 Equine rabies immunoglobulin (ERIG)

As stated earlier, equine rabies immunoglobulin assumes great importance in treatment of post-bite cases with suspected exposure to rabies virus. ERIG can be made available in much larger quantities and also at much lesser cost. The heterogeneous nature can cause allergic reactions but the technological developments in processing of horse immunoglobulins have rendered them less allergic and more stable. Purification techniques can be used to reduce the risk of sensitization to ERIG. Their objective of purification is to maximize the specific activity and to minimize the allergenic substances in the product. These techniques are implemented with careful adherence to the recommendations of the WHO Expert Committee on Biological Standardization. The clinical safety of Equine Rabies Immunoglobulin is also well established (Wilde and Chutivongse, 1990., Satpathy et al. 2005). In developing countries the use of highly purified
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horse immunoglobulin, are safer than the heterologous products of the previous generation. In the past few years, purified equine immunoglobulins have become available, and in recent studies, the incidence of serum sickness among recipients was reported to be as low as 1-6.2%. In India, manufacturers of Equine Rabies Immunoglobulin follow CPCSEA guidelines and cGMP norms (Hong et al. 1994., Meslin et al. 1996., CPCSEA, 2001., WHO, 2009).

In developing countries in general and India in particular, the need of RIG exceeds the production. In addition, the animal protection groups that are becoming more and more influential in developing countries, and they condemn animal rearing for serum production. In this context accelerating research and development of alternative products is very timely and using a limited number of carefully selected MAbs for therapeutic purpose is an attractive possibility. (WHO, 2002).

1.2.3 Administration of antirabies serum

Equine rabies immunoglobulin should be given as a dose of 40 IU per kg of body weight. Rabies immunoglobulin (RIG) should be infiltrated into the depth of the wound and around the wound as much as anatomically feasible. Any remainder should be injected at an intramuscular site distant from that of vaccine inoculation e.g. into the anterior thigh. Sensitivity to heterologous immunoglobulin must be determined before it is administered. The physician should be prepared to deal with anaphylactic shock reactions. Treatment should be started as early as possible after exposure, but in no case should it be denied to exposed persons whatever time interval has elapsed.

1.2.4 Production of equine rabies immunoglobulin

Equine antirabies immunoglobulin (ERIG) have been produced using various immunogenic preparations, consisting usually of a combination of inactivated and fixed strains of rabies virus. The animals are given a series of injections of the vaccine in increasing quantities. All the injections are given subcutaneously into
the lateral side of the neck. The general process of preparing equine antirabies serum is as follows (Fig. 1.8):

**Fig. 1.8 Antirabies serum manufacturing process** (at Haffkine Biopharmaceutical Corporation, Ltd., Pune, India)
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Horses are purchased and kept isolated for a period of one month under observation and only healthy horses are taken for production. During this period, equines are immunized with tetanus toxoid. Primary immunization with Rabipur vaccines begins after the completion of quarantine period. Horses are injected with gradually increasing doses of rabies antigen. Amount of antirabies antibodies in serum is determined using the Mouse Neutralization Test (MNT). After the required antirabies titer is obtained, the horses are taken for bleeding. Usually, 6-9 months are required for immunization.

Blood is withdrawn from each animal and kept for settling of the cells at (5±3) °C overnight. The supernatant plasma is siphoned into another sterile bottle and stored in cold room till processed. Diethyl ether and phenol are added as preservative. Hyperimmune plasma is diluted with sufficient quantity of distilled water to bring the protein content to 1%. The temperature of the mixture is then raised to 30°C, the pH is lowered to 3.2. At this pH and temperature, the proteolytic treatment with pepsin is carried out. After digestion with pepsin, pH is raised followed by addition of toluene and solid ammonium sulfate. The addition of ammonium sulphate is followed by thermocoagulation at 55°C for 1 hr with continuous stirring. Mixture is then cooled quickly and left at this temperature overnight. The slurry is micro filtered and the filtrate thus obtained is subjected to ultrafiltration. This results in removal of ammonium sulphate and also in concentration of filtrate by a factor of ten.

The ultrafiltered antirabies serum thus obtained is described as one “Lot”. Based on the appropriate titer requirements in the finished product, lots are pooled together, diluted, sterile filtered, containerized and lyophilized. After passing all the quality control tests as per Indian Pharmacopeial requirements, the batch is released into the market.
1.2.5 Potency of antirabies serum

The different methods by which the antirabies antibodies in antirabies serum can be quantified are described as under:

1.2.5.1 Virus neutralization test using mice

This test is a WHO approved test for antirabies serum because it uses a large number of mice, it is not encouraged either by WHO or OIE. This test forms a part of Pharmacopoeial requirement for antirabies serum testing in countries like India.

A constant dose of challenge virus (CVS) is mixed with a series of different dilutions of the antirabies serum under test and also with a reference serum standard which can be either a national or international standard antirabies serum. This antigen-antibody mixture is inoculated intracerebrally, in a set of mice (Swiss albino). The basis of the test is that after neutralization with the serum, the remaining free virus (if any) causes the death of the mice.

1.2.5.2 Rapid fluorescent focus inhibition test (RFFIT)

The RFFIT is performed by mixing test sera with rabies virus followed by addition of mouse neuroblastoma (MNA) or Baby hamster kidney cell lines (BHK-21). The cell cultures are incubated, washed, fixed and then stained with a antirabies immunoglobulin fluorescent dye conjugate and observed under a fluorescence microscope for the presence of fluorescing cells. Antibodies, if present in test serum would neutralize the virus resulting in failure of infection of cells. Such non infected cells would therefore show no fluorescence. This test is a WHO approved test, and is also prescribed for international trade by the OIE.

All the tests are standardized either using RFFIT or the VNT. A modified version of the RFFIT is known as “Fluorescent Inhibition Microtest” (FIMT), uses microtitration plates instead of tissue-culture chamber slides. It correlates well with VNT & RFFIT.
1.2.5.3 Fluorescent antibody virus neutralization test (FAVN)

This test is a slightly modified RFFIT. Here the virus is used to infect BHK-21 cells. These infected cells are appropriately diluted to give 100 TCID$_{50}$/cm$^3$ per well of microtiter plate. (In RFFIT to begin with virus is incubated with antirabies test serum). To each well, test serum, appropriately diluted is added and incubated for one hour. After one hour, fresh cell suspension is added to each well and incubated further for 48 h. The plates are washed, fixed and dried with acetone and stained with fluorescent anti rabies antibodies. The entire surface of the well is evaluated in terms of fluorescence and results are recorded using the “all or nothing” approach. The serum titer is the dilution at which 100% of the virus is neutralized in 50% of the wells showing no fluorescence at all. This titer is expressed in IUs by comparing it with the neutralizing dilution of a standard serum under the same experimental conditions (OIE serum of dog origin or WHO standard for rabies immunoglobulin or both). The serum titer is expressed in IU/ml by comparing it with neutralizing dilution of the reference standard.

The main application of this test is the detection of antibody response to vaccination, either in domestic or wildlife populations. This test is prescribed by the Office of International Epizootic (OIE) for international trade. The fluorescent antibody virus neutralization (FAVN) test correlates well and are in agreement with VNT using mice and RFFIT (Meslin et al. 1996, OIE, 2008., Shankar, 2009).

1.2.5.4 Radial immunodiffusion (RID) assay

Radial Immunodiffusion is a classical diagnostic method to determine antibodies. This reliable assay is widely used and easy to perform. The RID assay is performed in agar plates, containing the specific antigen. Test samples, standard and control sera are prepared and added to the plates. After 48-64 hours incubation at room temperature the diameters of the immunoprecipitation rings are measured. The antibody concentrations in the test samples are quantified. RID assay has been used along with VNT using mice, to determine the serum

1.2.5.5 Counter immunoelectrophoresis test (CIET)

Antigen-antibody diffuse through gels forming precipitin lines at the point where these meet in optimal concentrations and this reaction is specific. The reaction can be enhanced and expedited by use of electrophoresis. The immunoelectrophoresis technique reduces the time required for formation of these precipitin lines. Anti-rabies antibodies can also be assessed by use of counter immunoelectrophoresis serum neutralization test where residual amount of antigen is detected by using a known positive antirabies serum as indicator serum (WHO, 2007). Counter immunoelectrophoresis (CIET) along with MNT, has been used in serological monitoring of dog vaccination campaigns and antirabies antibody titer in humans (Diaz et al. 1995, and da Silva et al. 2002).

1.2.5.6 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay is a diagnostic method used in detecting the presence of antibody or an antigen in a particular sample. The basic principle of an ELISA is to use an enzyme-tagged antibody to detect the antigen (Ag)-antibody (Ab) reaction. The use of corresponding chromogenic substrate easily indicates antigen (Ag)-antibody (Ab) reaction. An ELISA can be used to detect either the presence of antigens or antibodies in a sample, depending on design of the test (Ma, 2006). Commercial kits for testing of antirabies serum antibodies in human and dogs or cats are available. The ELISA test is acceptable as a prescribed test by OIE, provided that it has been validated and adopted on the OIE register. Virus neutralization tests may be used as confirmatory tests if desired.

There are three main types of ELISA: Direct and Indirect ELISA and Sandwich ELISA (Fig. 1.9) (Crowther, 2000).
**Direct ELISA**

In direct ELISA, an antigen is adsorbed to a micro titer plate. An excess amount of other protein (bovine serum albumin) is added to block the remaining binding sites on plate. The enzyme-antibody conjugate is allowed to adsorb to the antigen and excess enzyme-antibody conjugate is washed off, leaving enzyme-antibody bound to antigen. The enzyme is detected by adding the proper substrate.

**Indirect ELISA**

In an indirect ELISA, the primary antibody is not enzyme tagged; instead the secondary antibody raised in heterologus species is enzyme tagged. The advantages of indirect ELISA are:

1) There are two antigen antibody reactions in indirect ELISA; first between the antigen and the primary antibody and second between the primary antibody and the secondary antibody (with reference to secondary antibody the primary antibody is an antigen). In each of these two reactions multiple antibody molecules bind to the corresponding antigen. Therefore, when the secondary antibody is enzyme tagged instead of primary antibody the amplified signal of enzyme-substrate reaction is obtained. This increases the sensitivity of the assay.

2) Moreover, the secondary antibody can be used by multitude of researchers as long as the primary antibodies (against different antigens) used by them are raised in the same species.

**Sandwich ELISA**

In this test microtitre plate is coated with primary antibody and not with antigen unlike Direct and Indirect ELISA. The plates are reacted with antigen followed by addition of primary antibody again. The antigen thus gets sandwitched between the plate-bound antibody and free antibody added afterwards. Appropriate use of bovine serum albumin is done to avoid nonspecific binding.
Fig 1.9 Various types of ELISA. The antigen of interest is immobilized by direct adsorption to the assay plate or by first attaching a capture antibody to the plate surface. Detection of the antigen can then be performed using an enzyme-conjugated primary antibody (direct detection) or a matched set of unlabeled primary antibody and conjugated secondary antibodies (indirect detection).

The advantages of the ELISA are specificity, sensitivity, inexpensiveness, safety and less time to perform the test. It aims doesn’t require the handling of live virus. Since the enzyme label is the critical portion of ELISA, the selection of enzyme is very important. The enzyme selected should be stable under the conditions used for storage, cross linking and the assay. The most effective enzyme should have a high specific activity and should be inexpensive. ELISA can detect a protein even in nanogram & picogram quantities. This test can be performed in various forms and is easily reproducible, standardized and validated.

Use of ELISA for determination of antirabies antibodies
ELISA tests are used in the detection and quantification of antirabies antibodies. To detect seroconversion of antirabies antibodies in dogs, sandwich ELISA and competitive ELISA has been used (Sugiyama et al. 1997., Inoue et al. 2003 and Yang et al. 2006). During oral immunization of swine, using the Vnukovo-32/107 rabies strain, excellent seroconversion was evidence by the ELISA test (Ondrejka et al. 2001). ELISA also has been used in field conditions, because of its
simplicity in performance (Bahloul et al. 2005). An ELISA has been developed either to detect seroconversion in humans vaccinated by cell-culture vaccine or suckling-mouse brain vaccine. ELISA also has been demonstrated to differentiate monoclonals against street and laboratory strains of rabies virus (Smith et al. 1984, and Piza et al. 2005), ELISA has proven to be a valuable tool in monitoring post immunization response in cattle with inactivated and attenuated rabies vaccines or in dogs and cows from rabies-endemic areas in Brazil (da Silva et al. 2002, and Fachin et al. 2005).

An ELISA is successfully developed to assess the potency of horse therapeutic polyvalent antitoxin antivenom (Heneine et al. 1998). The Centers for Disease Control (CDC) has successfully developed a specific, sensitive and a quantitative ELISA against the protective antigen Bacillus anthracis and has proved valuable for the confirmation of cases of cutaneous and inhalational anthrax patients (Quinn et al. 2002). ELISA test was modified suitably to detect antibodies against specific antigens like the glycoprotein I of the Pseudorabies virus in case of Aujeszky’s disease (Oirschot, 1991) and also in detection of class specific antibodies (IgG and IgM) against West Nile Virus (Malan et al. 2004). In a multicentric study, a highly sensitive capture ELISA has been developed and used along with indirect immunofluorescence technique (IFT) for detection of antineutrophil cytoplasmic antibodies directed against proteinase 3 in Wegener’s granulomatosis (Csernok et al. 2004). Many commercial ELISA kits are developed to detect various factors related to different diseases, e.g. IL6 in case of congestive heart failure and elevated concentrations of CCL2 and Tumor Necrosis Factor–α in Chagasic Cardiomyopathy (Gaertner et al. 2003., Talvani et al. 2004).

ELISA has proven to be an extremely useful tool to evaluate the immunogenicity of experimental vaccine candidates and strongly support the development of novel vaccines, especially malarial vaccine (Muira et al. 2008). ELISA has also been validated for the diagnosis of human “Trichinellosis” which is a zoonotic disease caused by the consumption of meat containing Trichienieella larvae. The validated ELISA shows good performance in sensitivity, repeatability,
reproducibility and better specificity, which are the key factors to validate a test (Morales, et al. 2008). In case of rabies neutralizing antibodies, after post exposure vaccination, a C-ELISA has been successfully developed and characterized, and is found to be 100% specific and sensitive (Muhamuda et al. 2007). A new ELISA, PLATELIA™ RABIES II kit has been developed for the detection and titration of anti-rabies glycoprotein antibodies in human samples from vaccinated and non-vaccinated people. In this case, ELISA proved to be extremely useful alternative to the neutralization test (Feyssaguet et al. 2007).

1.3 PROBLEMS ASSOCIATED WITH THE USE OF ANIMALS FOR ROUTINE EVALUATIONS

The VNT using mice has to be used as a mandatory test to quantify antirabies antibodies in antirabies serum, as per Indian Pharmacopeia. But VNT using mice suffers from the following drawbacks:

- VNT requires the use of live virus. There are potential hazards in working with challenge virus strain.
- Performing VNT using mice is extremely laborious. More skill is also required for intra-cerebral mice inoculations.
- Time required for the completion of test is fourteen days.
- Results inherently suffer from biological variations amongst individual mice.
- Personal or accidental errors cause wide deviations in the results. Moreover, inter-laboratory variations are observed.
- The test requires usage of large number of animals (mice). Around 350 mice are utilized taking into consideration all the in-process samples to be tested for the potency of rabies antibodies during production of ARS using equines. The crucial factors involved are the cost of mice and the ethical rationale behind using a large number of mice.
- The test requires utmost care during virus handling and maintenance. Virus handling demands stringent norms in working area with “Humidity,
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Ventilation and Air conditioning” (HVAC) system and a relatively negative pressure.

- The animals are subjected to considerable pain and suffering which should not be overlooked indefinitely (Halder et al. 2002).

The book entitled “The Principles of Humane Experimental Technique”, published by Russell and Burch in 1959, but recognized only in the late 1970s became a milestone in animal experimentation resulting in the 3Rs concept of animal tests:

**Reduction**

Reduce the number of animals used in a particular experiment or a test.

**Refinement**

In *in vivo / in vitro* tests involving the use of animals, modify or refine the method or technique in such a manner that, it will lessen or eliminate pain or distress caused to animals.

**Replacement**

During animal experimentations, modify the test in such a manner that it will totally replace the number of animals used.

The ‘European Centre for the Validation for Alternative Methods’ (ECVAM) has contributed greatly in implementation of these 3Rs in the field of biologicals (Cussler et al. 2002., Metz et al. 2002).

Various cell culture and immunoassays do not require animals. These tests can be standardized, statistically validated and applied at all the in-process levels and even for release of the product in trade. The validation should involve inter-laboratory, intra-national and international collaborative studies before being considered as a standard test by competent national and international regulatory authorities. (Hartung et al. 2004)

In case of antirabies serum, the only WHO approved test which complies as a replacement technique is the RFFIT, and the other one being ELISA, which can be implemented after appropriate discussions at the national and international levels.
Therefore a need for an alternative test like ELISA has always been felt. Moreover ELISA test complies with the three ‘R’ concept in relation to animal experimentation ECVAM. The ELISA test can be standardized using inactivated vaccine and does not require live virus. ELISA is shown to correlate excellently with VNT and RFFIT. The test can be performed in a normal GLP (Good Laboratory Practices) compliant laboratory. The test doesn’t require the use of animals and also reagents used for the test are not costly. Moreover, a large number of samples can be quantified using a microtiter plate, at a given time.

The standardization and validation of ELISA in place of VNT for potency testing of antirabies antibodies during antirabies serum production using equines is imperative for all manufacturers and necessity of the time.

1.4 SCOPE OF THE PRESENT WORK

As discussed earlier Rabies, is a fatal zoonotic infection of the Central nervous System transmitted by the bite of rabid animal. In India at least 20,000 human deaths occur per year. India, the country with highest rabies fatalities in Asia is second (only to Ethiopia) in the world. (Cliquet et al. 2007., WHO SEA report 2009, WHO, 2010).

In the treatment of rabies, antirabies serum is indispensible (WHO, 2009). ERIG is the preferred choice during the treatment of rabies (Hong et al. 1994., Hanlon et al. 2001., Folbe and Cooke, 2006., Sugiyama and Ito, 2007., WHO, 2010). HRIG is expensive (five times that of ERIG) and is available in limited quantities, because of involvement of human volunteers.

In the manufacture of antirabies serum, potency testing is of critical importance (Fitzgerald et al. 1975). Virus Neutralization Test (VNT) using mice has to be used as a mandatory test to quantify antirabies antibodies in antirabies serum, as per Indian Pharmacopeia. The method is approved by WHO. However this test, apart from usage of large number of animals viz mice, the test suffers from other serious limitations. Intracerebral inoculation into young mice is indeed a skilful job and therefore personal or accidental errors cause wide deviations in the results. It is further compounded by inherent biological variations amongst
mice. The animals are subjected to considerable pain and suffering (Halder et al. 2002).

The Standardization and Validation of an indirect ELISA and its comparison with the VNT was therefore the focus of the present thesis. Attempt has been made to establish that ELISA test can replace VNT for potency testing during production of antirabies serum using equines.

1.4.1 Objectives of the present study

The primary objectives of the study were:

- To standardize indirect ELISA for quantification of antirabies antibodies in serum, plasma or purified final product
- To compare indirect ELISA with VNT
- To establish reproducibility of ELISA for all kinds of samples viz serum, plasma or processed immunoglobulins as final product.
- To explore the use of ELISA for routine evaluation of antirabies antibodies at production stage, for frequent monitoring of primary response and for optimization of immunization & bleeding schedules.
- To explore the applicability of ELISA in evaluation of immunological status of horses with respect to antirabies as responders/poor responders.