

**INDIGENOUS PREPARATION OF EQUINE RABIES IMMUNE
GLOBULIN AND MOUSE ANTI-RABIES MONOCLONAL
ANTIBODIES**

**THESIS SUBMITTED
UNIVERSITY OF MUMBAI**

**FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
MICROBIOLOGY**

**SUBMITTED BY
RAJENDRA M. MAHAJAN**

**UNDER THE GUIDANCE OF
DR. (MRS.) R. A. DESHMUKH**

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MUMBAI – 400 012**

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A

Thesis Submitted to

The University of Mumbai

For

The Degree of

Doctor of Philosophy

In

The Faculty of Science

(Microbiology)



By

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STATEMENT BY THE CANDIDATE

As required by the university ordinances 770 and 771, I wish to state that the work embodied in this thesis titled ***“Indigenous Preparation Of Equine Rabies Immune Globulin And Mouse Anti-Rabies Monoclonal Antibodies”*** forms my own contribution to the research work carried out under the guidance of **Dr. (Mrs.) Ranjana A. Deshmukh** at the **Haffkine Institute for Training, Research and Testing, Mumbai-12**. This work has not been submitted for any other degree of this or any other University. Whenever reference have been made to previous works of others, it has been clearly indicated as such and included in the “Bibliography”.



**Signature of the Candidate
Rajendra M. Mahajan**

Certified by:



Dr. (Mrs.) R. A. Deshmukh

M.D., Ph.D.

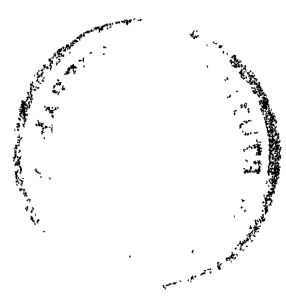
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INDIA.

Dedicated to my family...



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INTRODUCTION AND REVIEW OF LITERATURE

RABIES VIRUS - A VIRUS OF HISTORICAL SIGNIFICANCE:

Rabies, a disease of antiquity presents as a distinct major public health problem in different parts of the world. It stands apart as the most fatal of all the viral diseases. In the more affluent societies of the industrialized nations, the risk of rabies to human beings has minimized; albeit at a high cost. Nevertheless, rabies persists to cause numerous human deaths and immeasurable human anguish and suffering in the less industrialized societies, which comprise the developing nations. Despite, significant scientific breakthroughs and advances in its prevention and control, rabies remains one of the most dreadful and gruesome of the human communicable and infectious diseases. The mere thought of rabies itself produces both loathing and fascination in people. Rabies exposure on the worldwide scenario has both medical and economic implications, with as many as 4 million people receiving post-exposure treatment to prevent rabies (1).

The Latin word "rabies" seems to have originated from the Sanskrit word, "Rhabas" which means, "to be violent". The Greek word for rabies, Lyssa, also means madness, and is the source of the taxonomic identification of the viral genus (Lyssavirus). One of the earliest references to rabies is cited

1) Outbreak of animal disease may threaten human populations too 2) Control of rabies in animal populations must be based on the occurrence and behaviour of rabies in various animals and finally 3) animal rabies epidemiology profoundly influences choices regarding post-exposure prophylaxis of persons with potential rabies exposure.

Rabies primarily being a disease of terrestrial and airborne mammals involves families like *Canidae* (dogs, wolves, foxes, coyotes and jackals), *Felidae* (cat, bobcat and lion), *Viveridae* (mongoose), *Musteladae* (skunks, badgers and martens), *Chiroptera* (bat) and *Primates* (man and monkeys). Human infection with rabies is nearly always due to secondary bites, although others, such as inhalation, are also a possibility. In most parts of the world, the major reservoir for rabies is the domestic or the semi-domestic dog or cat. Data pertaining to rabies epidemiology are generally derived from public health sources, physicians, veterinarians, and laboratories reporting cases and epidemiological investigations. All age groups are at the risk of exposure to rabies; but the predominant group (>50%) is in the 6-15 years range. Males are four times more common victims than females (9).

Rabies is currently distributed worldwide except for Australia, Antarctica and few island nations. In 1992, ninety-six nations reported the presence of rabies, and 46 reported its absence (10). As per the World Health Organization (WHO) reports, more than 2.5 billion people are at risk in

over 100 countries (11). Of all the infectious diseases causing mortality worldwide rabies ranks number ten (11). The tropical countries like Africa, Asia, South America and Oceania are the most affected. 'Rabies-free' countries include island states like UK, Japan, Spain, Portugal, Sweden, Finland, Norway, Greece and some others (12). Globally, rabies is considered to be a relatively insignificant human disease, as it causes only 1% deaths (1). As per WHO, 36,001 cases of human rabies were reported in the year 1992 (13) and 33,212 cases of human rabies were reported in 1996 (14). Although effective vaccines are available, still there are 50,000-60,000 human deaths occurring annually (11). These reports probably estimate the worldwide incidence of disease, which under reprisal may cause as many as 1,00,000 deaths annually (15). From India alone 25,000-50,000 deaths are reported per year (16). Thirty lakhs individuals all over the world is administered post exposure rabies vaccine and 1 in 1000 individuals is admitted to hospital every year for animal bites (17, 18).

Rabies in India:

Rabies is prevalent in most parts of our country except in some islands like Lakshdweep-Minicoy, Andaman and Nicobar (19). In India, the stray dog bites is responsible for nearly 96 % of rabies cases. Annually about one million people receive post-exposure anti-rabies vaccination of which children constitute about 35-40%. Of these, half a million receive the Semple Vaccine free of cost from Government hospitals, while the rest

Institute, Kasauli were found to be positive for rabies. This definite evidence of rabies in mongoose suggests a possible transmission of rabies from the mongoose to other rodents, particularly in various species of rats that occupy the same ecological niche (21).

The possibility of rats becoming infected with rabies virus is most alarming. This aspect needs to be investigated further. So far, there has been no reported evidence of rabies infection in the various species of bats in India. Other animals like lizards, deer, squirrel, vultures and eagles seem to bite incidentally on provocation and the rabies-phobia leads the victims to take anti-rabies treatment. The number of monkey bite cases has been increasing in the last few years. But there is no epidemiological or clinical evidence that monkeys, free in nature, are infected with rabies virus. But their susceptibility to rabies infection is a known fact.

RABIES VIRUS CLASSIFICATION:

The viruses belonging to the family Rhabdoviridae are negative stranded RNA viruses and consist of mainly two genera – 1) Vesiculoviruses and 2) Lyssaviruses. Rabies is enzootic and sometimes epizootic, in a variety of mammalian species, including domestic Canids (dogs, foxes), Mustelids (skunks), Viverids (mongoose), Procyonids (raccoons), and insectivorous and hematophagus bats.

1. Vesiculoviruses:

The viruses in the vesiculovirus genus exhibit various degrees of serological cross reactivity, and gene analysis of a few species in this genus indicates sequence similarities between nucleoprotein (N) genes. Vesiculoviruses have many similar characteristics like that of lyssaviruses. They infect large numbers of animal and insect species; contacts with animals too infect humans occasionally, typically through respiratory secretion (22). Seven Vesiculoviruses such as Vesicular Stomatitis Virus (VSV), Chandipura, Cocal, Isfahan, Maraba and Piry are known to infect humans occasionally (23).

2. Lyssaviruses:

This genus includes only one serogroup. This group is determined by serological cross-reaction of viral antigens (by Complement fixation, immunofluorescence test), particularly antigenic sites on the N protein. Placement of virus species as rabies or rabies related viruses is determined by antigenic sites of the glycoprotein (G protein) as recognized in virus neutralization tests. The members of the Lyssavirus genus are rabies and rabies related viruses, which are:

- 1) Lagos bat virus isolated from pooled brains of frugivorous bat in Lagos Island, Nigeria (1957).
- 2) Makola virus isolated from the viscera of shrews captured near Ibadan, Nigeria (1968); this was isolated from human patients also.

- 3) Obodhiang - isolated from mosquitoes (1963)
- 4) Kotonkan- neutralizing substance for Kotonkan was found in high percentage of cattle, rodents, sheep, and horses in Nigeria (1967).
- 5) European bat Lyssavirus1 (EBLV1) 1985- almost all cases are in the common European house bat.
- 6) European bat Lyssavirus2 (EBLV2) 1985- First identified in Swiss bat biologist, who died in Finland.

BIOLOGY OF RABIES VIRUS:

Morphology and properties:

Rhabdoviruses are either bullet shaped or bacilliform particles that are 100 to 430 nm long and 45 to 100 nm in diameter (24). Virions, Defective-Interfering particles (DI) have the same configuration but are shorter (20 % - 50 % the length of standard infectious viruses). DI particles resemble standard viruses in their protein and lipid compositions, but are not infectious because 50 % to 80 % of the genome is deleted (25).

Gross chemical composition reveal presence of 22% lipid, 3% carbohydrates, 1% RNA, and 74% protein. The molecular weight range of rhabdovirus is $300 - 1000 \times 10^6$. Virus buoyant density in CsCl is 1.19 to 1.20 g/cm³, and in sucrose it is 1.17 to 1.19 g/cm³. The virus does not tolerate extremes of pH (below 3 or above 11) and it is relatively stable at

pH 5.0 to 10.0 (26) but is also rapidly inactivated at 50°C, or by exposure to ultraviolet or X-ray irradiation, lipid solvents, 45% - 70% ethanol, iodine preparations, quaternary ammonium compounds (27) and oxidizing agents. Viral RNA accounts for 1-4% of the particle weight and has an estimated molecular mass of 4.2 to 4.6 X 10⁶ daltons, for the virus Sedimentation coefficient is 600s. The virion RNA has a sedimentation coefficient of 42-45 Svedberg units and a buoyant density of 1.66 g/cm³. The RNA has 5' terminal triphosphate and is not polyadenylated of which the terminal sequences are complementary. Defective RNA's are significantly shorter than full-length RNA and replicate only in the presence of usually homologous helper rhabdovirus (28).

Virus infectivity is rapidly disrupted by the application of formalin, phenol, mercury- bichlorides, β-propiolactone, ether, trypsin and detergents (15). The virus is sensitive to pasteurization temperatures, U.V. light, drying, sunlight, and heat. These physical parameters also destroy the virus infectivity, but protein or glycerol suspensions allow the virus to be stored at -30 to -60°C or viability for long periods is achieved by maintaining at 4°C (29).

Biochemical Studies:

Biochemical studies have demonstrated that each virion is composed of single molecule of genomic RNA and five proteins showing post-

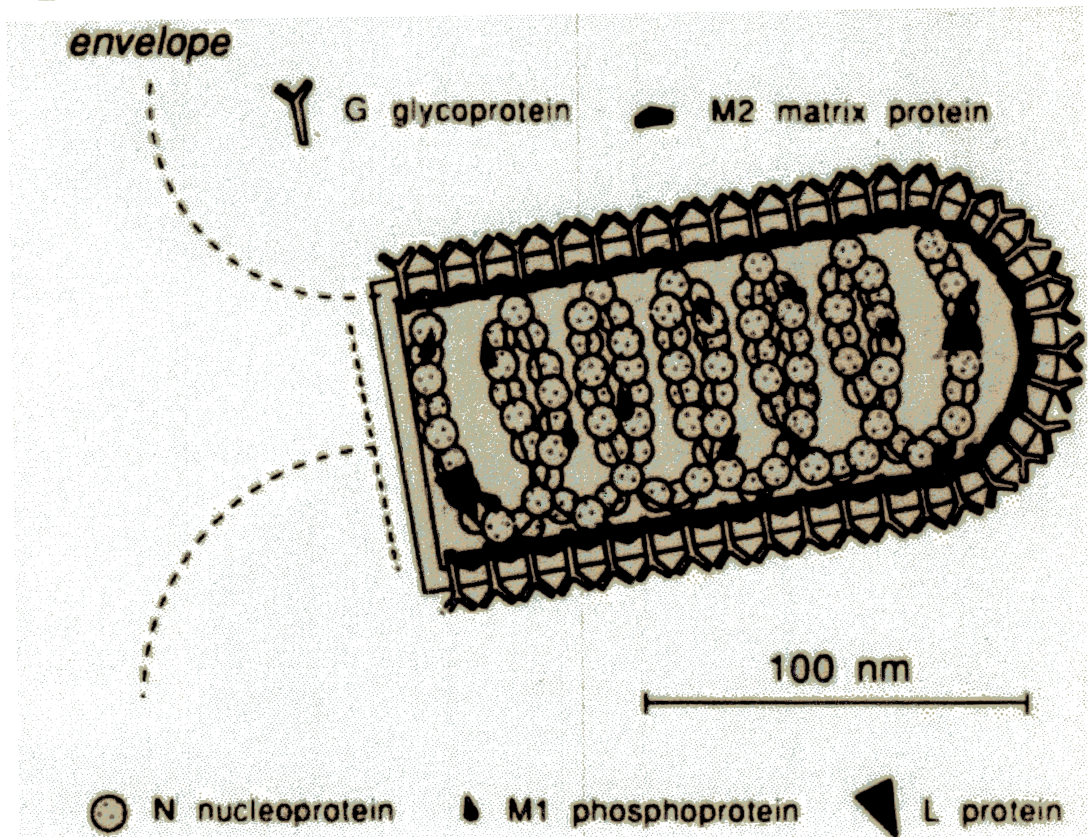
translational modification (30).

1. The RNA dependent RNA polymerase (L protein), which is responsible for duplication of the viral genome and also several structural proteins, having a high relative molecular mass 180 KD.
2. The Glycoprotein (G) which exists in two forms G I (65 KD) and G II (70 KD) with different extent of glycosylation, both of which are fatty acylated with palmitic acid.
3. The nucleoprotein (N), which is phosphorylated, has a molecular mass of 57 KD.
4. A phosphoprotein M1 having two forms M1 I (38 KD) and M1 II (41KD), with different extents of phosphorylation.
5. The matrix or membrane protein M2 having two confirmations M2 I (24KD) and M2 II (25 KD), differing in the number of internal disulphide bridges. These both forms are palmitoylated (30).

G (Surface) protein:

The G protein is the best studied at the structural and immunological levels. It is 524 and 522 amino acids long in rabies and Mokola virus respectively. The antigenic structure was determined by obtaining the limited number of fragments by using chemical cleavage and isolation by SDS-PAGE. Eight large peptides, ranging in size from 32-77 amino acid residues have been mapped within the amino acid sequence of glycoprotein by first determining N-terminal sequence of each fragment. Then

Figure: Structure of Rabies Virus



antigenicity and immunogenicity of these peptides were studied (31). The proteolytic treatment of the virion removes the spike like projections and selectively affects the G protein. Only a small G protein remains linked with the spikeless particle indicating that the G protein is membrane anchored and constitutes the main component of the viral spikes. The spikes that contain the envelope glycoprotein are attached to the core particles probably with the help of viral lipids. Each spike consists of three associated G proteins (trimers). There are about 1200 G proteins (400 trimers) per virus particle. There are three sugar chains that are N-glycosidically attached. Penetration of the virus into the cytoplasm takes place in the endocytic pathway and not at the plasma membrane. This is because the G protein trimer undergoes a change in conformation at pH 6.1, thus stabilizing the trimer and probably allowing the hydrophobic region of the molecule to become exposed and to get embedded in the membrane of the cell to be infected.

This virus glycoprotein G is responsible for induction of virus neutralizing antibody and protection of animals against challenge, stimulation of specific T cells, which express helper, suppressor or cytotoxic activities and determination of virulence. Because of its multiple immunogenic functions, this G protein is potentially suitable for vaccination purposes. Recently approaches have been made to develop second-generation vaccines (31).

N protein (Nucleoprotein):

This is a 450 amino acid long polypeptide that is phosphorylated on a serine residue in position 389. The functions of the N protein are to encapsidate and protect the genome. The Monoclonal antibodies (MAbs) directed against the N protein has been used to differentiate and classify rabies wild isolates into types of lyssaviruses.

M 1 protein:

This is highly hydrophilic protein having 303 amino acids. In the year 1981, it was suggested that, M1 protein is not a membrane protein but found to be occupying an internal position within the virion in association with N protein (32).

M2 protein:

This is a 202 amino acid long polypeptide. It plays an intermediate role between the ribonucleocapsid and the viral membrane, presupposing the presence of regions interacting with both elements. It is believed to play an important role in morphogenesis of the virus. The association of the two membrane proteins with the nucleocapsids results in folding of the M2 protein and its enclosure by membrane.

The L protein:

This is a giant protein consisting of 2142 and 2127 amino acids in the PV and SAD-B19 strains of rabies virus respectively and occupying more than half (50 %) of the genome.

Replication of Rabies Virus:

Rabies virus, although essentially neurotropic, replicates in wide variety of cells both in animal and tissue culture models (33). A number of laboratory host systems have proved satisfactory for the propagation of rabies virus. Virus replication and disease is elicited by intracerebral injections of virus containing substances into mouse or rabbit brain. Other laboratory animals that have been satisfactorily used include hamsters, rats, and guinea pigs. Tissue culture systems too have proved useful for virus cultivation. These systems provide large quantities of virus for vaccine preparation. Electron microscopy studies reveal that numerous viral particles can be attached to the cell plasma membrane or within intracytoplasmic vesicles just 5 minutes after exposure. In the first hour of infection of rabies virus, degradation of the viral particle, fusion of the virus to the cell membrane and release of internal structures can be observed. After 60 minutes, intact virions can no longer be seen within the cell protoplasm. Rabies specific antigen recognized by immune fluorescent reaction appears four hours after infection and by the end of eight hours all cells show presence of antigen.

Budding viral particles are visible by 6 hours and reach to a maximum within 24 hours.

Replication of rabies virus appears to be similar to VSV, which is intensely studied. The virion's transcriptase i.e. RNA dependent RNA polymerase transcribes messenger RNA (mRNA) and another polymerase (replicase) constructs a positive-strand RNA (40S) to serve as a template for virion RNA, and which infact has a polarity opposite to that of the transcribed mRNA. The transcriptase helps in the formation of mRNA at a single promoter site, resulting in a complimentary messenger (positive-strand) molecule derived from the negative viral RNA strand. This produces a long mRNA, which acts as an intermediate for replication of new virion nucleoproteins. Virus replication takes place in the cytoplasm of the host cell, where collections of the nucleocapsids are identified as inclusions termed as Negri bodies. The initial cycle of multiplication follows an eclipse period of about 6-8 hours and this process is completed by 19-20 hours. Budding from cytoplasmic membrane occurs, as the virions assemble and forms its envelope from the host cell membrane. However, *de novo* viral envelope synthesis also occurs within or at the border of host cytoplasmic membrane, without budding (34). Infection of cultured cells by itself is not lethal, but an endosymbiotic infection transpires (35), and results in continued infection to the cells and thus shedding of virus subsequently.

DISEASE MANIFESTATION:

The most common mode of transmission to man is by the bite of a rabid animal or its saliva coming in contact to the wounds or to bruises. Also other modes of infection reported are through the mucosal membranes of the mouth, conjunctiva and digestive tract but never through the intact skin (36). However, airborne natural infection is also possible in an exceptional circumstance, e.g. caves harboring large number of bats infected with the virus (37). Also, the transmission from human-human was reported (38). The incubation period of rabies virus varies from human to human from few days to 19 years. Seventy five percent patients show rabies symptoms within 18 – 60 days of exposure. Various studies generally report mean incubation period to be around 30 - 78 days (39). It is usually short when bitten on face or head, and long when bitten on legs. It also depends on site of the bite and dose of inoculum. The disease develops through a series of clinical stages such as incubation period, prodrome, acute neurological phase, coma, and finally death or rarely recovery.

The onset of the disease is usually initiated by prodromal symptoms such as fever, anorexia, nausea, vomiting, malaise, lethargy and parasthesia or pain at the site of bite. This prodromal period usually lasts for 2-4 days, followed by an acute neurologic phase, which generally lasts for 2-7 days. Two forms such as furious and dumb or paralysis, mark this phase. Signs of hyperactivity, disorientation, hallucination, period of excitement and

bizarre behaviour characterize the furious form. Of these, hydrophobia the most distinguishing characteristic feature for rabies identification (40) starts with painful contractions of the larynx and pharynx. These spasms last for about 5-15 seconds resulting in aspiration, coughing, sneezing, choking or finally vomiting. This is then followed by development of conditioned aversion to swallowing water. At this stage the patient develops the dread for even the sight or sound of water.

In contrast, paralytic rabies patients lack hydrophobia, aerophobia, hyperactivity or seizures. Their initial findings may suggest an ascending paralysis, resembling acute inflammatory polyneuropathy (Guillain Barre Syndrome) (41), or a symmetric quadriparesis. In some cases weakness is more severe in the extremity. Meningeal signs (headache, neck stiffness) may be prominent despite the normal sensorium. As the disease progresses, the patient with paralytic rabies becomes confused and progressively declines into coma within 2-10 days of onset of the acute neurologic phase. As a result of these various potential fatal complications death usually occurs.

LABORATORY DIAGNOSIS:

After a bite, laboratory tests can determine whether the animal bitten was indeed rabid or not. Animal and human rabies diagnosis can be made by four methods: i) Histopathology ii) Virus cultivation iii) Serology and iv)

Virus antigen detection.

Although each of the first three methods is advantageous, they do not provide a rapid definitive diagnosis. In the mid 1950's, rapid rabies antigen detection was first developed (42). The presence of rabies virus in an animal or an infected person can be determined by serology using Enzyme Linked Immunosorbant Assay (ELISA) (43) and Fluorescent Antibody Test (FAT) (44). Antigen determination is also done using biopsies of skin, brain or corneal specimens. Histologically, in 90% of infected humans rabies is detected mainly by the presence of Negri bodies, which are nothing but intracytoplasmic inclusions formed by aggregates of nucleocapsids in neurons by using Seller's staining method (45). The most definitive means of diagnosis of rabies is by virus cultivation from infected tissues using WI-38, BHK 21 and CER cell lines. Since rabies virus induces minimal cytopathic effect (CPE), direct fluorescent antibody staining is the only sensitive and rapid test (46). Also, intracerebral inoculation of mice or other laboratory rodents are used by injecting 20% brain suspensions of suspected specimens for virus cultivation. The inoculated mice are then observed for disease signs. Upon death, or after 28 days of inoculation, they are sacrificed and observed for virus by immunofluorescence. Neural tissue, spinal fluid, salivary gland and saliva are the most likely sources of virus.

Rabies infection or vaccination usually results in antibody production,

which can be measured by Mouse Neutralization Test (MNT) (47) or Rapid Fluorescent Focus Inhibition Test (RFFIT) (48). Additionally several new studies are also documented for the diagnosis of rabies. For detecting rabies tissue antigen in formalin fixed specimens, avidin biotin peroxidase system is more sensitive than immunofluorescence test (49). To detect rabies virus antigen in tissue suspensions, antinucleocapsid antibody is fixed to microtitre plates (50). Dot Immuno-binding assay is used for detection of rabies antibodies (51). Also Mouse Monoclonal Antibodies (MAbs), PCR (Polymerase Chain Reaction) techniques are used for the diagnosis of rabies (52).

PATHOGENESIS:

Pasteur et al (1881) first demonstrated the role of Central Nervous System (CNS) and especially the bulb, which joins the spinal cord to the brain in the progress of rabies infection. The virus at first completes the stages like entrance, absorption, and eclipse at the site of introduction before proceeding towards the CNS. Rabies virus injected into the footpads or muscle tissue of experimental animals can be recovered from the site of inoculation only if done within short periods of time. Dean et al (53), amputated the feet of mice inoculated with fixed virus and found that the procedure of removing the virus from the inoculated area was a life saving procedure if done within 4 hours. These results were found to be similar to

that found some 50 years ago by Fermi in 1928. Disease manifestation also depends on the site of infection, for example when guinea pigs were infected with a drop of fixed rabies virus on a small hole cut in the skin of the neck these died but none succumbed when the same dose was applied in the rear leg (54). Statistics on human disease also indicate that wounds on the head and neck are generally more lethal to that of hand and feet (55). After the short initial period of obvious viability the virus goes into an eclipse phase, where it cannot be recovered from any tissue. And during this period the virus may have remained locally or already proceeded on its pathway to or within the CNS. There is also evidence that virus stays locally for a long time at the site of wound or inoculation (56).

In 1973, Murphy and colleagues elucidated the pathogenesis of rabies infection in an exquisite series of studies (57). They reported that rabies infections are generally established by virus inoculation into peripheral tissues and ascend via afferent peripheral nerves and reach brain neurons. Although the usual method of transmission is inoculation of infected saliva into subcutaneous tissue, muscle or onto broken skin, the other modes of transmission have also been recognized Viz. virus inhalation, transplantation of corneal tissue containing virus and inoculation onto mucous membranes. On inoculation of the virus into the striated muscle cells the virus begins to replicate. Electron microscopy studies on myocytes revealed virus particles budding from plasma and sarcoplasmic

reticulum membranes of infected cells and amplifying of the number of virions locally. This remarkable finding that striated muscle probably is the site of virus sequestration in long incubation infection has a great importance in the study of post-exposure rabies prophylaxis. It is because; the efficacies of treatment procedures most probably depend on the accessibility of virus before it becomes insconced in the nervous system. Within 62- 72 hours of inoculation in dorsal root ganglion cells the virus ascents into spinal cord. Rapid infection of spinal cord, brain stem, cerebellum and other brain structures follows. The virus then descends via sensory and autonomous nervous system to retina, cornea, salivary glands, skin, nasal mucosa and other organs and tissues subsequently. Besides the direct viral replication in the salivary glands, the high concentration of virus in saliva results from viral shedding from sensory nerve endings in the oral mucosa (58).

It has been suggested that another major site of neuronal invasion is neuromuscular junction. In experimental models, within 1 hour after intramuscular or subcutaneous injection active rabies virus co-localizes with cholinesterase positive binding site (59). On inactivation, the virus can be prevented from binding. For example, pretreatment with α -bungarotoxin or d-tubocurarine blocks nicotinic acetylcholine receptor (60). A partial amino acid sequence homology exists between rabies virus glycoprotein and the sequences of several snake neurotoxins that bind

specially to the nicotinic acetylcholine receptor (61). In contrast to experimental infections, natural rabies infection may require a period of local viral replication, presumably to increase the concentration of inoculum, before nervous system infection can occur. During this period, the administration of antirabies immunoglobulins and the development of (antirabies) immunity by active immunization are able to prevent spread of the virus into the nervous system and are thereby able to prevent clinical disease. Once the virus enters the peripheral nerve, current therapeutic techniques may not be able to prevent subsequent replication of the virus. However, advanced research techniques hold the promise of antibody delivery across the blood brain barrier, which in turn may allow more effective treatment (62).

PATHOLOGY:

The gross appearance of the brain at autopsy is usually unremarkable, except for the vascular congestion that frequently occurs in patients dying after prolonged mechanical ventilation. The microscopic CNS pathology of rabies is described as 'encephalitis with Negri bodies'. All autopsy specimens do not show perivascular lymphocytic cuffings and necrosis that characterize encephalitis, and in some cases it appears histologically too as meningitis. Negri bodies are usually concentrated in the hippocampal pyramidal cells and less frequently in neurons and cerebral Purkinje cells

dependent cellular cytotoxicity (67). The Ribo Nucleo Protein (RNP) of rabies virus is also a major antigen that induces a virus specific antibody response, and antibodies directed against RNP also may contribute to protection against rabies virus infection (68). On absence of vaccination, antibodies in naturally acquired infection occur only after well establishment of the disease. Serum neutralizing antibodies generally do not appear until the 5th day of the onset of rabies symptoms (69). They may be also detected in cerebrospinal fluid (CSF)

Cellular Immune Responses:

Hale (1978), reports that rabies virus infection result in the generation of virus specific CD8⁺ and CD4⁺ T cells. The viral G protein appears to be the major antigen that mediates a cytotoxic T cell response and also is its major target (70). In animals, the live rabies virus develops a strong CTL (Cytotoxic T- Lymphocytes) response specific to NS protein. The rabies nucleocapsid is also a major antigen that activates CD4⁺ cells, and the majority of these T cells cross-react among different Lyssaviruses. The RNP specific T cells helps in production of VNA by intracellular antigen recognition and mediates the protective immune response induced by RNP. The immune mediated cytolysis of rabies infected cells results in death of neuronal cells, detrimental to host. Rabies infection usually induces the

production of interferon, which inhibits infection in tissue culture (71). And administration of exogenous interferon or interferon inducers demonstrated their ability to protect against rabies infection. The production of interferons in natural infection is not sufficient enough to protect an unvaccinated individual. Delayed processing and response by the immune system is due to the small amount of viral antigen early in the infection and also the intracellular location.

DISEASE PREVENTION:

As rabies is the most fatal illness, emphasis is laid on preventing human infection. Three aspects of prevention should be considered:

- 1) Prevention of exposure through animal contact.
- 2) Prophylactic treatment to those most likely individuals to be exposed to the virus and
- 3) Prevention of the onset of infection in those who have been exposed to the virus.

Since rabies is zoonosis, man becomes a casual host only by exposure to an infected animal. Human illness can be averted by elimination of this exposure through, control of rabies in wild, feral and domestic animals by various enforcements of rabies vaccination laws, capture of stray animals and providing rabies vaccine through low cost animal clinics. Persons likely to be exposed to rabies should receive pre-exposure prophylaxis.

Laboratory workers who, handle potentially infected tissue and virus, veterinarians, animal control officers working in rabies prevalent areas and individuals who travel to highly endemic areas ought to receive initial immunization followed by maintenance boosters (72). Tissue cultures Human Diploid Cell Culture Vaccine (HDCV), Purified Chick Embryo Cell Culture Vaccine (PCECV), Purified Vero Cell Rabies Vaccine (PVRV) etc. are some of the examples. These vaccines are preferred because of demonstrable safety and efficacy. But, Semple vaccine, Suckling Mouse Brain Vaccine and Duck Embryo Vaccines are discontinued in most of the developed countries due to their adverse side reactions.

The glycoprotein containing spikes that project from the viral envelope appears to be the sole antigen responsible for induction of neutralizing antibodies (73). When sub-virion components were used to inoculate mice or rabbits the purified envelope glycoprotein resulted in neutralizing titres 3.5 to 80 times higher than "core particles" or nucleocapsid material. Core particles, mixtures of envelop proteins and whole virus induced titers proportional to the amount of "contaminating" glycoprotein each contained. Purified glycoprotein had a neutralizing antibody binding activity value of 8 times higher than core particles, suggesting that neutralizing antibodies had a high degree of specificity for envelop glycoproteins. Nucleocapsid and core particles administered to mice

provided virtually no protection against rabies virus infection. Whole virion isolation was more protective than either glycoprotein or envelope preparations, probably due to interferon induction. These data suggests that protection due to whole virus or subunit vaccines depend on the presence of envelope glycoprotein. Finally, liposomally anchored spike glycoprotein induced both strong humoral and cellular immune responses. Pre and post exposure potency testing demonstrated high level of protection.

Pre-exposure prophylaxis includes series of three intramuscular injections administered as per the schedule such as – Day 0, Day 7 and Day 21, over a three-week course. The vaccine to be administered should have concentrated minimum potency of 2.5 IU/ml. Antibody response determination is not usually required in normal hosts. In every 2-3 years booster doses are usually recommended for individuals frequently at risk of exposure. As per the WHO recommendation, an adequate antibody response is generally considered to be complete neutralization at 1:5 level RFFIT and or by MNT, which is equivalent to 0.5 IU/ml concentration. Post-exposure prophylaxis is intended to prevent rabies in persons who have been exposed to virus. The treatment for each exposure situation is to be evaluated individually by a physician keeping in mind biting species animal, bite incidence circumstances and type of exposure. Preventive therapy should be instituted immediately as soon as possible following an exposure. The bite should be washed thoroughly with soap and debrided, if

there are six WHO recommended rabies vaccination schedules for cell culture vaccines. The standard post-exposure vaccination schedule is the 'Essen' 5 - dose intramuscular regimen on days 0, 3, 7, 14 and 30. The multisite intradermal administration of small doses of cell culture rabies vaccines (0.1 ml) was developed as an economical alternative to dangerous nerve tissue rabies vaccines being relatively low cost, despite variable potency and neurological complications risk. But intradermal applications do not give high and long lasting titres as compared to the intramuscular application (76).

When prescribed, Rabies Immune Globulin (RIG) and rabies vaccine should be used for passive and active immunization respectively. The serum-neutralizing antibody generated by vaccination is directly related to rabies protection in animal models and also in man. In 1950, Koprowsky et. al first used crude anti-rabies equine serum for this purpose (77). Even though the crude antisera were later replaced by purified and pepsin digested equine rabies immune globulin, it is still widely used in many parts of the world. In Iran (1954), the persons seriously wounded by rabid animals were administered post exposure treatment with anti rabies equine serum and vaccine and found to be successful (78). Passive immunization is generally recommended in case of immunocompromised patients, category III exposures and delayed treatment and also in case of vaccine failure. In such cases, WHO recommendations for post-exposure treatment

of rabies include Human, or Equine Rabies Immune Globulin (HRIG or ERIG) in combination with rabies vaccine (79-81). These immunoglobulins are purified from donor's sera, hyper immunized with rabies vaccine. HRIG and ERIG dosage is 20 IU/kg and 40 IU/kg respectively. Half of the dose is injected in the vicinity of the wound and the remaining is injected intramuscularly in the gluteal region.

HRIG:

In 1965, approximately 16% of persons treated with antirabies equine serum reported to have developed serum sickness: the incidence was 46 % in persons over 15 years of age (82). Recent studies on ERIG state incidence of <1-6.2% serum sickness among recipients (83-85). And so to avoid such reactions, developed countries prefer human rabies immunoglobulin preparations for post-exposure treatment. As HRIG is homologous, it causes no allergic reactions like anaphylaxis and serum sickness as compared to heterologous serum products. Since 1975, this product has been administered to more than 2,50,000 people in the United States and no cases of serum sickness have been reported.

Disadvantages of HRIG:

HRIG is expensive and available only in limited quantities, because the cost of HDCV is high, the time required for rabies virus-neutralizing

antibodies in immunized donors to be produced is much more and also the manufacturing process require supply of donors whose antibody titres are at least 15 IU / ml. Limited access to HRIG is probably a major contributing factor in the increasing number of post-exposure treatment failure (86, 87).

In spite of these disadvantages, the Advisory Committee for Immunization Practices (ACIP), United States recommends HRIG in preference to ERIG (88). Even though, human plasma proteins are important for prophylaxis or therapy of diseases, they bear potential risk of HIV (1 and 2), Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV) contamination.

ERIG:

Equine Rabies Immunoglobulin (ERIG) is a heterologous serum product. Different types of ERIG have been produced using inactivated and fixed strains of rabies virus (89). For example, a therapeutic antirabies immunoglobulin for human use is produced in Queen Saovabha Memorial Institute (QMSI), Bangkok Thailand, by first immunizing horses with a Purified Vero cell Rabies vaccine (PVR) subcutaneously into the lateral aspect of neck. This is then followed by serial injections of vaccine in increasing volumes. The immunization period lasts 105 days and the first bleeding is made after 14 days.

Factors affecting the Production of ERIG:

Selection of animals is most important because even if the horse is of same breed, serum production varies according to its age, nutritional status, general health (free of diseases) and immunization history. Antibody titre should be checked in donor animals at appropriate intervals and animals that do not produce high titre consistently should be withdrawn by day 56. Different vaccines like nerve tissue, as well as those prepared from tissue culture were used for the production of ERIG to induce high antibody levels. The former (nerve tissue vaccine) had side effects like anaphylactic and neuromparalytic reactions in vaccinated horses while no such reactions were reported following immunization with PVR vaccine prepared as per WHO requirements (90, 91). Recent data indicate that PVR vaccine is probably more immunogenic in humans than any other equi-potent cell-culture vaccine. Vaccine adjuvants of various types have been used in different institutions. It's not been yet established whether adjuvants are necessary. Studies are also needed to determine whether non-ulcerogenic adjuvants such as aluminium hydroxide or bentonite are as effective in inducing high antibody titres as complete Freund's adjuvant, which often causes severe local reactions. Studies have shown that aluminium hydroxide mixed with PVR vaccine and used for the intramuscular vaccinations of humans will enhance a significant antibody response (92). Similarly, controlled studies are also required to determine the optimal

schedule for vaccinating horses in-order to produce high ERIG titre.

The RFFIT and MNT are the most widely used potency tests for anti-rabies serum and immunoglobulins. When the potency of several lots of ERIG of different manufacturers was assayed at the Queen Saovabha Memorial Institute, wide variation in potency were found, ranging from 122% to 312% (93). Since high doses of ERIG may suppress rabies virus-neutralizing antibody response when vaccine is also given, manufacturers must ensure that ERIG preparations are calibrated against the international standard for Rabies Immunoglobulin (94) or a national reference preparation, and the potency (in IU per ml) as clearly stated on each ampoule or vial. The leaflet accompanying the package should also include storage information and date of expiry. ERIG preparations from various manufacturers also showed serum sickness in some recipients, which ranged from 0.82% to 6.19%, depending on the protein content (95, 96). Most cases are relatively minor and do not require alteration to the post-exposure treatment regimen. Nevertheless, the purification procedure employed by different manufactures appears to influence adverse reaction (96). But still the reported rates are much lower than those associated with the available preparations of ERIG (46%) (82). Further studies are also required to improve the safety of this product.

Need for ERIG purification:

In view of the high cost of the rabies immunoglobulin of human origin (HRIG), heterologous (mainly Equine) immunoglobulins are still frequently used for the prevention of rabies, even though they may cause sensitization and eliminated more rapidly than ERIG. Hence, purification technique is a must to reduce the high risk of sensitization to ERIG. The sole objective is to maximize its specific activity and minimize allergic substances in the product. When these techniques are employed, it is advisable to adhere to the WHO Expert committee recommendations on Biological Standardization (91). Although limited information is available on the manufacturing and purification procedures of commercially available ERIG and ERIG preparations produced in developing countries, most of these depend on the use of classical methods developed decades ago (84, 97).

Habel, first attempted protein fractionations in 1945 (98) using ammonium sulphate, and various other methods have since been performed (99, 100). The purification of immunoglobulins from human plasma is carried out in accordance with Cohn et al (99). This technique was thus adapted and used for the purification of heterologous immunoglobulins (101). The antirabies serum can also be purified by enzyme treatment and heat denaturation (102-105) which include: i) Cleavage of immunoglobulins by a proteolytic enzyme, pepsin followed by separation of the F (ab)² fragments ii)

Selective denaturation by heat iii) Fractionated precipitation by neutral salts, such as ammonium sulphate. Horejsi (1956), used ethicridine lactate and ethanol precipitation to purify serum (106). This method is adapted for the purification of both ERIG and HRIG. Purified ERIG causes serum sickness like reactions in less than 1% of the cases and anaphylactic reactions in 1 out of 45,000 cases (107-108).

MONOCLONAL ANTIBODIES AND ITS APPLICATIONS IN RABIES:

Conventional antisera contain products of hundreds or thousands of different antibody secreting clones, which are not specific for many different antigens (109). In hyper immune animals, the circulating antibodies for one specific antigen are very rare. The use of these mixed populations of antibodies creates a lot of problems. Therefore, the preparation of homogenous antibodies with a defined specificity was a long-standing goal of immunochemical researchers. This goal was then achieved by the development of hybridoma production technology. Kohler and Milstein (1975) developed a technique for the production of monoclonal antibody-secreting cell lines (hybridomas) formed by the fusion of myeloma cells with B-lymphocytes specific to a particular antigen (110). Continuous tissue culture cell lines are produced by fusing mouse myeloma cells and spleen cells from mice that have been

immunized with rabies vaccine / virus. The cell lines producing the monoclonal antibodies are continuously cultivated. The advantage being highly specific antibodies can be harvested cheaply in large quantities. Monoclonal antibodies react more specifically than polyclonal antibodies produced in hyper immunized animals and humans, hence they neutralize the virus more effectively.

Wiktor et al. produced these MAbs in 1978 (111, 112). These monoclonal antibodies displayed specific reactivity patterns, which are thus used to characterize and classify rabies, and rabies related viruses into groups corresponding to antigenic determinants. Since then other hybridomas have also been produced and different panels of monoclonal antibodies have been established to allow differentiation of rabies virus isolates from terrestrial and bat host species in USA, Western Europe and to lesser extent in Africa, Asia, Eastern Europe and Latin America (113-117). The studies coordinated between 1982 and 1990 by WHO (118-123) led to the establishment of two panels of monoclonal antibodies i.e., for the identification of various Lyssavirus serotypes and the differentiation of major field virus isolate strains used for vaccine production. An additional panel of monoclonal antibodies was also selected to differentiate rabies viruses isolated from terrestrial animal species to that of European bat species. MAbs have been widely used in identifying rabies virus strains, diagnosing rabies in humans and animals and the detection of virus strains

resistant to neutralization by murine neutralizing MAbs (124-128). Although, the monoclonal antibodies are mainly used for epidemiological investigations, they are also used for rabies diagnosis in circumstances of uncertain exposure (129, 130) and also routinely in countries where large-scale programmes for oral vaccination of foxes. This confirms no further infections from the vaccine strain to the foxes. MAbs have also been used to locate antigenic sites on the rabies virus N protein (131). Candidate vaccines have been prepared using anti-idiotypic MAbs directed against antigenic determinants in the rabies protein (132). Possible replacement of HRIG or ERIG by MAbs has been investigated in hamsters and mice treated after infection with field strains of rabies and Lyssaviruses; preliminary results appeared promising (133, 134).

CELL CULTURE AND RABIES VIRUS:

Rabies virus is highly neurotropic. But, in an infected animal, after the replication in the Central Nervous System (CNS) it spreads centrifugally to most organs of the body, where it often replicates efficiently. Therefore, it is not surprising that the rabies virus can be cultivated in a wide variety of host cells. Such cultivation is extremely important in, not only for obtaining knowledge about the virus itself but also to produce large quantities of virus for vaccine preparation (135, 136).

The first *in-vitro* cultivation of rabies virus dates back to 1936 (135).

Methods of Virus Propagation:

Many conventional methods of virus propagation can be employed for rabies virus propagation in tissue culture. Monolayer in stationary or revolving vessels and in suspension cultures are generally used. Some cells require specialized type of medium, but for most of the purposes, the propagation medium is based on Earle's salt solution supplemented with Eagle's amino acids and vitamins or with lactalbumin hydrolysate. The addition of serum appears to protect against thermal inactivation of the virus, rather than providing necessary ingredients for virus replication (142). The optimum growth of the cells can be obtained at low incubation temperature i.e., $\sim 32^{\circ}\text{C}$ (143). During the virus production the medium should be maintained at pH 7.4 – 7.6 by the addition of extra sodium bicarbonate (142). In general, no clearly definable Cytopathic Effect (CPE) accompanies the production of rabies virus in cell cultures. In case of infected BHK-21 cell monolayer, the cells merely begin to “age” and detach more quickly from the cell surface as compared to the uninfected cells (136). While, in case of murine neuroblastoma cells when infected with fixed rabies virus and rabies related viruses, the cytopathological effects were more severe than to BHK- 21 cells.

Aims

&

Objectives

AIMS AND OBJECTIVES

The Passive Immunization for rabies exposure plays a very important role in prevention of this dreaded disease. For grade III wounds, World Health Organization (W.H.O) recommends passive immunization. Unfortunately, many a times patients do not receive important passive immunization timely, for lack of hyper-immune sera, high cost of homologous HRIG, or may develop a serious adverse reaction to unpurified Equine Anti-Rabies Serum (ERS).

Hence the present work is envisaged with the following Aims and Objectives.

PART A

1. Indigenous production of Equine Anti-Rabies Serum (ERS)
2. Purification of ERIG by the following methods:
 - a. Ammonium sulphate precipitation
 - b. Caprylic acid.
 - c. Ion exchange chromatography
 - d. Affinity chromatography using protein G
 - e. Ammonium sulphate precipitation and pepsin digestion.
3. Comparative study of the yield and efficacy of purified immunoglobulins.

PART B

1. Production of anti-rabies monoclonal antibodies (MAbs) using hybridoma technology.
2. Testing the efficacy of MAbs.

Materials

&

Methods

MATERIALS AND METHODS

PART A

1. INDIGENOUS PRODUCTION OF EQUINE ANTI-RABIES

SERUM:

A. Horse:

Healthy Female horse aged 7 years and weighing 380 Kg was procured and immunized at National Research Centre on Equines (NRCE) Hissar, Haryana. The horse was tagged and numbered for the identification during the course of immunization. It was fed and watered ad libitum.

B. Vaccine:

The vaccine used for the immunization of the horse was Purified Vero Cell Rabies Vaccine (PVRV) Batch No.R0461, purchased from Pasteur Merieux India Ltd.

C. Mice:

Swiss Albino mice weighing 14-16 gm (Kasauli strain) were procured from Haffkine Biopharmaceutical Corporation Ltd., Mumbai and were fed and watered ad libitum. These were maintained with proper ventilation in the animal house.

D. Immunization Schedule:

The horse was inoculated subcutaneously with series of injections of PVRV with increasing volumes. The immunization schedule was followed as described by Luekerajang (93). On day zero, the horse was inoculated in cervical region with a suspension of 0.5 ml vaccine and 0.5 ml complete Freund's adjuvant (Sigma chemicals). On days 21st, 28th, 35th and 42nd, one ml vaccine without Freund's adjuvant was injected subcutaneously on the neck region. On day 63rd, 70th, 77th, 84th, 91st, 98th and 105th, 2.0 ml vaccine without adjuvant was injected subcutaneously on both sides of the neck. Further, on day 159, 2 ml of vaccine was injected subcutaneously and same dose was repeated weekly for subsequent three weeks.

E. Bleeding:

Before immunization on day zero, the horse was bled and 10ml blood was collected aseptically. From day 21st to day 56th approximately 10 ml of blood samples were taken at weekly intervals and the antibody titre from serum was determined by Mouse Neutralization Test (MNT) (144). Approximately, 200ml of blood samples were collected on day 119, 121 and 123. After the completion of last booster injection, the blood samples (200 ml approx.) were taken on day 194th, 196th and 198th. The sera were thus separated and pooled. Total 450 ml of antirabies serum was obtained from all six-blood samples. The aliquots of pooled serum sample were

made and stored at -20°C until further purification. Anti-rabies antibody titre was determined by the MNT for the pooled anti-rabies serum.

F. Mouse Neutralization Test (MNT) :

Principle:

The method involves neutralizing a constant dose of previously titrated challenge virus with a series of different dilutions of antirabies serum. This method is used mainly for the assay and potency testing of therapeutic anti-rabies serum and immunoglobulin, but also applicable to any serum containing rabies antibody. Thus, it can be used to determine the antibody titres of human sera collected during therapeutic trials of different vaccines.

The following method comprises three stages:

- i) Preparation and titration of the challenge virus
- ii) Serum-virus neutralization test: preparation of the serum and serum-virus mixtures, Inoculation of the indicator system (i.e., Mice)
- iii) Interpretation of the results.

i. Preparation and titration of the challenge virus:

CHALLENGE VIRUS:

The strain used for the test was Challenge Virus Standard (CVS), available and maintained at Department of Virology, Haffkine Institute, Parel, Mumbai-400 012.

TITRATION OF STOCK VIRUS:

The challenge virus was stored as a 20 % suspension or as the supernatant following low speed centrifugation of suspension. The aliquots of the 20% suspension were prepared and stored at -80°C . Shortly, before the test was carried out, an ampoule was taken out from the stock and thawed rapidly under the tap. Then serial tenfold dilution was prepared using the diluent i.e., 2 % Normal Horse Serum (2% NHS) inactivated at 56°C for 30 minutes.

The dilutions of the stock virus suspension (the tubes) were incubated for 1.0 -1.5 hours at 37°C . In order, to ensure that the conditions will be same as those of the serum virus neutralization step, the tubes were removed from the incubator and chilled in an ice-water bath. The groups of mice weighing 14-16 g were inoculated intra-cerebrally with 0.03 ml of each virus dilution and observed for 14 days. The results were recorded and the virus titre was calculated using the Reed and Muench method (145).

Based on the above results, the challenge dose (i.e., 200 -250 LD_{50}) was prepared and used for the serum-virus neutralization test. This challenge dose generally has to be prepared before performing the serum-virus neutralization test.

ii. Serum-Virus Neutralization Test:

INACTIVATION OF SERA:

The sera to be tested were inactivated for 30 minutes at 56 °C.

NEUTRALIZATION:

Serial twofold dilutions of the serum were prepared and 0.5 ml of each of these dilutions was transferred to each test tube. 0.5 ml of the CVS virus dilution corresponding to 200-250 LD₅₀ was added to each tube. The tubes were incubated at 37 °C for 1-1.5 hrs. The test was carried out against the reference serum. After incubation, the Swiss Albino mice weighing 14-16 gm. were inoculated with 0.03 ml of each dilution and were observed for 14 days.

iii. Interpretation of the results or Calculation of potency of the serum under test:

The ED₅₀ of the serum under test and reference serum (procured from the Central Research Institute Kasauli) was calculated from the above results. The potency of the test serum is expressed in International Units (I.U.), calculated by the difference between the logarithms of 50% end point dilutions of the two sera (serum under test and reference serum). This difference was then multiplied with the I.U of the reference serum in order

to calculate the potency of the test serum.

2. PURIFICATION OF ERIG BY FOLLOWING METHODS:

A. Ammonium Sulphate Precipitation:

The purification of the antirabies serum or horse immunoglobulins (IgG) was carried out according to the method described by Philips (146). Forty percent saturated solution of ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$, Qualigens-make) was prepared for the precipitation of immunoglobulins. Twenty ml. of anti-rabies serum was mixed with twenty ml. of saturated solution of $(\text{NH}_4)_2\text{SO}_4$ by stirring at 4^0 - 8^0 C for 30 minutes. After this it was centrifuged at 5000g for 30 min. The pellet obtained was then dissolved in 40 ml of distilled water and the resulting solution containing immunoglobulins was then subjected to dialysis against PBS pH 7.2 (Hi-Media Laboratories).

B. Purification by Caprylic Acid:

The anti-rabies serum obtained was purified by McKinney and Parkinson's method (147). Anti-rabies serum measuring 20 ml, was diluted with 80 ml of acetate buffer (pH 4.0). The suspension was then adjusted to pH 4.5. To the suspension, 2500 μl of Caprylic acid (MERCK) was added slowly and stirred for 30 minutes at 20^0C . This was later centrifuged at 10,000 g for 30

minutes and the supernatant was separated. The pH of the supernatant was adjusted to 7.4 and cooled to 4°C. This was then fractionated with ammonium sulphate by stirring for 30 minutes and subjecting to centrifugation at 5000g (REMI centrifuge) for 15 minutes. The precipitate obtained was then dialysed against PBS pH 7.2 to remove any traces of $(\text{NH}_4)_2\text{SO}_4$ left out .

C. Purification by Ion-Exchange Chromatography:

i. Batch Method:

Purification of the anti-rabies serum or immunoglobulins was performed batchwise as described by Avest et al. (148). The DEAE (Diethylaminoethyl – Bio-Rad Macroprep Ion Exchange supports) resin was washed separately twice with 0.5 M HCl, 0.5 M NaOH and Phosphate buffer pH 6.0. Five ml. of anti-rabies serum was mixed gently with five ml. of DEAE. The mixture was then stirred for 1 hour at 20 ° C and was later centrifuged at 4500g for 25 minutes. The supernatant separated was subjected to ammonium sulphate precipitation, as described above.

ii. Purification using Column:

Purification of the antibodies was done using column chromatography (columns- procured from Bio-Rad) as described by Andrew (149). The DEAE cellulose resin was swollen in 0.01 M Tris, pH 8.6. It was then allowed to stand for 10 minutes and the fine particles were decanted.

This process was repeated till most of the fine particles removed. The resin was gently poured into a sterile polypropylene column of 1.5X12 cm dimension. A bed of DEAE resin, 5 ml in volume was prepared. This bed was then washed twice and equilibrated with Tris Cl, pH 8.6. The baseline O.D at 280 nm was measured to check for any contaminant. Then 2.0 ml of the antiserum was loaded with the help of a syringe and allowed to run. The column was then washed with 0.01 Tris Cl, pH8.6 and fractions were collected successfully. The fractions were read spectrophotometrically at 280 nm. Fractions were collected till the baseline O.D. was reached. The column was eluted using a 100 ml. linear gradient of 0.01 M Tris Cl, pH 8.6 without NaCl and 0.5 M NaCl in Tris Cl, pH 8.6. The fractions were collected and read at 280 nm. Positive fractions were pooled and were subjected to ammonium sulphate fractionation.

D. Purification by Affinity Chromatography using G protein:

Purification of immunoglobulins was performed using Lindmark's method and Huet's method (150, 151). The anti-rabies serum 1.25 ml, was mixed with 3.75 ml of sodium phosphate buffer (pH 7.0-8.0). This diluted sample was passed through Protein G (Protein G Sepharose 4 fast flow- Pharmacia Biotech) column. The unbound material was washed from the column using sodium phosphate buffer pH 8.1, until the emission at 280 reached the baseline level. The bound immunoglobulins were eluted with 0.1 M

glycine buffer pH 2.7. The fractions containing antibodies were pooled and subjected for ammonium sulphate precipitation.

E. Purification by ammonium sulphate and pepsin digestion

(WHO method):

The anti-rabies serum was purified and the pepsin digestion of the immunoglobulins was performed according to method described by Gluck (152).

i. Elimination of albumin and pigments:

Twenty ml of anti-rabies serum was diluted with 20 ml the distilled water. To this solution, 9.24 gm of pure crystalline ammonium sulphate was added by slow stirring to give a final concentration of 1.75 moles per litre. The solution was then adjusted to neutral pH. After 6 hours, the precipitate was collected by centrifugation at 3000-4000 g for 10 minutes. The precipitate was then resuspended in 20 ml distilled water.

ii. Pepsin Digestion:

The above protein suspension was adjusted to pH 3.2 with 1 N Hydrochloric acid and to this 0.015 gm of pepsin (Hi-media) was added. The digestion was carried out on a magnetic stirrer at 32°C for 30 minutes.

iii. Selective Denaturation by heat:

After pepsin digestion, the solution was adjusted to pH 4.3 with dilute ammonia solution. Then 1.98 gm. of $(\text{NH}_4)_2\text{SO}_4$ was added to the above suspension to achieve a concentration of 0.75 Mol/l. To this mixture, 3 μl of toluene (Qualigens-Glaxo chemicals) and 0.1 g of charcoal (Qualigens-Glaxo chemicals) were added respectively. The temperature of the mixture was raised to 55°C in a water bath, followed by cooling and filtering. The filtrate was then diluted with distilled water to give 0.3 Mol/l of ammonium sulphate. It was then adjusted to neutral pH.

iv. Concentration of protective fragments:

After the pH adjustment, the concentration of $(\text{NH}_4)_2\text{SO}_4$ was increased to 1.75 Mol/l. The suspension was left for 10-12 hrs and then centrifuged at 5000g for 10 minutes to obtain the fractionated immunoglobulins precipitate.

v. Dialysis:

The concentrated solution of fractionated immunoglobulins was dialysed against PBS (pH 7.2) using dialysis bags (Hi Media) with a 10,000 Dalton cut-off.



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3. COMPARATIVE STUDY OF THE YIELD AND EFFICACY OF PURIFIED IMMUNOGLOBULINS:

All the purified samples were subjected to the following test procedures.

A. Protein estimation by Bradford test:

Protein concentration of the serum samples was measured according to the Bradford's method (153).

i. Reagents:

Coomassie Brilliant blue G- 250 (Hi-Media), 2–mercaptoethanol (Sigma), Triton X-100 (Hi-Media), Ethanol and Phosphoric acid (Qualigens-Glaxo Chemicals).

ii. Preparation of protein reagent:

Coomassie brilliant blue G-250 (100 mg) was dissolved in 50 ml of 95 % ethanol. To this solution, 100 ml of 85 % (W/V) phosphoric acid was added, and the mixture was diluted to a final volume of 1 litre.

iii. Preparation of Protein standards:

For this test, the Standard solution of Bovine Serum Albumin (BSA) was prepared. The various concentrations (100 µg/ml – 1000µg /ml) were prepared by diluting the BSA stock solution. From these diluted stocks, 0.1

ml of the protein suspension was pipetted out in to different tubes labelled respectively. Five-ml. protein reagent was added to each test tube and vortexed. After 2 minutes the absorbance was read at 595 nm in a 3ml cuvette. This was measured with reference to the blank (prepared with 0.1 ml of the 0.15 M sodium chloride and 5 ml of protein reagent). The protein concentrations were plotted against the corresponding absorbance (O.D). This standard curve was then used to determine the protein content in serum samples.

B. IgG detection using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

Immunoglobulins were detected using SDS-PAGE as described by Laemmli (154).

SDS-PAGE: Principle and Methodology

Sodium Dodecyl Sulphate-Polyacrylamide Gel electrophoresis involves the use of SDS. SDS, being a strong anionic detergent is used in combination with β -mercaptoethanol (reducing agent) to dissociate the proteins. The latter breaks disulphide linkages resulting in primary linear structure of the protein, whereas SDS imparts a net negative charge. Migration of the polypeptides is independent of shape and charge but separation is indeed based on the molecular weight / size. Thus, by using markers of known molecular weights it is possible to estimate molecular weight of

polypeptides or proteins. SDS-PAGE was performed using vertical slab gel apparatus. Here, resolving/separating gel (12 % acrylamide) and stacking gel (6% acrylamide) were used. The separating gel is of 14 cm long and 0.75mm thickness. The antibodies were stained using Coomassie Brilliant Blue (CBB-R 250).

All the reagents and buffers used for SDS- PAGE are described in Appendix.

C. Potency testing:

Potency testing of all the serum samples was carried out by MNT described earlier (144).

D. Sterility Testing:

Sterility testing of the purified serum samples was carried out as per the Indian Pharmacopoeia (155). The media used for sterility testing i.e., Fluid Thioglycolate Medium (FTG) and Soyabean Casein Digest (SCD) medium were procured in dehydrated form from Hi-Media Laboratories Mumbai. One-tenth ml. (0.1 ml.) sample was inoculated in to the tubes containing the sterility medium and kept at 30⁰C - 35⁰C for 7 - 14 days and 20⁰C - 25⁰C for 7 - 14 days respectively. At the same time, suitable positive and negative controls were incubated or maintained along with the test samples.

PART B

1. PRODUCTION OF ANTI-RABIES MONOCLONAL ANTIBODIES USING HYBRIDOMA TECHNOLOGY.

A. Cell culture:

i. Preparation of Media and buffers:

Media and buffers used in this study were prepared as per the protocols and references quoted. Few media and reagents were procured in ready to use form.

ii. Growth and maintenance:

BHK 21 clone 13 cells susceptible to rabies virus were obtained from National Centre for Cell Sciences (NCCS, Pune). These were grown and maintained in Roux-flasks (1 L capacity) in the presence of 80-100 ml Modified Eagles base Medium (MEM-Hi-Media) and 10 % Fetal calf serum (FCS- Sigma chemicals). The cells are fibroblastic having rapid growth rate, and run parallel to each other (140).

iii. Sub-cultivation:

The cells were routinely sub-cultured as described below. The roux flasks were drained off their growth medium. And the cell monolayer was washed couple of times with sterile phosphate buffered saline (PBS). One ml of

0.25% Trypsin Versene EDTA Glucose Phosphate (TPVG) was uniformly added on to the glass surface of the roux flasks. The Excess TPVG was drained out and the bottles were gently rotated for even spreading of the TPVG. Once the cells had started to detach from the glass surface, 5 ml fresh Eagles base medium with 10 % FCS was added to the bottles. The cell clumps were separated by repeated pipetting using sterile glass pipette and then collected in sterile 20 ml Laxbro culture vials and washed three times by spinning down and resuspending in 10 ml Eagles medium containing 10% FCS. Fresh culture flasks were seeded with these cells at a count approximately 10^6 cells/ml. The flasks were incubated at 37 °C until the confluent cell monolayer was formed.

B. Preparation of crude rabies Antigen:

i. Propagation of rabies virus in cell culture:

The rabies virus (RV-CVS) was adapted and propagated on BHK 21 cells (156). The cells were infected with rabies virus in Roux bottles having capacity of 1 litre and incubated for 1 hour at 37°C. The supernatant was discarded and the monolayer was rinsed with five ml of virus medium (MEM without FCS). The virus medium again was replaced with a fresh medium. The Roux bottles were sealed and incubated at 32°C for 60-72 hours in a humidified CO₂ incubator (3-5% Carbon dioxide). Cultures from the 33rd passage were pooled to give a total volume of 800 ml. These

incubator. One week before fusion, the cells were transferred to DMEM containing 10% horse serum to improve the fusion efficiency. The cells were maintained in stationary suspension culture at concentration of 1×10^6 cells /ml and 5×10^6 cells/ml for fusion respectively. After 3-4 days of complete immunization, splenocytes from the rabies-immunized mouse were fused with SP2/0 mouse myeloma cells using PEG 1500 (Polyethylene Glycol).

iii. Rabies antigen:

Rabies virus (RV-CVS) was propagated in BHK 21 cell culture monolayer. The crude antigen was inactivated by β - propiolactone. The method of immunization and production of monoclonal antibodies was followed as described by Lafon (157).

iv. Immunization of Animals:

A group of six mice were primed with three injections of rabies antigen at a weekly interval. For the first injection 0.2 ml of antigen was mixed with 0.2 ml of complete Freund's adjuvant (Sigma Chemicals) and injected subcutaneously. A booster injection was given intravenously 3-4 weeks later without adjuvant.

v. Fusion:

A mouse was killed by dislocating the neck and then the body was

immersed in 70% ethanol. The abdominal skin was opened and the exposed tissue was washed with 70% ethanol. Peritoneal cavity was also then opened. The spleen was removed and transferred into a petri dish containing 10ml PBS and rough surface sterile slides. Pressing it through rough surface slide disrupted the spleen. The cell suspension was then transferred to a 15ml centrifuge tube. The spleen cells were centrifuged at 200g for 10 minutes and then washed twice with PBS. Red blood cells were removed by suspending the pellet in 5 ml NH_4Cl (Haemolysing Solution) and kept in a standing position at room temperature for 5 minutes. Seven ml of FCS was added and centrifuged at 200g for 10 minutes. This was again washed twice with DMEM without serum. The above suspension was finally suspended in 10 ml DMEM without serum.

In the mean time the myeloma cells grown in DMEM-10 HS (DMEM with 10% Horse Serum) were centrifuged at 200g for 10 minutes and washed twice and resuspended with DMEM without serum. The myeloma cells and spleen cells were mixed together in ratios of 1:1, 1:4 and 1:10. The myeloma cells and spleen cells were centrifuged together at 200g for 5 minutes in 45 ml conical centrifuge tubes. The supernatant was discarded and the tube was taped gently to spread the pellet. One ml of PEG 1500 was added over a period of 1 minute and mixed gently by tapping the tube. The tube was then incubated at 37°C for 2 minutes with 1 ml DMEM without serum overlaying the tube. Two ml of DMEM without serum was

then mixed gently. Another 4 ml of DMEM-20 FCS was mixed to the above solution with the help of a pipette. In the same tube of suspension, again 20 ml of DMEM -10 FCS added and centrifuged at 200g for 5 minutes. The supernatant was discarded and the pellet was resuspended in 100 ml of DMEM-10 FCS and from this 100 μ l of the cell suspension was distributed into each well of the microtitration plate. The plates were incubated for 24 hours at 37 ° C in a humid 5 % carbon dioxide incubator. To each well 100 μ l of DMEM -10 FCS - HAT (2X) concentrate was added. In a week, 100 μ l of supernatant was replaced with 100 μ l of fresh DMEM- 10 FCS - HAT. This was repeated for a span of 2 weeks, after which the culture supernatant was tested or screened for the presence of MAbs.

vi. Screening of Hybridoma Supernatants:

Enzyme Immunoassay:

Microtitration plates precoated with rabies glycoprotein G (Platelia) were used for detection of MAbs, as described by Lafon (157).

At first, the plates were washed with 0.05 mol/l PBS polysorbate 20. To this 300 μ l of the blocking medium (polysorbate 20 supplemented with 10 % normal serum of the species in which anti-mouse IgG was prepared) was added to each well and incubated at 37°C in a 5% CO₂ humid incubator. On aspiration of the plates, 100 μ l supernatant of hybridoma colonies were

precipitation of immunoglobulins. Equal quantity clarified ascitic fluid and saturated solution of $(\text{NH}_4)_2\text{SO}_4$ was mixed by stirring at $4^\circ - 8^\circ \text{C}$ for 30 minutes. After this the precipitate was centrifuged at 5000g for 30 min. The pellet was dissolved in sufficient quantity of distilled water and the resulting solution containing immunoglobulins was then subjected to dialysis against PBS pH 7.2.

ix. Estimation of proteins:

Purified sample containing MAbs were subjected to Bradford test (described earlier) for estimation of proteins.

2. TESTING THE EFFICACY OF MAbs:

A group of six Swiss albino mice (4-6 week old) were injected intramuscularly with 0.1 ml of diluted MAbs. After 24 hrs of MAb treatment the mice were inoculated intramuscularly with 10^6LD_{50} challenge virus standard (CVS). The animals were observed for 3 weeks and the deaths were reported daily. The effective dose (ED_{50}) was calculated (158).

Results

RESULTS

PART A

Estimation of Proteins:

The protein content in the serum was estimated by using the standard curve. This was obtained, using the protein standards (Bovine serum albumin-BSA) such as 100 µg/ml, 200 µg/ml, and 300 µg/ml, upto 1000µg/ml. The optical density of the protein standard was plotted against the concentrations mentioned in the graph. There is a slight non-linearity in the response pattern and also scattering of points around the line drawn in the graph. The protein content of the serum samples was calculated by using the line of equation (refer graph).

Immunization of Horse:

During the course of immunization, no adverse reactions such as anaphylactic or neuromuscular were observed in immunized horse. The fact that the horse remained healthy suggests that the PVR vaccine is safe for immunization purpose. Also the rabies antibody titre was found to be zero in the horse before immunization, this indicates that the horse was not previously immunized with any rabies vaccine. The horse immunized as per the schedule described by Luekerajang (As per WHO) was bled on day 56 and the anti-rabies antibody titre in the horse serum was found to be

more than 70 ml i.e. 78 IU/ml. This also makes the horse suitable for further immunization. After the completion of immunization schedule, the anti-rabies antibody titre and protein concentration in pooled anti-rabies serum was determined and found to be 261 IU/ml and 91.6 mg/ml respectively. This anti-rabies antibody titre is considered as the initial potency of the serum and the protein content in it is considered as an initial protein.

Purification of ERIG by the following methods:

The comparative study of different ERIG purifying methods is illustrated in the table.

A. Ammonium Sulphate Precipitation:

The antirabies serum was purified by the ammonium sulphate precipitation method. By using this method the final protein concentration and final potency obtained were 56 mg/ml and 255 IU/ml respectively. This method resulted in 97% recovery, with a specific activity ratio of 1.59.

B. Purification by Caprylic Acid:

The anti-rabies serum was purified using the McKianey method. This method mainly consists of two steps. The final protein concentration and potency of the serum were obtained as 40 mg/ml and 207 IU/ml respectively. Purification by Caprylic acid resulted in a recovery of 79% with specific activity ratio 1.76.

C. Purification by Ion-Exchange Chromatography:

i. Batch Method:

The DEAE ion exchange batch wise purification followed by ammonium sulphate precipitation of anti-rabies serum resulted in final protein and potency, 31 mg/ml and 180 IU/ml respectively. The recovery and specific activity ratio obtained by this method was 68% and 2.02 respectively.

ii. Purification by using Column:

In order to get better resolution, the anti-rabies serum was purified by the DEAE column chromatography followed by ammonium sulphate precipitation. Using this method the final protein and the final potency of the purified serum or immunoglobulins obtained was 27.8 mg/ml and 189 IU/ml. The recovery and specific activity ratio was 72 % and 2.37 respectively.

D. Purification by Affinity Chromatography using G protein:

The affinity chromatography purification of anti-rabies serum with protein G Sepharose 4 fast flow gels followed by ammonium sulphate resulted in final protein and final potency, 20mg/ml and 207 IU/ml respectively. The recovery and specific activity ratio obtained by this method was 79% and 3.64 respectively.

E. Purification by Ammonium Sulphate and Pepsin Digestion

(WHO) method:

This method for purification of anti-rabies serum consists of many steps.

The purified serum (before pepsin digestion) showed final protein and final potency as 51 mg/ml and 231 IU/ml. respectively. Recovery and the specific activity ratio of purified serum was 88% and 1.58 respectively.

- **Comparison of Batch purified and pepsin digested sample with purified sample obtained by using WHO method:**

The pepsin-digested sample by WHO method resulted in the potency of 127 IU/ml with the recovery of 49 % and the specific activity ratio of 3.12, while the DEAE Batch purified and pepsin digested method resulted in the potency of 157IU/ml with the recovery of 60% and the specific activity ratio 3.57.

- **IgG Detection by SDS–PAGE:**

The serum or the solution containing antibodies was subjected to SDS-PAGE detection as shown in the figure. Approximately 10 µl samples were loaded on the gel, as follows: (1) Protein Standards (Molecular Weight Markers) (2) Pasteur antirabies serum (pepsin digested ERIG) (3) Test sample (4) Unpurified antirabies serum.

The results revealed complete digestion of IgG, giving F (ab')₂. Hence, it suggests that 30 minutes incubation is sufficient for the complete digestion of the immunoglobulins.

- **Sterility Testing:**

The sterility testing of each purified serum was carried out in duplicate as per IP (Indian Pharmacopoeia, 1996).

All the serum-inoculated media were found to be free from fungal and bacterial contaminants, following 14 days incubation.

PART B

Propagation of Rabies Virus in Cell Culture:

The BHK-21 cells were found extremely susceptible to the rabies virus. Culture from the 33rd passage inoculated was 0.03 ml in 14-16 g mice. After 14 days of inoculation, the virus titre was calculated by Reed and Muench method, and found to be $10^{-6.16}$.

Inactivation of Rabies Virus:

The rabies virus was inactivated by β -propiolactone, described in earlier methods. Inactivation of the virus was confirmed by intracerebral inoculation of groups of 6 mice. After 14 days of observation, mice showed no symptoms of rabies and the fact that they survived indicated complete inactivation of the virus.

Production of MAbs:

Using the technique described by Lafon, total four fusions were obtained. Of which, three hybridomas were established and maintained in our laboratory. The monoclonal antibodies were produced by injection of hybridoma into the peritoneal cavity of syngenic mice. After 12-15 days, the mice were tapped and ascitus containing the antibodies was collected and purified by ammonium sulphate precipitation. The purified MAbs were

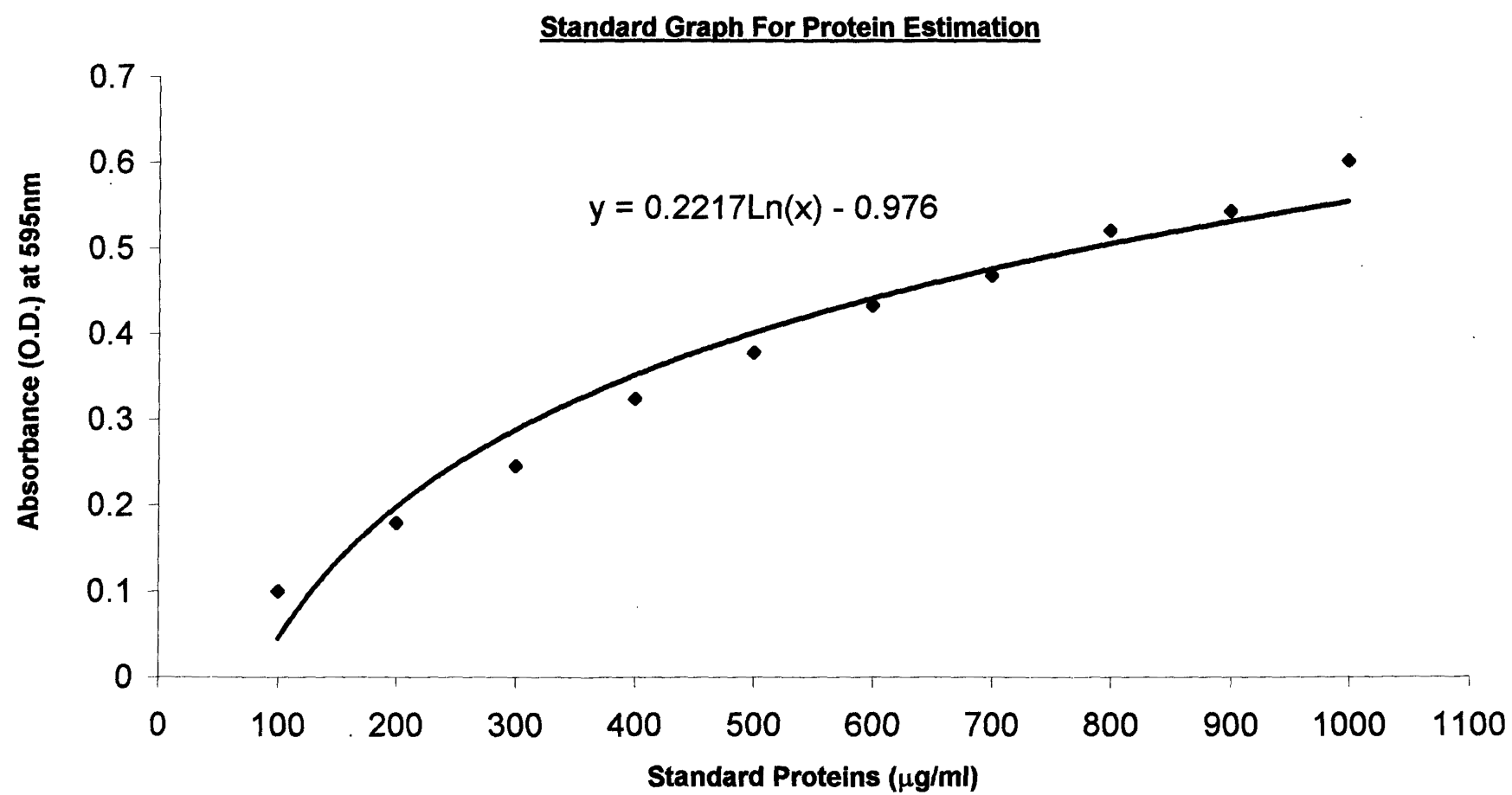


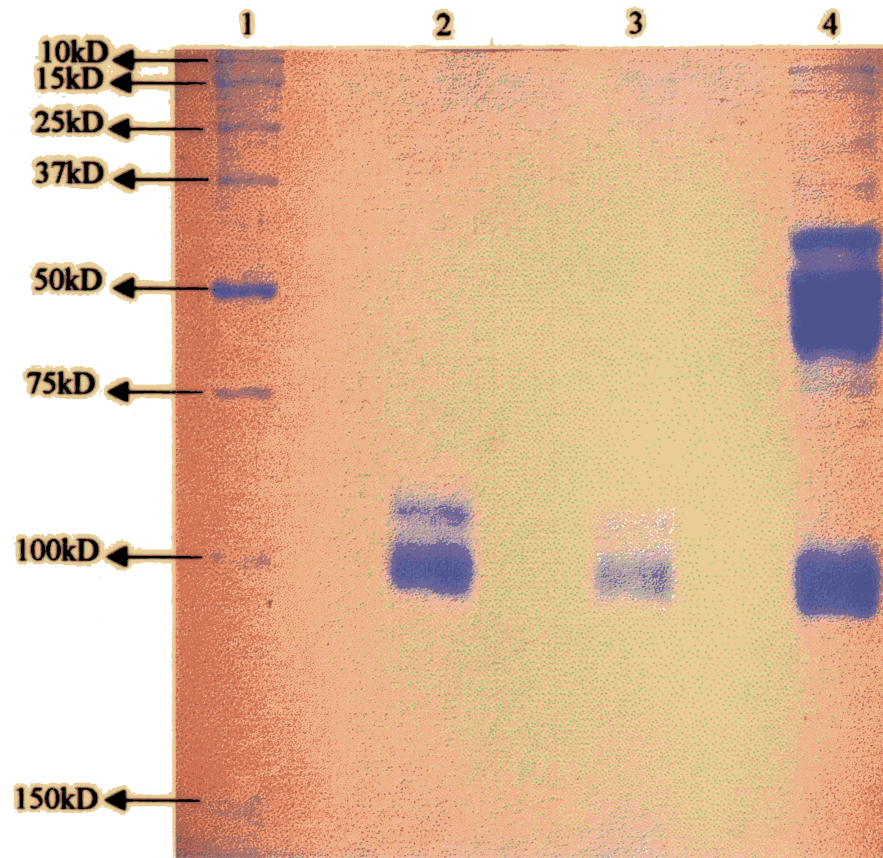
Table: Comparative study of different ERIG purifying methods

Purification method	DEAE Column	Protein G	DEAE Batch	WHO	C.A	A.S.
Initial Protein (mg/ml)	91.6	91.6	91.6	91.6	91.6	91.6
Final Protein (mg/ml)	27.8	20.0	31.0	51.0	40.0	56.0
Initial potency (IU/ml)	261	261	261	261	261	261
Final potency (IU/ml)	189	207	180	231	207	255
Percentage recovery Activity	72	79	68	88	79	97
Specific Activity Ratio	2.37	3.64	2.02	1.58	1.76	1.59

A.S: Ammonium Sulphate Precipitation

C.A.: Purification by Caprylic Acid.

Figure: SDS-PAGE



Legend:

1. Protein Standards (Molecular Weight Markers)
2. Pasteur antirabies serum (pepsin digested ERIG)
3. Test sample
4. Unpurified antirabies serum

Discussion

DISCUSSION

Rabies, still a major public health problem remains the cause of thousands of human deaths in several developing countries. The disease can be prevented by pre-exposure and post-exposure treatments. World Health Organization recommending post exposure treatment includes either prompt use of vaccines or the combined treatment of vaccines and rabies immunoglobulins in the severe cases. The implementation of this prophylaxis of vaccines and immunoglobulins is hampered by their high costs, so the need for the development of safe and cheap vaccines and immunoglobulins arise. The Human Rabies Immunoglobulin (HRIG) is safe to use but costly. While, the Equine Rabies Immune Globulin is cheaper as compared to HRIG. But, the crude antirabies serum from equine origin causes some adverse reaction, such as anaphylaxis or serum sickness in patients. These adverse reactions can be minimized to a larger extent, by purification and fragmentation of anti-rabies antibodies.

The production of antirabies serum or ERIG depends on the type of animals and the vaccine used. The antirabies serum can also be produced in laboratory animals and livestock. Since, substantial amount of serum can be obtained from equines as compared to other animals, the present study was carried out by immunization of the horse. Different immunization schedules have been tried for the production of anti-rabies serum (ARS)

such as, 1. Pasteur Institute method comprising (159) Pasteur strain of fixed rabies virus inactivated with β -propiolactone. Here, the vaccine was used in large quantity i.e., 20 ml/injection. 2. Fuenzalida and Palacios method (160) (or “accelerated method”) comprising usage of fixed rabies virus and the vaccine was developed in brain tissue. 3. In USSR method, the antirabies serum was prepared by immunizing the horse with 10 % sheep brain suspension containing fixed rabies virus (161). These methods of immunization using vaccine prepared in nerve tissues, showed anaphylactic and neuromparalytic reactions in vaccinated horses. In the present study using tissue culture vaccine, such reactions did not occur in the immunized horse. Hence, PVR (Purified Vero cell Rabies) vaccine is safe for immunization and also substantial amount of antibody titers were found in the serum.

After the bleeding of the horse, the serum was separated and stored at -20°C for future use. This storage condition was found to be suitable than conventional method of adding antiseptics in the serum or plasma. Thus the interference and separation of antiseptics during serum purification is avoided. Various methods are available for the determination of the antirabies antibodies. WHO has recommended the most popular tests, such as Mouse neutralization test (MNT) and Rapid Fluorescent Focus Inhibition Test (RFFIT). In the present study, the antibody titre was determined by MNT test and found to be suitable and convenient. At

present, commercially available kits, such as Platelia Rabies Kit, also determine anti-rabies antibody titers, but this is applicable only for human and few animals' antibodies determination. Equine antirabies antibodies cannot be determined as the antibodies do not bind or poorly bind to protein A.

In protein purification studies, it often requires rapid and sensitive methods for protein quantitation. Mainly three methods are used to quantify proteins viz., Bradford test, Folin-Lowry method and Biuret method. The disadvantage of standard Lowry method and Biuret method is that the result is affected by the use of certain interfering compounds like Potassium, Magnesium ions, EDTA, Thiol reagents, Carbohydrates, Tris, Ammonia and Glycerol respectively (162-163). In the present study, the Bradford Test was used to estimate serum proteins. The binding of Coomassie Brilliant blue G 250 and protein causes a shift in the absorption maxima from 465 to 595 nm, but the final reading is taken at 595 nm. This method of estimation is found to be very reproducible and rapid since the dye binding process is completed in 2-3 minutes and also the colour is stable for an hour.

Monoclonal and Polyclonal antibodies have many applications as stated earlier. Monoclonal antibodies may be used either as ascitus fluid or as tissue culture supernatant whereas polyclonal antibodies are used as antiserum. Purified antibodies are used, however, when accurate

concentrations are required, or labeling with fluorescent and radioactive probes for binding, or structural modifications involving removal of the Fc portion to produce the Bivalent F (ab')₂ or monovalent Fab. There are different methods available for purification of immunoglobulins but the choice depends on intended use of antibodies and also its available resources.

The present study was aimed to prepare and purify antibodies so as to reduce the risk of adverse reactions. Different methods have been tried for the purification of antirabies serum or ERIG however, there is not much or very less data available on commercial methods of purifying equine antirabies antibodies.

The conventional method of purifying antibodies by salt precipitation is well known, and also an established technique. The Mammalian sera are usually precipitated by ammonium sulphate method whereas, Fowl sera is precipitated using sodium sulphate method (164). Temperature is not a critical factor for salt precipitation of proteins as compared to that of ethanol. In the latter method, the proteins are precipitated at 0 to -4°C (99). Routinely, ammonium sulphate precipitation is carried out at 4°C to avoid possible chances of bacterial contamination. The advantage of this method is that it is simple, removes unwanted proteins maximally and also gives high recovery percentage in a single step. In spite of yielding a low purity product it is still used as a primary or supportive method for the

purification of proteins.

The plasma proteins could also be precipitated by short chain fatty acids (165). This method has wide applicability and can be used for the fractionation of rabbit, sheep, goat, horse, rat and mouse sera and also to purify monoclonal antibodies from mouse ascitus fluid. The present study has described a simple, non-chromatographic procedure for the purification of immunoglobulins Viz., combinational use of Caprylic acid and ammonium sulphate precipitation step. Moreover, immunoglobulins recovered were in high yield i.e., 79% with a better purity as compared to independent ammonium sulphate salt precipitation. Similar results were obtained by McKinney (1987) while purifying monoclonal antibodies using Caprylic acid. The disadvantages of this method are:

- 1. Large volume of supernatant has to be handled during the purification process.**
- 2. Caprylic acid is toxic, so it has to be removed completely from the final purified ERIG product.**

Chromatographic techniques for protein purification of immunoglobulins have contributed greatly to advances made in immunology. Among these, Ion-Exchange Chromatography and Affinity Chromatography have wide applications in protein purification. In the former, the immunoglobulins are purified by selective adsorption and desorption of proteins to an

insolubilized matrix by charge interaction. The substituted groups such as diethylaminoethyl (DEAE) and carboxymethyl (CM) cellulose determine the functional characteristics of the adsorbent. For IgG separation, the weak anion exchangers bearing the basic DEAE functional groups are almost universally used. In this method, one can choose whether to bind the substances of interest and allow the contaminants to pass through the column, or to bind the contaminants and allow the substance of interest to pass through. Generally, the first method is more useful since it allows a greater degree of fractionation and concentration of the substance of interest. There is absolutely no difference in separation procedures between column developed by stepwise elution and batch procedure. The horse serum proteins fractionated by column chromatography using anion exchange cellulose adsorbents DEAE-polycel yielded proper resolution with promising results (166). Later, James attempted horse serum purification by batch method and found it convenient and reliable (167).

In the present study, the antibodies were separated using DEAE resins and serum purification was carried out with both batch and column chromatography methods. The results suggest that, the DEAE column chromatography gives better resolution and purity than DEAE batch method. The advantage and disadvantages pertaining to both methods are compared. Even though the batch method is less efficient than column chromatographic technique, it has advantages in particular cases. When

very large sample volumes with low protein concentrations have to be processed, the sample application time on a column can be very long and filtration of such large sample can also be rather difficult to perform and so batch method is preferred in such instances. Batch separation method is very rapid and no technical difficulties like swelling or shrinkage of ion-exchangers. Also, it is used for concentrating dilute solutions and separating substances of interest from gross contaminants during initial purification stages. The other advantages of batch method include, quicker sample binding and no need to remove particulate matter. But, in case of column chromatography the serum sample should be free from any particulate matter or other precipitates. Even though, column chromatography gives better resolutions, there are chances of considerable inactivation of antibodies during second purification step.

Affinity purification isolates either antibody of interest from antigen used for immunization or antigen of interest from antibody. The molecules used as ligands are linked by chemical reactions to an activated chromatography matrix. Protein A and Protein G have strong affinity for the Fc part of immunoglobulins from certain species. Protein A is derived from the strain of *Staphylococcus aureus*. It consists of six regions, of which five bind to IgG. Being affinity ligand, protein A is immobilized to the matrix so that the remaining five regions are free to bind. One molecule of immobilized protein A can bind at least two molecules of IgG. Whereas, Protein G is a

surface protein from group G streptococci which is type III receptor, but binds with non immune mechanism similar to that of protein A. Studies reveal stronger binding of Protein G to polyclonal IgG derived from cow, sheep and horse (168). Also Polyclonal rat IgG, human IgG3 and mouse IgG1 are bound by protein G but not by protein A. Protein G's greater affinity and also minimal albumin binding results in cleaner preparation and greater yield.

In the present study, the antibodies were purified by Protein G affinity chromatography. In addition to high and pure product yield, it is also most reliable and gives best antibody purification results as compared to all other results. But due to the high cost, this method cannot be scaled up for the purification of large amount of anti-rabies serum. In this study, the antirabies serum was also purified by WHO method, which is based on the conventional methods. Parfentjev studied the use of ammonium sulphate, refining antitoxins in immune sera by use of enzymes in the year 1936. (Parfentjev I U.S patents nos. 2065196 and 2123198). In 1938, Pope developed a procedure for diphtheria and other antitoxin solutions. The results of these methods are still now helpful in fragmentation of antibodies. In the present study, the antibodies were completely digested using pepsin at pH 3.3 and temperature 32⁰ C. After the enzyme digestion of antibodies and partial denaturation of proteins, the results were relatively good. Also the amount of recovery and specific activity ratio was high.

For analytical purposes, SDS–PAGE has exceptionally high-resolution power. The electrophoretic mobility is inversely proportional to the molecular weight of the protein (169). The sodium dodecyl sulphate moieties bind to protein and denature so that the molecule assumes an extended configuration. The SDS also masks the net native charge on the molecule. In the present study, the fragmented antibodies were detected using SDS PAGE, which was simple and gave high resolution. The fragmented antibodies F(ab')₂ were compared with the standard molecular weight markers and Pasteur antirabies serum (or ERIG). The results showed similarity when compared to each other.

In the present study, the DEAE purified and enzyme digested antibodies were compared to the purified antibodies obtained from WHO method. The recovery of fragmented antibodies F(ab')₂ were comparatively greater than WHO method. This could be due to the involvement of more purification steps in WHO method. For large-scale serum purification, preferably DEAE batch, WHO method and Caprylic acid have to be studied in detail. Because protein G even though yields highest purity is highly costly.

Monoclonal Antibodies (MAbs):

Rabies virus–specific antibodies represent important factors in protective immunity against rabies. Also, rabies virus neutralizing antibodies have been considered as a primary basis of effective protection against death in

animals and humans from street rabies virus infection (77, 170). Therefore, anti-rabies serum as an adjunct to vaccination is an essential component in the prophylaxis of rabies after exposure. But, because of the potential risks associated with the use of immune sera derived from animals and also the high cost of HRIG, the bioefficacy of the MAbs was determined against the lethal rabies virus infection.

In the present study, monoclonal antibodies (MAbs) were produced using rabies antigen. Even though, the rabies virus is neurotropic it grows on non-neural cells or tissues (143). In the present study, after the propagation of virus upto 33 passages the rabies virus was found to be virulent and well adapted. The cell line susceptible to this virus was BHK 21. During denaturation of rabies virus or vaccine preparation inactivating agents such as phenol and β -propiolactone are generally used. Keeping in mind phenol's adverse effects, β -propiolactone was used in this study. The result revealed complete inactivation of the virus and so was safe for use. Also, β -propiolactone is routinely used for virus inactivation produced in brain tissue or other cell culture methods.

In case of hybridoma technique, the fusion of Sp2/0-Ag14 myeloma cells with splenocytes obtained from mice immunized with inactivated rabies antigen resulted in production of anti-rabies antibody producing cells. Large amounts of monoclonal antibodies can be produced by methods involving 1. Production of ascitus fluid obtained by injecting hybridoma

into peritoneal cavity of syngenic mice 2. Production of large volumes of hybridoma supernatants of cell cultures 3. Dialysis tubing 4. Continuous perfusion culture systems (171). In the present study, the monoclonal antibodies were produced by production of ascitus fluid. The advantage of this method is that, the protein content is high as compared to other methods. But, non-relevant antibodies present in the peritoneal fluid are the only disadvantage, as they interfere in purification procedure. This method of producing MAb's in large scale has got some limitations due to ethical problems and so, other techniques are under-development.

The mouse MAbs used in post exposure treatment of rabies in mice and hamsters has proved to be effective. In humans, the mouse MAbs although being xenogenic bear low risk of severe allergic reaction (172). To obtain identical or similar activities as that of HRIG, several MAbs are to be selected to meet the criteria: a) The MAbs should be of IgG isotype, since IgM antibodies are not effective *in vivo* (173) b) Majority of MAbs should recognize G protein and also neutralize a broad spectrum of rabies and rabies-related virus strains. These G specific antibodies in turn must differ in their epitope specificities to prevent the escape of neutralization-resistant variants c) Some MAbs should recognize the internal RNP protein of rabies virus. This cocktail of MAbs, consisting of rabies G protein and RNP-specific mouse MAbs is highly effective in post exposure treatment of rabies in infected mice and hamsters. Such a cocktail has several

advantages over hyperimmune sera: a) specific virus neutralizing activity and protective activity is much higher than that of HRIG b) Based on the protein content, only relatively small quantity of MAbs have to be injected c) The use of MAbs may be superior for local wound treatment, where only small volumes can be inoculated at the bite site. The disadvantages of mouse MAbs for therapeutic use in human are possible side effects like anaphylactic reactions and hypersensitization.

In the present study Polyclonal Antibodies (PABs) and Monoclonal Antibodies (MAbs) are prepared. Polyclonal antibody solution contain mixture of antibodies which are against unrelated antigens, several epitopes, and having differing affinity. In contrast, MAbs solution contains antibodies having identical specificity, equal affinity for antigens and also has better ability to engage biological process like opsonisation. The PABs from animals differ from lot to lot whereas in case of MAbs it is identical throughout. A strong reaction to antigen is higher in PABs as compared to that of MAbs, because antibodies are directed against several different epitopes. The production method involving PABs is comparatively simple and predictable as opposed to more complex, time consuming and unpredictable in case of MAbs.

*Summary
&
Conclusion*

SUMMARY AND CONCLUSIONS

Rabies being 100% fatal, is still a major public health problem. So the use of active immunization and or passive immunization prevents the disease. Passive immunization plays a very important role in severe cases or class III exposures, immunocompromised patients, delayed treatment and also in cases of vaccine failure. Passive immunization includes use of vaccines and anti-rabies antibodies or rabies immune globulin. Tissue culture vaccines are found to be safer than neural cell culture vaccines and hence formers are commonly preferred. Rabies immune globulin are of two types: Human Rabies Immune Globulin (HRIG) and Equine Rabies Immune Globulin (ERIG). As compared to ERIG, HRIG is safe, but costly and available in a limited quantity. Crude anti-rabies serum causes allergic reactions in patients while purified ERIG rarely react. Monoclonal antibodies have proven to be useful in passive immunization as well as epidemiological studies.

The present study was aimed to purify equine anti-rabies serum by different methods and also to get fragmented immunoglobulins F(ab)₂.

The following conclusions were drawn:

1. The Purified Vero cell Rabies (PVR) vaccine is safe for horse immunization.
2. Ammonium sulphate precipitation method can be used as a

preliminary or a supportive to other methods.

3. In the present study, the purification of anti-rabies serum or ERIG by DEAE Batch and WHO method gives promising results. Thus, further studies need to be carried out to ascertain beneficial effects.
4. The Protein G chromatography method is the best alternative to purify ERIG, inspite of its high cost.
5. The purified anti-rabies antibodies can be completely pepsin digested at 32 °C for 30 minutes.
6. The rabies virus adapts well on BHK-21 cells.
7. Even though Monoclonal antibodies (MAbs) preparation is more complex, time consuming and unpredictable method, proper skill and due care gives promising results.
8. In the present study, the MAbs proved their bioefficacy in mice. Thus, further studies are needed to prepare or develop MAbs against different virus strains or specific antigens, so that the MAbs can be used as a cocktail for passive immunization or in epidemiological studies.

Appendix

APPENDIX

1. Acrylamide Stock Solution:

1.0 gm. of Bisacrylamide was added to 30 ml Distilled water and heated upto 50 ° C to 70 ° C to dissolve. After this, 29 g of acrylamide was added and the final volume was made upto 100 ml.

2. Stacking gel Buffer pH 6.8:

6.0 gms. of Tris was dissolved in 40 ml Distilled water. The pH was then adjusted to 6.8 with 1 N HCl and the volume was made upto 100 ml.

3. Resolving gel buffer pH 8.8:

36.3 gms. of Tris was added to 48 ml of Distilled water. The pH of the solution was adjusted to 8.8 and then the volume was made upto 100 ml using D.W.

4. Tank Buffer:

0.1 % SDS was added to the mixture containing 3 gms. of Tris and 14.4 gms of glycine and the final volume was made upto 1000ml.

5. Gel Loading Buffer Preparation:

0.5 M Tris (pH 6.8)	1 ml
Glycerol	2 ml

β -Mercaptoethanol	1 ml
10 % SDS	4 ml
Bromophenol Blue (0.25 %)	2.5 mg
Distilled Water	1.5 ml

6. Stacking Gel (6% acrylamide) Preparation:

Ammonium per sulphate (APS)	50 μ l from 10% stock
Distilled Water	2.69 ml
Stacking Gel Buffer	1.25 ml from stock
Acrylamide (6%)	1 ml from stock
10 % SDS	50 μ l
TEMED	5 μ l

7. Resolving gel (12 % acrylamide) Preparation:

Ammonium per sulphate (APS)	100 μ l from 10% stock
Distilled Water	3.39 ml
Resolving gel Buffer	2.5 ml from stock
Acrylamide (12%)	4 ml from stock.
10 % SDS	100 μ l
TEMED	10 μ l

8. Staining solution (Coomassie Brilliant Blue) Preparation:

0.2% Coomassie Brilliant Blue (CBB-R 250) was dissolved in Methanol:Glacial Acetic Acid:Distilled water (5:5:2 ratio) and filtered and stored in amber colored bottle at room temperature.

9. Destaining solution Preparation:

10 ml. Glacial acetic acid was mixed in 30 ml. of methanol and the volume was made upto 100 ml using distilled water.

10. 2% NHS:

2 ml. of Normal Horse Serum was diluted to 100ml. using distilled water.

11. Preparation of 1 L Phosphate buffered Saline (PBS):

Sodium Chloride	8.0 g
Potassium Chloride	0.2 g
Na ₂ HPO ₄	1.4 g
KH ₂ PO ₄	0.2 g
Phenol Red	4.0 ml
pH	Adjusted to 7.4

Method:

The above chemicals were dissolved in 800ml. Distilled water. The pH of the solution was adjusted to 7.4 and finally made upto 1000 ml.

12. Preparation of 250 ml. Antibiotics solution (PSN):

Penicillin	25.0 g
Streptomycin	50.0 g
Neomycin	1.75 g
Distilled Water	Adjust level to 250 ml

The above solution was filtered through 0.2-micron Millipore filter and the sterility was observed for a week for any contamination. This antibiotic solution was then stored at -20°C .

13. Preparation of 1 L TPVG:

NaCl	8.0 g
KCl	0.4 g
Glucose	1.0 g
Phenol Red	4.0 ml
Antibiotics (PSN)	2.0 ml
Trypsin (Difco)	1.5 g
Versene (EDTA)	1.5 g

Method:

Trypsin and Versene were dissolved separately in distilled water and kept in an ice bath. The final solution of TPVG was filtered through 0.2-micron Millipore filter. The aliquots of the same were prepared and stored at 4°C .

Abbreviations

LIST OF ABBREVIATIONS

ABTS	2,2-Azino-di-3-ethyl-benzthiazolin-sulphonate
ACIP	Advisory Committee for Immunization Practices
ARS	Anti-Rabies Serum
BHK-21 clone 13 cells	Baby Hamster Kidney 21 Clone 13 cells
BSA	Bovine Serum Albumin
CBB	Coomassie Brilliant Blue
CER	Chick Embryo Related
CNS	Central Nervous System
CVS	Challenge Virus Standard
DEAE	Diethyl- aminoethyl
DMEM	Dulbecco's Minimum Essential Medium
EBLV	European Bat Lyssa Virus
ED ₅₀	Median effective dose, 50% end point dilution
ELISA	Enzyme Linked Immunosorbent Assay
ERIG	Equine Rabies Immune Globulin
ERS	Equine Rabies serum
F(ab)	Fragment- antigen binding
FAT	Fluorescent Antibody Test
Fc	Fragment- crystalline
FCS	Fetal Calf Serum
FTG	Fluid Thioglycolate medium
HAT	Hypoxanthine-Aminopterin-Thymidine

HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HDCV	Human Diploid Cell Culture Vaccine
HEL	Human Embryonic Lung
HIV	Human Immunodeficiency Virus
HRIG	Human Rabies Immune Globulin
HRP	Horse Radish Peroxidase
IFN	Interferon
IgG	Immunoglobulin G
IP	Indian Pharmacopoeia
IU	International Units
LD₅₀	Median lethal dose
MAbs	Monoclonal Antibodies
MIT	Mouse Inoculation Test
NCCS	National Centre for Cell Sciences
NHS	Normal Horse Serum
NRCE	National Research Centre on Equines
O.D	Optical Density
PAbs	Polyclonal Antibodies
PAGE	Poly-acrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCECV	Purified Chick Embryo Cell culture Vaccine
PCR	Polymerase Chain Reaction
PEG	Poly Ethylene Glycol
PVRV	Purified Vero Cell Rabies Vaccine

QMSI	Queen Sovabha Memorial Institute
RFFIT	Rapid Fluorescent Focus Inhibition Test
RIG	Rabies Immune Globulin
SCD	Soyabean Casein Digest medium
SDS	Sodium Dodecyl Sulphate
TEMED	<i>N,N,N',N'</i>- Tetramethelene diamine
TPVG	Trypsin Versene – EDTA Glucose Phosphate
VNA	Virus Neutralizing Antibodies
WHO	World Health Organization
WI-38	Wistar Institute-38

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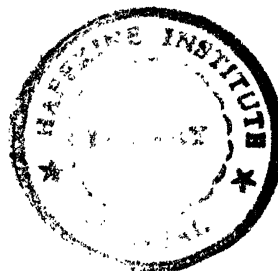
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Synopsis

SYNOPSIS OF THE THESIS TO BE SUBMITTED
TO THE
UNIVERSITY OF MUMBAI
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
MICROBIOLOGY

TITLE OF THE THESIS : INDIGENOUS PREPARATION OF
EQUINE RABIES IMMUNE
GLOBULIN AND MOUSE ANTI-
RABIES MONOCLONAL
ANTIBODIES.

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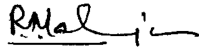
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INTRODUCTION:

Rabies is one of the oldest recognised diseases of mankind and one of the most important zoonotic diseases in India. It has been known in India since Vedic periods, as corroborated in Atherva Veda. "Yama", The God of Death has been shown with dogs as his constant companions and as emissaries of death.

The Latin word "rabies" seems to have originated from the Sanskrit word, "rhabas" which means, "to be violent". The Greek word for rabies, Lyssa, also means madness, and is the source of the taxonomic identification of the viral genus (Lyssavirus).

All age groups are exposed to rabies; but the predominant group (50%) is in the 6-15 years range. Males are four times more common victims than females. Epidemiologically it is described to occur as urban and sylvatic varieties; however, this is of no help in understanding the Indian epidemiology of the disease. A description of the disease can be found even as early as 100 A. D (1).

It was proved experimentally by Zinke in 1804 and Gruner and Sahn-Refferscheidt in 1814 that, the disease was transmitted from animal to animal through the inoculation of saliva of the biting beast (1)

Pasteur and his group of workers established the etiology of the disease in 1881. They paved the way for an intensive study of the disease and suggested that; etiological agent was not a bacterium but a 'Virus', which could be modified by serial passages in an animal other than natural host (1). In 1885, Pasteur developed and administered the crude rabies vaccine. The first human case to receive the vaccine was Joseph Meister- a 9-year-old boy bitten by a dog, diagnosed as having rabies.

In 1992, ninety-six nations reported the presence of rabies, and 46 reported its absence e.g. Australia, Antarctica etc. Rabies is prevalent in most parts of our country except in some islands like Lakshwdweep- Minicoy, Andaman and Nicobar. Most of rabies deaths occur in India, Bangladesh and Pakistan, where rabies is endemic and healthcare is poor.

As per World Health Organization (W.H.O), 36001 cases of human rabies were reported in 1992 (2). These reports probably estimate the worldwide incidence of disease, which under reprisal may cause as many as 1,00,000 deaths annually (3). Since 25,000-50,000 deaths per year are reported from India alone (4).

In India nearly 96 % of rabies cases are due to bites from stray dogs. Annually about one million people receive post-exposure anti-rabies vaccination. Children constitute about 35-40% of those, who receive post-exposure treatment. Half a million receive the Semple Vaccine free of cost, another half receive the cell culture vaccines through private hospitals (5).

The stray dogs constitute the main reservoir of infection. Moreover, very few epidemiological studies have been undertaken in different parts of the country to assess the role of other animal species that may play a role in disease

transmission. Figures of death of human rabies and livestock losses are poorly documented; except a few human rabies carried out by the WHO in Veterinary Public Health; and reports by Haffkine Institute, Mumbai; Pasteur Institute, Coonoor and Central Research Institute, Kasauli.

The dog has been, and still is the major reservoir of rabies in India. A steady increase in number of dog bite cases and ever increasing demand of anti-rabies vaccine poses the question: are we in the midst of epizootic incidence of rabies in dogs? Or is it merely a manifestation of the uncontrolled growth of dog population and the greater number of humans exposed to them? This needs a detailed study, constant surveillance and vigilance.

The importance of the species particularly wild animals such as jackals, wolf, fox, mongoose etc can not be properly assessed until the data for the total dog bite cases is segregated from all the other exposure cases. This may become more obvious when rabies is controlled in dogs. A limited survey about evidence of rabies infection in various species of rodents has been carried out in Mumbai and Kasauli in the early seventies. A later survey showed rabies in bandicoots, mongoose and rats (6).

There have been human as well as animal deaths following mongoose bites. A few mongoose brains examined at Central Research Institute, Kasauli were found to be positive for rabies. This definite evidence of rabies in mongoose suggests a possible transmission of rabies from the mongoose to other rodents, particularly in various species of rats that occupy the same ecological niche (6).

The possibility of rats becoming infected with rabies virus is most alarming. This aspect needs to be investigated in-details as it is an alarm to Public Health authorities. So far, there has been no evidence of rabies infection in the various species of bats in India. Other animals like lizards, deer, squirrel, vultures and eagles seem to bite incidentally on provocation and the rabies-phobia leads the victims to take anti-rabies treatment.

The number of monkey bite cases has been increasing in the last few years. But there is no epidemiological or clinical evidence that monkeys, free in nature, are infected with rabies virus. But their susceptibility to rabies infection is a known fact.

Rabies related viruses: -

Rabies virus and rabies related viruses belong to a family Rhabdoviridae and genus Lyssaviruses that are characterized by their bullet-like shape. Composed of over 20 animal viruses, that share their morphology, the rhabdoviruses are usually longer and bacilliform in shape.

Besides rabies, other rabies like viruses are:

- 1) Lagos bat virus isolated from pooled brains of frugivorous bat in Lagos Island, Nigeria, 1957;
- 2) Makola virus was isolated from the viscera of shrews captured near Ibadan, Nigeria (1968); this was isolated from human patients also.

- 3) Obodhiang - isolated from mosquitoes in 1963
- 4) Kotonkan - high percentage of neutralizing substance for Kotonkan were found in high percentage of cattles, rodents, sheep, horses in Nigeria in 1967.
- 5) European bat Lyssavirus 1 (EBLV1) 1985- almost all cases are in the common European house bat.
- 6) European bat Lyssavirus 2 (EBLV2) 1985- First identified in Swiss bat biologist who died in Finland.

Morphology: -

Rabies virions are cylindrical, with one round or conical end and the other planar, giving each the appearance of a bullet. The intact particle measures 180 X 75 nm. Regularly placed projections with knob like structures (spikes) at the distal end cover the surface of the rabies virion except at the flat end. In most preparations, these surface projections are randomly spaced. Occasionally, they are organized in rows, a feature that seems to give a honey comb-effect. It probably reflects asymmetry in the underlying layers.

The flat end of each virion has a depression at the axis of its internal helical nucleocapsid. The nucleocapsid, thus symmetrically wound within the envelope lies closely opposed to internal constituents of the virus and may be invaginated at variable distance to form an axial channel.

Properties of virions:

Gross chemical composition show the presence of 22% lipids; 3% carbohydrates; 1% RNA; and 74% protein; for this virus Sedimentation coefficient is 600s. Protein kinase is the only virion-associated enzyme, which has been detected in purified virus preparations. Four major polypeptide components of different molecular sizes form the protein moiety of rabies virus. These protein moieties are nucleoprotein, glycoprotein and two membrane proteins. These are of different molecular weights, molecular sizes and their relative proportions are constant.

The major nucleocapsid protein corresponds to the virion polypeptide that has the second largest molecular weight. The glycoprotein (80,000 Dalton) is the largest polypeptide. The two envelope proteins (Mol. wt. 40,000 and 25,000 Dalton respectively) are closely associated with the virus nucleocapsid.

The viral RNA is surrounded by a phosphoprotein to form a helical nucleocapsid. The association of the two membrane proteins with the nucleocapsids results in a folding of the latter and its enclosure by membrane. The spikes that contain the envelope glycoprotein are attached to the core particles probably with the help of viral lipids.

This virus is sensitive to lipid solvents, 45% -70% ethanol, iodine preparations and quaternary ammonium compounds (7). It is relatively stable at pH 5.0 to 10.0 but is virus is sensitive to Pasteurization temperatures and U.V. light. It is readily inactivated by β -Propiolactone.

Replication of rabies virus:

The infectivity of saliva from rabid animals has been long suspected and was demonstrated in the beginning of 19th century. In most instances, natural infection occurs from contamination of external wounds with saliva with rabies virus. The exact mechanism by which the virus penetrates the nerve is not fully understood

at present, but evidence of centripetal spread is generally accepted. Adsorption and penetration of rabies virus occurs relatively rapidly only a few seconds of contact are needed for the virus to attach itself irreversibly. Penetration of the attached virus begins within minutes after the initial exposure and is at the maximum level in about one hour.

Indeed electron microscope studies reveal that numerous viral particles can be attached to the cell plasma membrane or are within intracytoplasmic vesicles just 5 minutes after exposure. In the first hour of infection, degradation of the viral particle, fusion of the virus to the cell membrane and release of internal structures can be observed. At 60 minutes, intact virions can no longer be seen within cell protoplasm. Rabies specific antigen recognised by immune fluorescent reaction appears four hours after infection and by the eight hours all cells show presence of antigen. Budding viral particles are noted at 6 hours and reach maximum within 24 hours.

Rabies is primarily a disease of terrestrial and airborne mammals involving canidae (dogs, wolves, foxes, coyotes and jackals); Felidae (cat, bobcat and lion); viveridae (mongoose); musteladae (skunks, badgers and martens); Chiroptera (bat) and primates (man and monkeys). Human infection with rabies is nearly always secondary to bites, although others, such as inhalation, are also a possibility. In most parts of the world, the major reservoir for rabies is the domestic or the semi-domesticated dog or cat.

Incubation period is between 20 to 60 days; occasionally, cases also occurs in individuals who have had recognised exposure to rabies. Incubation periods are shorter when the size of the bite is on or near the head in contrast to extremity; the incubation period is shorter in children than in adults. The incubation period for rabies related virus infection of man remains uncertain because the means of exposure are not known.

Clinical Symptoms:

Symptoms of rabies have been best described for man, the major animal host. Although there is some variation in species, clinical symptoms usually begin with non-specific lassitude, loss of appetite, or change in behaviour. In a variable proportion of cases, the animal develops aggressive or combative behaviour and irritability, viciousness and hyper-reaction when exposed to external stimuli. In these cases, the clinical course is described as furious rabies. A paralytic phase then develops; this is characterized by weakness of one or more limbs, jaws drop due to paralysis of muscles of the head and neck; difficulty in phonation and respiration leading ultimately to death. In a small number of animals, it appears that spontaneous recovery may occur. Infection occasionally produces no obvious clinical signs, even though it may produce sero conversion and subsequent immunity to rabies.

The clinical illness in man may be divided into five stages; the incubation period, prodrome, acute neurologic phase, coma and very very rarely recovery. During the incubation period, there are generally no symptoms and clinical illness begins with non-specific complaints of malaise, anorexia, fatigue, headache and fever. Pain or paresthesia at or close to the site of exposure are reported in 50 to 80% of

cases. Apprehension, anxiety, agitation, irritability, nervousness, insomnia or depression may be prominent during the prodrome period; less commonly cough, chill, sore throat, abdominal pain, nausea, vomiting and diarrhoea have been reported as early symptoms in clinical rabies cases. After the prodrome of 2 - 10 days, objective signs of nervous system involvement develop like hyperactivity, disorientation, hallucinations, seizures, bizarre behaviour, nuchal stiffness or paralysis.

In man hyperactivity is intermittent and consists of periods of agitation, thrashing or other bizarre behaviour lasting from 1-5 min. This may occur spontaneously or may be precipitated by a variety of tactile, auditory, visual or other stimuli. Hydrophobia, the fear of water-appears to develop in most cases of human rabies. Attempts to drink or eat may produce severe painful spasms, which seem to be extremely frightening to the patient; subsequent sight of water or liquid may precipitate a similar episode of pharyngeal spasms. Muscular fasciculations, particularly near the site of the exposure hyperventilations, hypersalivation- focal on generalized convulsions and rarely priapism.

Paralysis generally becomes the major problem. Paralysis may be generalized, asymmetric with maximum involvement of the bitten extremity or ascending as in Landry Guillain- Barr's syndrome. This is commonly found in rabies due to vampire bat bites. During the acute neurologic phase, the mental status of the patient gradually deteriorates over a period of 2-10 days. Cardiac or respiratory arrest, coma occur in final stage.

Laboratory Diagnosis:

The laboratory diagnosis of rabies includes staining of the brain tissue impression smear by Seller's method showing Negri bodies.

The virus can be identified by fluorescent antibody method, compliment fixation test. The tissue specimen can be examined for the presence of virus by direct intracerebral inoculation into laboratory mice or into highly susceptible tissue culture cells and other molecular biology techniques like polymerase chain reaction (PCR)

Several antibody tests have been developed to detect antibodies against rabies virus such as mouse neutralization test (MNT), rapid fluorescent focus inhibition test (RFFIT), and enzyme linked immunosorbent assay (ELISA)

Prevention and Treatment:

Since rabies is almost invariably fatal illness, emphasis has been placed on preventing human infection. Three aspects of prevention should be recognised.

- 1) Prevention of exposure by contact with animals.
- 2) Prophylaxis of those individuals most likely to be exposed to the virus and
- 3) Prevention of the onset of infection in those who have been exposed to the virus.

Since rabies is a zoonosis, man becomes a casual host only by exposure to an infected animal. Human illness can be prevented by elimination of this exposure through control of rabies in wild, feral and domestic animals by various enforcements of rabies vaccination laws, capture of stray animals and providing

rabies vaccine through low cost animal clinics.

Persons likely to be exposed to rabies should receive pre-exposure prophylaxis. Laboratory workers who, handle potentially infected tissue and virus, veterinarians, animal control officers working in rabies prevalent areas and individuals who travel to areas where rabies is highly endemic ought to receive initial immunization followed by maintenance boosters with killed rabies vaccine e.g. tissue culture human diploid cell culture vaccine (HDCV), purified chick embryo cell culture vaccine (PCEC), purified Vero cell rabies vaccine (PVRV) etc. are preferred because of demonstrable safety and efficacy of these vaccines. Post-exposure prophylaxis is intended to prevent rabies in persons who have been exposed to virus. Each exposure situation is to be evaluated individually by a physician and considering the species of biting animal, circumstances of the bite incidence and type of exposure the treatment is initiated.

Preventive therapy should be instituted immediately as soon as possible following an exposure. The bite should be washed thoroughly with soap and debrided if necessary. Animal studies have clearly demonstrated the protective effects of washing rabies infected wounds with 20% soap solution or various concentrations of benzalkonium chloride which decreases rabies mortality by 35 % to 90 % (8). Reduction in mortality is greater with mechanical scrubbing than only irrigation of the wound. Prompt and thorough scrubbing of wounds with soap is recommended.

Rabies immune globulin (RIG) and rabies vaccine should be used for passive and active immunization respectively when indicated. Serum neutralizing antibody generated by vaccination appears to have a direct relationship to protection against rabies in several animal models and probably represents the protective antibody in man.

The glycoprotein containing spikes that project from the viral envelope appears to be the sole antigen responsible for induction of neutralizing antibodies (9). When sub-virion components were used to inoculate mice or rabbits the purified envelop glycoprotein resulted in neutralizing titres 3.5 to 80 times higher than "core particles" or nucleocapsid material. Core particles, a mixture of envelop proteins and whole virus induced titers proportional to the amount of "contaminating" glycoprotein each contained. Purified glycoprotein had a neutralizing antibody binding activity value that was 8 times higher than core particles, suggesting that neutralizing antibodies had a high degree of specificity for envelop glycoproteins. Nucleocapsid and core particles administered to mice provided virtually no protection against rabies virus infection. Whole virion isolation was more protective than either glycoprotein or envelop preparations, probably due to interferon induction. These data suggests that protection due to whole virus or subunit vaccines depend on the presence of envelope glycoprotein. Finally liposomally anchored spike glycoprotein induced both strong humoral and cellular immune responses. Pre and post exposure potency testing demonstrated high level of protection

Semple vaccine, suckling mouse brain vaccine and duck embryo vaccines have been discontinued in most of the developed countries due to their adverse side

reactions. Five doses of tissue culture vaccines (concentrated minimum potency 2.5 I.U) on day 0,3,7,14 and 30th and a booster on day 90, is recommended for persons who can afford these vaccines.

Antirabies serum of heterologous origin :

ARS and its globulin fractions are of proven effectiveness as an adjunct to vaccine treatment. Antirabies serum of proven potency has been produced in horses, mules donkey and other animals, using various methods of hyperimmunization and of purifying and concentrating the resulting sera. It is suggested that the antigen used for immunizing the animal should be of cell culture origin. Horse serum hyperimmunized by vaccination is concentrated and refined to produce equine antirabies serum. The neutralizing antibody content is standardized to 1000 I.U./5 ml vial. Although effective 40 % of adults who receive ARS will experience serum sickness. Hence the purification of equine rabies immune globulin (ERIG) is advocated.

Human rabies immune globulin (HRIG) is prepared by cold ethanol fractionation of plasma to obtain a concentrated gammaglobulin fraction from hyper immunized human donors. Each ml of HRIG contains 150 IU. Rare adverse reactions to HRIG make it preferable to ARS.

Although the production of monoclonal antibodies (MAbs) to rabies virus in tissue culture hybridoma will be the ultimate answer to the growing needs of RIG, this needs investigation and perfection of techniques. These antibodies may ultimately replace equine and human antirabies globulin for passive immunization.

Continuous tissue culture cell lines are produced by fusing mouse myeloma cells and spleen cells from mice that have been immunized with rabies vaccine / virus. The derived cells are cloned and cell lines which produce the desired antibody are continuously cultivated. Highly specific antibodies can be harvested cheaply in large quantities. Monoclonal antibodies react more specifically than polyclonal antibodies produced in hyper immunized animals and humans, hence they neutralize the virus more effectively.

On this background the present study has been planned. The main aims being preparation and purification of ERIG to eliminate the components which are responsible for adverse reactions like serum sickness. The second aim is production of MAbs by applying the hybridoma technology.

THE PROBLEM AND AN APPROACH TOWARDS ITS SOLUTION:

The passive immunization for rabies exposure plays a very important role in prevention of this dreaded disease. World Health Organization (W.H.O) recommends passive immunization for grade III wounds. Unfortunately many a times, patients do not receive timely important passive immunization for the lack of hyper-immune sera, high cost of homologous HRIG, or may develop serious adverse reactions to unpurified equine antirabies serum (ERS).

Hence the present work is envisaged with the following Aims and Objectives.

PART A

- 1) Indigenous production of equine antirabies serum (ERS)**
- 2) Purification of ERIG by the following methods:**
 - a) Ammonium sulphate precipitation**
 - b) Caprylic acid.**
 - c) Ion exchange chromatography**
 - d) Affinity chromatography using protein G**
 - e) Ammonium sulphate precipitation and pepsin digestion.**
- 3) Comparative study of the yield and efficacy of purified immunoglobulins.**

PART B

- 1) Production of anti-rabies monoclonal antibodies (MAbs) by using hybridoma technology.**
- 2) Testing the efficacy of MAbs.**

MATERIALS AND METHODS:

PART A

INDIGENOUS PRODUCTION OF EQUINE ANTI-RABIES SERUM:

Horse:

Healthy Female horse aged 7 years and weighing 380 Kg was procured and immunized at National Research Centre on Equines (NRCE) Hissar, Haryana. The horse was tagged and numbered for the identification during the course of immunization. The horse was fed and watered ad libitum.

Vaccine: The vaccine used for the immunization of the horse was Purified Vero Cell Rabies Vaccine (PVRV) Batch No.R0461 and it was purchased from Pasteur Merieux India Ltd.

Mice: Swiss Albino mice weighing 14-16 gm (Kasauli strain) were procured from Haffkine Biopharmaceutical Corporation Ltd. Mumbai and they were fed and watered ad libitum and maintained with proper ventilation.

IMMUNIZATION SCHEDULE:

The horse was inoculated with series of injections of PVRV with increasing volumes. The immunization schedule was followed as described (10). On day zero, the horse was inoculated in cervical region with a suspension of 0.5 ml vaccine and 0.5 ml complete Fraeund's adjuvant. On days 21st, 28th, 35th and 42nd, one ml vaccine without Fraeund's adjuvant was injected subcutaneously on the neck region. On day 63rd, 70th, 77th, 84th, 91st, 98th and 105th, 2.0 ml vaccine without adjuvant was injected subcutaneously on both sides of the neck. On the day 159, 2 ml of vaccine was injected subcutaneously and same dose was repeated weekly for subsequent three weeks.

BLEEDING:

Before immunization on day zero, the horse was bled and 10ml blood was collected aseptically. From day 21st to day 56th approximately 10 ml of blood samples were taken at weekly intervals and Serum was separated and the antibody titre was determined by mouse neutralization test (MNT) (11).

Approximately 200ml of blood samples were collected on day 119, 121 and 123. After the completion of last booster injection, the blood samples (Appx. 200 ml) were taken on day 194th, 196th and 198th serum was separated and pooled. Total 450 ml of antirabies serum was obtained from all six-blood samples. The aliquots of pooled serum sample were made and stored at -20 °C until further purification. Anti-rabies antibody titre was determined by the MNT for the pooled anti-rabies serum.

Mouse neutralization test (MNT):

The sera to be tested were inactivated for 30 minutes at 56⁰ C. Serial twofold dilutions of the serum were prepared and 0.5 ml of each of these dilutions was transferred to test tube. The 0.5ml of the CVS virus dilution corresponding to 200 -250 LD₅₀ was added to each tube. The tubes were incubated at 37⁰ C for 1-1.5 hrs. The test was carried out with the reference serum.

After incubation, the Swiss albino mice weighing 14-16 gm were inoculated with 0.03 ml of each dilution and were observed for 14 days and then the antibody titre was determined.

2) PURIFICATION OF ERIG BY THE FOLLOWING METHODS :

a) AMMONIUM SULPHATE PRECIPITATION (12):

Forty percent-saturated solution of ammonium sulphate ((NH₄)₂SO₄) was prepared for the precipitation of immunoglobulins. Twenty ml of anti-rabies serum was mixed with twenty ml of saturated solution of (NH₄)₂SO₄ by stirring at 4⁰ - 8⁰ C for 30 minutes. After this the precipitate was centrifuged at 5000g for 30 min. The pellet was dissolved in 40 ml of distilled water and the resulting solution containing immunoglobulins was then subjected to dialysis against PBS pH 7.2.

b) PURIFICATION BY CAPRYLIC ACID (13):

The antirabies serum 20 ml, was diluted with 80 ml of acetate buffer (pH 4.0) The pH of the suspension was then adjusted to 4.5. To the suspension, 2500 :1 of caprylic acid was added slowly. The suspension was stirred at 20⁰ C for 30 minutes. The suspension was centrifuged at 10,000 g for 30 minutes and the supernatant was separated. The pH of the supernatant was adjusted to 7.4 and was cooled at 4⁰ C. Then it was fractionated with ammonium sulphate. The sample was stirred for 30 minutes and subjected to centrifugation at 5000g for 15 minutes using Remi centrifuge. The precipitate obtained was dialysed against PBS pH 7.2 to remove traces of (NH₄)₂SO₄.

c) PURIFICATION BY ION- EXCHANGE CHROMATOGRAPHY

i) BATCH METHOD (14):

The DEAE (Diethylaminoethyl) resin was washed twice with 0.5 M HCl. It was then washed with 0.5 M NaOH and twice with Phosphate buffer pH 6.0. Five ml of anti-rabies serum was mixed gently with five ml of DEAE. Then the mixture was stirred for 1 hour at 20⁰ C. then it was centrifuged at 4500g for 25 minutes. The supernatant was separated and subjected for ammonium sulphate precipitation described in above method.

ii) PURIFICATION BY USING COLUMN (15):

The DEAE cellulose resin was swollen in 0.01 M Tris, pH 8.6. The suspension was allowed to stand for 10 minutes and the fine particles were decanted. This

process was repeated till most of the fine particles were removed. The resin was gently poured into a sterile poly propylene column of 1.5X12 cm dimension. A bed of 5 ml volume was prepared. This bed was washed twice and equilibrated with Tris Cl, pH 8.6. The baseline O.D at 280 nm was measured. Then 2.0 ml of the antiserum was loaded with the help of a syringe on to this bed and allowed to run. The column was washed with 0.01 Tris.Cl; pH8.6 and fractions were collected. The fractions were read spectrophotometrically at 280 nm. Fractions were collected till the baseline O.D. was reached. The column was eluted using a 100 ml linear gradient of 0.01 M Tris Cl, pH 8.6 without NaCl to 0.5 M NaCl in Tris. Cl, pH 8.6. The fractions were collected and read at 280 nm. Positive fractions were pooled and were subjected to Ammonium sulphate fractionation.

d) PURIFICATION BY AFFINITY CHROMATOGRAPHY BY USING G PROTEIN (16):

The anti-rabies serum 1.25 ml, was mixed with 3.75 ml of sodium phosphate buffer (pH 7.0 -8.0). This diluted sample was passed through Protein G column. The unbound material was washed from the column with sodium phosphate buffer pH 8.1 until the emission at 280 reached the baseline level. The bound immunoglobulins were eluted with 0.1M glycine buffer pH 2.7. The fractions containing antibodies were pooled and subjected for ammonium sulphate precipitation.

e) PURIFICATION BY AMMONIUM SULPHATE AND PEPSIN DIGESTION (17):

Elimination of albumin and pigments:

Twenty ml of anti-rabies serum was diluted with 20 ml the distilled water. To this solution, 9.24 gm of pure crystalline ammonium sulphate was added slowly while stirring to give a final concentration of 1.75 moles per liter. The pH of the solution was adjusted to neutral. After 6 hours, the precipitate was collected by centrifugation at 3000-4000 g for 10 minutes and the supernatant was discarded. The precipitate was then resuspended in 20 ml distilled water.

Pepsin Digestion:

For the pepsin digestion process the pepsin was purchased from Hi-media. The pH of the above protein suspension was adjusted to 3.2 with 1 N Hydrochloric acid. 0.015 gm of pepsin was added to the suspension and the digestion was carried out at 32⁰ C with stirring on magnetic stirrer for 30 minutes.

Selective Denaturation by heat:

After digestion with pepsin, the pH of the solution was adjusted to 4.3 with dilute ammonia solution. Then 1.98 gm of Ammonium sulphate (NH₄)₂SO₄) was added to the above suspension to achieve a concentration of 0.75 Mol/l. To the solution, toluene and charcoal was added as 3 :1 and 0.1 g respectively. The temperature of the solution was raised to 55⁰ C in water bath and it was cooled immediately and filtered. The solution was diluted with distilled water to give 0.3 Mol/l of ammonium sulphate. The pH was then adjusted to neutral.

Concentration of protective fragments:

After adjusting pH of the filtrate to neutral, the concentration of $(\text{NH}_4)_2\text{SO}_4$ was increased to 1.75 Mol/l. The suspension was kept standing for 10-12 hrs and was centrifuged at 5000g for 10 minutes to obtain the precipitate containing fractionated immunoglobulins.

Dialysis:

The concentrated solution of fractionated immunoglobulins was dialysed against PBS, pH 7.2 using dialysis bags with cut-off 10,000 Dalton.

3) COMPARATIVE STUDY OF THE YIELD AND EFFICACY OF PURIFIED IMMUNOGLOBULINS.

All the purified samples were subjected to the following testing procedures.

i) PROTEIN ESTIMATION AND IgG DETECTION:

Protein concentration of the serum samples was measured according to the method described by Bradford (18). The absorbance of diluted serum samples were measured at 595nm and plotted against a standard curve.

Immunoglobulins were detected using SDS-Polyacrylamide gel electrophoresis (19) with a vertical slab gel apparatus using 12.0% acrylamide gel and standard molecular weight markers.

ii) POTENCY TESTING:

Potency testing of all the serum samples was carried out by MNT test (11).

iii) STERILITY TESTING:

The test was carried out as mentioned in Indian pharmacopoeia (1996). The purified samples were subjected for the sterility test to check the microbial contamination. 0.1 ml of sample was inoculated in the tubes containing Fluid Thioglycolate medium and soyabean casein digest medium. The tubes were incubated at 30°C - 35°C for 7 - 14 days and 20°C- 25°C for 7- 14 days respectively

PART B

1) PRODUCTION OF ANTI-RABIES MONOCLONAL ANTIBODIES BY USING HYBRIDOMA TECHNOLOGY.

Laboratory animal: 10-12 week- old BALB/c mice were procured from Intervet India Ltd. Pune and they were fed and watered ad libitum in separate animal house with proper ventilation.

Cell Line: Myeloma Cell line, SP2/0 was procured from National Centre for Cell Sciences (NCCS) Pune. And it was maintained in our tissue culture Laboratory.

Rabies antigen: Rabies virus (RV-CVS) was propagated in BHK 21-cell culture monolayer. The crude antigen was inactivated by β -propiolactone.

The method of immunization and production of monoclonal antibodies was followed as described (20)

IMMUNIZATION OF ANIMALS:

A group of six mice were primed with three injections of rabies antigen at weekly interval. For the first injection 0.2 ml of antigen was mixed with 0.2 ml of complete Fraeund's adjuvant and injected subcutaneously. A booster injection was given intravenously 3-4 weeks later without adjuvant

MYELOMAS:

Myeloma cells were maintained in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 10 ml of 100 mmol/l sodium pyruvate with 10% fetal calf serum in a humid incubator with 5% carbon dioxide. One week before fusion, the cells were transferred to DMEM containing 10% horse serum to improve the efficiency of fusion. The cells were maintained in stationary suspension culture at concentration of 1×10^6 cells /ml and 5×10^6 cells/ml for fusion.

FUSION:

After 3-4 days of complete immunization, spleenocytes from the rabies immunized mouse were fused with SP2/0 mouse myeloma cells using PEG 1500 (Polyethylene glycol).

2) TESTING THE EFFICACY OF MABs:

A group of six Swiss albino mice (4-6 week old) were injected intramuscularly with 0.1 ml of diluted MABs. After 24 hrs of MAB treatment the mice were inoculated intramuscularly with 10^6 LD₅₀ of challenge virus standard (CVS) the animals were observed for 3 weeks and the deaths were reported daily. The effective dose (ED₅₀) was calculated (21).

RESULTS AND DISCUSSION:

The horse which was immunized as per W.H.O schedule, was bled on day 56 and the antirabies antibody titre in the horse serum was found to be more than 70 ml i.e. 78 IU/ml. This made the horse suitable for further immunization

After the completion of immunization schedule, the antirabies antibody titre and protein concentration in pooled antirabies serum was determined and found to be 261 IU/ml and 91.6 mg/ml respectively.

The following table illustrates the data obtained from the comparative study for different methods of purifying ERIG.

Purification method	DEAE Column	Protein G	DEAE Batch	WHO	C.A	A.S.
Initial Protein (mg/ml)	91.6	91.6	91.6	91.6	91.6	91.6
Final Protein (mg/ml)	27.8	20.0	31.0	51.0	40.0	56.0
Initial potency (IU/ml)	261	261	261	261	261	261
Final potency (IU/ml)	189	207	180	231	207	255
Percentage recovery Activity	72	79	68	88	79	97
Specific Activity Ratio	2.37	3.64	2.02	1.58	1.76	1.59

A.S: Ammonium sulphate precipitation

C.A.: Purification by Caprylic Acid.

The serum purification by WHO method (before pepsin digestion) resulted in a recovery of 88% with a specific activity ratio of 1.58, While the ammonium sulphate precipitation method resulted in a recovery of 97% with specific activity ratio of 1.59. The serum purified by the Caprylic acid, resulted in a recovery of 79% with specific activity ratio 1.76

The purification of serum using batchwise and column DEAE chromatography resulted in a recovery of 68% and 72% with a specific activity ratio of 2.02 and 2.37 respectively. While the serum purification by Protein G Affinity chromatography resulted in a recovery of 79% with a specific activity ratio of 3.64.

The advantage of ammonium sulphate is that it can be carried out at 4⁰ C and it is simple and one step method, while the drawback of this method is that it yields a product of low purity. Therefore it can not be used as an independent method. While caprylic acid method gives better results compared to independent salt precipitation. This method can not be used because antibodies are in large volume of supernatant.

The DEAE column chromatography showed better resolution and purity than DEAE batch method but can not be used for large-scale purification of antibodies. While DEAE batch method is easy and can be used for large-scale purification of antibodies.

In case of Protein G Affinity purification, the product yield is high with a good purity comparing to all other methods. Due to the high cost, this method can not be scaled up.

From the above results, Further process of pepsin digestion for 30 minutes was carried and fragmented antibodies were detected with standard molecular weight markers.

This suggests that 30 minutes for digestion is sufficient time period.

DEAE batch purified sample was compared to WHO (After pepsin digestion) method. The recovery of fragmented antibodies $F(ab)_2$ was comparatively more in DEAE batch than WHO method. This could be due to involvement of more steps in WHO method.

No microbial contamination was found in final purified immunoglobulins.

The MAbs produced by the method described in this thesis yielded antibodies more than 2 I.U./ml. The bioefficacy of MAbs in preventing the disease in mice was more than 50%.

Hence passive immunity with MAbs can be very important in clinical setting.

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