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ASSESSMENT OF POST EXPOSURE ANTI-RABIES THERAPY IN CATTLE

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Thesis submitted in partial fulfilment of the requirement for the degree of

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DECLARATION

I hereby declare that the thesis entitled **"ASSESSMENT OF POST EXPOSURE ANTI-RABIES THERAPY IN CATTLE"** is a record of research work done by me during the course of research and this thesis has not previously formed the basis for the award of any degree, diploma, fellowship or associateship or other similar title, of any other University or Society.

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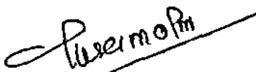

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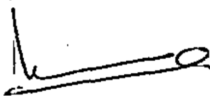

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Introduction

1. INTRODUCTION

Rabies is an ancient infectious disease of man and animal with a rich and fanciful history, related partly to the rise of civilization, the growth of cities, the gradual demise of superstition, and the domestication and movement of animals. Unfortunately, it is still a wide spread uncontrolled killer of human and animal in the developing world.

Rabies is distributed all over the world. In about 100 countries with more than 2.5 billion population, rabies is endemic in wild and domestic animals. Every year, four million people are exposed to rabies worldwide after animal bites and 50,000-60,000 humans fall victims to it. Rabies mortality ranks about 10 among all infectious diseases. Even by rudimentary surveillance, one person dies from the disease each 15 minutes, and more than 300 others are exposed.

More than 99% of the fatalities occur in developing countries in Asia and Africa where there are large populations of stray dogs. The highest numbers of human rabies deaths are reported from Asia. In India alone 17,800 human deaths occurs annually which accounts to 40% of global report of 50,000 deaths. (WHO, 2004).

Rabies is an acute viral disease that attacks the central nervous system of its victim. It is invariably fatal once clinical symptoms have occurred. Rabies is passed from animal to animal or animal to human through bites or scratches. But it can also be transmitted by contamination of wounds, the virus can cross mucous membranes and infections through inhalation of aerosol contaminated with the virus have also occurred. (Haupt, 1999)

In India Stray dogs play a crucial role as vectors in the spread of disease. In Kerala up to 97% the disease is transmitted by dogs and the rest by cats and other animals (Saseendranath, 1996).

All the warm blood animals are susceptible to this killer disease and the cattle are highly susceptible. Domestic livestock are rarely a source of infection although chance of transmission to humans may occur if the mouth of rabid animal is manipulated during treatment or examination. (Radostitis *et al.,* 1996)

Rabies in cattle remains a serious economic problem in tropical country like India as cattle are used for milk production and in agricultural operations. Although, the post exposure treatment of unvaccinated animals exposed to rabies has been discouraged by W.H.O and recommended that such unvaccinated farm animals should be slaughtered immediately or, if the owner is unwilling to do so then the animals should be kept under close observation for 6 months. (Clark and Wilson, 1996)

This recommendation has been largely disregarded by owners who will not agree to euthanize or prolonged isolation of their animals especially in India due to socio economic conditions and religious sentiments where farmers are totally depending on their livestock.

Several challenge studies were carried out with Essen's schedule post exposure treatment in animals and demonstrated effectiveness. Few works have already reported the effectiveness of post exposure anti-rabies therapy in cattle (Ramanna *et al.,* 1991b and Basheer *et al.,* 1997b). Hence the present study was undertaken to assess the effectiveness of a tissue culture anti-rabies vaccine and a combined DNA vaccine in two schedules.

Considering these situations the present study was conducted to assess the efficacy of two anti-rabies vaccines in two different schedules for post exposure therapy in cattle.

Review of Literature

2. REVIEW OF LITERATURE

2.1 HISTORY

In the beginning of the time, Homer's *Iliad* refers to rabies when he mentions Sirus, the Dog star of Orion, exerting a malignant influence on the health of mankind. Sirus was associated with mad dogs through out the Egypt, Rome and the Eastern Mediterranean. The Greeks and Aristaeus, Son of Appolo, to counteract the effect of Rabies.Artemis is also represented as an early healer of rabies. (Baer, 1975)

The origin of Rabies virus appears to be in Africa and that this oldest disease was first described in the Sumerian law code from the city of Eshunna dating from about 1885 B.C in Mesopotamia. (Beran, 1981)

In Greece, the Rabies was described in dogs by the philosopher Democritus (500 B C) and in human patients by Hippocrates about 400BC. (Smith, 1996)

The word "Rabies" comes from the Sanskrit word "*rabhas*" which means, "to do violence". It refers to the Vedic period of India (30th century BC), when the God of Death was depicted being attended by a dog, his constant companion and the emissary of death. (Fu, 1997)

Rabies is derived from the Latin word "*rabere*" which means "*madness*". Rabies was present in Egypt before 2300 B.C and also in ancient Greece (Murphy *et al.*, 1999).

The term *rabies* has been variously attributed to derivation from the Sanskrit, *rabhas* ("to rage") or the Latin *rabere* ("to rave"). Rabies was referred to in the Eshunna Code in Babylon more than 4000 years ago. (Daniel *et al.*, 2004)

2.2. ETIOLOGY

Smith (1996) recorded that rabies virus is the prototype member of the genus *Lyssavirus* of the family *Rhabdoviridae*, Order *Mononegavirales*. The genetic features of rabies virus were similar to those of other members of the *Mononegavirales* in that a nonsegmented, negative stranded RNA genome is tightly encapsidated into ribo-nucleocapsid structures.

Bourhy *et al.* (1999) opined that rabies is caused by *Lyssaviruses*, and are negative stranded RNA viruses that can be divided into seven genotypes. Viruses of genotypes 1, 5 and 6 are characterized by their natural and stable association with specific mammalian species that act as the vectors for their transmission.

Morimoto *et al.* (1999) provided evidence that a particular rabies virus strain consists of variants with different biological properties and that changes in the host environment rapidly results in shifts in the dominant variant

The rabies virus is classified as a *Rhabdovirus* in the Family *Rhabdoviridae*, genus *Lyssavirus*, which are rod or bullet-shaped ultrastructually (*rhabdos* = rod in Greek), and composed of a single stranded RNA (ribonucleic acid) genome, 11-15 kb in size. The external surface of the viral envelope is covered with 10 nm long projections or spikes comprised of glycoprotein. These glycoprotein spikes constitute rabies viral antigen that induce neutralizing antibody production which may confer immunity to the disease (Murphy *et al.*, 1999).

On the basis of nucleotide sequence comparison and phylogenetic analysis, the *Lyssavirus* genus has been divided into seven genotypes (GTs). GT_1 includes the classical rabies viruses and vaccine strains, whereas GT_2 to GT_7 correspond to rabies-related viruses, including Lagos bat virus (GT2), Mokola virus (Mok) [GT3],

Duvenhage virus (GT4), European bat lyssavirus 1 [EBL1 (GT5)], and EBL2 (GT6). A new Australian bat Lyssavirus that belong to new genotype (GT7) (Jallet *et al.*, 1999)

Classical rabies is generally a fatal encephalitis of all mammals, caused by *Lyssa virus* genotype 1 and the virus has a genomic structure of single stranded, negative sense, non-segmented RNA which codes for five separate proteins designated nucleoprotein (N), phosphoprotein (M1 or NS), matrix protein (M2 or M), glycoprotein (G) and polymerase (L) (Davies and Lowings, 2000a).

Molecular epidemiology based on RT-PCR is an important tool for the classification of animal virus diseases, including rabies virus, and provides a better understanding of epidemiological relationships (David *et al.*, 2000).

Roux *et al.* (2000) investigated the role of phosphoprotein (involved in viral transcription and replication) by searching for cellular partners by using a two-hybrid screening of a PC12 cDNA library and results speculate that dynein may be involved in the axonal transport of rabies virus along microtubules through neuron cells.

2.3. EPIDEMIOLOGY

Tierkel (1975) observed that the typical incubation period for rabies in dogs ranged from 21 to 56 days.

Rabies is endemic in India and poses a serious health problem where in dogs are the principal reservoirs but the infection is also maintained by mongooses, jackals, and wolves and in limited areas by foxes and hyenas. (Beran, 1981)

Kieny *et al.* (1987) opined that dogs are the major vector of rabies in Asia whereas Beran (1981) was of the opinion that across the temperate and subtropical continental Asia both dogs and wildlife act as reservoirs.

Kandavel *et al.* (1989) reported that the occurrence of furious form of rabies was higher than the paralytic form in calves.

Dutta and Dutta (1994) recorded that rabies kills more than 25,000 people each year in India, where stray dogs are mainly responsible for transmission and almost half a million people receive prophylaxis after being bitten.

According to Saseendranath (1996) rabies is endemic in India except in Lakshadweep and Andaman and Nicobar islands.

Smith (1996) opined that a concise description of the epidemiology of rabies requires data from molecular typing methods and case surveillance. Further the author stressed that characteristic nucleotide analysis like pseudogene analysis permit identification of rabies virus variants associated with different outbreaks, but without case surveillance the presumed phylogenies of these variants are of little value to identify the animal contributing to disease maintenance, and the circumstances promoting the outbreak.

Mahendra *et al.* (2000) emphasised that ignorance, poverty and lack of proper medical advice were the main reasons for inadequate management of rabies in India wherein more than 30000-35000 deaths occurs annually.

As per Hostnik *et al.* (2001) there are two forms of epidemiological status urban rabies where dogs are responsible for the maintenance and transmission of infection to man and in sylvatic form of rabies where it spread among wild animals.

Wilson and Clark (2001) stated that reporting of possible post exposure prophylaxis failure by the attending veterinarians and owners has been considered as an acceptable and active rabies surveillance system.

Jackson (2002) reported that rabies remains as an important public health problem due to uncontrolled stray dog rabies in developing countries.

According to Pavlinic and Hostnik (2002) the existence of a huge reservoir of rabies in domestic and wild animals in certain European countries and around the world was the main reason for the need to continue surveillance necessitating health education

The incubation period of rabies is variable, usually weeks to months, but in extreme cases months to years and it depends on the site of inoculation, viral dose and host (Calle, 2003)

The incubation period of rabies usually 3-8 weeks, rarely as short as nine days or as long as seven years. It depends on the severity of the wound, site of the wound in relation to the richness of the nerve supply and its distance from the brain, and the amount and strain of virus introduced. (CDC, 2004)

2.3.1 Vectors

Kaplan (1969) stated that cats usually serve as secondary hosts and were seldom involved in the intraspecies maintenance of transmission.

Across temperate and subtropical continental Asia both dogs and wildlife serve as reservoir hosts (WHO, 1973).

In India, dogs are the principal vectors of rabies but the disease is maintained by jackals, foxes, mongooses and wolves (Beran, 1981).

Fekadu (1993) opined that dog rabies is still epizootic in most countries of Africa, Asia and South America and in these countries dogs are responsible for most human deaths

Stray dogs play a major role as vectors in the spread of rabies in India (Saseendranath, 1996)

Villa *et al.* (2002) reported that epizootiological data for rabies and the molecular typing of the virus have shown that there are several reservoirs for genotype 1 whose variants remain in nature by independent cycles

2.3.2 Susceptibility

Worldwide dogs remain as the major host of rabies in animals and are intermediate in susceptibility to infection while cats and cattle are highly susceptible. Further foxes of any kind, skunks and raccoons are highly susceptible and play a major role in maintenance of infection in wildlife (WHO, 1973)

Bhatia *et al.* (1988) in their study on canine rabies in and around Delhi for a period of 16-year observed that the younger animals were found to be more

susceptible and the percentage positivity of disease did not vary much between the sexes.

Greene and Dreesen (1998) opined that the degree of species susceptibility varies and that cattle are more susceptible than dog whereas goat and the cats are comparatively resistant.

2.3.3 Transmission

Barnard *et al.* (1982) studied the non-bite transmission of rabies in kudu (*Tragelaphus strepsiceros*) and observed a high susceptibility of kudu to rabies when the virus is applied to their mucous membranes in comparison with cattle and mice wherein they resisted similar exposure.

Transplacental infections in skunks, bats and cows have been reported (Greene and Dreesen, 1990).

Transmission of rabies is nearly always due to the bite of an infected animal that has rabies virus in its saliva. (Greene and Dreesen, 1998)

The usual route of infection is the transdermal inoculation of infected saliva. (Davies and Lowings, 2000a)

Though there are various routes of transmission that include contamination of mucous membranes (i.e., eyes, nose, and mouth), aerosol transmission, and corneal transplantations, the most common mode of rabies virus transmission is through the bite of an infected host-containing virus in its saliva (CDC, 2003).

Bertolini (2004) opined that non-bite exposures include being scratched, or licked over an open wound or mucous membrane or exposure to the brain tissue or cerebrospinal fluid (CSF) of a rabid animal.

Rabies virus transmission can occur among solid organ transplant recipients. (CDC, 2004).

2.4. PATHOGENESIS

Rabies virus binds to nicotinic acetylcholine receptors at the neuromuscular junction and recent studies using nerve-muscle co-cultures indicate that the neuromuscular junction is the major site of entry into neurons (Lentz *et al.,* 1982)

After a bite, the rabies virus enters either unmyelinated nerve fibres or muscle endplates and travels by retrograde axonal transport to the nearest sensory or motor neuron in the dorsal root ganglion or anterior horn of the spinal cord, where it replicates. After replication, the virus may return to the site of the bite by orthograde axonal transport, or may travel along the corticospinal tract to the brain where it infects neurons in almost all brain regions. (Fu, 1997)

Thoulouze *et al.* (1998) stated that the Rabies virus attaches specifically to two other receptors on neuronal cell membranes: the neural cell adhesion molecule and the p75 neurotropin receptor (p75NTR). Two neurotransmitter receptors in the central nervous system, for N-methyl-D-aspartate subtype R1 and gamma amino butyric acid (GABA) have been possible receptors for rabies virus.

Casta *et al.* (2002) studied that the neuronal nicotinic acetylcholine receptor plays in the adsorption process of rabies virus , by using adult dorsal root ganglion dissociated cultures.

Rabies Viral Glycoprotein (G) is responsible for rabies pathogenesis by interacting with neuronal receptor(s), contributes to the high neurotropism of the virus and a mutation on the G, particularly at arginine at position 333, plays an important role in rabies pathogenesis and determines the virulence of rabies virus. (Yan *et al.*, 2002)

Rabies virus spreads from sites of peripheral inoculation to the CNS by fast axonal transport. Rabies virus spreads in peripheral nerves and in the CNS within axons by fast axonal transport at a rate of 12 to 100mm per day. (Jackson, 2003)

Rabies virus virulence is influenced by its glycoprotein envelope. Factors associated with increased virulence experimentally are the presence of the surface amino acid residue arginine-333, the very low external expression of viral glycoprotein on infected cells (Warrell and Warrell, 2004)

2.5. POST EXPOSURE TREATMENT

According to Kaplan *et al.* (1962) the urgency and benefit of wound cleaning is an essential step in the post exposure therapy and proved experimentally, scrubbing with soap and water can increase survival by 50%.

Cho and Lawson (1989) conducted the experimental trial on post exposure vaccination in dogs and indicated that the animals can be protected from rabies by post exposure treatment and the route of exposure and timing of the administration of vaccine and hyper immune serum would seem to be important.

Blancou *et al.* (1991) proved that the post-exposure rabies vaccination with tissue culture vaccine and rabies immune globulin was effective in sheep.

Based on severity and exposures to potentially rabid animals the exposures are classified into category-I, II and III and the post-exposure treatment consists of three important elements, Wound cleaning, Active immunization with vaccine, passive immunization with rabies immune globulin (WHO, 1992)

Clark and Wilson (1996) opined that an effective post exposure rabies prophylaxis schedule for domestic animals includes immediate rabies vaccination, with a minimum of one booster vaccination, and 90 days strict isolation.

Post -exposure treatment should begin as soon as possible (preferably within 24 to 48 h of an animal bite), but it should be initiated even if a lengthy delay has occurred. (Smith, 1996)

Elimination of rabies virus at the site of infection by chemical or physical means as immediate vigorous washing and flushing of bite wound with soap and water, detergent or water alone are the most effective mechanism of protection, with the application of either ethanol (70%) or tincture or aqueous solution of iodine or povidone iodine. (WHO, 1996a)

Basheer *et al.* (1997a) studied the clinical observation in cattle immunized with different post exposure schedules with different anti-rabies vaccines and found that tissue culture anti-rabies vaccine with usual Essen's schedule of post exposure vaccination was effective than other schedules and nervous tissue vaccine.

Basheer *et al.* (1997b) studied the post exposure anti- rabies vaccination in bovines by using three different vaccines with three schedules and concluded that tissue culture rabies vaccine was superior to nervous tissue vaccine in eliciting satisfactory immune response required to protect animals exposed to virulent rabies virus

Goswamia *et al.* (2005) compared the Zagreb intramuscular scheme with the Essen's intramuscular scheme by evaluating four rabies post-exposure regimens in two dog bite centers and four local health centers in India and observed a similar antibody response in Zagreb scheme as that of Essen's scheme with comparatively economical in using four dose of vaccine for post exposure therapy.

2.6. VACCINES

The first modified rabies vaccine for animal use was the low egg passage (LEP) vaccine using Flury strain isolated and adapted through 138 serial intra-cerebral passages in day-old chicks and further modified by 40-50 serial intra-yolk sac passages in embryonating hen's eggs (Koprowski and Cox 1948).

Wiktor *et al.* (1972) compared the rabies virus-inactivating ability of the chemical agents β-propiolactone (BPL) and acetylethyleneimine (AEI) and by physical treatment with ionizing radiation and stated that the vaccine prepared by ionizing radiation was equal or superior to that prepared by β - propiolactone.

Although Pasteur demonstrated the possibility of vaccinating Dogs between1884-1885, it was only in the 1920s that animal vaccination was developed and used in practice. The first vaccine widely used was the Semple type anti-rabies vaccine. (Kieny *et al.,* 1987)

Vaccines with a potency at least 2.5 International Units (IU) per single intramuscular dose should be applied for the Post exposure therapy according to one of the recommended schedules. (WHO, 1992)

The mean potency should not be less than 1.0 IU per single dose that has been shown to be efficacious in all species of animal for which the vaccine is intended. (WHO, 1994)

As like the anti rabies vaccines for use in humans, vaccines for veterinary use have been prepared from cell cultures mainly BHK21 cell line and Vero cell line. (Jayakumar, 1995)

Tissue Culture Vaccine is the most popular among various types of rabies vaccines and has been proved to be effective in various species of domestic animals. (Kariath, 1995)

According to Smith (1996) currently available animal rabies vaccines are potent and safe, and only rarely does rabies occur in a vaccinated animal.

In India, The vaccines for veterinary use are of three different types: a 5% nervous tissue vaccine for post-exposure vaccination, a 20% nervous tissue vaccine for pre-exposure vaccination and tissue culture vaccines for both pre- and post exposure vaccination of animals. All these vaccines are produced in the country. (WHO, 1996b)

In India about one million people each year are injected with rabies vaccine, two-thirds of these receive the Semple vaccine, and about one-third cell culture vaccines. (John, 1997)

2.6.1. Cell culture vaccine

Fenje (1960) reported the preparation of tissue culture anti-rabies vaccine from the hamster kidney cells and their effectiveness in the animals.

The preparation of a rabies vaccine from virus propagated in tissue culture and inactivated by the various chemical agents like phenol, β-propiolactone (BPL) or ultraviolet (UV) light has been reported by Kissling and Reese (1963)

Petermann (1967) used the NIL line of hamster fibroblasts to prepare a rabies vaccine inactivated with BPL and used in cats, dogs and cattle. Several tissue culture vaccines are now available for domestic animals

Larghi *et al.* (1976) studied the replication of seven rabies virus strains (CVS, HEP, PV, ERA, WIRAB, CPZ, and BOLIVAR) in BHK cells and the inactivation dynamics of these strains by beta-propiolactone, acetylethyleneimine, and ethylenimine to find the most immunogenic strain and the most economic and stable inactivating agent for the production of an inactivated tissue culture rabies vaccine for animal use and found that an inactivated, stable, economic, and easy-to-prepare rabies vaccine can be produced in BHK cells by using the PV strain and ethylenimine as an inactivating agent.

Strating *et al.* (1979) tested an inactivated, nonadjuvanted tissue culture-origin rabies vaccine in dogs for its ability to provide protection against challenge of immunity one year after vaccination

Lin *et al.* (1983) proved that the primary hamster kidney cell rabies vaccine (PHKC) are effective and safe from several field trials with both pre and post exposure vaccination.

Larghi and Nebel (1985) studied the duration of immunity produced in cattle by a binary-ethylenimine inactivated tissue culture rabies vaccine and observed the satisfactory level of antibody level maintained up to one year of the study period.

Pay *et al.* (1985) described the process for the production of an inactivated rabies vaccine from BHK 21 suspension cell culture for animal use

Seroconversion of skunks and raccoons intramuscularly vaccinated against rabies with inactivated rabies vaccine was studied by Rosatte *et al.* (1990) and detected the 100% seroconversion on 314 to 757 days post vaccination. Five of six skunks vaccinated in the laboratory-survived challenge with rabies virus 90 days post vaccination.

Ramanna *et al.* (1991a) described the method of preparation and use of tissue culture inactivated rabies vaccine.

Ramanna *et al.* (1991b) used the tissue culture inactivated Rabies vaccine during an outbreak of rabies in cattle and proved to be effective as post exposure vaccination in exposed cattle and prophylacticaly in unexposed cattle in that area.

Palanisamy *et al.* (1992) used the tissue culture inactivated rabies vaccine for the evaluation of immune response in cattle.

Ramanna and Srinivasan (1992) used the tissue culture rabies vaccine to study the serological response in cattle vaccinated against rabies.

The use of vaccines prepared in cell culture should replace those derived from brain tissue as soon as possible. (WHO, 1992)

Inactivated rabies vaccine adjuvanted with aluminium hydroxide provoked a high level of antibody response in cattle and were protected against experimental challenge one year after revaccination (Cortes *et al.*, 1993)

The serological response in cattle vaccinated against rabies by using inactivated cell culture vaccines was studied by Sihvonen *et al.* (1994) and observed

the Rabies neutralizing antibody titre (> or = 0.5 IU/ml) in 80% of 163 animals tested about one month and in 42% of 133 animals tested about one year after primary vaccination and indicated that booster was always necessary after primary vaccination to ensure that all animals were protected.

Dreesen (1997) stated that the rabies vaccines have been developed and used with varying degrees of effectiveness and safety. When used appropriately, new cell culture vaccines provide nearly 100% protection with a high degree of safety.

Reddy and Srinivasan (1997) studied the performance of Aluminium hydroxide gel and oil adjuvanted rabies tissue culture vaccines in bovines and found to be effective in inducing satisfactory neutralizing antibody levels.

Lalosevic *et al.* (1997) indicated that the rabies vaccine from cell culture, were safe, with minimal post-vaccinal reactions

Albas *et al.* (1998) used tissue culture inactivated anti-rabies vaccine in cattle for the assessment of immune response and the used vaccine was constituted of a PV virus, BHK - 21 clone 13 replicated and Bromoethileinemine - inactivated vaccine and adsorbed in aluminium hydroxide gel and observed the protective antibody titre of >0.5 IU/ml from first month of study to 360 days post vaccination.

Kalanidhi *et al.* (1998) used the tissue culture inactivated rabies vaccine for the evaluation of seroconversion and duration of immunity after prophylactic anti-rabies vaccination in camels

Modern rabies vaccines produced on cell cultures or embryonating eggs are safe and effective. (WHO, 2001)

2.6.2. DNA vaccine

A DNA vaccine against rabies, using a plasmid encoding the rabies virus glycoprotein and driven by the SV40 Promoter/Enhancer, was previously evaluated in mice and was shown to induce potent immune responses protecting mice against an intracerebral rabies virus challenge was proved by Xiang *et al.* (1994)

The immune response to the DNA vaccines expressing two forms of glycoprotein, secreted and membrane bound were compared and found to be similar in magnitude and the DNA vaccines produced a long lasting immunity against rabies virus. (Xiang *et al.*, 1995)

Bahloul *et al.* (1998) observed that a single intramuscular injection of DNA plasmid encoding the rabies glycoprotein induce an early, strong and long-lasting production of neutralizing antibodies (>1 IU/ml), as well as specific T helper, T cytotoxic and NK cells resulting in full protection against an intracerebral challenge of rabies virus in mice.

Biswas *et al.* (1999) observed that the DNA rabies vaccine comprising plasmid DNA encoding rabies virus surface glycoprotein protect mice against intracerebellar challenge

Osorio *et al.* (1999) observed that the intra-muscular route of DNA vaccination elicit a stronger and more durable virus neutralizing antibody titres in dogs

Perrin *et al.* (2000) studied the neutralizing antibodies in Dogs that were immunized by intramuscular injection with a plasmid encoding the rabies virus

glycoprotein DNA-based immunization with a plasmid encoding the antigen responsible for inducing protection

The process of combined DNA vaccine involves inoculating a DNA vaccine and a low dose of an inactivated virus vaccine. This can be developed into a novel, cost-effective vaccination strategy for rabies. The DNA vaccines can be produced at a low cost and stored at room temperature, they are ideal for prophylactic immunization and post exposure therapy against rabies in developing countries. (Rangarajan *et al.*, 2000)

Biswas *et al.* (2001a) observed production of stronger and more durable viral neutralizing antibodies in mice and bonnet monkeys after intramuscular inoculation of rabies DNA vaccine.

Biswas *et al.* (2001b) studied the pre-exposure efficacy of a novel combined rabies vaccine containing a low dose of inactivated rabies virus vaccine and DNA rabies vaccine and found to be induced an anamnestic antibody response in mice as well as in cattle.

Lodmell *et al.* (2002a) studied the induction of neutralizing antibody by varying the route and site of DNA vaccination and booster frequency by employing pre and post exposure vaccination in non-human primates and found that enhanced antibody response in DNA vaccine with gene gun method of injection.

Lodmell *et al.* (2002b) studied the comparison of neutralizing antibody responses and protection against rabies virus in non-human primates vaccinated with One-time gene gun or intramuscular rabies DNA vaccine and found that long-term protection of non-human primates against rabies is obtained by using a DNA vaccination protocol that did not include a booster immunization.

Bahloul *et al.* (2003), observed that rabies post-exposure vaccination in mice, based on a single administration of rabies DNA vaccine was as effective as five injections of cell culture-derived vaccine and comparatively evaluated the two rabies post-exposure therapies in mice that were challenged at day 0 with rabies virus and then received either a single dose of rabies DNA vaccine administered at day 0, or five doses of cell culture-derived rabies vaccine administered at days 0, 3, 7, 15 and 28. Both regimens rapidly triggered protective levels of neutralizing antibodies against rabies virus in vaccinated mice

Excellent seroconversion in vaccinated horses after a primary course of two injections of DNA vaccine has produced a very strong impact on both onset and intensity of serological responses was observed by Fischer *et al.* (2003).

Garmory *et al.* (2003) opined that the DNA vaccination is a relatively recent development in vaccine methodology and the strategies may include the incorporation of immunostimulatory sequences in the backbone of the plasmid, co-expression of stimulatory molecules, utilisation of localisation signals, and utilisation of the appropriate delivery system.

Lodmell *et al.* (2003) opined that a single-dose of DNA vaccination to elicit enhanced levels of neutralizing antibody. Intradermal vaccination into ear pinnae elicited elevated and long-lasting levels of neutralizing antibody and could aid in the control of canine rabies in developing countries.

Nel *et al.* (2003) studied the comparison of DNA vaccines for the rabies-related Mokola virus by using different promoters and DNA backbone compositions and found that no cross protection between rabies virus and Mokola virus.

Cupillard *et al.* (2005) proved that a single rabies DNA vaccination fully protected cats against a lethal rabies challenge as early as three weeks post

vaccination and a single rabies DNA vaccination with plasmids containing at least 70% of super coiled molecules triggered significant specific antibody titres and specific Th-1 oriented cell-based immunity as early as two and three weeks post vaccination, respectively.

2.7. ASSESSMENT OF IMMUNE RESPONSE

Detection and quantification of rabies antibodies is intended in the first place for checking the immunity to rabies or effectiveness of rabies vaccines. Detection and quantification of virus neutralisation rabies antibodies in the serum is based on inhibition of rabies infection *in vivo* in animals or *in vitro* in cell cultures (Atanasiu, 1973)

The World Health Organization recommended that the mouse neutralization test (MNT) and the rapid fluorescent focus inhibition test (RFFIT) are the standard techniques for the detection of anti-rabies neutralizing antibodies (Habel, 1996).

Rabies antibodies can be quantified by more than 14 various techniques. At present, classic virus neutralization test on mice – VNT, RFFIT and ELISA test are the most frequently used ones. (Beniek *et al.,* 2000)

Khawploda *et al.* (2005) opined that to evaluate new vaccines or administration schedules investigating immunogenicity and efficacy, the measurement of virus-neutralizing antibody (VNA) against rabies virus was an indispensable and important technology.

2.7.1. Mouse neutralization test (MNT)

Baer *et al.* (1977) inoculated intracerebrally or in the footpad of mice with 11 salivary gland suspensions that differed in their ratios of intracerebral titre to footpad

titre from rabid foxes, skunks, and bobcats and the result indicated that the "invasiveness" of different isolates varies markedly. The inoculation of the less invasive isolates resulted in an appreciable number of permanently paralysed animals with high titres of neutralizing antibody in serum but no neutralizing antibody in the brain, a finding suggesting that virus had invaded only the peripheral nervous system or the spinal ganglia.

Coe and Bell (1977) studied the antibody response elicited in Syrian hamsters vaccinated with inactivated, attenuated, and virulent rabies virus , by using mouse neutralization test

Koprowski (1996) described in detail about the methodology of standard mouse inoculation test.

Webster and Casey (1996) opined that the mouse inoculation test traditionally known as the golden method in diagnosing rabies has been replaced by the virus isolation in cell cultures in many of the laboratories since it is more sensitive, easy to perform, less time consuming and more humane

Katz *et al.* (2000) conducted a survey to assess the efficiency of anti rabies vaccination and the efficacy of different routes of anti-rabies vaccination in cattle and good correlation was observed in tests performed with 45 bovine serum of vaccinated cattle between tissue culture neutralization assay based on enzyme immunoassay – (NTCEIA) and the standard mouse neutralization test.

Arai *et al.* (2002) studied the immunogenicity of a Japanese purified chick embryo cell culture rabies vaccine (PCECV) by determining the Rabies antibody titres by using mouse neutralization test and enzyme-linked immunosorbent assay

(ELISA) from the serum samples obtained from 86 subjects after pre-exposure or post-exposure prophylaxis.

2.7.2. Rapid fluorescent focus inhibition test (RFFIT):

Louie *et al.* (1975) compared the RFFIT with the MNT for measuring rabies antibody and found that the RFFIT was more sensitive, reproducible, convenient and accurate than the MNT and concluded that the RFFIT should be substituted for MNT for measurement of rabies antibody.

Lee *et al.* (1977) studied the comparison of rabies humoral antibody titres in rabbits and humans by Indirect radio immunoassay (RIA), RFFIT, and Indirect fluorescent antibody assay and opined that both RIA and RFFIT effectively differentiated anti-rabies positive sera from anti-rabies negative sera.

Blancou *et al.* (1983) studied comparison of four different serological techniques (MNT, RFFIT, plaque reduction test and immunoenzymatic test) for the determination of antibody levels against rabies virus in vaccinated street dogs and indicated that the other three techniques may each be used as an alternative to the mouse neutralization test for routine titration.

The RFFIT is a significantly better reproducible test system than the MNT and excellently correlated with MNT for the titration of anti-rabies virus neutralization antibodies (Kurz *et al.*, 1986)

Lyng *et al.* (1989) studied the relative potencies of a number of rabies immunoglobulin preparations by using a virus neutralization test in mice (MNT) and a virus neutralization test in cell culture (RFFIT) and indicated that the RFFIT is a more reliable method than the MNT.

Gelosa and Borroni (1990) studied the immunological response in people vaccinated against rabies by using four serological methods, the immunoenzymatic test with HDCV virus vaccine (EIA-V) and with purified viral glycoprotein (EIA-P), the neutralizing in vitro RFFIT and the indirect immunofluorescent test (IFI) and observed that the RFFIT as the most sensitive and specific method for rabies antibodies.

Kitala *et al.* (1990) studied the antibody responses to a conventional rabies pre-exposure regimen of a new purified Vero cell rabies vaccine (PVRV) and a human diploid cell vaccine (HDCV) in human by using the RFFIT and an inhibition enzyme immunoassay (INH EIA) on days 0, 7, 28, and 49 days of vaccination

Serum neutralizing antibody to rabies virus was determined by Tepsumethanon *et al.* (1991) in previously unvaccinated dogs after receiving one subcutaneous dose of inactivated tissue culture rabies vaccine, by employing the rapid immunofluorescent focus inhibition test on 14, 30, 60, 180 and 360 days of vaccination and suggested that one dose of tissue culture vaccine in dogs by the subcutaneous route of injection is not adequate to maintain rabies neutralizing antibody in serum for one year.

RFFIT is highly sensitive *in vitro* test for the detection and quantification of rabies antibodies and it is advantageous because of its less expensive and more rapid and the application of the RFFIT requires an OIE standard (WHO, 1992)

WHO and the Office International des Epizooties (OIE) recognized the RFFIT to evaluate and to certify the level of VNA (>0.5 IU/ml) prior to allowing animals to enter rabies-free countries, which can significantly reduce, quarantine periods (OIE, 1996)

Zavadova *et al.* (1996) studied the titration anti-rabies virus neutralization antibodies in serum by RFFIT method and compared results with virus neutralisation test on mice and indirect haemagglutination test and opined that the RFFIT has many advantages when using highly attenuated strain as the challenge virus in RFFIT method, the potential risk of laboratory exposition is absent.

Cliquet *et al.* (1998) developed a micro-test named the fluorescent antibody virus neutralisation test (FAVN), which is an adaptation of the original RFFIT and the test has the ability to distinguish negative sera from positive sera with low titres much than RFFIT.

Beniek *et al.* (2000) demonstrated the post vaccination rabies antibodies by using the ELISA method and by the RFFIT on days 14, 28, 60 and 180 after immunization with inactivated purified concentrated adjuvant rabies vaccine in cattle.

Strady *et al.* (2000) studied the neutralizing antibody response following pre-exposure rabies immunization in human by using RFFIT.

Ndrejkova *et al.* (2001) studied the detection and quantification of rabies antibodies by an immunoenzymatic assay – ELISA, RFFIT and VNT on mice on days 30 and 90 post-immunization orally in swine using Vnukovo-32/107 vaccination strain

Khawploda *et al.* (2005) described the methodology of a novel rapid fluorescent focus inhibition test for rabies virus using a recombinant rabies virus visualizing a green fluorescent protein and indicated that the novel method is convenient, economical and a reliable tool not requiring instead of expensive FITC-conjugated antibody for routine rabies VNA assays

2.8. SEROLOGY:

Lavender (1973) studied the immune response in adult rhesus monkeys vaccinated with four inactivated rabies vaccines, including two cell culture vaccines, one zonal purified cell culture vaccine, and a 10% extracted duck embryo vaccine.

Haddad (1987) studied the serological response to the efficacy of an anti-rabies vaccine in field dogs.

Shankar *et al.* (1991) stated that neutralizing antibodies play a major role in preventing a fatal rabies virus infection at least upon peripheral challenge, presumably by preventing the virus from entering the central nervous system.

Aubert (1992) opined that the cats and dogs which develop antibodies after anti rabies vaccination and have a very high probability of surviving any challenge, no matter how strong the dose and which virus strain was used

Efficacy of rabies vaccines can be evaluated on the basis of their immunogenic activity or antigenic activity by determination of humoral but also cellular immune response after vaccination of target animals. (WHO, 1992 and OIE, 1996)

titres occurs, but after 90 to 180 days, there is a fast decrease, but maintaining a detectable level of titre for a year

Cabasso *et al.* (1974) studied antibody response to a human diploid cell rabies vaccine with pre- or post exposure immunization

Lee *et al.* (1977) studied the rabies humoral antibodies in rabbits by a single intramuscular injection of inactivated suckling mouse brain rabies vaccine and the primary response to immunization was measured in blood samples taken at selected intervals for 6 months

Diaz and Lambardo (1982) studied the duration of immunity in calves immunized with suckling mouse brain rabies vaccine, and found that calves were protected against the challenge dose virus injected one year after vaccination and indicated that the suckling mouse brain rabies vaccine can be used successfully to immunize calves.

Titoli *et al.* (1982) observed the circulating antibodies against ERA rabies virus in cattle and dogs vaccinated with multiple doses of ERA strain vaccine intramuscularly in the gluteal or masseter region and found to be poor virus neutralizing titre elicited in animals vaccinated in masseter muscle.

Prosperi *et al.* (1983) studied the immune response in vaccinated wild ruminants (22 fallow deer and 10 mouflons) against rabies with an inactivated vaccine and found to be all the animals developed sufficient antibody titres and were protected after 24 months.

Antibody responses to a conventional rabies pre-exposure regimen of a new purified Vero cell rabies vaccine (PVRV) and a human diploid cell vaccine (HDCV)

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Efficacy of rabies vaccines can be evaluated on the basis of their immunogenic activity or antigenic activity by determination of humoral but also cellular immune response after vaccination of target animals. (WHO, 1992 and OIE, 1996)

The G protein is the only rabies antigen that consistently induces virus-neutralizing antibodies and induces a cellular immune response involving both T-helper cells and cytotoxic T cells. (Tordo, 1996)

Hooper *et al.* (1998) proved that the rabies virus neutralizing antibodies was an absolute requirement for clearance of an established rabies virus infection and for the latter to occur in a timely fashion, collaboration between the virus neutralizing antibodies and an inflammatory mechanism is necessary and also indicated that any use of anti-inflammatory agents concomitant with rabies post exposure prophylaxis may lead to delay in the clearance of rabies virus and ultimately to the failure of rabies post exposure treatment.

Rabies vaccines induce an active immune response that includes the production of neutralizing antibodies. This antibody response requires approximately 7-10 days to develop and usually persists for greater than or equal to two years (CDC, 1999).

The principle use of serological testing is a measure of successful vaccination. (Davies and Lowings, 2000a)

2.9. ANTIBODY RESPONSE:

The Serum neutralization antibody and immunoglobulin responses in human were studied by Cho *et al.* (1972) in paired serum samples and opined that the immune reaction consisted of a predominantly immunoglobulin G (IgG) antibody response and also, a significant increase in neutralizing antibody titres was observed.

Netto *et al.* (1973) observed that the patterns in antibody responses after administration of anti-rabies vaccine in cattle as initially a rapid rise in neutralizing

titres occurs, but after 90 to 180 days, there is a fast decrease, but maintaining a detectable level of titre for a year

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Antibody responses to a conventional rabies pre-exposure regimen of a new purified Vero cell rabies vaccine (PVRV) and a human diploid cell vaccine (HDCV)

were compared by Kitala *et al.* (1990) and stated that both vaccines elicited a rapid antibody response.

Blancou *et al.* (1991) observed the efficacy of post exposure vaccination in experimentally infected 68 sheep an found to be effective in 100% of the animals. The animals were given a cell-culture vaccine on the day of infection, then at day three, seven and 14.

Dutta *et al.* (1992) opined that the prophylactic immunization is an important in rabies control and the development of adequate level of antibody (greater than or equal to 0.5 IU/ml serum) is necessary for protection against the disease.

There was no significantly different serological response in cattle administered with Foot and Mouth disease vaccine only, rabies vaccine only or combined foot and mouth disease + rabies vaccine as observed by Palanisamy *et al.* (1992).

Ramanna *et al.* (1991b) reported the protective serum neutralizing antibody titres were observed in cattle thirty days after vaccinated with cell culture rabies vaccine on days 0, 3,7,14 and 28 after bite by a rabid dog.

Ramanna and Srinivasan (1992) studied the serological response in cattle to tissue culture rabies vaccine and observed the presence of satisfactory neutralizing antibody levels after vaccination

The vaccinated animals are protected sufficiently when their level of rabies antibodies equals to or exceeds 0.5 IU/ml (WHO, 1992)

Cortes *et al.* (1993) studied the Immune response in cattle induced by inactivated rabies vaccine and recommended that the optional revaccination of young

animals at six months of age with, annual revaccination should be sufficient to ensure high levels of antibody between vaccination cycles

Good but short-duration of immunity induced in 100% of the 50 semi-domesticated reindeer after administration of one dose of inactivated, adjuvanted rabies vaccine of cell culture origin was observed by Sihvonen *et al.* (1993).

Sihvonen *et al.* (1994) studied the immune response produced cattle vaccinated against rabies using inactivated cell culture vaccines and stated that the booster dose is necessary after primary vaccination to ensure that all animals are protected with the rabies neutralizing antibody titre of (> or = 0.5 IU/ml)

Baltazar and Blancou (1995) examined the humoral immune response of sheep to experimental infection of rabies virus and analyzed the efficacy of vaccination on the day of infection and at 3, 7, 14 and 30 days post-infection

Basheer *et al.* (1997b) studied the immune response in cattle vaccinated with post exposure therapy against rabies with different schedules of anti rabies vaccines and observed the satisfactory neutralizing antibodies for a period of 150 days.

In the post exposure antibody response, a rapid appearance of antibodies of the IgG class is desired, as antibodies of the IgM class do not leave the vessels and thus have difficulty in reaching the locally introduced virus. Immunization with 14 daily doses of vaccine has been shown to prolong IgM antibody production, probably due to the persistence of the antigen. (Grandien, 1997)

Albas *et al.* (1998) studied the humoral immune response produced in 35 bovines after using inactivated rabies vaccine with a booster dose at 30 days after primary vaccination and observed that, 13 (92.8%) animals presented titres of 0.5 IU/ml in ninety days after vaccination. After 180 days, nine 64.3%) animals showed

titres of 0.5 IU/ml, after 270 days, only one (7.1%) showed a titre of 0.51 IU/ml, and after 360 days, all animals showed titres < 0.5 IU/ml.

Katz *et al.* (2000) studied the efficacy of anti-rabies vaccination in calves and cows for the rapid determination of the neutralizing anti-rabies antibody titre by using the neutralization tissue culture enzyme immunoassay (NTCEIA).

Kalanidhi *et al.* (1998) studied seroconversion and duration of immunity after prophylactic anti-rabies vaccination in camels using the tissue culture inactivated rabies vaccine and indicated that protective titres were present for a period of 48 months.

Andrade *et al.* (1999) evaluated the immune response produced by rabies vaccines in new world nonhuman primates and stated that the vaccine produced in NIL-2 cell culture induced high antibody levels in all vaccinated animals and all animals survived the viral challenge.

Beniek *et al.* (2000) evaluated the antigenic activity of the experimental and commercial vaccines in cattle and the immune response tested by RFFIT method after vaccination reveals the protective antibody titre.

Lodmell and Ewalt (2000) evaluated the neutralizing antibody in mice after a single immunization with experimental DNA or recombinant vaccinia virus (RVV) vaccines encoding the rabies virus glycoprotein(G), or the commercially available inactivated virus human diploid cell vaccine (HDCV) and concluded that all three vaccines elicited long-term levels of neutralizing antibody that exceeded 0.5 IU/ml.

Oliveira *et al.* (2000) studied the immune response produced against inactivated and live attenuated vaccine in cattle and stated that high titres were obtained in the cases of booster with inactivated vaccine.

Rodrigues da Silva *et al.* (2000) compared the antibody response by using cell-culture neutralization test and ELISA in cattle after vaccination with an attenuated vaccine and an inactivated adjuvanted vaccine and observed that there were no significant differences between the Virus Neutralisation Antibody titres and seropositivity rates obtained with two vaccines.

Knowlton *et al.* (2001) studied the serological responses of coyotes vaccinated with two commercial rabies vaccines by testing for rabies virus neutralizing antibodies with the rapid fluorescent focus inhibition test (RFFIT) at 30, 90, 180, 270, and 365 days post-vaccination.

The determination of an antibody response after immunization against rabies is an acceptable index of the efficacy of vaccine and successful treatment in both animals and humans. Among the different antibodies elicited after immunization, those specific for the virus glycoprotein (neutralizing antibodies) are considered the most important to provide protection (Bordignon *et al.,* 2002).

Piza *et al.* (2002) analyzed the association among potencies of rabies vaccines tested by the NIH test, the contents and form (virus-attached total-glycoprotein or free-soluble) of rabies glycoprotein (G) in the vaccines, and the VNA titres elicited in cattle. They observed that the quantification of virus-attached rabies glycoprotein has a strong correlation with VNA elicited in cattle.

Takayama *et al.* (2002) studied the anti-rabies antibody titters in human vaccinated with rabies post- exposure prophylaxis with foreign-made rabies vaccines at the beginning and followed with Japanese rabies vaccine

The epidemiological aspects and immune response in patients attacked by domestic and wild animals submitted to post-exposure rabies treatment was studied

by Ayres (2003) and suggested that the post-exposure rabies treatment induced significant alterations in the patients' immune response characterized by increase in cytokine and serum levels of specific antibodies to rabies virus

Cardoso *et al.* (2004) studied the indirect immunoperoxidase virus neutralization (IPVN) and MNT to detect antibodies against rabies virus from vaccinated dogs and cattle to measure 0.5 IU /ml of antibody required by the World Health Organization and the Office International des Epizooties as the minimum response for proof of rabies immunization.

2.10. MONITORING:

Beniek *et al.* (2000) opined that in cattle the rate of immunity onset after vaccination and preservation of sufficient levels of specific antibodies or cellular immunity for a minimum of six months is important

In post exposure treated patients, a survival assessment at six months is sufficient to establish the efficacy of rabies vaccine. (Quiambao *et al.*, 2004)

2.11. CONTROL:

Cattle are the most frequent livestock species infected with rabies. To control rabies in Cattle in endemic areas, dog rabies in these areas should be eliminated. In endemic areas of wildlife rabies, animals at risk should be vaccinated (Greene and Dreesen, 1990).

Wandeler *et al.* (1993) opined that the domestic-dog strains of rabies virus account for more than 90% of human disease worldwide and the rabies in stray dogs can be reduced by parenteral vaccination, fertility control, and clearing rubbish to reduce the food supply.

Narayanan (1995) stated that epidemiological surveillance, community education and participation, immunization, dog control, organization and implementation are the elements of control of rabies

Since the dog is the principle reservoir of rabies in India and is responsible for 96% of persons undergoing anti-rabies treatment, all effort must be made to control the rabies in dogs. (Sahu, 1995).

Local programs of vaccination of dogs and cats, restriction of movement (leash laws), and removal of stray or unwanted animals are very effective measures of rabies control. (Smith, 1996)

Singh *et al.* (1999) indicated that the animal and human vaccines provide efficient weapons for the prevention and control of rabies.

Rabies can be effectively prevented following a recognized exposure in humans with wound cleansing, immunization with cell culture rabies vaccine, and by administration of human rabies immune globulin (Jackson, 2000)

In much of the developed world the use of modern vaccines has led to effective rabies control in domestic animals and man, although reservoirs of disease remain in a number of wildlife species. (Davies and Lowings, 2000b)

Rabies control in dogs remains the only long-term, cost-effective means of eliminating or preventing most human cases. Human public health preventive measures should be paralleled by programmes for dog rabies control. (WHO, 2001)

To save human lives the most cost-effective and foremost step would be educating the general public and healthcare physicians regarding prompt, appropriate wound care and rabies post exposure therapy. (Parviza *et al.,* 2004)

Materials and methods

3. MATERIALS AND METHODS

The present study was conducted at the Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Sciences, Mannuthy, Thrissur during the period from January 2004- January 2005

3.1. GLASSWARE AND CHEMICALS

The glassware used in the study was made of Borosil brand and the chemicals were of laboratory grade. The materials were processed using standard methods (Hoskins, 1967) and sterilized either in hot air oven or autoclaved depending upon the materials to be sterilized.

3.2. BIOLOGICAL MATERIALS

3.2.1. Experimental animals

Eighty-two cattle were subjected to the study, which were reported with the history of suspected rabid animal exposure in and around Thrissur district. They were grouped randomly in to four groups as 23 animals in group one, 21animals in group two, 15 animals in group three, and 23 animals in group four.

3.2.2. Vaccines.

Following anti rabies vaccines were used.(Fig. 1.)

3.2.2.1. Inactivated tissue culture anti-rabies vaccine (Raksharab)

Anti-rabies vaccine having the potency of 2.5 IU/ml prepared from fixed rabies virus (CVS-11) grown on BHK-21 cell lines and inactivated by aziridine, adjuvanted with aluminum hydroxide, manufactured by M/s., Indian Immunologicals Ltd., Hyderabad.

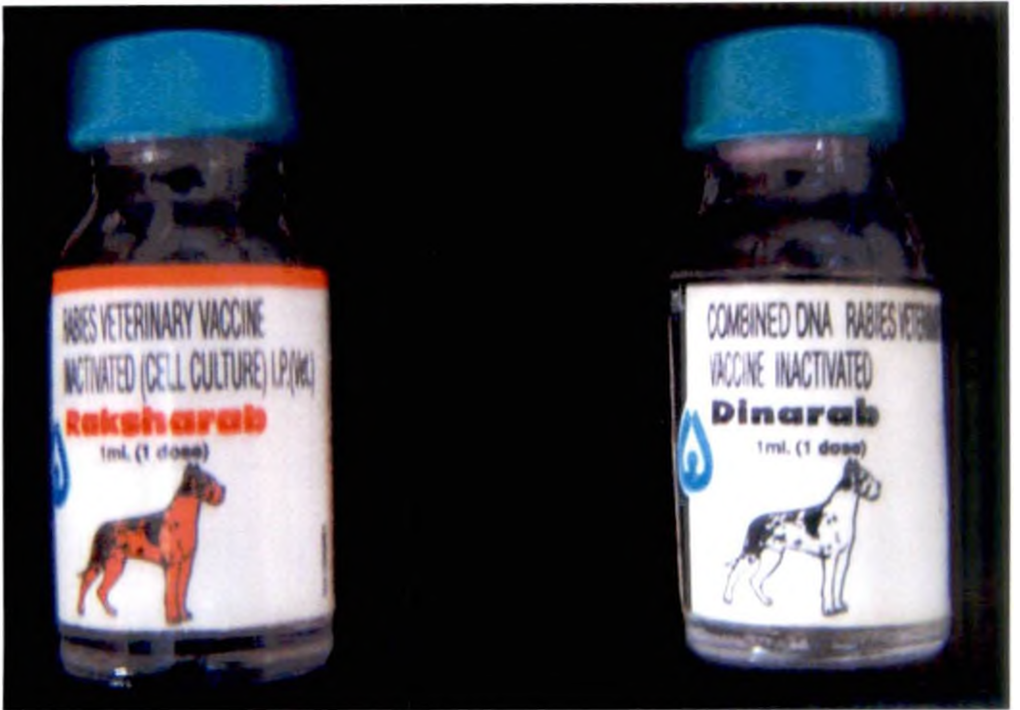

Fig.1.Anti-rabies vaccines: Raksharab and Dinarab

3.2.2.2. DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab).

The anti-rabies vaccine having the potency of >2.5 IU/ml adjuvanted with aluminum hydroxide gel manufactured by M/s. Indian Immunologicals Ltd., Hyderabad.

3.2.3. Rapid fluorescent focus inhibition test –RFFIT.

3.2.3.1. Reagents

3.2.3.1.1. Diagnostic conjugates: Fluorescein isothiocyanate (FITC) conjugated anti rabies antibodies. (Bio-Rad,USA)

3.2.3.1.2. Acetone: AXO120-6 (500ml) GR ACS,stored at -20°C.

3.2.3.1.3. Gelatine

3.2.3.1.4. Disinfectant: Lysol I.C., (diluted 1: 256).

3.2.3.1.5. Cell culture Media (a-e)

a. Distilled water: Distilled deionized water, sterile.

b. Foetal bovine serum: Foetal Bovine Serum (500ml), 40nm filtered.

c. Minimum Essential Medium: Minimum Essential Medium (MEM) (10X).

500ml liquid,contains Earle's salts but no L-glutamine or sodium bicrbonate.

Components	Concentration (mg/L)
Calcium chloride (CaCl2) (Anhyd.)	2000.00
Potassium chloride (KCl)	4000.00
Magnesium Sulfate (MgSO4)	976 .70
Sodium chloride (NaCl)	68000.00
Sodium phosphate –H2O (NaH2PO4-H2O)	1400.00

Other components	
D-glucose	10000.00
Phenol Red	100.00
Amino acids	
L-Arginine hydrochloride	1260.00
L-Cystine -2Na	286.00
L- Histidine – HCl –H2O	420.00
L- Isoleucine	520.00
L- Leucine	520.00
L- Lysine hydrochloride	720.00
L- Methionine	150.00
L- Phenylalanine	320.00
L- Threonine	480.00
L- Tryptophan	100.00
L- Tyrosine – 2Na -2H2O	520.00
L- Valine	460.00
Vitamins	
D- Ca Pantothenate	10.00
Choline chloride	10.00
FolicAcid	10.00
I – inositol	20.00
Niacinamide	10.00
Pyridoxal HCl	10.00
Riboflavin	1.00
ThiamineHCl	10.00

d. Antibiotic – Antimycotic: (100x), (Invitrogen life technologies, Gibco) Contains - 10,000 units of penicillin (base), 10,000µg of streptomycin sulfate, Amphotericin B as Fungizone Antimycotic in 0.85 %saline.

e. Sodium bicarbonate; sodium bicarbonate solution, 7.55 (w/v).

3.2.3.1.6. Trypsin: Trypsin- EDTA 0.05 per cent Trypsin, 0.053 mM EDTA 4Na (10x),

3.2.3.1.7. Phosphate buffered saline (PBS): Two PBS formulas are for RFFIT:

a. For rinsing cell monolayer, Ca_2+ and Mg_2+ free PBS, 0.01m, pH =7.40

Formulation:

Sodium chloride	8.0	gm
Potassium chloride	0.2	gm
Sodium phosphate dibasic anhydrous	1.15	gm
Potassium Phosphate Monobasic anhydrous	0.21	gm
Water reagent-grade Type I, QS	1000.0	ml

Adjusted to pH 7.4 with Hydrochloric acid.

b. For immunoflourescence.0.01M, pH 7.4 – 7.6, pH =7.50 ± 0.1

Formulation:

Sodium chloride...............................8.50 gm

Potassium phosphate monobasic...........0.23 gm

Potassium phosphate Dibasic ....1.46 gm

Water, Reagent – Grade Type I, qs.........1000.0ml

Adjusted to pH 7.50 with Hydrochloric Acid or Sodium Hydroxide.

3.2.3.1.8. Dimethylsulfoxide (DMSO): - for cryopreservation of cell line stored at 2-8°C.

3.2.4. Supplies

3.2.4.1. Lab-tek chamber slides with coverslip.

Lab-Tek chamber slide with cover glass 8-well slide, sterile.

3.2.4.2. Sterile Plasticware and glassware

3.2.4.2.1. Disposable pipette tips: Ranin pipette tips for pipetman, 200µl and 1000µl

3.2.4.2.2. Serological pipette: Serological pipette, 5ml, 10ml, and 25ml, plystyrene, nonpyrogenic, sterile, individual packaged.

3.2.4.2.3. Cell culture flask: Flask, 25 cm^2, 150 cm^2, cell culture flask, treated, non-pyrogenic, polystyrene, and sterile.

3.2.5. Equipment

3.2.5.1. CO_2 incubator: CO_2 water-jacketed incubator.

3.2.5.2. Water bath: set point temperature 56°C.

3.2.5.3. Inverted microscope: Nikon model.

3.2.5.4. Fluorescent microscope: Zeiss, Axioskop with 160x or 200x magnification.

3.2.6. Standards and Reference

3. 2.6.1. Mouse neuroblastoma cells:

3. 2.6.2. Rabies challenge virus:

3. 2.6.3. Reference serum standard:

The standards and references kept at M/s. Indian Immunologicals Laboratory, Hyderabad were used for the present study.

3.3. METHODS:

3.3.1. Selection of animals:

Cattle reported with history of rabid animal bite in and around Thrissur district were subjected to this study. Selection of animal was based on the exposure with rabid animal. Exposures include being bitten by rabid animal or suspected to be rabid. Non-bite exposure like mucous membrane contamination with infected saliva.

3.3.2. Post exposure therapy:

3.3.2.1. Wound management: advocated for thorough wound cleaning with soap and water and application of antiseptics like iodine solution or 70% alcohol.

3.3.2.2. Immunization:

Post exposure immunization was carried out in all the four-study group of cattle in two different schedules with two vaccines.

3.3.2.2.1. Schedule of vaccination:

The schedule-I and II were derived based on preliminary studies conducted in the Department of Veterinary Epidemiology and Preventive Medicine,

Schedule – I: Essen's Schedule of post exposure regimen:

1st dose: On 0 day (day of first injection)
2nd dose: 3rd day
3rd dose: 7th day
4th dose: 14th day
5th dose: 28th day

Schedule- II:

1st dose: On 0 day (day of first injection)

2nd dose: 1st day

3rd dose: 2nd day

4th dose: 3rd day

5th dose: 4th day

Exposed cattle were randomly allotted in to four groups.

Each animal was vaccinated with 1 ml of either vaccine deep intramuscularly as follows,

Group-1 (23 Animals): Inactivated Tissue culture anti-rabies vaccine (Raksharab) - Schedule I

Group-2 (21Animals): DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) - Schedule I

Group-3 (15Animals): Inactivated Tissue culture anti-rabies vaccine (Raksharab) - Schedule II.

Group-4 (23 Animals): DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) - Schedule II

3.3.3. Collection of serum samples:

Blood samples were collected from all the vaccinated animals in both the groups on the day of immunization (0 day) and on 7th, 14th, 28th, 60th and 90th days of vaccination. From the collected blood samples, serum was separated and labeled accordingly with group and schedule details. Serum samples were inactivated at 56°C

for 30 minutes in water bath and stored in freezer at -20°C until tested for the rabies virus neutralizing antibodies.

3.3.4. Procedure for Rapid Fluorescent Focus Inhibition Test –RFFIT.

Rabies virus neutralizing antibodies were assessed by the Rapid fluorescent focus inhibition test- (RFFIT) as described by Smith *et al.* (1996).

3.3.4.1. Dilution of test serum in the Minimum Essential medium-10:

Serum end point titration were tested at 8 serial five fold dilutions in MEM-10 using an 8 well Tissue –Tek slide.0.075 ml of MEM-10 added in the first well by using micro titre pipette and 0.1ml of MEM-10 to the seven other wells of the slide.0.05ml of test serum was added to the first well (1:5 dilution) and mixed several times, then transferred 0.025 ml of the 1:5 dilution to the second well and continued the transformation to consecutive wells up to the final well with the dilution of 1:390625 and discarded 0.025 ml at the end.(Fig. 2)

3.3.4.2. Preparation of control slide:

Control slide prepared by using standard reference serum control, a virus back titration and a cell control.0.075 ml of MEM-10 was added to the first well of reference serum dilution on the left of the slide and 0.1 ml MEM-10 of to the remaining wells of the reference serum dilution wells (1:25 to 1:625) and to the three wells of the back titration. The cell control well received 0.2 ml of MEM-10 and the 0.05 ml of reference serum containing 2.5 IU/ml is added to the 1:5 dilution well on the bottom left of the slide (well-1) mixed several times and transferred 0.025 ml 1:5 dilution of reference serum (well-1) to the 1:25 dilution well (well-2) and continued through the 1:635 dilution well (well-4) discarded 0.025 ml at the end.

3.3.4.4. Preparation of challenge virus and back titration:

1. The amount of virus used in the test has 50 FFD_{50} /0.01ml.(FFD - Fluorescent foci doses)
2. 2 -serial 10 fold dilutions of CVS-11 from the 50 FFD_{50} /0.1ml as 5 FFD_{50} and 0.5 FFD_{50} /0.1ml was made by using MEM-10 as a diluent.
3. 0.1 ml of the 0.5 FFD_{50}, 5 FFD_{50}, and 50 FFD_{50} of virus is added in sequential chambers.
4. 0.1 ml of virus preparation containing 50 FFD_{50} / 0.1 ml is added to all chambers of the test sera and reference serum dilutions.
5. All the slides were incubated for 30 minutes at 37°C in a CO_2 incubator with 0.5 % CO_2.

3.3.4.5. Preparation of the mouse neuroblastoma (MNA) cells:

Suspension of 10 ml of MEM-10 is transferred to a 25 ml conical centrifugal tube(Fig.3) and counted the cells by using haemocytometer 0.2 ml of $6x10^5$ cells/ml is added to each chamber of the slide, starting with the cell control well on the bottom right corner of the control slide. Then the slides were incubated for 20 hrs at 37°C in a CO_2 incubator with 0.5 % CO_2.

3.3.4.6. Fixation of slides:

After incubation, slides were taken and discarded the medium in virucidal solution. The slides were rinsed once in PBS and then fixed with cold acetone (-20°C) for 10 minutes at room temperature. After that acetone was removed and the slides were air-dried at room temperature.

Fig.2. Diluted serum samples in an eight well Lab-Tek chamber slide

Fig.3. Mouse neuroblastoma cells suspended in MEM-10 in a trypsinization flask

3.3.4.7. Staining of slides:

1. Diluted Rabies conjugate (Fig.4) was added to each chamber of the monolayer sufficient to cover the entire monolayer.
2. Slides were incubated in moist chamber at 37°C for 30 minutes.
3. After incubation conjugate was discarded from the slides and rinsed with PBS (4558) for 10 minutes.
4. Rinsed slides were placed in the slide holder for reading with fluorescent microscope. (Fig.5)

3.3.5. Interpretation:

1. Each of the 8-well Tissue-Tek slides chambers contains 25 to 50 distinct microscopic fields when observed at 160-200-x magnification.
2. In each chamber 20 microscopic fields were observed for the presence of fluorescing cells and counted the number of fields containing fluorescing cells. (Fig.6 & 7).
3. By comparing the control slide and test serum values, the test serum end-point titre and international units were calculated.

3.3.6. Statistical analysis:

Statistical analyses of the results obtained were done by students Paired t-test and Kruskal Wallis methods as per Snedecor and Cochran (1994).

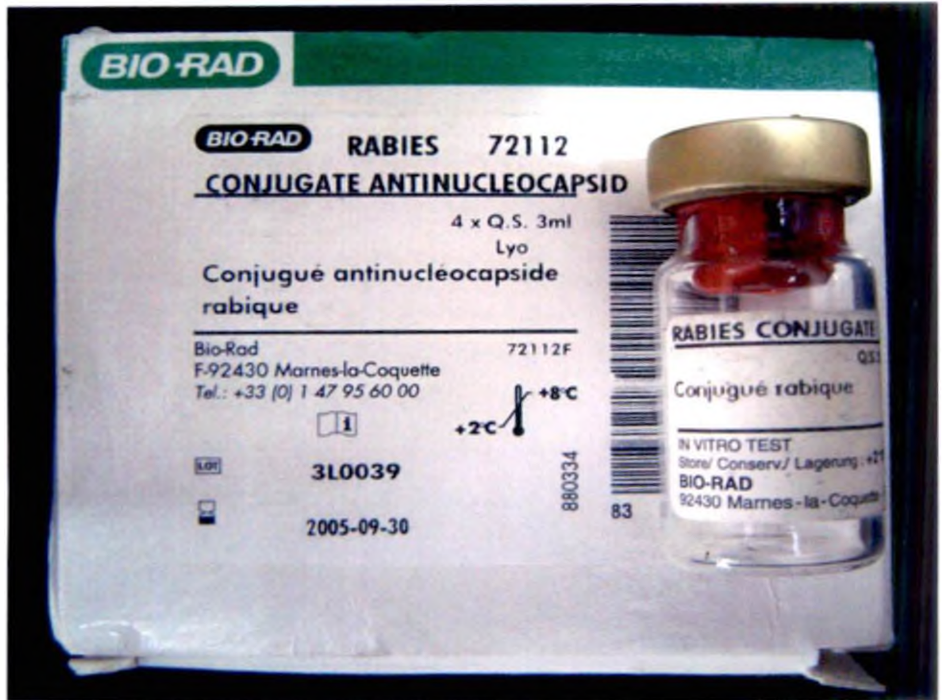

Fig. 4. The fluorescein isothiocyanate conjugated anti-rabies serum

Fig. 5. Fluorescent microscope attached to the computer

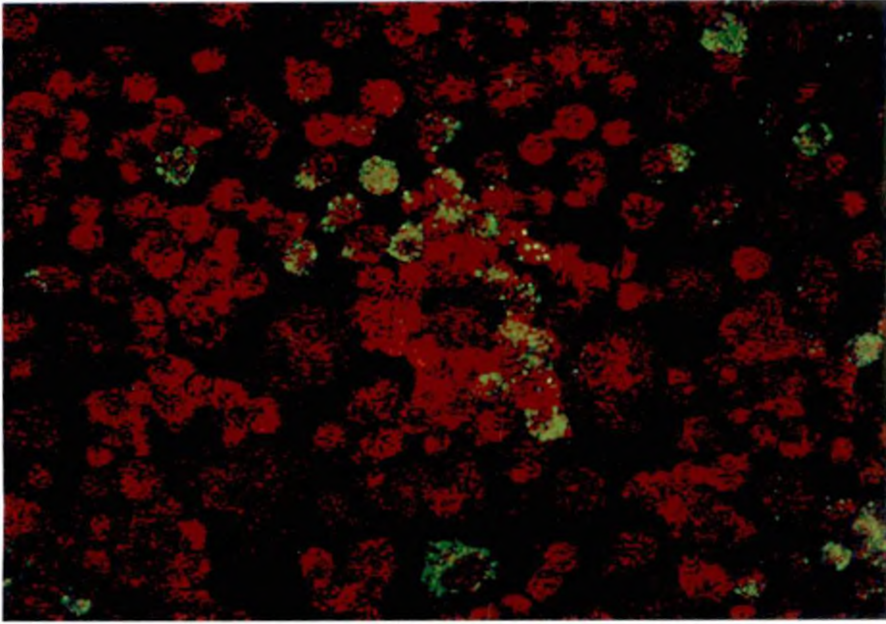

Fig. 6. Fluorescent foci observed under a fluorescent microscope

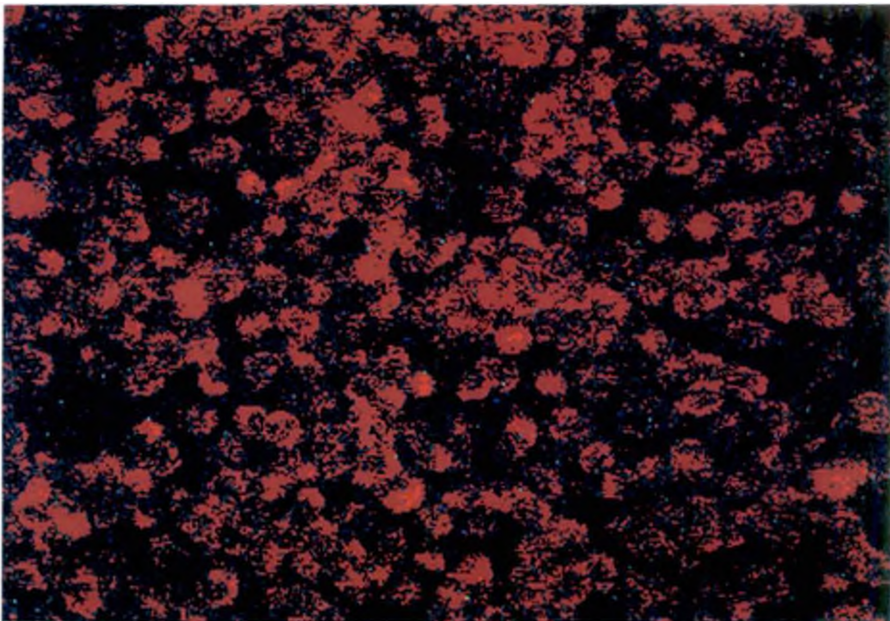

Fig. 7. No fluorescent foci observed under a fluorescent microscope

Results

4. RESULTS

All the serum samples collected from the study animals were subjected to Rapid fluorescent focus inhibition test (RFFIT) for estimation of rabies virus neutralizing antibody titres. By comparing the test serum with the reference control serum, the values were calculated in IU/ml. The obtained antibody titres are presented in Table (1 to 4).

Comparison of results within a group was done by students Paired t-test and the P-values obtained are presented in table (5). As the numbers of study animals were differed in each group with randomised selection of animals, the statistical analysis of results between groups were done by Kruskall Wallis method and presented in table (6).

4.1. IMMUNE RESPONSE PRODUCED FOLLOWING POST EXPOSURE VACCINATION IN FOUR DIFFERENT GROUPS.

4.1.1. Group-I

The rabies virus neutralizing antibody titres of all the animals belonging to group I from the day zero to 90th day are presented in table (1). The Geometric mean titres of group-I cattle on day zero was 0.02 IU/ml and raised to 1.38 IU/ml on 90th day of post vaccination. The highest mean antibody titre of 19.08 IU/ml was obtained on the 14th day of post vaccination.

There was a significant rise in rabies antibody titre ($P < 0.01$) from 0.14 IU/ml to 19.08 IU/ml from the 7th day to 14th day of study. But significant ($P<0.5$) reduction of mean rabies antibody titre was observed from 14th to 90th day. The mean rabies antibody titre of 7.97 IU/ml on 28th day was significantly ($P<0.5$) lowered to 3.83

IU/ml on 60th day. The reduction of antibody titre was observed between 60th and 90th day also, though the titre was above protective level.

4.1.2. Group-II

The rabies virus neutralizing antibody titres of group-II animals are shown in table (2). The mean antibody titres of group-II animals on day zero was 0.01 IU/ml and the mean titre during 90th day was 1.69 IU/ml. The highest mean antibody titre of 21.28 IU/ml was observed on 14th day of study.

There was a significant rise in rabies virus neutralizing anti body titres ($P < 0.01$) from 0.23 IU/ml to 21.28 IU/ml on the 7th day to 14th day of post vaccination. There was a significantly high fall ($P < 0.01$) in mean rabies antibody titre from 21.28 IU/ml to 5.28 IU/ml on the 14th day to 28th day and from 5.28 IU/ml to 2.86 IU/ml ($P < 0.05$) on the 28th day to 60th day of study. (Table 5)

4.1.3. Group-III

The rabies virus neutralizing antibody titres of group-III animals are presented in table (3). The mean antibody titres of group-III animals before first injection of vaccine was 0.01 IU/ml and the mean titre during 90th day was 0.83 IU/ml. The peak mean antibody titre of 20.89 IU/ml was observed on 14th day of post vaccination.

There was a highly significant increase in mean antibody titres ($P < 0.01$) from 0.13 IU/ml to 20.89 IU/ml from the 7th day to 14th day of study. A significant fall ($P < 0.01$) in mean antibody titre from 20.89 to 2.91 during the 14th day to 28th day and from 2.91 IU/ml to 1.18 IU/ml ($P < 0.05$) during the 28th day to 60th day of study as shown in table (5). The antibody titre remained above protective level even on 90th day.

4.1.4. Group-IV.

The rabies virus neutralizing antibody titres of group-IV animals are presented in table (4). The mean rabies antibody titres of group-IV animals before first injection of vaccine was 0.01 IU/ml and the mean titre on 90th day was 0.71 IU/ml. The peak mean antibody titre of 27.61 IU/ml was observed on 14th day of post vaccination.

There was a highly significant increase in mean antibody titres ($P < 0.01$) from 0.15 IU/ml to 27.61 IU/ml on the 7th day to 14th day of study. A significant fall ($P < 0.01$) in mean antibody titre from 27.61 IU/ml to 3.50 IU/ml on the 14th day to 28th day and from 3.50 IU/ml to 1.23 IU/ml ($P < 0.01$) on the 28th day to 60th day of study was observed. (Table, 5)

4.2. COMPARISON OF ANTIBODY TITRES BETWEEN GROUPS.

The comparisons of rabies virus neutralizing antibody titres of animals of all the four groups are presented in table (6) and Fig. (12 & 13). In all the four groups the peak mean antibody titre was observed on 14th day of post vaccination.

4.2.1. Group I and Group II

There was no significant difference between the groups I and II during the entire period of study (both the groups were vaccinated with schedule - I)

4.2.2. Group I and Group III

No significant difference ($P < 0.05$) was observed in between the groups I and III, on zero, seven, 14th and 90th day of observation. There was significant ($P < 0.01$) difference in the mean antibody titre of group-I and III on 28th and 60th day of study.

4.2.3. Group I and Group IV

No significant difference ($P < 0.05$) was observed in between the groups I and IV, on zero, seven and 14th day of observation. There was significant ($P < 0.01$) difference in the mean antibody titre of group-I and IV on 28th and 60th and 90th day ($P < 0.05$) of study.

4.2.4. Group II and Group III

No significant difference ($P < 0.01$) was observed in between the groups II and III, on zero, seven and 14th day of observation. There was significant ($P < 0.01$) difference in the mean antibody titre of group-II and IV on 28th and 60th and o 90th day ($P < 0.05$) of observation.

5.2.5. Group II and Group IV.

No significant difference ($P < 0.05$) was observed in between the groups II and IV on zero, seven and 14th day of observation. There was significant ($P < 0.01$) difference in the mean antibody titre of group-II and IV on 28th, 60th and 90th ($P < 0.05$) day of study.

5.2.6. Group III and Group IV

There was no significant difference between the groups III and IV during the entire period of study. (Both the groups were vaccinated with schedule - II)

4.3. COMPARISON OF IMMUNE RESPONSE PRODUCED FROM SCHEDULE I AND SCHEDULE II.

Schedule I and Schedule II used in this study were effective in inducing a protective immune response. In both schedules detectable level of antibody titre were observed from day seven and maintaining the mean rabies virus neutralizing antibody

titre above the protective level of titre (>0.5 IU/ml.) up to the 90th day of the study period.

In both set of groups (I, II and III, IV) which were vaccinated with schedule I and schedule II respectively obtained the peak level of antibody titre on 14th day of study period and there was no greater difference in the pattern of immune response in all the group of animals vaccinated with both the schedules.

During the entire period of study, immune response produced from group I and II, which were vaccinated with schedule I were comparatively higher than that of group III and IV, which were vaccinated with schedule II except on14th day, group IV (schedule II) got the higher antibody titre level (27.61 IU/ml) than that of group I (19.08 IU/ml) and II (21.28 IU/ml) which were vaccinated with schedule I.

4.4. PROTECTION ATTAINED BY VACCINATION:

The number of animals obtained the protective antibody titre (0.5 IU/ml) in all the four groups are presented in table (7).

4.4.1. Group-I

The mean rabies virus neutralizing antibody titres (IU/ml) of group-I animals and protective level required are shown in Fig. (8).The mean antibody titres of group-I animals were above protective level from 14th to 90th day of study period. Out of 23 animals five animals (21.74%) showed the protective antibody titre of >0.5 IU/ml at 7th day (Table 7). From 14th day onwards all the animals in this group maintained protective level till the end of the study.

4.4.2. Group-II

The mean rabies virus neutralizing antibody titres (IU/ml) of group-II animals are presented in Fig. (9) along with the antibody level required for protection. The mean antibody titres of group-II animals were above protective level from 14th to 90th day of study period. Out of 21 animals in group-II the required protective antibody level of >0.5 IU/ml was observed in five animals (23.81%) on 7th day of post vaccination. Out of 21 animals 20 retained the protective titre up to the 90th day of post vaccination. (Table 7).

4.4.3. Group-III

The mean rabies virus neutralizing antibody titres (IU/ml) of group-III animals with protective level required are plotted in Fig. (10).The mean antibody titres of group-III animals were above protective level from 14th to 90th day of post vaccination. In group-III out of 15 animals, three (20%) were shown protective antibody level at 7th day of post vaccination. From 14th day onwards all the animals in group III maintained protective titre till the end of the study. (Table 7).

4.4.4. Group-IV.

The mean rabies virus neutralizing antibody titres (IU/ml) of group-IV animals with protective level are presented in table (4) and Fig. (11).The mean antibody titres of group-IV animals were above protective level from 14th to 90th day of post vaccination. Out of 23 animals, five animals (21.74 %) shown the protective antibody titre of >0.5 IU/ml at 7th day and five animals (21.74 %) shown the antibody level lower (<0.5 IU/ml) than that of required level for protection on 90th day of study. (Table 7).

4.5. OBSERVATION OF TREATED ANIMALS.

All the vaccinated animals were observed for the period of six months from the day of first vaccination for the development of any signs suggestive of rabies. Six months after exposure, all the eighty-two cattle were alive and no animal had succumbed to rabies or died of other causes. The vaccines used were well tolerated by all the animals. There was no serious adverse effect caused by the vaccine. All the animals were found to be healthy in the monitoring period of six months.

Table.1. Rabies virus neutralizing antibody titres (IU/ml) of Group-I.

S.NO	ANIMAL. NO	0-DAY	7th -DAY	14th -DAY	28th -DAY	60th -DAY	90th -DAY
1	BT1-I	0.01	0.56	16.59	12.88	13.8	2.81
2	BT2-I	0.01	0.56	9.33	12.88	2.81	2.81
3	BT3-I	0.11	0.51	2.81	3.54	2.57	0.51
4	BT4-I	0.45	0.51	69.18	64.56	2.88	2.81
5	BT5-I	0.01	0.1	4.78	0.95	0.51	0.51
6	BT6-I	0.07	0.41	20.89	2.81	1.25	0.56
7	BT7-I	0.32	0.09	38.9	3.01	1.99	0.56
8	BT8-I	0.45	0.61	69.18	13.8	13.8	2.81
9	BT9-I	0.01	0.06	7.58	3.98	2.81	0.56
10	BT10-I	0.01	0.1	30.9	57.54	4.78	1.25
11	BT11-I	0.01	0.32	64.56	3.01	12.88	2.81
12	BT12-I	0.01	0.11	69.18	48.97	11.48	2.81
13	BT13-I	0.1	0.21	69.18	3.38	2.81	2.81
14	BT14-I	0.01	0.39	44.66	13.8	13.8	2.81
15	BT15-I	0.01	0.01	64.56	64.56	2.81	0.95
16	BT16-I	0.01	0.13	10	2.81	2.81	2.81
17	BT17-I	0.01	0.12	64.56	16.98	13.8	6.3
18	BT18-I	0.01	0.01	2.51	0.56	2.29	0.51
19	BT19-I	0.01	0.45	3.8	8.12	0.95	0.56
20	BT20-I	0.01	0.28	2.81	3.16	0.51	0.51
21	BT21-I	0.01	0.43	69.18	57.54	13.8	2.81
22	BT22-I	0.01	0.01	2.18	2.29	2.81	0.51
23	BT23-I	0.01	0.01	64.56	13.8	13.8	2.81
GMT		**0.02**	**0.14**	**19.08**	**7.97**	**3.83**	**1. 38**

GMT: Geometric mean titres.

Table.2. Rabies virus neutralizing antibody titres (IU/ml) of Group-II.

S.NO	ANIMAL NO	0-DAY	7th -DAY	14th -DAY	28th -DAY	60th -DAY	90th -DAY
1	BD1-I	0.01	0:11	13.8	2.81	2.81	0.95
2	BD2-I	0.01	0.11	2.81	15.13	2.29	1.25
3	BD3-I	0.01	0.11	11.48	0.54	0.56	3.38
4	BD4-I	0.01	0.56	17.78	20.89	13.8	13.8
5	BD5-I	0.01	0.01	64.56	12.88	2.81	2.81
6	BD6-I	0.01	0.25	48.97	1.25	0.95	0.56
7	BD7-I	0.01	0.12	64.56	12.88	13.8	6.3
8	BD8-I	0.01	0.11	40.73	2.81	0.56	0.56
9	BD9-I	0.01	0.25	40.73	12.88	2.57	0.51
10	BD10-I	0.01	0.14	13.8	3.38	2.81	2.29
11	BD11-I	0.01	2.81	30.9	2.81	0.56	0.11
12	BD12-I	0.01	1.62	0.85	6.3	3.98	2.29
13	BD13-I	0.01	0.25	30.9	13.8	2.81	2.81
14	BD14-I	0.01	0.06	2.29	2.81	2.57	0.56
15	BD15-I	0.01	0.01	69.18	12.88	2.81	1.25
16	BD16-I	0.01	0.32	26.3	11.48	13.8	2.81
17	BD17-I	0.01	0.28	25.7	2.81	2.81	2.57
18	BD18-I	0.01	0.39	19.49	0.95	13.8	2.81
19	BD19-I	0.01	2.81	36.3	2.81	2.81	2.57
20	BD20-I	0.01	0.56	51.54	12.88	2.81	2.57
21	BD21-I	0.01	0.39	64.56	12.88	2.81	2.81
GMT		**0.01**	**0.23**	**21.28**	**5.28**	**2.86**	**1.69**

Table.3. Rabies virus neutralizing antibody titres (IU/ml) of Group-III.

S.NO	ANIMAL NO	0-DAY	7th -DAY	14th -DAY	28th -DAY	60th-DAY	90th-DAY
1	BT1-II	0.01	0.12	13.8	1.62	0.95	0.95
2	BT2-II	0.01	0.15	36.3	13.8	2.81	1.25
3	BT3-II	0.01	2.81	11.48	2.81	0.95	0.56
4	BT4-II	0.01	0.79	23.98	2.81	1.99	0.56
5	BT5-II	0.01	0.14	25.7	2.81	0.56	0.51
6	BT6-II	0.01	0.11	57.54	13.8	2.57	0.56
7	BT7-II	0.01	0.07	11.74	2.81	0.95	0.51
8	BT8-II	0.01	0.39	16.98	2.57	1.62	0.56
9	BT9-II	0.01	0.11	10	0.95	0.95	0.51
10	BT10-II	0.01	1.25	11.48	2.81	2.81	2.29
11	BT11-II	0.01	0.12	12.3	1.62	0.67	1.25
12	BT12-II	0.01	0.01	69.18	3.38	0.56	0.51
13	BT13-II	0.01	0.01	69.18	4.78	0.56	1.99
14	BT14-II	0.01	0.1	4.78	2.81	0.95	1.99
15	BT15-II	0.01	0.01	40.73	0.95	0.61	0.56
GMT		**0.01**	**0.13**	**20.89**	**2.91**	**1.18**	**0.83**

Table.4. Rabies virus neutralizing antibody titres (IU/ml) of Group-IV.

S. NO	ANIMAL NO	0-DAY	7th -DAY	14th -DAY	28th -DAY	60th -DAY	90th -DAY
1	BD1-II	0.01	0.01	30.9	0.56	0.56	0.56
2	BD2-II	0.11	0.01	11.74	2.81	2.57	2.57
3	BD3-II	0.01	0.45	12.58	1.25	0.11	0.11
4	BD4-II	0.01	0.19	13.8	2.81	0.79	0.56
5	BD5-II	0.01	0.56	36.3	2.81	1.25	0.95
6	BD6-II	0.01	0.79	11.48	2.81	0.61	0.56
7	BD7-II	0.01	0.01	64.56	10	2.81	0.95
8	BD8-II	0.01	0.01	11.48	11.48	2.81	0.95
9	BD9-II	0.01	0.51	64.56	2.81	0.56	0.25
10	BD10-II	0.01	0.12	11.48	3.38	2.81	2.81
11	BD11-II	0.11	0.01	30.9	6.3	2.81	2.81
12	BD12-II	0.01	0.11	3.01	2.81	0.95	0.51
13	BD13-II	0.01	0.45	12.88	1.99	0.56	0.25
14	BD14-II	0.01	0.25	2.57	2.57	0.56	0.11
15	BD15-II	0.01	0.39	69.18	15.13	0.95	0.56
16	BD16-II	0.01	0.25	69.18	1.25	0.56	0.45
17	BD17-II	0.01	0.19	69.18	2.57	2.57	0.56
18	BD18-II	0.01	2.29	64.56	3.38	2.57	0.56
19	BD19-II	0.01	1.25	69.18	12.88	3.01	0.56
20	BD20-II	0.01	0.07	69.18	12.88	2.57	2.57
21	BD21-II	0.11	0.05	69.18	0.56	0.56	0.56
22	BD22-II	0.01	0.09	64.56	12.88	2.81	2.57
23	BD23-II	0.01	0.32	69.18	3.98	2.57	2.29
GMT		**0.01**	**0. 15**	**27.61**	**3.50**	**1.23**	**0.71**

Table.5.Comparision of mean rabies virus neutralizing antibody titres (IU/ml) between days within the Group.

Probability values of Paired t-test.

Animals	Days post vaccination				
	0 & 7	7 & 14	14 & 28	28 & 60	60 & 90
Group-I	0.001197**	0.0000**	0.032421*	0.01829*	0.00007**
Group-II	0.00587**	0.0000**	0.0000**	0.04416*	0.13249 NS
Group-III	0.04574*	0.0000**	0.00032**	0.01741*	0.23585 NS
Group-IV	0.000279**	0.0000**	0.0000**	0.00089**	0.75042 NS

* Significant at 5 % level (P< .05)

** Significant at 1 % level (P< .01)

NS – Non significant.

Table.6.Comparision of mean rabies virus neutralizing antibody titres between the Groups.

Animals	Days post vaccination					
	0-DAY	7th-DAY	14th -DAY	28th-DAY	60th -DAY	90th -DAY
Group I	0.02_{a}	0.14_{a}	19.08_{a}	7.97^{**}_{a}	3.83^{**}_{a}	1.38^{*}_{ab}
Group II	0.01_{a}	0.23_{a}	21.28_{a}	5.28^{**}_{a}	2.86^{**}_{a}	1.69^{*}_{b}
Group III	0.01_{a}	0.13_{a}	20.89_{a}	2.91^{**}_{b}	1.18^{**}_{b}	0.83^{*}_{ac}
Group IV	0.01_{a}	0.15_{a}	27.61_{a}	3.50^{**}_{b}	1.23^{**}_{b}	0.71^{*}_{c}

Values in the same column bearing same subscript do not differ significantly.

* Significant at 5 % level ($P < .05$)

** Significant at 1 % level ($P < .01$)

Table.7. Number and percentage of animals seroconverted above protective titre (0.5 IU/ml) in four groups undergone post exposure anti-rabies therapy.

Groups	n	Days post vaccination									
		7th day		14th day		28th day		60th day		90th day	
		SC (%)	**NS (%)**	**SC (%)**	**NS (%)**	**SC (%)**	**NS (%)**	**SC (%)**	**NS (%)**	**SC (%)**	**NS (%)**
I	**23**	5 (21.74)	18 (78.26)	23 (100)	0 (0)	23 (100)	0 (0)	23 (100)	0 (0)	23 (100)	0 (0)
II	**21**	5 (23.81)	16 (76.19)	21 (100)	0 (0)	21 (100)	0 (0)	21 (100)	0 (0)	20 (95.24)	1 (4.76)
III	**15**	3 (20)	12 (80)	15 (100)	0 (0)	15 (100)	0 (0)	15 (100)	0 (0)	15 (100)	0 (0)
IV	**23**	5 (21.74)	18 (78.26)	23 (100)	0 (0)	23 (100)	0 (0)	23 (100)	0 (0)	18 (78.26)	5 (21.74)

n: Total number of animals in each group.

SC: Number of animals seroconverted above 0.5 ML/ml.

NS: Number of animals not seroconverted above 0.5 ML/ml.

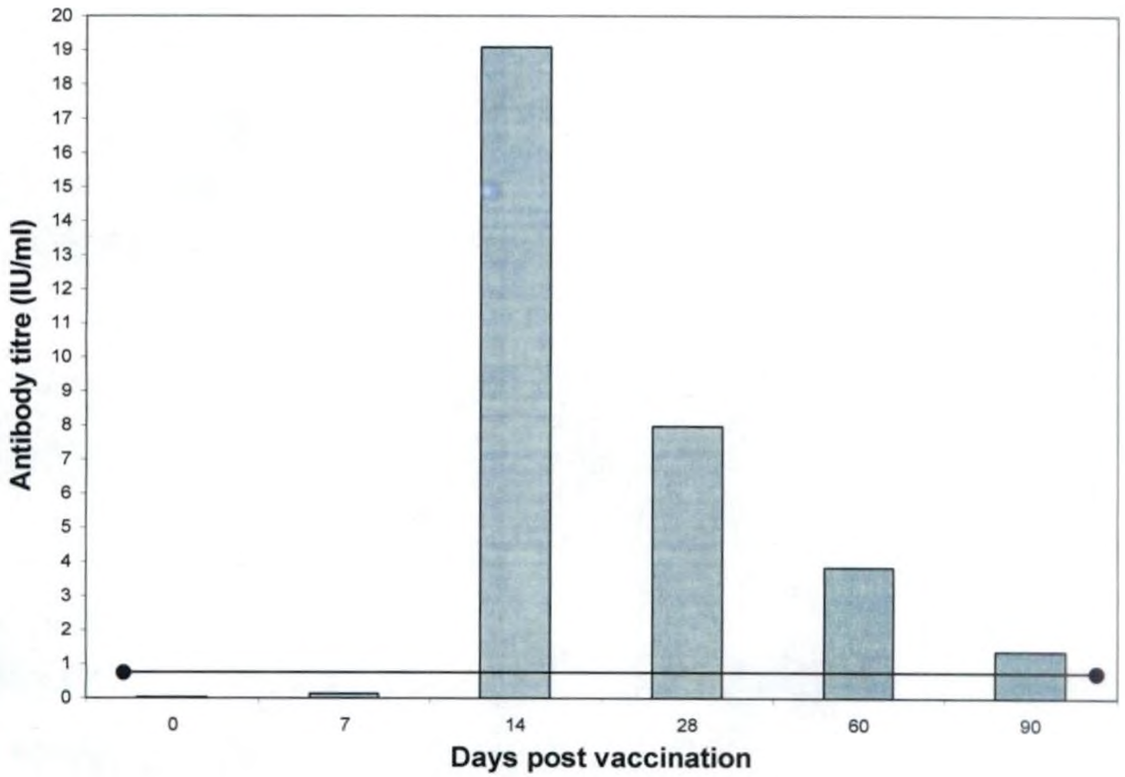

Fig.8.Mean rabies virus neutralizing antibody titres (IU/ml) of group-I.

The black line indicates the level of protecting antibody titre: 0.5 IU/ml)

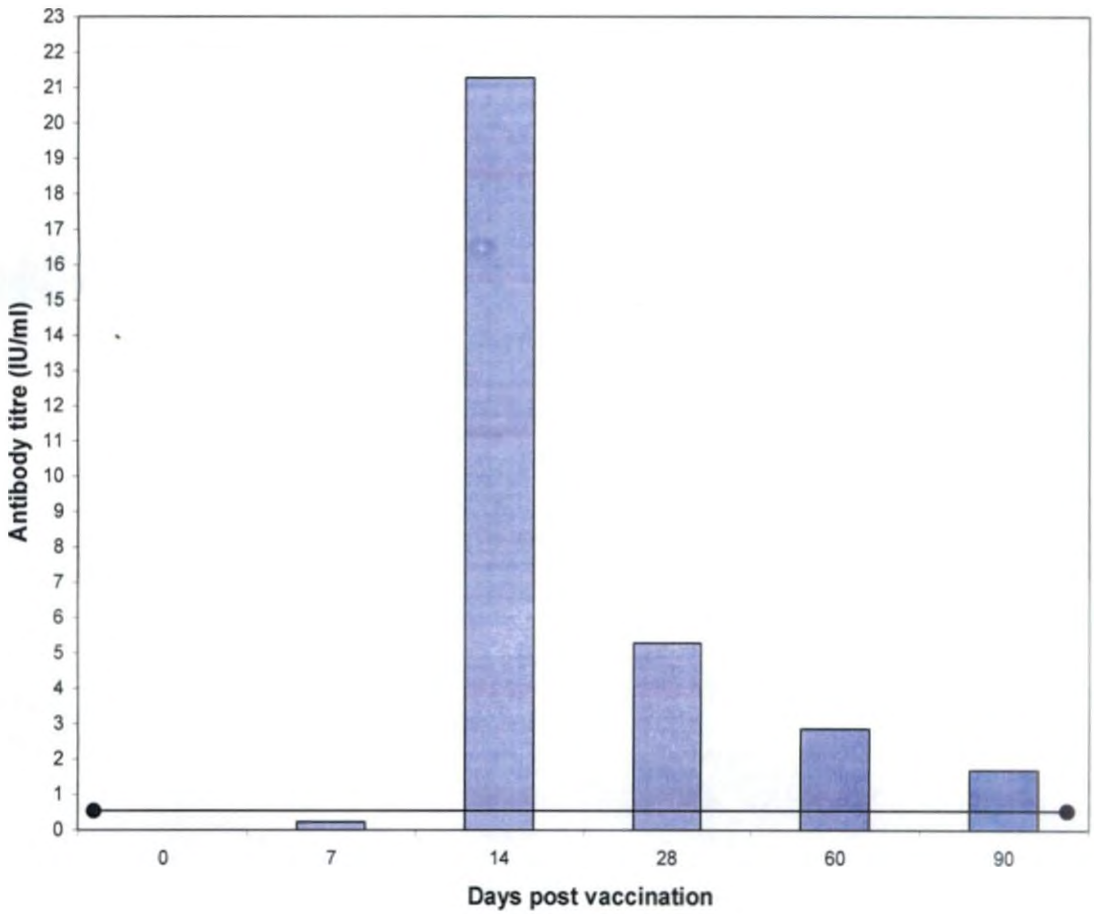

Fig.9.Mean rabies virus neutralizing antibody titres (IU/ml) of group-II.

(●——● The black line indicates the level of protecting antibody titre : 0.5 IU/ml)

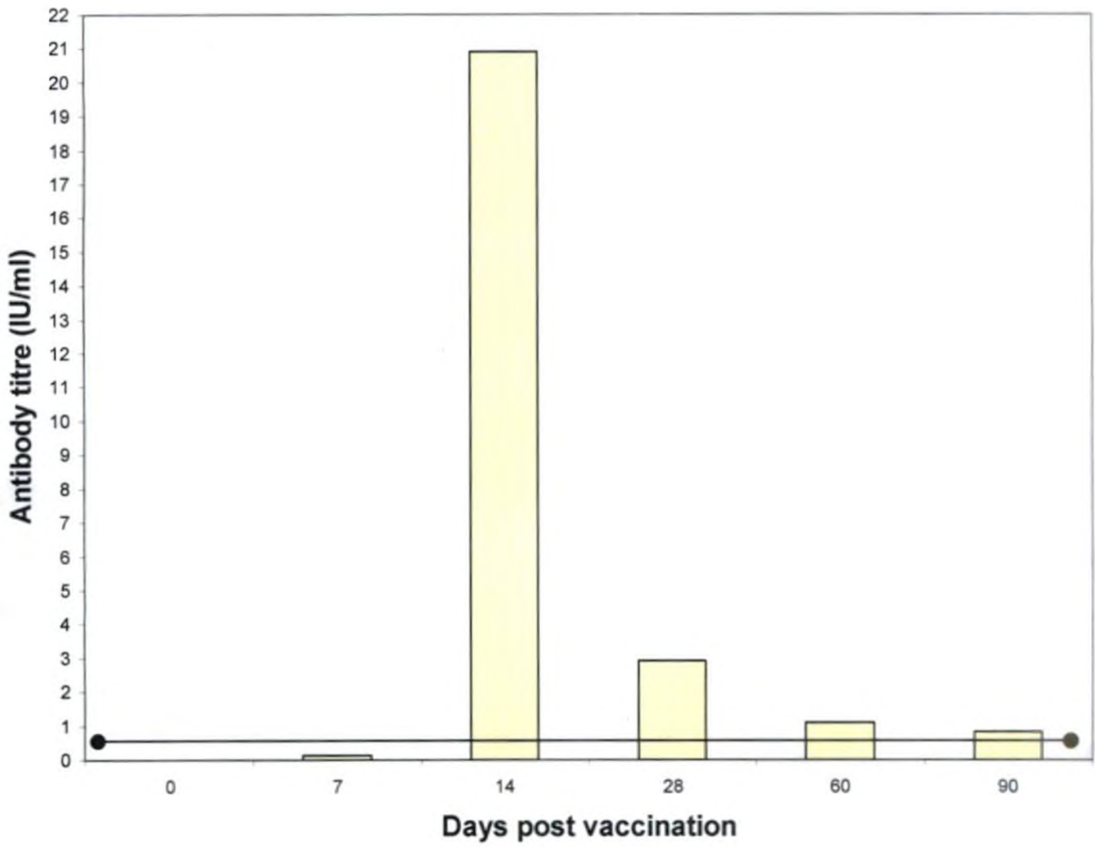

Fig.10.Mean rabies virus neutralizing antibody titres (IU/ml) of group-III.

The black line indicates the level of protecting antibody titre : 0.5 IU/ml)

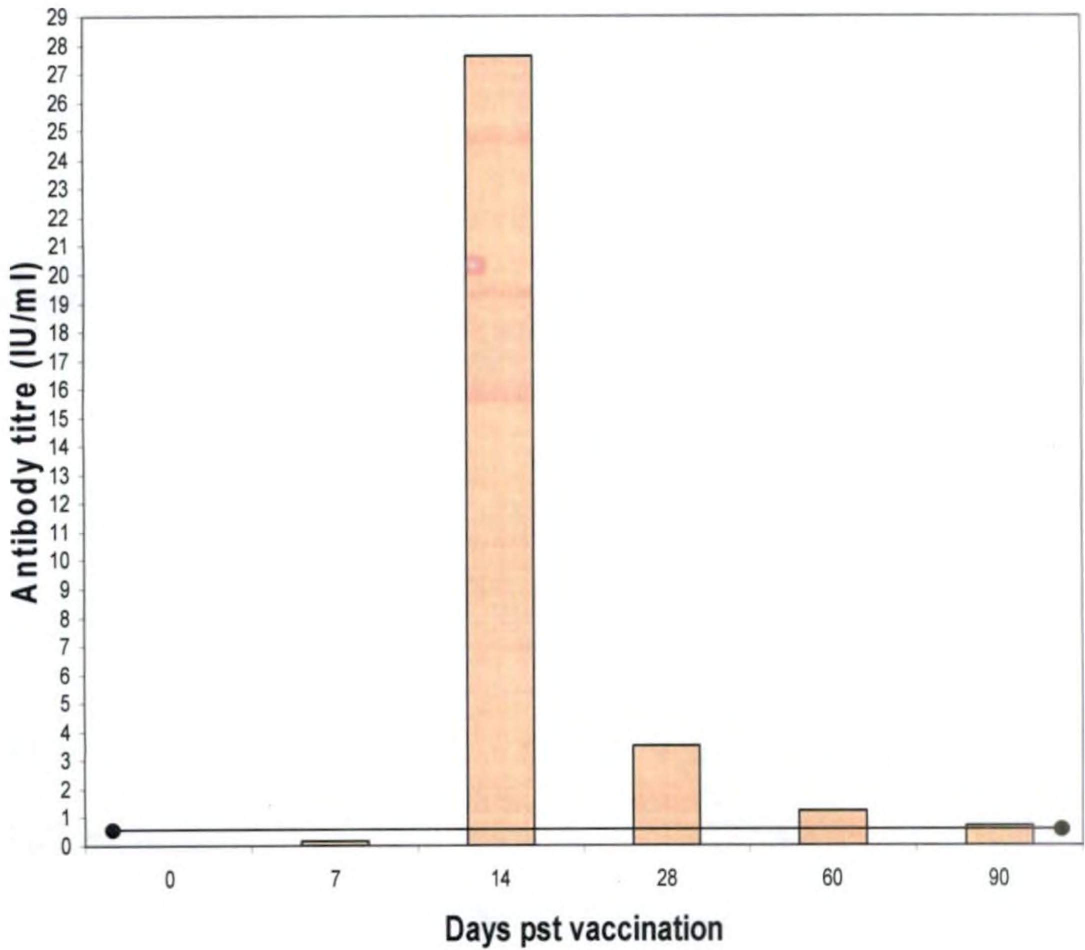

Fig.11.Mean rabies virus neutralizing antibody titres (IU/ml) of group-IV.

(●—● The black line indicates the level of protecting antibody titre : 0.5 IU/ml)

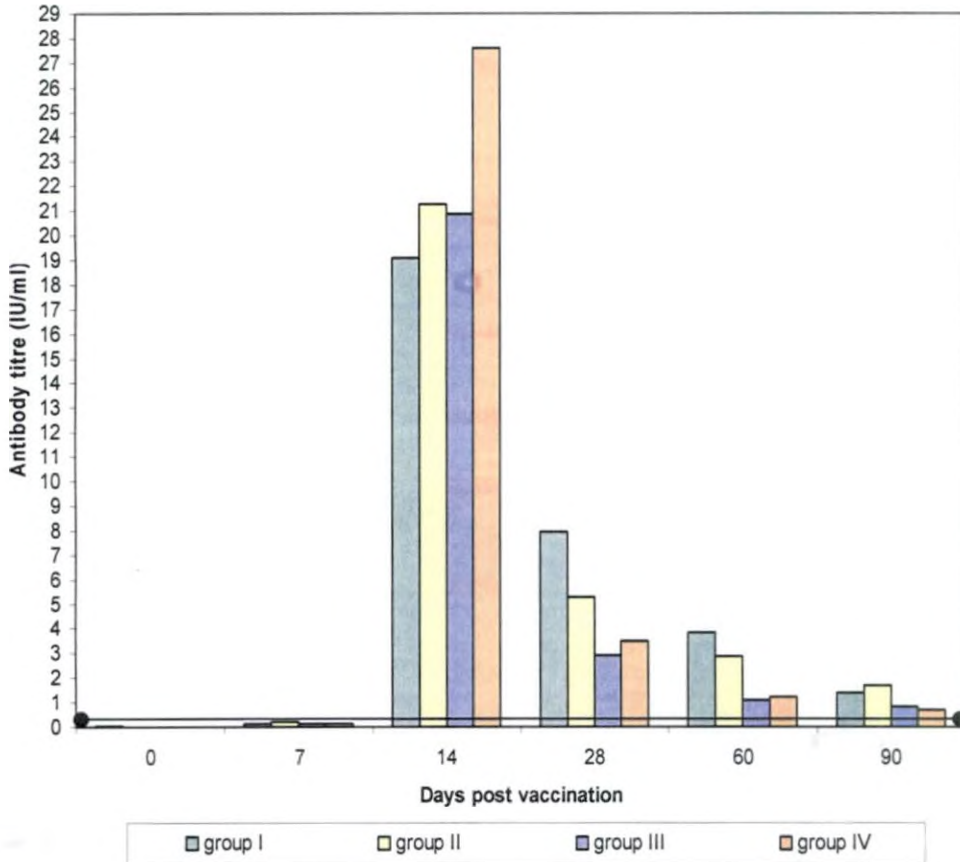

Fig.12.Comparision of mean rabies virus neutralizing antibody titres (IU/ml) in cattle in four groups undergone post exposure anti rabies therapy. (●——● The black line indicates the level of protecting antibody titre: 0.5 IU/ml)

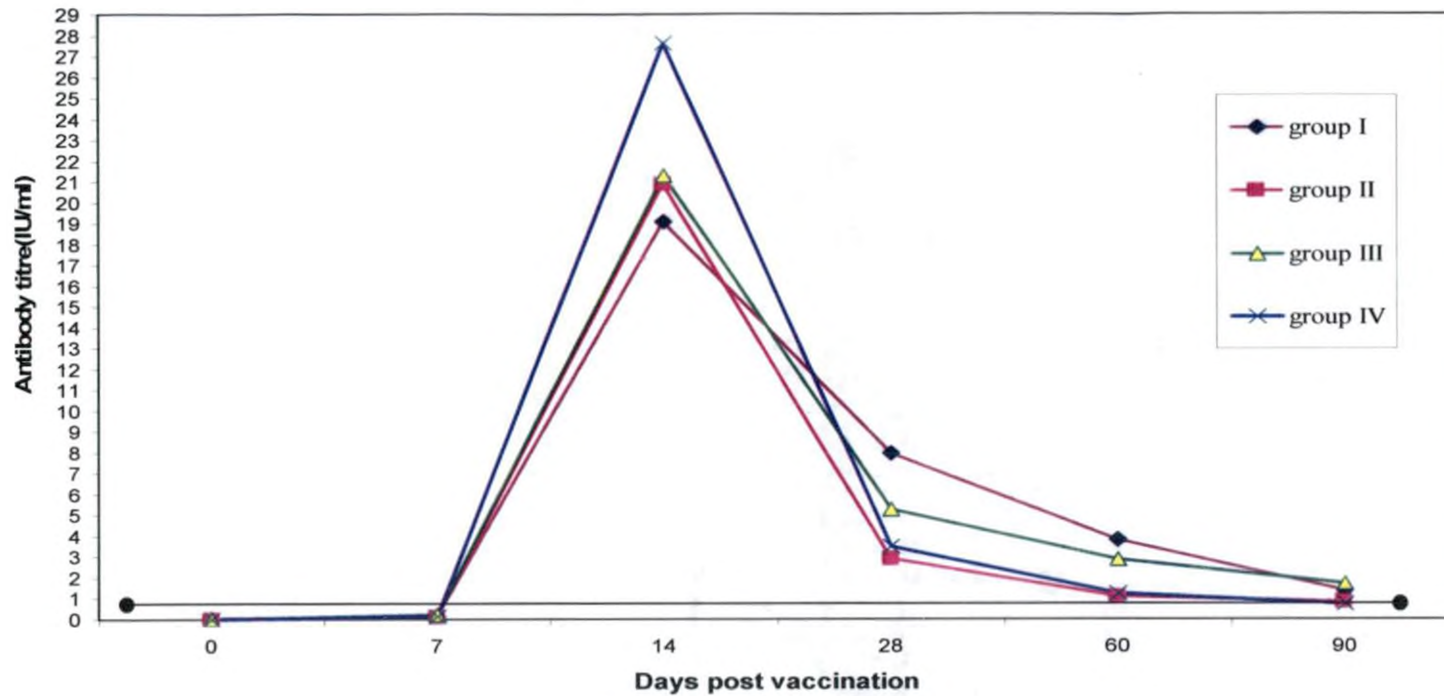

Fig.13.Comparision of mean rabies virus neutralizing antibody titres (IU/ml) in cattle in four groups undergone post exposure anti-rabies therapy.(●——● The black line indicates the level of antibody titre required for the protection: 0.5 IU/ml)

Discussion

5. DISCUSSION

In the present study, the immune response produced by the two different anti-rabies vaccines in two different post exposure schedules were assessed in cattle, of which one vaccine was inactivated tissue culture anti-rabies vaccine (Raksharab) and the other one was DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab). Each vaccine was administered in two different post exposure schedules in four different groups of exposed cattle. Schedule - I was the classical "Essen" Schedule with the injection of vaccine on 0, 3rd, 7th, 14th and 28th days and the Schedule – II consisted of administration of five dose of vaccine on 0, 1st, 2nd, 3rd and 4th days. Rabies virus neutralizing antibodies were assessed on 0, 7th, 14th, 28th, 60th and 90th days of post vaccination by employing Rapid fluorescent focus inhibition test (RFFIT).

5.1. IMMUNE RESPONSE PRODUCED FOLLOWING POST EXPOSURE VACCINATION IN FOUR DIFFERENT GROUPS.

5.1.1. Group I

All the animals in group-I were vaccinated with inactivated tissue culture anti-rabies vaccine (Raksharab) with Schedule - I. In this group a significant difference in geometric mean rabies virus neutralizing antibody titre was observed in between the adjacent sampling days. The geometric mean antibody titre ranged between 0.02 to 19.08 IU/ml. with the peak titre observed on 14th day of study period.

All the animals of group-I were sero-negative on zero day (0.02 IU/ml) of study period as prophylactic anti-rabies vaccination in cattle is not a common practice in India and the present study area in and around Thrissur.

Reddy *et al.* (2001) observed the mean rabies virus neutralizing antibody titres of 0.12 and 11.00 IU/ml on zero and 21st days of post vaccination in cattle vaccinated against rabies prophylactically with tissue culture inactivated anti-rabies vaccine, which correlates with the present result obtained in zero and 28th days of post vaccination in group I animals as 0.02 and 7.97 IU/ml respectively.

The geometric mean antibody titres of 7.97 IU/ml obtained on 28th day of post vaccination in group I correlates with the results obtained by Kalanidhi *et al.* (1998) in camels vaccinated with the inactivated tissue culture anti-rabies vaccine. The protective titre obtained on the 28th day of post vaccination also agrees with the Ramanna *et al.* (1991a) who observed the protective serum neutralizing antibody titres in cattle thirty days after vaccinated with inactivated tissue culture anti-rabies vaccine (Raksharab) on days 0, 3,7,14 and 28 as post exposure vaccination.

The present study antibody titre of 7.97 IU/ml on 28th day post vaccination in group I animals vaccinated with tissue culture inactivated anti-rabies vaccine correlates with Piza *et al.* (2002) who observed the virus neutralization antibody titres ranging from 7.45 to 12.16 IU/ml on day 30 in cattle vaccinated with experimental tissue culture vaccine produced from BHK 21 cell lines.

The geometric mean antibody titre values obtained in the group I on zero, 7th and 60th days are similar to Mitmoonpitak *et al.* (2002) who observed the geometric mean antibody titre of 0.03, 0.18 and 0.69 IU/ml on 0,7th and 60th days of post vaccination respectively in pigs vaccinated with a tissue culture anti-rabies vaccine on 0,3,7,14,28 and 60th day of exposure.

In the present study peak antibody titre recorded on 14th day of post vaccination (19.08 IU/ml.) which agrees with the findings of previous workers with same schedule in cattle (Plotkin *et al.*, 1976) and in pigs (Mitmoonpitak *et al.*, 2002). Quiambao *et al.* (2004) recorded highest antibody titre on 14th day of post vaccination

in human beings treated with human diploid cell culture vaccine as per "Essen" schedule. But Basheer *et al.* (1997b) in a similar study observed the peak antibody titre of 37.33 IU/ml on 95th day of post vaccination in cattle.

The mean antibody titre of 2.42 ± 0.60 and 2.18 ± 0.43 IU/ml on 60th and 90th day of post vaccination in cattle vaccinated with experimental tissue culture anti-rabies vaccine by Beniek *et al.* (2000) correlates with geometric mean antibody titres obtained in group I as 3.83 and 1.38 IU/ml on 60th and 90th day respectively and also agrees with the result obtained by Ramanna and Srinivasan (1992) as 2.02 IU/ml on 60th day of post vaccination in cattle vaccinated with tissue culture inactivated anti-rabies vaccine (Raksharab)

The antibody titre declined further and reached 1.38 IU/ml on 90th day in the present study, which was well above the protective level in a similar study of Basheer *et al.* (1997b) who observed the protective (10 IU/ml) titre up to 150 days. Albas *et al.* (1998) also observed the mean antibody titre of 1.814 IU/ml on 90 days of post vaccination in cattle vaccinated with prophylactic tissue culture anti-rabies vaccine.

Group I animals, which were vaccinated with inactivated tissue culture anti-rabies vaccine (Raksharab) showed the anamnestic increase in the mean antibody titre on 14th day after vaccination. This anamnestic reaction resulting in high antibody titre on 14th day could be due to the repeated injection of antigen, which produces immune response with shorter lag period of time than single inoculation. (Tizzard, 1998)

There was a gradual fall in antibody titre level from 14th day to 28th, 60th and 90th day of post vaccination. Kalanidhi *et al.* (1998) was of the opinion that this could be due to the difference in the breeds and age of cattle used in the study or the individual animal response to the vaccine. It is possible that factors involving genetics, nutrition, or parasitic infections may contribute to the poor immune response (Delgado and Carmenes, 1997). The important point was the interval

between the last dose of the vaccine and the time of sampling which is inversely correlated to the antibody titre. In other words, the levels of antibody titre decreases as the time between the last doses increased as observed by Simani *et al.* (2001) in an efficacy study on rabies vaccine in humans.

None of the animals in this group developed clinical signs for a period of six months indicated that this "Essen schedule" is also effective as post exposure therapy for rabies among cattle bitten by suspected rabid animals.

5.1.2. Group II

All the animals in group-II were vaccinated with DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) with Schedule - I. In this group also there was a significant difference in mean rabies virus neutralizing antibody titre in between the adjacent sampling days except between the 60th and 90th days of observation.

The geometric mean antibody titre ranged between 0.01 to 21.28 IU/ml with the peak titre observed on 14th day of study period. Subsequently declining to 1.69 IU/ml on 90th day vaccination.

All the group-II animals were having sero-negative antibody titre on zero day (0.01 IU/ml) of study period before the first injection of vaccine as prophylactic anti-rabies vaccination in cattle is not a common practice in India and the present study area in and around Thrissur.

In this group of animals, the geometric mean antibody titre raised above protective level on 14th day of vaccination i.e., 21.28 IU/ml and the protective level maintained till the 90th day.

Protective titre was maintained up to 90th day in the present study. Bahloul *et al.* (1998) also observed an early, strong and long-lasting immune response in mice vaccinated with single dose of DNA vaccine. The mean antibody titre obtained on 7th and 14th day post vaccination with DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) in the continuous five doses of vaccination schedule was similar to that of "Essen" post-exposure schedule. This observation is in accordance with the observation of Tizzard (1998), who described that repeated injection of antigen produces immune response with shorter lag period of time than single inoculation. There was a gradual reduction in anti body titre level from 14th day to 28th, 60th and 90th day of post vaccination.

All the animals in this group were having protective titre till 90th day of bleeding correlates with the observation of Fischer *et al.* (2003) who observed the strong onset and intensity of serological response in horses vaccinated with DNA vaccine and recorded the maintenance of protective level of anti body titre (1.30 IU/ml) up to the 56th day of post vaccination.

Biswas *et al.* (2001b) observed rabies virus neutralizing antibody titre of 5.4 IU/ml on 21st days of post vaccination in cattle vaccinated with two doses of DNA combined tissue culture inactivated anti-rabies vaccine on zero and 14th day. This endorses the finding of the present study where the titre was 5.28 IU/ml on 28th day of vaccination.

The present study value on 7th day of post vaccination, which got the protective level of antibody titre of >0.5 IU/ml in 23.81% of group II animals which correlates with the observation of Bahloul *et al.* (1998) who observed an early, strong and long-lasting production of neutralizing antibodies in mice vaccinated with single dose of DNA vaccine with plasmid encoding the glycoprotein and found to be protected against intra cerebral challenge of rabies virus.

The group II animals had an increased antibody titre from 7th day. (0.23 IU/ml) which conforms with the findings of Lodmell and Ewalt (2000) observed the robust increase in antibody titre from 7th day of post vaccination in mice vaccinated with two dose of DNA vaccine on day zero and day 7.

Bahloul *et al.* (2003) studied the immune response in mice vaccinated with single dose of DNA vaccine as post exposure vaccination and observed that the mice were protective up to 50th day of study period and elicited the peak level of antibody titre (17 IU/ml)on day 14 after vaccination. This result correlates the present findings where the animals on 14th day showed the peak mean antibody titre of 21.28 IU/ml and maintained the protective level up to 90th day of study period. Whereas of Perrin *et al.* (2000) observed the peak rabies virus neutralizing antibody titres on 28th day of post vaccination in dogs immunized with a DNA vaccine encoding rabies virus glycoprotein.

On 28th day of post vaccination, the group II animals had a mean rabies virus antibody titre of 5.28 IU/ml. which agrees with the observation of Bahloul *et al.* (2003) who also recorded the rabies virus neutralizing antibody titre of 6.8 IU/ml on 30th day of post vaccination.

In the present study in group II animals, on 28th day the rabies virus neutralizing antibody titre was 5.28 IU/ml which declined to 1.69 IU/ml on 90th day. Similar findings were observed by Perrin *et al.* (2000) who recorded significant decrease in the antibody level from 28th to 70th day of study period.

The mean rabies virus neutralizing antibody titre obtained in the animals of group II up to the 90th day of study period correlates with the results of Lodmell *et al.* (2002a) who studied the induction of neutralizing antibody with DNA vaccination by

employing post exposure vaccination on 0, 3, 7, 14, and 28th days of post exposure in non human primates.

In the present study the mean antibody titre was raised to protective level (0.05 IU/ml) on 14th day and maintained till the end of the study period i.e., 90th day. Similar findings were reported by Perrin *et al.* (2000) on post exposure therapy with DNA vaccine and Lodmell *et al.* (2003) on single intra-dermal vaccination in dogs, where the protective titre was maintained up to 175 days of vaccination.

5.1.3. Group III.

All the animals of group-III were vaccinated with inactivated tissue culture anti-rabies vaccine (Raksharab) with Schedule - II. In this group a significant difference in mean rabies virus neutralizing antibody titre was observed in between the adjacent sampling days except between the 60th and 90th days of observation. The geometric mean antibody titre of this group ranged between 0.01 to 20.89 IU/ml with the peak titre observed on 14th day of study period. Then declined to 0.83 IU/ml maintaining a protective titre on 90th day. This findings correlates with the result of Lavender (1973) who observed the peak titre on 14th day and declining of neutralizing antibody titre on 60th day onwards in monkeys vaccinated with 7 consecutive doses of cell culture vaccine.

All the animals of group-III were sero-negative on zero day (0.01 IU/ml) of study period before the first injection of vaccine. Because prophylactic anti-rabies vaccination in cattle is not a common practice in India and the present study area in and around Thrissur.

In this group animals also showed the rise in the mean antibody titre on 14th day after vaccination as five doses of vaccines were given continuously on five days

of exposure. This observation is in accordance with the observation of Tizzard (1998), who described that repeated injection of antigen produces immune response with shorter lag period of time than single inoculation. There was a drastic fall in anti body titre level from 14th day to 28th, 60th and 90th day of post vaccination. Though the titre remained above protective level from 14th day onwards the titre has fallen drastically, and this could be probably due to five continuous vaccination and lack of subsequent stimulation of immune system. However the protective level was maintained till 90th day. The reason for this could be the difference in the breeds and age of cattle used in the study or the individual animal response to the vaccine (Kalanidhi *et al.*, 1998)

The maximum antibody titre in this group was recorded as 20.89 IU/ml on 14th day. Similar findings of maximum titre on 14th day were also reported by Drings *et al.* (1999) in mice vaccinated with inactivated purified rabies vaccine on days 0 and 7, whereas Lodmell and Ewalt (2001) reported maximum titre of 100 IU/ml on 10th day of post vaccination in mice administrated with a tissue culture inactivated rabies vaccine (HDCV) as five consecutive doses for post-exposure study.

All the animals were alive and active till 180th day of this study indicated that though the titre was not up to the group I, protective level was maintained. This schedule has got the advantage of continuous vaccination, often when following "Essen schedule" even in human beings missing of some injections in between leads to the breakdown of immunity and resulting in rabies.

5.1.4. Group IV.

All the animals in group-IV were vaccinated with DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) with Schedule - II. The geometric mean antibody titre was between 0.01 to 27.61 IU/ml with the peak titre observed on 14th day of study period.

In this group a significant difference in mean rabies virus neutralizing antibody titre was observed in between the adjacent sampling days except between the 60th and 90th days of observation.

All the animals of group-IV were sero-negative (0.01 IU/ml) on zero day of study period before the first injection of vaccine. Probably, because prophylactic anti-rabies vaccination in cattle is not a common practice in India and the present study area in and around Thrissur.

In the group IV animals also showed the rise to the peak in the mean antibody titre on 14th day after vaccination with DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) as five doses of vaccines continuously on 0, 1st, 2nd, 3rd and 4th days of exposure. This observation is in accordance with the observation of Tizzard (1998), who described that repeated injection of antigen produces immune response with shorter lag period of time than single inoculation. There was a drastic fall in anti body titre level from 14th day to 28th, 60th and 90th day of post vaccination. The reason for this could be the difference in the breeds and age of cattle used in the study or the individual animal response to the vaccine (Kalanidhi *et al.*, 1998) and lack of subsequent stimulation of immune system. However the protective titre was maintained till 90th day of study period and none of the animals developed the disease also.

Cupillard *et al.* (2005) observed the protection against a lethal rabies challenge as early as 21 days post vaccination with a single rabies DNA vaccine in cats, but in this study, protective level of rabies virus neutralizing antibody titre (>0.5 IU//ml) was obtained with in 14th day of post vaccination in group IV animals.

In this group also, the mean rabies virus neutralizing antibody titres significantly decreased from 14th day onwards. The reason for this could be that there

was no constant stimulation of the immune system after 5th day and no persistent level of glycoprotein protection by the injected plasmid DNA as explained by Perrin *et al.* (2000)

The induction of rapid, strong antibody response more than 0.5 IU/ml raised within 7 days after vaccination correlates with the observation of Lodmell and Ewalt (2001) who vaccinated mice with five consecutive doses of DNA vaccine for post-exposure study.

All the animals were alive and active till the end of observation period of 180 days.

5.2. COMPARISON OF IMMUNE RESPONSE IN BETWEEN THE GROUPS.

5.2.1. Group I and Group II

All the animals vaccinated with inactivated tissue culture anti-rabies vaccine (Raksharab) in group-I and with DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) in group-II were having the minimum protective antibody titre (0.5 IU/ml) from 14th day to 90th day of study period.

In group I and II there was no significant difference in mean antibody titres between each other during the entire study period from day zero to 90th day. The reason for this may be the same post exposure schedule used in both the groups as administration of vaccine on 0,3rd ,7th ,14th and 28th days of exposure.(Schedule I)though different vaccines were administered.

In both the groups the peak rabies virus neutralizing antibody titres observed on 14th day of study period as 19.08 and 21.28 IU/ml in group-I and II respectively.

Group I animals maintained the lower mean rabies antibody titre level than that of group II animals during the entire study period except on 28th and 60th days of post vaccination. Possible reason for this may be that the factors involving genetics, nutrition, or parasitic infections, which contribute to the poor immune response (Delgado and Carmenes, 1997) and the probable synergistic action of DNA and tissue culture vaccine.

Group II animals which were vaccinated with DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) showed early protective antibody titre level in 23.81% animals on 7th day of post vaccination than group I animals where it was observed in 21.7% animals. Since the primary importance of the early induction of rabies virus neutralizing antibodies is to offset the risk of infection due to a possible short incubation period of rabies (Vadopija *et al.*, 1999), the early response in group II has a significant effect on preventing the disease.

5.2.2. Group I and Group III

All the animals vaccinated with inactivated tissue culture anti-rabies vaccine (Raksharab) in group-I and III were having the minimum protective antibody titre (0.5 IU/ml) from 14th day to 90th day of study period. In group I and III there was no significant difference in mean antibody titres between each other during the study period of day zero, 7, 14, and 90 of post vaccination whereas there was a significant difference in antibody titres on 28th and 60th days of post vaccination.

The difference in production of antibody in between these groups may be due to the difference in the vaccination schedules in each group. But in both the groups the vaccine used was inactivated tissue culture anti-rabies vaccine (Raksharab) and the peak rabies virus neutralizing antibody titres observed on 14th day of study period as 19.08 and 20.89 IU/ml in the animals of group-I and III respectively.

Group I animals maintained the highest antibody titre than that of group III animals till the end of the study period (90th day) except on 14th day. The present result observed in group I animals is correlating with the finding of Basheer *et al.* (1997b) who obtained the increased level of rabies virus neutralizing antibody titres with inactivated tissue culture anti-rabies vaccine in Essen schedule. The author studied the immune response in cattle vaccinated with inactivated tissue culture anti-rabies vaccine (Raksharab) and compared the different post exposure schedules like six doses of vaccine on 0,3,14,28 and 90 days of exposure with Raksharab in first group and Vero cell vaccine in second group, five doses of vaccines on 0,10,20,30,90 days post exposure with Raksharab in third group and Vero cell vaccine in forth group and fifth group was vaccinated with ten doses Nervous tissue vaccine daily for ten days.

The geometric mean antibody titres obtained in group I and III were 7.97 IU/ml and 2.91 IU/ml respectively on 28th day of post vaccination agrees with the results obtained by Kalanidhi *et al.* (1998) in camels vaccinated against rabies by using two doses of inactivated tissue culture anti-rabies vaccine on day zero and 30.

The protective titre obtained on the 28th day of post vaccination in group I and III also agrees with the Ramanna *et al.* (1991a) who observed the protective serum neutralizing antibody titres in cattle thirty days after vaccinated with inactivated tissue culture anti-rabies vaccine (Raksharab)

Albas *et al.* (1998) observed the mean antibody titre of 1.814 IU/ml on 90th day of post vaccination in cattle vaccinated with tissue culture anti-rabies vaccine, which correlates with the mean antibody level, obtained on 90th day in group I and III.

Animals of group I and III, which were vaccinated with inactivated tissue culture anti-rabies vaccine (Raksharab) showed the anamnestic increase in the mean antibody titre on 14th day after vaccination (19.08 and 20.89 IU/ml) and in group III

there was a drastic fall in the mean antibody titre on 28th day itself, where in group I there was gradual fall in titre from 14th day to 28th, 60th and 90th day of post vaccination. The reason for declining antibody titre may be due to decrease in the number of memory cells. (Oliveira *et al.*2000) and lack of further stimulation of immune system.

Comparatively lower mean antibody titres were observed in group III animals than group I during the entire study period up to 90th day.

Kitala *et al.* (1990) observed the 100% seroconversion in subjects vaccinated with purified Vero cell rabies vaccine in one group and human diploid cell vaccine in another group. But in the present study both in group I and III, 100 % seroconversion achieved on 14th day of post vaccination.

5.2.3. Group I and Group IV

All the animals vaccinated with inactivated tissue culture anti-rabies vaccine (Raksharab) in group-I and with DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) in group-IV were having the minimum protective antibody titre (0.5 IU/ml) up to 90th day of study period.

In group I and IV there was no significant difference in mean rabies antibody titres between each other during the study period of day zero, 7th, and 14th days of post vaccination and there was a significant difference in antibody titres on 28th and 60th and 90th days of post vaccination.

The difference in production of antibody in between these groups may be due to the difference in the vaccination schedules and different in vaccine used in each group.

The peak rabies virus neutralizing antibody titres observed on 14th day of study period were 19.08 and 27.61 IU/ml in group-I and IV respectively.

Group I animals maintained the highest antibody level than that of group IV animals on 28th, 60th and 90th days of study period and there was an increased level of mean rabies virus neutralizing antibody titres on day 7th and 14th day of post vaccination in group IV than that of group I. This correlates with the observation of Bahloul *et al.* (2003), who proved that the DNA vaccine induced a rapid protective level of neutralizing antibodies against rabies virus than tissue culture vaccine. The reason for the increased level of antibody production from the DNA vaccine may be the concentration of processed antigen, which results in an enhanced stimulation of T lymphocytes by antigen loaded dendritic cells. (Lodmell *et al.*, 2003)

5.2.4. Group II and Group III

All the animals vaccinated with DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) in group-II and with inactivated tissue culture anti-rabies vaccine (Raksharab) in group-III were having the minimum protective antibody titre (0.5 IU/ml) up to 90th day of study period.

In group II and III there was no significant difference in mean antibody titres between each other during the study period of day zero, 7th, and 14th days of post vaccination and there was a significant difference in antibody titres on 28th and 60th and 90th days of post vaccination.

The reason for difference in production of antibody in between these groups may be due to the difference in the vaccination schedules and difference in vaccine used in each group. But in both the groups, the peak rabies virus neutralizing antibody titres observed on 14th day of study period as 21.28 and 20.89 IU/ml in animals of group-II and III respectively.

Group II animals maintained the highest antibody titre level than that of group III animals during the entire study period. The increased level of rabies virus neutralizing antibody titres in cattle vaccinated with inactivated tissue culture anti-rabies vaccine using Essen's post exposure schedule was proved by Basheer *et al.* (1997b). In the present finding, group II animals were vaccinated with schedule I, had increased level of antibody titre than cattle vaccinated with schedule II in group III. This finding conforms the observation of Bahloul *et al.* (2003), who observed the higher level of immune response in mice vaccinated with DNA vaccine as post exposure vaccination than tissue culture rabies vaccine.

5.2.5. Group II and Group IV.

All the animals vaccinated with DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) in group II and IV shown the minimum protective mean antibody titre up to 90th day of study period.

In group II and IV, there was no significant difference in mean antibody titres between each other during the study period of day zero, seven, and 14th days of post vaccination and there was a significant difference in antibody titres on 28th, 60th and 90th days of post vaccination.

The difference in production of antibody in between these groups may be due to the difference in the vaccination schedules with each group. But in both the groups the vaccine used was DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) and the peak rabies virus neutralizing antibody titres observed on 14th day of study period as 21.28 and 27.61 IU/ml in animals of group-II and IV respectively.

Animals of group II maintained the highest antibody titre level than that of group IV animals during the entire study period except on 14th day of post vaccination.

Biswas *et al.* (2001b) observed the higher production of rabies virus neutralizing antibody on vaccination with DNA combined tissue culture inactivated anti-rabies vaccine than that of tissue culture vaccines in cattle. The results obtained in the animals of group IV on 14th day correlates with his observation. As per his study the satisfactory rabies virus neutralizing antibody titres were maintained up to the 60th day of study period, which correlates with the group II and IV animals that maintained the protective antibody level up to 90th day of study period. And the protective antibody level (0.5 IU/ml) obtained earlier in 7th day of post vaccination in group III animals

The results obtained in animals of group II and IV on 14th day which shown the peak mean antibody titre of 21.28 and 27.61 IU/ml respectively correlates with Bahloul *et al.* (2003) who observed that the mice were protective up to 50th day of study period and elicited the peak level of antibody titre on day 14 after vaccination with single dose of DNA vaccine.

The mean rabies virus neutralizing antibody titre obtained in the group II and IV in the study period correlates with the results of Lodmell *et al.* (2002a) who studied the induction of neutralizing antibody with DNA vaccination by employing post exposure vaccination in non human primates.

The protective mean antibody titres obtained in all animals in the group II and IV were at satisfactory level, which correlates with the observation of Perrin *et al.* (2000) who vaccinated the dogs intramuscularly with DNA vaccine.

The ideal vaccine for field use is that which elicit maximum immune response in large population of animals for very long period (Reddy and Srinivasan, 1999.) In the present study, group II cattle were obtained the highest antibody level till the end of the study period (90th day) than other groups, which also induced protective titre on 7th day of vaccination.

5.2.6. Group III and Group IV

All the animals vaccinated with inactivated tissue culture anti-rabies vaccine (Raksharab) in group-III and with DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) in group-IV were having the minimum protective antibody titre (0.5 IU/ml) up to 90th day of study period.

In group III and IV there was no significant difference in mean antibody titres between each other during the entire study period from day zero to 90th day. The reason for this may be in both the groups same post exposure schedule was used as administration of vaccine on 0,1^{st}, 2nd, 3rd and 4th days of exposure, though the vaccine was different.

In both the groups the peak rabies virus neutralizing antibody titres observed on 14th day of study period as 20.89 and 27.61 IU/ml in group-III and group-IV animals respectively. Here the high rabies antibody titre in group IV on 14th day correlates with the finding of Lodmell and Ewalt (2001) who compared the efficacy of DNA vaccine and a tissue culture inactivated rabies vaccine (HDCV) in mice with five consecutive doses of vaccination and obtained the peak antibody titre on 10th day of post vaccination in both the vaccinated groups.

Group IV animals maintained the highest antibody titre level than that of group III animals during the entire study period except on 7th and 90th day of post vaccination.

In both the groups the reduction in antibody titre noticed in later stages of study period (60th and 90th day) which correlates with the result of Lavender (1973) who observed the declining of neutralizing antibody titre on 60th day onwards in monkeys vaccinated with 7 consecutive doses of cell culture vaccine. The reason for decline of titres may be due to decrease in the number of memory cells. (Oliveira, 2000) as well as the lack of further stimulation of immune system in schedule II. However, both the groups III and IV maintained the protective titre till the 90th day of study period.

The high level of antibody titre observed in group IV animals vaccinated with DNA combined tissue culture inactivated anti-rabies vaccine than group III animals correlates with the finding of Bahloul *et al.* (2003), who observed the higher level of immune response in mice vaccinated with DNA vaccine as post exposure vaccination than tissue culture rabies vaccine.

5.3. COMPARISON OF IMMUNE RESPONSE IN SCHEDULE - I AND SCHEDULE - II.

Schedule I and II used in this study were effective in inducing a protective immune response and maintaining the mean rabies antibody titre above protective level of antibody titre (>0.5 IU/ml).

During the entire period of study, immune response produced from group I and II, which were vaccinated with schedule I were comparatively higher than that of group III and IV, which were vaccinated with schedule II except on14th day where in high titre was observed.

Group I animals produced comparatively higher antibody level than group III animals during the entire period of study. This results conforms with the observation

of Basheer *et al.* (1997b) who proved that 0,3,7,14,28 and 90 days of vaccination is effective in eliciting satisfactory neutralizing antibody against rabies in cattle.

Several studies (Plotkin *et al.*, 1976; Ramanna *et al.*, 1991a; Kalanidhi *et al.*, 1998; Quiambao *et al.*,2000; and Mitmoonpitak *et al.*,2002.) proved the advantage of Essen schedule in post exposure anti-rabies therapy which is used in Schedule – I.

Group II animals produced comparatively higher antibody level than group IV animals during the entire period of study except on 14th day of post vaccination. In group II animals 23.81 per cent animals elicited an early protective antibody level with in 7 days of post vaccination and maintained the higher level of antibody titre up to the 90th day of study period.

Group IV animals produced comparatively higher antibody level than group III animals during the entire period of study except on 7th day of post vaccination. This is in agreement with Lodmell *et al.* (2002b) who stated that lengthy rest period was not necessary to accelerate and augment the neutralizing antibody response.

5.4. PROTECTION ATTAINED BY VACCINATION

5.4.1. Group-I

The mean antibody titres of group-I animals were above protective level from 14th day to 90th day of study period. The detectable level of antibody titre observed on 14th day of post vaccination correlates with previous works with same schedule in cattle (Plotkin *et al.*, 1976) and in pigs (Mitmoonpitak *et al.*, 2002). Quiambao *et al.* (2004) recorded highest antibody titre on 14th day of post vaccination in human beings treated with HDCV as per "Essen" schedule.

5.4.2. Group-II

The mean antibody titres of group-II animals were above protective level from 14th to 90th day of study period. This finding agrees with the result of Lodmell *et al.* (2002a) who observed the protective level of neutralizing antibody elicited on 14th day in monkeys vaccinated with DNA vaccine as post exposure vaccination in "Essen" schedule

5.4.3. Group-III

The mean antibody titres of group-III animals were above protective level from 14th to 90th day of post vaccination. The protection level attained on 14th day of post vaccination is not correlating with the observation of Lodmell and Ewalt (2001) who vaccinated mice with five consecutive doses of a tissue culture inactivated rabies vaccine (HDCV) and observed the protective level elicited with in 7th day of post vaccination.

5.4.4. Group-IV

The mean antibody titres of group-IV animals were above protective level from 14th to 90th day of post vaccination. The induction of rapid, strong antibody response more than 0.5 IU//ml raised within 14 days after vaccination is not correlating with the observation of Lodmell and Ewalt (2001) who vaccinated mice with five consecutive doses of DNA vaccine for post-exposure study and observed the protective titre on 7th day of post vaccination.

5.5. OBSERVATION OF VACCINATED ANIMALS.

All the vaccinated cattle in the four groups were observed for the period of six months from the day of first injection of vaccine (zero day) and are found to be healthy, without development of any signs suggestive of rabies. The result obtained in

the present study correlates with the statement of Beniek *et al.* (2000) who opined that in cattle the rate of immunity onset after vaccination and preservation of sufficient levels of specific antibodies for a minimum of six months is important.

According to Quiambao *et al.* (2004) in post exposure treated patients, a survival assessment of six months is sufficient to establish the efficacy of rabies vaccine. This agrees with the observed results in all the vaccinated animals which were protective up to the six month of study period and the used vaccines and schedules were protective for the post exposure treatment in cattle.

The present result obtained from the observation of study animals for the period of six months correlates with Basheer *et al.* (1997b) who observed the post exposure vaccinated animals for the period of five months to evaluate the efficacy of different vaccines and different post exposure schedules.

Various studies were proved the efficacy of tissue culture vaccine in cattle by observation of vaccinated animals up to one-year period. (Netto *et al.*, 1973; Lee *et al.*, 1977; Prosperi *et al.*, 1983; Ramanna and Srinivasan 1992; and Kalanithi *et al.*, 1998).

Lodmell *et al.* (2002a) studied the effect of post exposure vaccination in monkeys with human diploid cell vaccine and a DNA vaccine and observed the survival of animals for a period of six months

From this study, it is concluded that both the inactivated tissue culture anti-rabies vaccine (Raksharab) and DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) were effective in inducing the protective rabies antibody titre up to 90th day of post vaccination. It is also observed that either inactivated tissue culture anti-rabies vaccine or DNA combined tissue culture inactivated anti-rabies vaccine when administered as per Schedule I or Essen schedule provides better antibody titre

till 90th day, whereas if these two vaccines when administered as per Schedule II produces a better response from 7th to 14th day of vaccination and then declines drastically, though the antibody titre was well above the protective level (>0.5 IU/ml). The antibody titres were comparatively higher in Schedule I on 90th day of vaccination.

In the present study, all the vaccinated animals were observed for a period of six months and all of them were healthy and active, suggesting the efficacy of post exposure therapy. Both the serum titre and the observation conforms the efficacy of both the vaccines.

It is concluded that though both the vaccines and both schedules were protective, as the farmers are likely to miss the vaccination if followed schedule I due to difference in dates of vaccination, as Bahloul *et al.* (2003) stated that drawback in schedule I was two consequences, the first was economic and the second practical, As rabies is endemic mainly in countries where medical/veterinary infrastructures are scare, especially in rural areas, the compliance of patients to the full course treatment is frequently impossible, creating the risk of treatment failure. Schedule II can be recommended as the anti-rabies post exposure therapy in cattle, which can be conducted on continuous five days, where there is less chance of missing.

From this study it was observed that,

i. Both the inactivated tissue culture anti-rabies vaccine (Raksharab) and DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) were providing protective titre (>0.5 IU/ml) from 14th day to 90th day of observation in cattle.

ii. Both the schedules I and II were providing protective titre from 14th to 90th day of observation in cattle.

iii. Early and high antibody titres were observed in schedule II where five consecutive injections were given. Though above protective titre, the fall in antibody level was also drastic in schedule II.

iv. In schedule I, protective antibody titre was observed on 14th day and the maintained at a fairly good level till 90th day.

It is concluded that the post exposure anti-rabies therapy in cattle could be conducted in both schedule using any of these vaccine. From farmers' practical point of view, continuous five doses of injections will be of much use. The fall in antibody titre beyond 14th day in schedule II could be avoided, if another injection on 21st or 28th day is given, which needs further studies.

Summary

6. SUMMARY

The immune response of two different anti-rabies vaccines with two different post exposure schedules were studied in cattle. For this study, 82 cattle, which were reported with the history of suspected rabid animal exposure in and around Thrissur district, were used. An inactivated tissue culture anti-rabies vaccine (Raksharab) and a DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) were used for this study. The vaccinations were carried out in Schedule - I & II. Schedule - I was the classical "Essen," schedule with the vaccination on 0, 3rd ,7th ,14th and 28th days and the Schedule – II consists of administration of five doses of vaccine on 0,1^{st} ,2nd ,3rd and 4th days continuously. The study animals were grouped randomly in to four groups. Group I and III animals were vaccinated with inactivated tissue culture anti-rabies vaccine (Raksharab) with schedule I and II respectively and group II and IV animals were vaccinated with DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) with schedule I and II respectively. The rabies virus neutralizing antibody titres were assessed on day zero, seventh, 14th, 28th, 60th and 90th days of post vaccination by employing Rapid fluorescent focus inhibition test- (RFFIT).

Mean rabies virus neutralizing antibody titres in animals of all the four groups were found to be below the protective level before the first vaccination (Zero day). All the four groups showed mean protective antibody titre level from 14th to 90th day of study period.

In all the four groups the peak antibody titre level were observed on 14th day of post vaccination and protective level of rabies virus neutralizing antibody titre were maintained up to the 90th day of study period. Group II animals showed the highest mean antibody titres on 90th day of post vaccination(1.69 IU/ml) than all other three groups and among the four groups, the group IV animals showed the lowest mean antibody titres(0.71 IU/ml) on 90th day post vaccination.

Group I and II animals which were vaccinated with schedule-I with inactivated tissue culture anti-rabies vaccine (Raksharab) and a DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) respectively, obtained the highest mean rabies virus neutralizing antibody titres than that group III and group IV during the study period. Among these two groups, group I obtained the highest antibody titre than group II during the entire study period except on day 14, whereas group II obtained the highest antibody titre on day 14 of the study.

From the group III and group IV animals which were vaccinated with schedule II with inactivated tissue culture anti-rabies vaccine ((Raksharab) and a DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) respectively, group IV animals obtained higher mean rabies virus neutralizing antibody titre than that of group III during the study period except on 90th day of post vaccination.

In all the four groups there was a fall in mean antibody titre level from 14th to 90th day of study period but maintained protective level of antibody titre for rabies.

In group I, a significant difference in mean rabies virus neutralizing antibody titre was observed in between the adjacent sampling days throughout the study period. Whereas in all other three groups significant difference in mean rabies virus neutralizing antibody titre was observed in between the adjacent sampling days except between the 60th and 90th days of titre.

There was no significant difference between the groups I and II (Both the groups were vaccinated with schedule – I) during the entire period of study and there was no significant difference between the groups III and IV (Both the groups were vaccinated with schedule – II) during the entire period of study.

Observations of all the vaccinated study animals were made for the period of six months from the day of first vaccination (zero day) for the development of any signs suggestive of rabies. All the animals were found to be healthy in the monitoring period of six months.

From this study it was concluded that,

1. Both the inactivated tissue culture anti-rabies vaccine (Raksharab) and DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) were providing protective titre (>0.5 IU/ml) from 14th to 90th day of observation in cattle.

2. Both the schedules I and II were providing protective titre from 14th to 90th day of observation in cattle.

3. Early and high antibody titres were observed in schedule II where five consecutive injections were given. Though above protective titre, the fall in antibody level was also drastic in schedule II.

4. In schedule I, protective antibody titre was observed on 14th day and the titre was maintained at a fairly good level till 90th day.

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ASSESSMENT OF POST EXPOSURE ANTI-RABIES THERAPY IN CATTLE

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ABSTRACT

The immune response and efficacy of two different anti-rabies vaccines in two different post exposure schedules were studied in rabies exposed cattle. An inactivated tissue culture anti-rabies vaccine (Raksharab) and a DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) were used. The study animals were grouped in to four groups. Animals of group I and III were vaccinated with inactivated tissue culture anti-rabies vaccine (Raksharab) with schedule I (injection of vaccine on 0, 3rd, 7th, 14th and 28th days) and schedule II (administration of five doses of vaccine on 0, 1st, 2nd,3rd and 4th days continuously) respectively and animals of group II and IV were vaccinated with DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) with schedule I and schedule II respectively. The rabies virus neutralizing antibody titers were assessed on day zero, 7th, 14th, 28th, 60th and 90th days of post vaccination by employing Rapid fluorescent focus inhibition test - (RFFIT).

In all the four groups the peak antibody titer level were observed on 14th day of post vaccination and protective level of rabies virus neutralizing antibody titer were maintained up to the 90th day of study period.

Group I and group II animals which were vaccinated with schedule-I with inactivated tissue culture anti-rabies vaccine (Raksharab) and a DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) respectively, obtained the high mean rabies virus neutralizing antibody titers than that of group III and IV during the study period. Among these two groups, group I obtained the highest antibody titer than group II on day zero, 14th, 28th and 60th days of study period. Whereas group II obtained the highest antibody titer than group I on day seven and 90th day of study period.

All the animals were found to be healthy in the monitoring period of six months in all four groups vaccinated with two antirabies vaccines with two different post exposure schedules.

Both the inactivated tissue culture anti-rabies vaccine (Raksharab) and DNA combined tissue culture inactivated anti-rabies vaccine (Dinarab) were providing protective titre (>0.5 IU/ml) from 14th to 90th day of observation in cattle. Both the schedules I and II were providing protective titre from 14th to 90th day of observation in cattle. Early and high antibody titers were observed in schedule II where five consecutive injections were given. Though above protective titre, the fall in antibody level was also drastic in schedule II. In schedule I, protective antibody titre was observed on 14th day and maintained at a fairly good level till 90th day.

It is concluded that the post exposure anti-rabies therapy in cattle could be conducted in both schedules using any of these vaccines. From farmers' practical point of view, continuous five doses of injections will be of much use.