# Combined effect of LPS and rabies immunoglobulin in pathogenesis of rabies in mouse model and study on spontaneous fetal brain affections in cattle and buffaloes


*Thesis*

*Submitted in partial fulfilment of the requirement for the degree of*

**MASTER OF VETERINARY SCIENCE**

*in*

**VETERINARY PATHOLOGY**

***By***

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Roll No. 4837

**To**

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**Dated:** 18.8. **2011**

# *Certificate*

*Certified that the research work embodied in this thesis entitled* **"Combined effect of LPS and rabies immunoglobulin in pathogenesis of rabies in mouse model and study on spontaneous fetal brain affections in cattle and buffaloes"** *submitted by* **Dr. Awadh Narayan Kushwaha, Roll No. 4837,** *for the award of* **Master of Veterinary Science** *degree in* **Veterinary Pathology** *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.*

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*Certified that the thesis entitled, "**Combined effect of LPS and rabies immunoglobulin in pathogenesis of rabies in mouse model and study on spontaneous fetal brain affections in cattle and buffaloes**" submitted by* **Dr. Awadh Narayan Kushwaha,** *Roll No. 4837, in partial fulfilment of the requirement of* **Master of Veterinary Science** *degree in* **Veterinary Pathology,** *Deemed University,* **Indian Veterinary Research Institute,** *Izatnagar, embodies the original work done by the candidate. The candidate has carried out his work sincerely and methodically.*

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Signature of the External Examiner

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Date : **(Awadh Narayan Kushwaha)**

Place :

# Abbreviations

| | |
|---|---|
| % | Percent |
| ABC | Avidin biotin conjugation |
| ACTH | Adreno corticotropic hormone |
| AEC | 3-amino-9-ethyl carbazole |
| BBB | Blood brain barrier |
| BHK | 21 Baby Hampster Kidney cells 21 |
| bp | Base pair |
| CA | Cornua ammonis |
| CD | Cluster of Differnetiation |
| cDNA | Complementary deoxyribonucleic acid |
| cm | Centimetre |
| CMI | Cell mediated immunity |
| CNS | Central nervous system |
| CVS | Challenge virus standard |
| DEPC | Diethyl pyro carbonate |
| dFAT | Direct Flouroscent antibody test |
| DNA | Deoxy ribo nucleic acid |
| dNTPs | Deoxy nucleotide triphosphate |
| DPI | Days post infection |
| *e.g.* | *exampli gratia* (for example) |
| EB | Ethidium bromide |
| EDTA | Ethylene diamine tetra acetic acid |
| *et al.* | et alii / Alia (and other people) |
| *etc.* | *et cetera* (and other things) |
| FACS | Fluorescence Activated Cell Sorter |
| Fig. | Figure |
| gm | gram |
| GMEM | Glassglow minimum essential media |
| HPA | Hypophyseal hypothalamic thymic axis |
| HPS | Histopathological scores |
| hr | Hour |
| HRPO | Horseradish peroxidase |
| *i.e. Idest* | (used to explain exactly what the previous thing mentioned means) |
| IFN | Interferon |
| IHC | Immunohistochemistry |
| IL | Interleukin |
| iNOS | Inducible Nitric oxide synthase |
| IVRI | Indian Veterinary Research Institute |
| kb | Kilo base |
| LD | Lethal dose |

| | |
|---|---|
| LPS | Lipopolysaccahride |
| M | Molar |
| Mab | Monoclonal Antibody |
| mg | Milligram |
| min | Minute |
| ml | Millilitre |
| mm | Millimetre |
| mM | Millimolar |
| MNC | Mono nuclear cell |
| NK | Natural Killer |
| NO | Nitric oxide |
| $^{o}$C | Degree centigrade |
| OD | Optical Density |
| OIE | *Office des International Epizooties* |
| PAMPs | Pathogen- associated molecular patterns |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| pmol | Picomoles |
| Poly I:C | Polyinosinic-polycytidylic acid |
| RBC | Red blood corpuscles |
| RFFIT | Rapid fluorescent focal inhibition test |
| RIG | Rabies immunoglobulin |
| RNA | Ribonucleic Acid |
| rpm | Revolutions per minute |
| RT | Reverse transcription |
| RT-PCR | Reverse transcription - polymerase chain reaction |
| RV | Rabies virus |
| s | Seconds |
| TBE | Tris Borate EDTA |
| TLR | Toll like receptor |
| $T_m$ | Melting Temperature |
| TNF | Tomour necrosis factor |
| U | Units |
| WHO | World Health Organization |
| µg | Microgram |
| µl | Microlitre |

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# 1 Introduction

Brain monitors and regulates all the body's actions and reactions. The brain, consisted of forebrain (olfactory bulb and tract, cerebral hemisphere and diencephalon), midbrain (corpora quadrigemina, crura cerebri) and the hindbrain (pons, cerebellum, medulla oblongata), together with spinal cord, peripheral nerves and ganglia regulates sensory, motor, cognitive, memory, and autonomic functions through complex network of neuronal cells connections, located in different anatomical sites. The functional properties of the brain are topographically localized. The brain cells, spanning within the white and the gray matter exhibit limited cytopathological and inflammatory responses and their susceptibility to the stimuli vary (Getty, 1975). The cerebrospinal fluid present inside the brain maintains internal homeostasis. It is separated from the blood by selectively permeable blood-brain barrier (BBB).The anatomical structure of the BBB is the cerebral microvasculature endothelium, which, together with astrocytes, pericytes, neurons and the extracellular matrix, constitutes a neurovascular unit ( Minagar and Alexander, 2003 ). Tight junctions between endothelial cells of the BBB restrict para-cellular diffusion of water soluble substances from blood to brain.

The brain may be affected either directly or indirectly by various kinds of disease causing agents like virus, prion protein, bacteria, rickettsia, chlamydia, fungus, protozoa, parasite, toxins, nutritional and metabolic disorders, etc. The infectious agents break open the brain barriers and reach the brain by way of the blood stream, or may pass along the axis cylinders of motor or sensory nerves, establish the infection and produce pathological changes. These changes are limited, diffuse in nature, and produce clinical signs more or less similar and overlapping (Sullivan, 1985). Further, the disturbance in one anatomical part of the brain may affect the other parts; thereby it becomes difficult to pinpoint the exact location of the lesion

based on the clinical signs. Similarly, the neurological disorders/ diseases are regionally distributed due to varying degree of vulnerability of different cell types and regions of the brain to various etiological agents. Therefore, systematic examination of the brain is recommended to find out the pattern and extent of lesions in knowing the known and documentation of new diseases (Jubb and Huxtable, 1993). Among the various affections of the brain, malformations occur disproportionately high in frequency and variety in domestic animals than in other tissues, because of high degree of complexity and differentiation of nervous tissue. The malformations are caused by number of teratogens (toxins, infectious agents and genetic causes), which affect specific cell population of the brain at particular developmental stage and result into morphological, microscopic or functional disturbances. Many of the agents like BVD, Bluetongue, Akabane virus, fungal and plant toxins, drugs, etc are known to cause developmental disturbances like microencephaly, hydrocephalous, cyclopia and dysraphic states, etc in domesticated animals (Binkhorst *et al*.,1983; Cornillie e*t al.*, 2004). The cytopathology of various cell types of the brain is also special which is reflected by the presence of specific changes within the neurons, the glial cells and the vascular endothelium (Jubb and Huxtable, 1993). In animals many etiological agents have been reported which result in brain defect, yet thorough histopathological study of fetal brain in aborted cases is seldom practiced. As a result, information on fetal brain pathology is scanty. A few workers have described cattle and buffaloes fetal brain malformations such as congenital hydrocephaly, anencephaly, agenesis of corpus callosum and other cavitating lesions. (Cho and Lei pold, 1978; Cho *et al*., 1985; Guarda, 1977; Cornillie *et al*., 2004). Protozoan infections like *Neospora caninum, Toxoplasma gondii* and *Sarcocytis* infections in brain have been infrequently reported (Barr *et al*., 1990; Thornton *et al*., 1991; Dubey *et al*., 1998).

Furthermore, amongst various infectious diseases of central nervous system (CNS), rabies is one of dreadful disease of warm blooded animals, including human. It is caused by a single stranded, non segmented, negative sense RNA virus of the genus *Lyssavirus* under the family *Rhabdoviridae*. The virus is transmitted mainly through bite by rabid animals (mainly dog in India) to cause acute encephalitis. Each year at least 55,000 people die of rabies worldwide (WHO, 2005), of which approximately 20, 000 human deaths account for India (WHO SEA report, 2009). Although these deaths are preventable, but because of prohibitive

cost of post bite prophylaxis, inadequate availability of vaccines and ignorance about the disease, deaths are bound to occur. Moreover, if all the bitten human are given post-exposure treatment, it will cause $25million expenditure to India (Sudarshan, *2007*). Under natural rabies cases, after the appearance of clinical signs, death is almost certain within days to weeks. Currently no validated therapy available once disease symptoms are developed (Jackson, 2003). Only six individuals were reported to have recovered after the development of clinical signs (Jackson, 2007). Among 7 genotypes of rabies virus, classical rabies virus (genotype-1) is responsible for causing majority of human rabies cases and remaining genotypes 2-7, which are restricted to certain geographical regions of the world (other than India), are also capable of causing rabies-like disease in human and animals.

The huge body of literature on rabies pathogenesis derived from experimental studies using fixed virus strains and laboratory mouse model showed that rabies virus completes its cycle by evading the host immune system. This virus cycle is completed by the help of 5 structural proteins named Nucleoprotein (N), Phosphoprotein (P), Matrix protein (M), Glycoprotein (G) and RNA dependent RNA polymerase (L) (Goto *et.al*., 1995) encoded by a rabies virus genome (3'-N-P-M-G-L-5'). The G, L and P proteins are important determinants in rabies pathogenesis (Lentz *et al.,* 1982). G protein is a determinant of virus entry and interaction with cell receptors and stimulation of neutralizing antibody, etc. (Dietzschold *et al*., 2005).

The pathological changes in rabies are not matched with clinical signs (Murphy, 1977). In addition, there is minimal immune response detectable in most cases of human rabies at 7–10 days after onset of clinical signs (Hattwig *et al.,* 1975). A possible explanation for the low degree of immune responses is that rabies virus uses a subversive strategy including the prevention of apoptosis and the destruction of invading T cells (Lafon, 2004) and interference in production of interferon by posphoprotein (Brzozka *et al.,* 2005). Most of the antibodies produced peripherally can neutralize the virus prior to nerve entry in the periphery, but not the virus in brain, as these cannot cross the BBB (Jackson, 2007; Roy *et al.,* 2007). The role of antibody in virus clearance from CNS was explored in different viral infections. Virgin IV *et al.* (1988) reported that antibody protects mice from the neurally spreading reovirus type 3. Antibody can act both early in the course of reovirus type 3 infections and after substantial

amounts of virus have entered the CNS. The fact that antibody protected against established CNS infection indicates that antibody inhibits steps in reovirus pathogenesis subsequent to viral entry into the CNS. Passive immunization of nude mice with specific antibodies after infection with influenza A virus induced only a transient recovery (Kris *et al.,* 1988). Sindbis virus infection of neurons is controlled via an Ab-mediated mechanism distinct from Ab-dependent cell-mediated cytotoxicity or C-dependent lysis (Levine *et al.,* 1991). Rabies virus neutralizing antibody can result in viral clearance from the central nervous system of experimentally infected mice (Dietzschold, 1993).

Similarly, the NK cells and activated lymphocytes reach the brain late in the infection due to delayed opening of the BBB by the action of inflammatory cytokines, by the time severe damages to the brain has already done. It is intriguing that rabies neutralizing antibody can be demonstrated in only 20% of humans with rabies (Hemachudha *et al.,* 1988). The majority of human rabies cases do not have detectable antibody responses until some days after the development of acute disease. In a study of rabies admissions in Thailand only 3 of 11 cases had detectable neutralizing antibody to the viral glycoprotein. Even in these cases the titres were particularly low, varying from 0.26 to 3.42 international units per ml serum. In 6 of these patients, where cerebrospinal fluid (CSF) was investigated, no anti-rabies antibody was detected (Kasempimolporn *et al.,* 1991). A similar review of human cases in the USA showed that none of the patients had detectable neutralizing antibody against rabies on presentation to hospital, although over half seroconverted within 10 days (Noah *et al.,* 1998).

However, the recovered rabies patients showed high anti rabies antibody titer in CSF (Jackson, 2007). Roy *et al.* (2007) reported that mice infected intradermally $10LD_{50}$ with Silver haired bat rabies virus showed that peripheral immune response is generated but unable to deliver effector cells and molecules to CNS because of impermeability of BBB. Shan (2010) reported around 42% survivability of CVS inoculated mice by modulating BBB permeability using Lipopolysaccharide compound which transiently opened the BBB. Therefore, keeping above facts in mind, we hypothesized that during the early phase of clinical signs of rabies, if BBB is opened using LPS followed by anti-rabies antibody administration, it may help in prolonging the survival of the infected host by some degree of viral clearance. Further, in spontaneous study of foetal brain, it was expected to have some baseline information on

possible etio-pathology of brain affections for the purpose of distinguishing lesions from real lesions during experimentation. Therefore, the present study was conducted with the following objectives:

- **To study spontaneous brain affections in aborted fetuses of cattle and buffaloes.**
- **To study the combined effect of LPS and rabies immunoglobulin in pathogenesis of CVS infected mice.**

# 2 Review of Literature

## A. Spontaneous brain affections in aborted foetuses

Various congenital anomalies of brain such as hydraencephaly, porencephaly in bluetongue, hypomyelinogenesis in border disease in sheep; cerebellar hypoplasia in bovine viral diarrhoea (BVD) in cattle and sheep; microcephaly, hypomyelinogenesis in hog cholera in swine have been reported (Jubb and Huxtable, 1993). Guarda (1977) examined 310 aborted bovine fetuses, of which 55 showed congenital malformations. Malformations such as hydrocephaly, microencephaly, cyclopia and cerebellar hypoplasia, have been reported in cattle and buffaloes (Cho and Leipold 1978; Cho *et al.,* 1985). Among various viral causes of developmental defects of the CNS, Akabane virus causes arthrogryposis and hydranencephaly in calves. Aino virus produce arthrogryposis, hydranencephaly and cerebellar hypoplasia in bovine foetuses infected experimentally (Tsuda *et al.,* 2004). Chuzan virus has been incriminated as teratogen of fetal calves with features similar to those of Akabane virus infection. Maclachlan *et al.* (1985) reported hydranencephaly in fetal calves caused by blue tongue virus infection. BVD virus infection in the first 100 days of fetal life tends to be lethal resulting in abortion or mummification. In such cases necrotizing inflammation can be observed in variety of tissues. The teratogenic effects of BVD virus is manifested during the 100-170 days period with cerebellar hypoplasia as the most characteristic defect. Encephaloclastic lesions, cavitations, and formation of pseudocysts, as well as rarefaction, occurred after infection at 75 days of gestation with BVDV (Montgomery *et al*., 2008). Munday and Black (1976) and McCausland *et al.* (1984) described multifocal nonsuppurative encephalitis and sarcocystis like protozoa in brain of aborted fetuses. Encephalitis in aborted bovine foetuses with histological lesions of

glial nodules, mononuclear infiltration, hemorrhage, neuronal necrosis, and cerebral cortical cavitation reported in Bovine herpesvirus 1 infection (Brower *et al.*, 2008).

Hong *et al.* (1991) reported meningitis in aborted foetuses naturally infected with *B. abortus*. Guarda (1977) reported brain lesions in *B. abortus* infected fetuses. The lesions described included non-suppurative leptomeningitis, nonsuppurative encephalitis, nonsuppurative ependymitis, and non-suppurative choriormeningitis. The pathogenesis of *B. abortus* associated meningitis in the bovine fetus may be related to, the predilection of *B. abortus* for the reticulo-histiocytic system, and the propensity for the bacteria to disseminate hematogenously from the placenta to many fetal organs (Hong *et al.*, 1991). *Neospora caninum* infected fetuses showed characteristic multifocal encephalitis (Otter *et al.*, 1995). Multifocal nonsuppurative encephalitis, with focal necrosis and gliosis has been seen in abortions of sheep due to *T. gondii* (Hartley and Cater, 1963).

McCausland *et al*. (1984) reported multifocal nonsuppurative encephalitis, myocarditis and hepatitis in aborted fetuses of cows. Sarcocystic like protozoa were found in the brains of fetuses from 2 of the outbreaks. Dogs and cats fed and affected fetus and new born calves from the affected herds failed to excrete protozoa in their faeces.

Hattel *et al*. (1998) found N.caninum in fetal tissues of 34/688 cases of bovine abortions (3-8 months gestation) during May 1994 to November 1996. Microscopic lesions primarily consisted of encephalitis and myocarditis. A labeled strept avadinbiotin staining procedure using anti N. caninum, polyclonal rabid serum revealed N. caninum within the fetal brain (27/27), heart (10/13), placenta (5/6), kidney (2/2), liver (1/4) and skeletal muscle (1/1).

Baszler *et al*. (1999) reported routine diagnosis of N.caninum abortion by histopathology and identification of tissue parasites by IHC. Confirmation of N.caninum infection by immunohistochemistry was found low insensitivity. Therefore, the authors used PCR on 61 bovine fetuses (fresh and formalin fix tissues). The results indicated a higher sensitivity of PCR in comparison to that of IHC. N.caninum DNA was amplified most consistently from brain tissue. PCR detection of N.canimum DNA in formalin fix, paraffin embedded tissues was superior to that of fresh tissue, presumably because of the increased accuracy of sample selection inherent histologic specimens.

Canada *et al.* (2002) isolated N. Caninum from the fetal brain homogenate inoculated intraperitoneally first into outbreak swiss mice given dexamethasone and then peritoneal exudate into mice co-inoculated with mouse sarcoma cells in the mice given dexamethasone of the 4 month old aborted fetus from a cow herd with endemic neosporosis in Porto, Portugal. the brain homogenate was inoculated intraperitoneally into outbreak Swiss Webster mice given dexamethasone and then the peritoneal exudates from these mice was coinoculated with mouse sarcoma cells in the peritoneal cavity of the mice given dexamethasone. The tachyzoites were seen in peritoneal exudate of the second passage. The tachyzoites reactive positive with anti-N caninum antibodies and not with anti-T. gondii antibodies. The dam of aborted fetus had an antibody titer of 1:10240 in the Neospora agglutination test (NAT). Antibodies to N. caninum were found in 76 to 106 cows in titre of 1:40 in 31, 1:80 in 22 more than 1:160 in 23 in NAT.

Canada *et al.* (2004) isolated N. Caninum from the brain of a 6 month old aborted bovine fetus from Galicia Spain. The fetal brain homogenate was inoculated intraperitoneally into cortisonized mice. The peritoneal exudate from the infected mice along with mice sarcoma cells was inoculated into a second group of mice and parasites were harvested from the peritoneal exudate. The parasites were adapted to invitro growth in vero monolayers. The tachyzoites from the peritoneal exudates reacted positively with N.caninum, antibodies and not with anti-Toxoplasma gondii antibodies on indirect FAT. The parasite was also confirmed by PCR amplification of N.caninum specific fragments.

Maanen *et al*. (2004) evaluated PCR and IHC testing on 36 infected and non infected fetuses. The results showed a relative low sensitivity but a high specificity of an IHC, however, PCR methods generally had a higher sensitivity then an IHC method and also a high specificity.

Medina *et al*. (2006) determined *N. caninum* in fetal brain tissue by nested PCR probe using single tube nested PCR probe with primers NF1, NS2, NR1 and SR1 in 44 fetal brains (8 dairy farms) and compared with histopathology. 35/44 (80%) bovine fetuses diagnose as being infected by N.caninum using PCR probe and 20 (45%) were considered positive by histopathology. The age of aborting cows ranged from 1.6 to 2.9 years old in 17 (49%) of the positive cases diagnosed by the PCR.

Corbellini *et al*. (2006) surveyed to determine the distribution pattern of infectious abortions. A total of 161 bovine fetuses from 149 farms were analysed using 1.5 year period.

The causes of abortion were identified in 51.5% of the cases overall 23% (37/161) were infected with N.caninum, bacterial infection for 17.4% (28/161) of cases, fungal infection for 3.1% (5/161) and viral etiology for 1.8% (3/161), 6 fetuses had concurrent infection.

Pescador *et al.* (2007), observed lesions of multifocal necrotizing non-suppurative encephalitis in 89 (34%) out of 258 aborted bovine fetuses due to tachyzoites and cysts of N.caninum by IHC. The study concluded that when the fetal brain is autolyzed, the lungs may be used for the presumpative diagnosis of N.caninum infection. Even in autolyzed brain the organism is suitably detected by anti-N.caninum IHC.

Kizaki *et al.* (2007) reported 2/15 aborted fetuses brain showing Neospora cysts and bradyzoide clusters. These results, coupled with neurological clinical symptoms in new born calves, implicated *N.caninum* as the major cause of abortions in dairy herd in Portugal.

Salehi *et al.* (2009) evaluated the presence of *N. caninum* in 12 brain samples of aborted fetuses and 07 placenta of full term calves borne of seropositive cows. The samples were examined by nested PCR and histopathology. All of 12 aborted fetal brain samples and 5 of 7 placentas were tested positive for nested PCR. The palcenta showed mild to severe placentites in 5 placentas. Severe hyperemia and perineuronal oedema revealed in all fetal brain. In 3/12 brains, scattered foci of hemorrhages, neuropilar necrosis and glyosis were present, in 1 case non purulent encephalitis with severe lymphohistiocytic PVC and a small cyst like *N. caninum* was observed in other calves.

## B. Rabies pathogenesis with reference to LPS and rabies immunoglobulin

### Rabies virus

Rabies virus (RV) is the prototype virus of the genus *Lyssavirus* in the family R*habdoviridae.* Rabies virus is a bullet shaped virus with a single stranded, non segmented negative sense RNA genome of 12kb length causes invariably a fatal encephalomyelitis in humans as well as in domestic and wild animals (Tordo *et al.*, 1986). Based on the RNA sequencing and phylogenetic analysis, lyssaviruses delineated as classical rabies virus (CRV) genotype-1(field and laboratory fixed strains) (Bourhy *et al.*,1993) and rabies related viruses (RRVs), such as Lagos bat virus (genotype-2), Mokola virus (genotype-3), Duvenhage virus

(genotype-4), European bat Lyssavirus 1 and 2 (genotypes-5 and-6, respectively ) and Australian bat Lyssavirus (genotype -7). Aravan, Khujand, Irkut, and West Caucasian bat lyssavirus are other tentative genotypes. The RRVs cause illness indistinguishable from classical rabies (Smith, 1996).

The genome consists of five genes (namely N, M, P, G and L) which encode five structural proteins.The highly conserved and abundant nucleoprotein (N), have a key structural component (62000 daltons) of the viral ribonucleoprotein core essential to viral propagation, constitutes the main target for rabies diagnosis (Dean *et al.*, 1996). The G protein controls major aspects of host cell infection, such as receptor binding, antigenicity and host adaptation (Badrane and Tordo, 2001). Variability in the sequence of this protein appears to be responsible for the serotypic differences among lyssaviruses (Rupprecht *et al*., 2002). Rabies virus G protein, the only protein exposed on the surface of the viral particle, is mediator of both binding to cellular receptors and entry into host. The ectodomain of G protein is involved in the induction of both virus neutralizing antibody production and protection after pre- and post-exposure vaccination (Perrin *et.al.*, 1986). G protein induces cytotoxic T lymphocytes and T helper cells (Celis *et al.*, 1988a). M proteins (25000 daltons) are a membrane protein and serve as a bridge between the G protein and the N protein. L protein is the largest of the rabies virus structural proteins with a molecular weight of approximately 240,000 Daltons. L (Large) protein and NS (nonstructural, otherwise known as P (phospho) protein together form the RNA-dependent RNA polymerase or transcriptase.

**Pathogenesis**

Despite increases in our understanding of rabies pathogenesis, the pathogenic mechanisms by which street (wild type) rabies virus (RV) infection results in neurological disease and death in mammals are not well understood. Understanding of the pathogenesis of this disease has been achieved to a greater extent mostly from studies using experimental animals, particularly rodents, and fixed strains of rabies virus (Jackson, 2002). Pathogenic variants of RABV tend to invade the CNS and cause an acute, progressive infection, whereas less virulent viruses may have limited invasion of the CNS and cause abortive infection (Dietzschold *et al.*, 2008; Wang *et al.*, 2005).Under natural conditions, rabies virus infection of the CNS causes only relatively mild neuropathological changes without prominent evidence

of neuronal death (Murphy, 1977; Iwasaki and Tobita, 2002). Laboratory attenuated RV, on the other hand induces extensive inflammation and neuronal degeneration in experimental animals (Yan *et al.*, 2001). The pathogenesis of rabies includes encephalitis and as a consequence of the direct effect of the virus on infected neurons combined with activation of immune mediators. There is no evidence of necrosis or neuronophagia in the infected brains of immunosuppressed mice (Smith *et al.*, 1982). However, direct damage by rabies virus on neural cells has been shown *in vitro*.

The pathogenesis of rabies begins with the replication of virus in the myocytes, local sensory and motor neurons. The replication in the myotomes may represent an amplification step necessary to yield sufficient virus to invade the peripheral nervous system (PNS) (Murphy and Bauer., 1974). There is evidence that the virus remains close to the site of inoculation during most of the long incubation period in natural rabies (Charlton *et al*, 1997). Laboratory models with street virus, the virus remains at or near the site of entry for most of the incubation period (Baer and Cleary, 1972). However, the time periods in which the procedure were life saving in rodents infected with fixed rabies virus strains were relatively short (Dean *et al.*, 1963; Baer *et al.*, 1965), suggesting that a different mechanism of viral entry for fixed virus than in natural rabies. The sequential events taking place in natural rabies case is inoculation of virus at the site of bite followed by viral replication in muscle. Then virus binds to receptor at neuromuscular junction and enters into axons and reaches to motor neurons of the spinal cord and local dorsal root ganglia through retrograde axonal transport. After that virus reaches to brain and centrifugal spread along nerves to salivary glands, skin, cornea and other organs occurs. The infection of muscle cell and occasional fibroblasts is a critical step in the pathogenesis of street virus, but that was not observed following inoculation of fixed virus strain (Johnson, 1965; Coulon *et al.*, 1989).

RV invades the nervous system by binding to neural receptors, such as nicotinic acetylcholine receptor (nAchR) (Lentz,*et al.*, 1982), neural cell adhesion molecule (NCAM CD56) (Thoulouze *et al.*, 1998) or nerve growth factor receptor (NTR75) (Tuffereau *et al.*, 1998). RV is transported to the central nervous system (CNS) by retrograde transportation, possibly by binding to cytoplasmic dynein (Raux *et al.*, 2000). CD56 is expressed on neurons, astrocytes, myoblasts, myotubes, activated T cells and NK cells. NCAM is expressed in

three major isoforms and expression occurs in adult muscle and at the neuromuscular junction. The trimeric G protein exposed on the surface of the virion is responsible for the attachment to the target cell by interaction with several cell membrane components. After binding with nAChRs virus reaches to postsynaptic cells which in turn facilitate subsequent uptake and transfer of the virus to peripheral motor nerves (Jackson, 2002). The spread between neurons and distribution of RV infection in CNS are influenced by G protein. Overall, RV G protein is important for uptake, transport, transynaptic spread and topographic distribution of the infection in nervous system. Following attachment to the G protein to the cell membrane, RV endocytosed in an early endosomal compartment, where fusion of the viral membrane with the endosomal membrane takes place (Jackson, 2002). This causes release of nucleocapsid to escape into the cytosol, where transcription and replication occurs (Thoulouze *et al*., 1998).The pathogenic mechanism that might contribute to the profound CNS dysfunction, characteristic of rabies, could be the impairment of neuronal functions. It has been shown that expression of housekeeping genes is markedly decreased in RV-infected neurons, resulting in a generalized inhibition of protein synthesis (Fu *et al.*, 1993).

Recent studies suggested that an interaction between RV phosphoprotein and the dynein light chain links the RV RNP to the host cell transport system, thereby facilitating retrograde axonal virus transport. However, it is not clear whether this alone accounts for the viral transport mechanism. Mazarakis *et al.* (2001) demonstrated that rabies virus G protein pseudotyped lentivirus based vectors enhance gene transfer to neurons by facilitating retrograde axonal transport. Hence, the rabies virus G protein may play a more important role than phosphoprotein. After infection develops in spinal cord or brainstem neurons, rabies virus disseminates rapidly throughout the CNS by fast axonal transport along neuro-anatomical connections. Rabies virus spreads within the CNS as in the peripheral nervous system, by fast axonal transport. Evidence was provided for axonal transport using stereotaic brain inoculation in rats (Gillet *et al.*, 1986) and by the administration of colchicine, which inhibited virus transport within the CNS (Ceccaldi *et al.*, 1989, 1990).

**Neuronal dysfunction**

Natural rabies is characterized by severe neurologic signs and a fatal outcome. However, neuropathological changes in the CNS are relatively mild, consisting of mild

inflammation with little neuronal degeneration, supporting the concept that neuronal dysfunction, rather than neuronal cell death, plays an important role in producing the disease (Jackson and Rossiter, 1997; Iwasaki and Tobita, 2002; Jackson, 2007). Studies of RV infection in experimental animals and *in vitro* have provided evidence of abnormalities in neurotransmission involving acetylcholine (Tsiang, 1982; Jackson, 1993), serotonin (Ceccaldi *et al.*, 1993; Bouzamondo *et al.*, 1993) and gamma-amino-n-butyric acid (GABA) (Ladogana *et al.*, 1994; Warrell and Warrell, 2004). Dysfunction of ion channels has been shown in infected neuroblastoma cells (Iwata *et al.*, 1999). Koprowaski *et al*. (1993) have hypothesized that nitric oxide neurotoxicity may mediate neuronal dysfunction in rabies and induction of iNOS mRNAs was observed in mice experimentally infected with street RV (Sukathida *et al.*, 2001 and Nakamichi *et al.*, 2004). iNOS mRNA was detected using RT- PCR amplification in the brains of 3/6 paralyzed mice 9 to 14 days after the inoculation of RV in masseter muscles. Ubol *et al*. (2001) and Madhu (2008) have reported that treatment of mice with the inducible nitric oxide synthase inhibitor aminoguanidine delayed the death of rabies infected mice compared with controls. Because nitric oxide also has antiviral activity, the role of nitric oxide in rabies pathogenesis still needs to be explored.

**Apoptosis**

Street RV strains, which are considerably more pathogenic than tissue culture-adapted strains, express very limited levels of G and do not induce apoptosis until late in the infection cycle, suggesting that the pathogenicity of a particular virus strain correlates inversely with RV G expression and with the capacity to induce apoptosis (Morimoto *et al.*, 1999). The mechanism by which the RV G gene mediates the apoptosis signaling process remains largely unknown. It has been speculated that RV G expression exceeding a certain threshold severely perturbs the cell membrane, resulting in the activation of proteins that trigger apoptosis cascades (Faber *et al.*, 2002). The challenge virus standard (CVS) of RV has been observed to induce apoptotic cell death in rat prostatic adenocarcinoma cells (Jackson and Rossiter, 1997), mouse neuroblastoma cells (Therasurakarn and Ubol, 1998) and in mouse embryonic hippocampal neurons (Morimoto *et al.*, 1999). Different neuronal cell types respond differently to RV infection i.e., hippocampal neurons show 90% apoptosis. Prominent apoptotic neurons have been observed in brains of mice of various ages inoculated intracerebrally (I/C) with the CVS

strain, and immunosuppression of adult mice did not reduce the apoptotic process (Jackson and Rossiter, 1997 and Jackson and Park, 1998). On the contrary, peripherally inoculated animals with CVS strains do not show the prominent apoptosis (Reid and Jackson, 2001).

Rabies virus induced apoptosis is activated by caspase dependent and caspse independent pathways (Thoulouze *etal.,* 2003; sarmento *et al.,* 2006). Rabies encephalitis in adult, suckling, and neonatal mice inoculated I/C with CVS is associated with marked neuronal apoptosis in multiple brain regions (Jackson and Rossiter, 1997; Jackson and Park, 1998 and Theerasurakarn and Ubol, 1998). In contrast, adult animals infected with CVS by peripheral routes of inoculation demonstrate severe and fatal encephalitis without prominent neuronal apoptosis (Reid and Jackson, 2001 and Jackson, 2003), and $CD3^{+}$ T cells are the main contributor to the pool of apoptotic cells in CNS (Baloul and Lafon, 2003).

Both beneficial and detrimental effects of apoptosis have been suggested in RV infections. In experimental animals infected I/C with mouse-adapted CVS strain virus, extensive apoptosis was observed in the CNS (Jackson and Rossiter, 1997; Theerasurakarn and Ubol, 1998). These observations led to the hypothesis that apoptosis plays an important pathogenic role in experimental RV infections. Thoulouze *et al*. (2003) observed an inverse correlation between the induction of apoptosis and the capacity of a RV strain to invade the brain, suggesting that inhibition of apoptosis could be a strategy employed by neurotropic virus to favour its progression through the nervous system. Thus, induction of apoptosis is a host defense mechanism in RV infections. This hypothesis is further supported by the finding that recombinant RV expressing cytochrome-c induced more apoptosis than parentral virus (Pulmanausahakul *et al*., 2001). CD8+ lymphocytes and certain cytokines like TNF have been shown to induce apoptosis in target cells (Ware *et al.,* 1996).

**Pathology**

Gross pathological lesions are unremarkable or relatively mild and non-specific (Love and Wiley *et al.,* 2002). There may be congestionof leptomengial and parechymal blood vessels, sometimes associated with multiple petechiae. Histopathologically, diffuse encephalitis with mononuclear cell perivascular cuffing and focal gliosis are reported. Neurones may contain the intracytoplasmic eosinophilic inclusions: 'Negri bodies'. Degenerative neuronal changes

are not usually prominent, which suggests that neuronal dysfunction of unknown cause is likely important in the pathogenesis of rabies. In some paralytic rabies cases there was extensive neuronal degeneration and loss in the anterior and posterior horns of the spinal cord and to a lesser extent in the medulla (Chopra *et al.,* 1980), whereas in others there was marked neuronal central chromatolysis, but only occasional or no neuronophagia (sheikh *et al.,* 2005). In addition to chromatolysis, vacuolation of neuronal cytoplasm and degenerative changes in nuclear chromatin have been reported.

**Immune regulation**

A limited natural human immune response to rabies does exist. The immunological response to peripheral RV infection includes both innate and adaptive immune responses (Lafon, 2007). Helper and cytotoxic T cells are activated to recognize and clear virus particles, respectively, outside and inside infected cells, which presumably primes both cell types for any subsequent response to infection. However, once the virus gains access to the CNS, the host adaptive immune response may be severely limited (Lafon, 2007). Uncontrolled replication in brain leads to disease and ultimately death (Jackson, 2003). The adaptive immune response often develops late in the clinical course that fails to control the disease. However, prophylactic and post-exposure vaccination provides effective immune protection through the induction of neutralizing antibodies (Hooper, 2005). In addition to the adaptive immune response, host defence against the early stages of infection is provided by the innate immune response (Haller *et al*., 2006). High levels of IFN are detectable in the serum of mice inoculated with RV by the peripheral or intracerebral routes (Marcovistz *et al*., 1994). Inoculation of the IFN inducer poly (I: C), immediately after RV challenge reduces mortality and improves RV vaccine efficacy (Harmon and Janis, 1975). These results demonstrate the importance of innate immune responses in protection against rabies. In mouse models of RV infection, levels of a number of gene transcripts associated with innate defences, including STAT1, IFN-γ, TNF-α, interleukin 6 (IL-6), IL-1β, T-cell growth factor â and Toll-like receptors (TLRs), have been shown to increase (Saha and Rangarajan, 2003; McKimmie *et al*., 2005).

**Innate immunity**

When neurons or other CNS cells become infected, changes quickly occur to initiate protective responses. Rapid production of type 1 interferon (IFN) is important for host survival.

IFN-β an immediate early IFN, which is produced by neurons as well as glial cells, is the main type 1 IFN that is produced by the CNS and is required for efficient induction of IFN-α *in vitro* (Erlandsson *et al.,* 1998). Inoculation of RV in mice result in progressive infection to spinal cord and brain and accompanied by the production of inflammatory cytokines IL-1, TNF-α and IL-6 as well as chemokines CCL-5,CXCL 10 (Baloul and lafon,2003). Prehaud *et al.* (2005) also observed inflammatory, chemo-attractive and antiviral response, including expression of antiviral IFN-β, Mx-1 and 2' 5' OAS genes and expression of the inflammatory TNF-α, IL-6 and chemokines. Inoculation of the IFN inducer poly (I: C), immediately after RV challenge reduced mortality and improved RV vaccine efficacy (Harmon and Janis, 1975). IFN establish a link between innate immunity and adaptive immunity (Le Bon and Tough, 2002). It has been proposed that the phosphoprotein of RV could control type 1 IFN production by interfering with the phosphorylation of IRF-3 (Brzózka *et al.,* 2005).

**Adaptive immunity**

The normal brain is relatively deficient in expression of MHC molecules, and the structure of the blood-brain barrier (BBB) inhibits the entry of circulating proteins and cells from the blood to the brain parenchyma. In acute rabies virus strain infection nervous system is progressively invaded by T and B lymphocytes. While in abortive rabies virus infection brain is protected against virus invasion by the T cell mediated response which kills infected neurons. As a consequence abortive rabies virus does not reach the brain and individual survive. The T cell-dependent destruction of motor neurons induces polio-like paralysis (Galelli *et al.,* 2000). In human neurons, rabies virus upregulates the expression of HLA-G, a non-classical human MHC class I molecule which may promote tolerance leading to acceptance of the semi-allogenic fetus and tumor immune escape (Lafon *et al.,* 2005). Rabies virus G protein is the mediator of both binding to cellular receptors and entry into host cells. The ectodomain part is involved in the induction of virus neutralizing antibody production and protection after pre- and post- exposure vaccination. The virus neutralizing antibodies induced against this highly immunogenic protein protect against rabies virus infection. G protein also induces cytotoxic T – lymphocytes (CTL) and T- helper cells (Th). Infection of adult animals with laboratory adopted attenuated strains can induce a strong CMI and antibody production, therefore, results in non lethal infection. However, lethal infection of mice with street rabies virus results in severe suppression of CMI (Wiktor *et al*., 1977).

**Cytokines of innate and adaptive immunity**

RV induces inflammation of the CNS associated with gliosis and perivascular lymphocytes infiltration. In the periphery, an over production of ACTH and glucocorticoides due to an alteration of hypothalamo- hypophyseal axis (HPA) has been described (Torres-Anjel *et al.*, 1988). This dysregulation is likely to be responsible for the alteration of the immune parameters and the immunosuppression. These effects have been related to the critical role of cytokines produced in plasma and brain during viral infection (Hirai *et al.*, 1992). Cytokines are key mediators involved in the host response to antigen challenge, but also in brain injury and in the communication between the immune system and CNS. Their expression in both systems is under a tight regulatory control. However, under pathologyical conditions, a dysregulation may lead to an overproduction of cytokines.

One of the cytokines known to play an important role in the CNS functions and HPA regulation is the IL-1. During rabies infection, IL-1 is produced in brain which activates HPA axis by stimulation of corticotrophin releasing factors (CRF) and secretion of ACTH leading to suppression of peripheral immune response. An important role for IL-1 in brain and pituitary functions is also suggested by the presence of specific IL-1 receptors (IL-1Rs) in mouse brain; the anterior cortex, the granular and molecular layers of dentate gyrus (hippocampal formation ), the choroids plexus, the meninges and the anterior pituitary.

The possible role of cytokines in rabies pathogenesis in relation to the concentration of IL- 1α, IL-1β and TNF-α and their brain receptors has been evaluated (Marquette.,1996). Shivasharanappa (2008) demonstrated role of TLR-3 and found upregulation of IL- 1α, IL-2 ,TNF-α and IFN-α and reduced pathology using poly I:C in CVS infected mouse model. TLR- 3 on glial cells and neurons detect ds RNA generated during replication, and involve in antiviral IFN response. Early signaling events after TLR-3 stimulation include the activation of NF-κβ and the phosphorylation of IRF-3, leading to the production of IFN-β, IL-6, IL-1α, TNF-α and some chemokines. Madhu (2008) demonstrated role of IL-1 α, IL-1β and TNF-α in reducing the severity of brain pathology using iNOS inhibitor in mouse model infected with CVS. Immunohistochemistry for IL-1β and TNF-α revealed expression of these cytokines in 96% of cases in microglial cells, macrophages and lymphocytes with a strong positive correlation between IL-1β and TNF-α immunopositivity and degree of perivascular and

parenchymal inflammation. In addition, expression of IL-1β and TNF-α also correlated positively with each other. However, there was no direct correlation of viral antigen load with grading of cytokine expression. These findings indicate that IL-1β and TNF-α together may play an important role to coordinate the dramatic inflammatory response associated with rabies viral encephalopathy (Anjali *et al*., 2009).

TNF-α and other cytokines such as IL-1α, IL-2 and IFN-α mediate their protective activity through different mechanisms, such as (i) direct inhibition of virus replication in neurons; (ii) accelerated clearance of RV infection via the induction of brain inflammatory processes, which open the BBB to allow access of immune effectors such as RV-neutralizing antibodies and T cells to the infected neurons; and (iii) enhanced microglial activation, which likely contribute to the immune defence (Lokensgard *et al*.,2001).

**Toll -like receptors**

TLR's are an expanding family of receptors that recognize unique molecular patterns characteristics of different types of pathogens. TLR's are expressed on antigen presenting cells and phagocytes. TLR 1-9 are expressed in both mice and human. TLR-3 can recognize dsRNA. TLR 4, 7, 8 can recognize ssRNA. There is little information available on TLR expression function in CNS. TLR 4 is involved in the recognition of lipopolysaccharide (LPS) and viral envelope proteins (Rassa *et al.*, 2002).

TLR3 along with TLR 7 and 8 is the member of TLR 9 subfamily which is mainly concerned with the recognization of nucleic acids and related structures. TLR3 recognizes the dsRNA generated as an intermediate in the viral replication (Alexopoulov *et al.*, 2001). The recognization of dsRNA by the TLR 3 lead to the secretion of type I interferon i.e, IFN-α and β and thus responsible for inducing potent antiviral response. TLR's upregulation was critically dependent on presence of a functional IFN α/β system. It has been reported that viral surface glycoproteins can activate TLR's (Boehme and Compton, 2004). Glial cells have been identified as the major producers of TLR-3 and initiators of inflammation in the CNS (Farina *et al*., 2005).

**Role of anti-rabies antibody in virus clearance from CNS**

Clearance of virus from cells in the brain parenchyma is a multistep process. First, there is inhibition of virus spread to new cells, then elimination of cell-free infectious virus.

Subsequently, virus-infected cells must be eliminated or intracellular virus replication permanently suppressed. The most frequently measured parameter of virus clearance is the amount of infectious virus that is present in CNS tissue. Humoral immunity plays an important role in the course of rabies virus infection. Immunoglobulin of the G isotype (IgGs), but not IgM confer passive protection against rabies virus (Turner, 1978). Antibodies against the N protein can be detected in human sera after immunization with inactivated rabies vaccines or after natural infection (Herzog *et al.*, 1992). These antibodies cannot neutralize the virus. Mice lacking B cells, such as Jhd knock-out mice, develop a progressive disease when infected intranasaly with an abortive RABV and they succumb to infection (Hooper *et al.*, 1998). Mode of action of antibody remains to be determined and could be linked to their ability to block virus replication inside the cell as observed *in vitro* after antibodies were artificially injected into infected cells (Lafon and Lafage, 1987). Dietzschold *et al*. (1992) have reported inhibition of both viral spread and RNA transcription *in vitro* as well as *in vivo* using Mab against rabies viral glycoprotein, resulting in virus clearance from CNS and protection in challenged laboratory rat. It was shown that this particular antibody mediates complete clearance of RABV from the NS without the help of antibody-dependent cell-mediated cytotoxicity or complement dependent lysis.

**Blood-Brain Barrier and Lipopolysaccharide**

Contact between circulating cells and factors and CNS tissues are restricted by selectively permeable neurovasculature, collectively referred to as the blood brain barrier (BBB). BBB consists of a continuous layer of endothelial cells joined by tight junctions at the cerebral vasculature. It represents a physical and enzymatic barrier to restrict and regulate the penetration of compounds into and out of the brain and maintain the homeostasis of the brain microenvironment (Davson and Segal, 1995). The BBB is formed by continuous layer of endothelial cells that are connected to each other through different junctional molecules; adherens junctions, gap junctions and tight junctions (TJ). So far, occludin, junctional adhesion molecule (JAM), endothelial-specific adhesion molecule (ESAM), and claudin-1, 3, 5 and 12 have been reported to be co-localized at cell-cell borders of brain capillary endothelial cells (Nitta *et al*., 2003; Wolburg *et al*., 2003 and Dobrogowska and Vorbrodt, 2004). Among these putative TJ proteins, claudins comprise a multigene family, and are believed

to be major constituents of the TJs, which are very important in restricting the passage of most substances from the blood to the brain extracellular environment (CSF) to maintain homeostasis, and in essence functions as a molecular switchboard (Weksler *et al*., 2005 and Ohtsuki *et al*., 2008). Although, the endothelium is the principal barrier and communication interface in the BBB, the local microenvironment is modulated by perivascular cells including neurons, astrocytes, pericytes, basement membrane and smooth muscle cells (Hawkins and Davis, 2005). The BBB also participates in regular immune surveillance of the brain and responds to proinflammatory cytokines to help mediate recruitment and transmigration of immune cells. BBB permeability has been seen in protective CNS immune responses in case of infection with the attenuated strain of the neurotrophic rabies virus CVS-F3, where clearance of the virus from the mouse CNS follows increased BBB permeability and the infiltration of T and B cells into CNS tissues (Phares *et al*., 2006).

Delivery of therapeutic agents to brain presents a major challenge to treatment of most brain disorders due to BBB. In its neuroprotective role, the BBB functions to hinder the delivery of many potentially important diagnostic and therapeutic agents to the brain. Therapeutic molecules and genes that might otherwise be effective in diagnosis and therapy do not cross the BBB in adequate amounts. BBB impermeability can be modulated in disease conditions or by using several chemicals like alkyl glycerol, sodium caprate and lipopolysaccharide. Erdlenbruch *et al*. (2000) reported enhancement of the BBB permeability by administration of alkyl glycerol and effective delivery of the antineoplastic agents cisplatin and MTX and the antibiotics in brain.

Lipopolysaccharide (LPS), an endotoxin and the main component of the cell wall of Gram-negative bacteria, is responsible for alteration in BBB permiablility (Olson *et al*., 1995). LPS binds to an LPS-binding protein in blood and activates monocytes, macrophages and leukocytes through CD14 receptors. Peripheral endothelial cells, which do not express CD14, are activated by LPS-soluble CD14 complex which leads to the production of proinflammatory cytokines interleukin-1, -6, -8, and tumor necrosis factor-a (Bannerman and Goldblum, 1999 and Rosenberg *et al*., 2007). The increased level of these cytokines during sepsis leads to the dysfunction of several organs, called multiorgan failure. Part of LPS signaling is mediated through Toll-like receptors (TLR), NFκB release and gene transcription. Brain endothelial

cells express LPS receptors TLR-4, TLR-2 and CD14 (Quan *et al*., 2002 and Singh and Jiang, 2004) and mediate the effects of peripheral LPS in the CNS (Singh and Jiang, 2004). LPS increases BBB permeability in different animal models, including newborn pigs, rats and mice (Temesvari *et al*., 1993; Veszelka *et al*., 2003; Qin *et al*., 2008 and Christopher *et al*., 2008). Rodents are highly insensitive to LPS (Redl *et al*., 1993). In studies using repeated high doses of LPS, BBB opening was observed in mice (Xaio *et al*., 2001 and Veszelka *et al*., 2003), while 3 h after a single lower dose of LPS no effect was seen in rats (De Vries *et al*., 1995). Experimental infection with CVS-F3 which is a less pathogenic variant of CVS induces an immune response that clears the virus without sequelae. This is due to antiviral immunity, an innate immune response of CNS-resident cells to the virus, together with enhanced BBB permeability might have contributed to the CNS inflammatory response that has been associated the clearance of CVS-F3 form CNS tissues (Phares *et al*., 2006). In contrast to mice infected with CVS-F3, CNS inflammation is rarely seen in human dying from infection with pathogenic strains of RV (Murphy, 1977) and it has been shown that the BBB integrity do not changes during experimental pathogenesis of silver Haired Bat rabies virus in mice (Roy *et al*., 2007).

**Diagnosis**

Rabies diagnosis contributes to control and prevention of disease. Diagnosis of rabies based upon clinical presentation is very difficult, as there are no pathognomonic symptoms or signs for the disease. Although the detection of Negri bodies using Sellers stain is a rapid, but there is a fairly high rate of false negatives when compared to antigen detection by immunofluorescence or virus isolation. Although, hydrophobia is indicative of this disease in human but can be confused with Guillain-Barre syndrome, poliomyelitis and encephalitis of other viral aetiology (Plotkin, 2000). Park *et al*. (1998) demonstrated the RV antigen in pyramidal neurons of the hippocampus and in the cerebral cortex of mice inoculated with CVS by immunoperoxidase test (IPT) and evidence of oligonucleosomal DNA fragmentation was detected *in situ* using TUNEL method. In this study, neurons of dentate gyrus of hippocampus demonstrated expression of viral antigen, apoptotic changes and positive TUNEL staining. Jackson (2003) demonstrated RV antigen in brains of CVS infected mice by avidin-biotin complex (ABC) method using monoclonal anti-RV immunoglobulin G (IgG). Pamini *et al*.

(2005) studied the distribution of RV antigen in cerebral hemisphere, pyramidal neurons of the hippocampus and Purkinje cells of cerebellum, by avidin-biotin peroxidase complex method, using mouse monoclonal antibodies 5DF12 in CVS infected mice and demonstrated extensive apoptosis by TUNEL staining method in these areas.

The preferred diagnostic tests for the detection of rabies are the FAT and mouse inoculation test (MIT) (Hemachudha and Wacharapluesadee, 2004). A modified method of PCR, Reverse transcriptase PCR (RT-PCR) has been used to detect rabies in infected decomposed brain tissues (Nadin-Davis, 1998). Problems associated with sensitivity of the FAT and decomposed brain tissues have been overcome with the adaptation of RT-PCR. Jackson and Wunner (1991) detected rabies viral RNA in the CNS of experimentally infected mice using *in situ* hybridization with RNA probes, whereas Warner *et al*. (1997) used *in situ* hybridization for detection of rabies virus antigen mRNA and genome in formalin-fixed tissues samples.

Sacramento *et al*. (1991) used PCR technique as an alternative method for the diagnosis and molecular epidemiology of RV. Tordo *et al*. (1994) used PCR technology for Lyssavirus diagnosis. Heaton *et al*. (1997) studied hemi nested PCR assay for detection of six genotypes of rabies and rabies related viruses. Nadin-Davis (1998) worked on PCR protocols for RV discrimination. Gupta *et al*. (2001) used single tube non-interrupted RT-PCR for the detection of RV in brain tissue. Heaton *et al*. (1999) and Picard *et al*. (2004) developed a hemi-nested RT-PCR method for the specific determination of EBL-1 and compared with other diagnostic methods. In recent years genetic tests, in which a portion of the viral genome is amplified by reverse transcription (RT)-PCR followed by sequence characterization of the resulting product have been applied extensively (Tordo *et al.*, 1994). Recently, *in-situ* PCR has been used to detect the rabies virus RNA both in cell culture (neuroblastoma cells) as well as in brain tissues (Jayakumar *et al*., 2003 and Praveena *et al*., 2007). Boldbaatar *et al*. (2009) reported rapid detection of rabies virus by reverse Transcription loop-mediated isothermal amplification. This method is rapid, easy and simple compared to other molecular diagnostic methods.

# 3 Materials and Methods

## A. Fetal brain pathology

### Collection of brains

A total of 21 fetal brains of cattle (19 cases) and buffaloes (2 cases) of either sex were collected from the Institute postmortem facility. The information with respect to breed of aborting animals gestation length, etc. was recorded on a proforma (Table 1). Among 19 cattle fetuses, 9 were of aborted type, 7 dystocia and 3 premature births (Table 2).

### Fixation of the brain

Fetal brains are found mostly soft during post-mortem examination due to autolysis and often damaged when removed from the cranium. In cases of maceration, the brain becomes semi-liquid and cannot be removed intact. Hence, for obtaining intact brain *in situ*, the head of the fetus was disarticulated from rest of the body at atlanto-occipital joint and the scalp was reflected. Anterior and posterior fontanelles were palpated and nick was made with a scalpel blade in the anterior fontanelle 2 to 3 mm lateral to the midline. By using a syringe, the 10% neutral buffered formalin (NBF) was injected into subarachnoid space. The volume depended on the gestation of the fetus. Fetuses of 16 weeks' gestation perfused with 5 ml, while those of term gestation perfused with about 40 ml of 10% NBF. The injection was performed until the fontanelles became taut but not distended. In larger foetuses, the posterior fontanelle was injected in a similar way. The skin was returned to the normal anatomical position and the fetus head was left overnight in the 10% NBF formalin in a tub (Nicholls, 1988). The following day, the skull was opened and brain taken out. It was noted that the brain is often a bright white colour instead of the usual light tan colour. After removal, the brain was placed in an adequate

**Table 1: Case details of bovine foetuses.**

| S No. | Necropsy No. | Species | Breed | Sex | Gestation age (Months) | Wt. of carcass (Kg) | Length of carcass (poll – base of tail) (inch) | Clinical history | Gross lesion in brain |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 567A/10 | Bovine | V | F | 8 | 17 | 20 | Abortion | Nothing specific detected (NSD) |
| | **Post-mortem findings**: carcass mildly autolyzed, flippers intact, hairs on body coat, straw colour fluid in peritoneum, enlarged liver with dark red colour, foetalic lungs, other organs did not reveal any specific gross changes. | | | | | | | | |
| 2 | 590A/10 | Bovine | V | F | 8 | 17 | 21 | Dystokia | NSD |
| | **Post-mortem findings**: carcass mildly autolyzed, flippers intact, hairs on body coat, straw colour fluid in peritoneum, amniotic viscous fluid in stomach, enlarged liver with dark red colour, kidney cortex dark red in colour, foetalic lungs, other organs did not reveal any specific gross changes. | | | | | | | | |
| 3 | 604A/10 | Bovine | M | M | 9 | 26 | 25 | Abortion | NSD |
| | **Post-mortem findings**: carcass moderately autolyzed, flippers intact, hairs on body coat, straw colour fluid in peritoneum, amniotic viscous fluid in stomach, enlarged liver with dark red colour, kidney medulla dark red in colour, foetalic lungs, other organs did not reveal any specific gross changes. | | | | | | | | |
| 4 | 660A/10 | Bovine | Th | F | 8 &1/2 | 18 | 22 | Premature birth | NSD |
| | **Post-mortem findings**: carcass mildly autolyzed, flippers intact, hairs on body coat, straw colour fluid in peritoneum, foetalic lungs, other organs did not reveal any specific gross changes.. | | | | | | | | |
| 5 | 662A/10 | Bovine | Th | M | 8 | 17 | 21 | Premature birth | NSD |
| | **Post-mortem findings**: carcass mildly autolyzed, flippers intact, hairs on body coat, foetalic lungs, other organs did not reveal any specific gross changes.. | | | | | | | | |
| 6 | 4A/11 | Bovine | V | M | 8 | 19 | 20 | Abortion | NSD |
| | **Post-mortem findings**: carcass mildly autolyzed, flippers intact, hairs on body coat, Straw colour viscous fluid was present in stomach.liver enlarged, turgid and dark red colour, foetalic lungs, other organs did not reveal any specific gross changes. | | | | | | | | |
| 7 | 17A/11 | Bovine | Th | M | 9 | 27 | 27 | Stillbirth | NSD |
| | **Post-mortem findings**: carcass moderately autolyzed, flippers intact, hairs on body coat, foetalic lungs, other organs did not reveal any specific gross changes. | | | | | | | | |
| 8 | 27A/11 | Bovine | Th | M | 5 &1/2 | 3.5 | 13 | Abortion | NSD |
| | **Post-mortem findings**: carcass moderately autolyzed, flippers intact, foetalic lungs, Coronary blood vessels were slightly congested. other organs did not reveal any specific gross changes. Feet were ill formed. | | | | | | | | |
| 9 | 32A/11 | Bovine | V | M | 9 | 22 | 24 | Stillbirth | Lateral ventricles of brain filled with redish colour fluid. |
| | **Post-mortem findings**: Carcass mildly autolyzed, flippers intact, hairs on body coat, foetalic lungs, Thoracic cavity and pericardial sac was filled with straw colour fluid. other organs did not reveal any specific gross changes. | | | | | | | | |
| 10 | 36A/11 | Bovine | V | M | 9 | 23 | 25 | Dystokia | NSD |
| | **Post-mortem findings**: carcass mildly autolyzed, flippers intact, hairs on body coat, thoracic cavity filled with reddish colour fluid, stomach content was viscous and yellowish in colour. foetalic lungs, other organs did not reveal any specific gross changes. | | | | | | | | |

**Table 1 : Contd...**

| S No. | Necropsy No. | Species | Breed | Sex | Gestation age (Months) | Wt. of carcass (Kg) | Length of carcass (poll – base of tail) (inch) | Clinical history | Gross lesion in brain |
|---|---|---|---|---|---|---|---|---|---|
| 11 | 41A/11 | Bovine | V | F | 9 | 20 | 24 | Stillbirth | NSD |
| | **Post-mortem findings**: carcass mildly autolyzed, flippers intact, hairs on body coat, foetalic lungs, other organs did not reveal any specific gross changes. | | | | | | | | |
| 12 | 45A/11 | Bovine | V | M | 9 | 18 | 22 | Stillbirth | NSD |
| | **Post-mortem findings**: carcass moderately autolyzed, flippers intact, hairs on body coat, abdominal and thoracic cavity filled with reddish colour fluid, stomach content was viscous and reddish in colour. foetalic lungs, other organs did not reveal any specific gross changes. | | | | | | | | |
| 13 | 55A/11 | Bovine | V | M | 5 | 2.5 | 16 | Abortion | Brain was mushy and semisolid. |
| | **Post-mortem findings**: carcass severly autolyzed, flippers intact, hairs not present on body coat, Abdominal and pelvic cavity viscera were missing, foetalic lungs, other organs did not reveal any specific gross changes. | | | | | | | | |
| 14 | 78A/11 | Bovine | V | F | 9 | 24 | 33 | Dystokia | NSD |
| | **Post-mortem findings**: carcass mildly autolyzed, flippers intact, hairs on body coat, Face and fore limb was swollen, Liver enlarged, turgid and dark red in colour, cut surface showed oozing of blood, Straw colour viscous fluid was present in stomach.foetalic lungs, other organs did not reveal any specific gross changes. | | | | | | | | |
| 15 | 123A/11 | Bovine | V | M | 4 | 1 | 6 | Abortion | Brain liquefied. |
| | **Post-mortem findings**: carcass severly autolyzed, flippers intact . Visceral organs showed advance stage of autolysis.. | | | | | | | | |
| 16 | 139A/11 | Bovine | V | M | 7 | 8 | 22 | Abortion | NSD |
| | **Post-mortem findings**: carcass mildly autolyzed, flippers intact, hairs on body coat, foetalic lungs, other organs did not reveal any specific gross changes | | | | | | | | |
| 17 | 175A/11 | Bovine | V | M | 4 | 1 | 6 | Abortion | NSD |
| | **Post-mortem findings**: carcass severly autolyzed, aborted fetus was small in size, having ruptured cavities exposing viscera which were contaminated with dung and dirt. The trunk and appendages were appearing crushed with absence of hind limbs and left fore limb. The carcass was not fit for diagnosis. | | | | | | | | |
| 18 | 190A/11 | Bovine | V | F | 7 | 9 | 22 | Abortion | Autolyzed |
| | **Post-mortem findings**: carcass severly autolyzed | | | | | | | | |
| 19 | 297A/11 | Bovine | V | F | 6.5 | 5 | 15 | Premature birth | NSD |
| | **Post-mortem findings**: carcass moderately autolyzed, flippers intact, hairs on body coat, Kidneys congested, Ecchymotic haemorrhage on serosal surface of the stomach. Cranio-ventral congestion of lungs. other organs did not reveal any specific gross changes. | | | | | | | | |
| 20 | 311A/11 | Bovine | Th | M | 7 | 8 | 21 | Abortion | Autolyzed |
| | **Post-mortem findings**: carcass severly autolyzed | | | | | | | | |
| 21 | 325A/11 | Bovine | M | M | 10 | 30 | 27 | Premature birth | . NSD |
| | **Post-mortem findings**: carcass moderately autolyzed, flippers intact, hairs on body coat, Kidneys congested, Lungs were inflated and mildly congested. other organs did not reveal any specific gross changes. | | | | | | | | |

V = Vrindavani; M = Murrah; Th = Tharparkar

**Table 2: Distribution of samples based on age and species wise**

| Category | Gestation time | Cattle brain | Buffalo brain |
|---|---|---|---|
| Aborted foetuses | 0-90 days | - | - |
| | >90-180 days | 4 | - |
| | >180 days | 5 | 1 |
| Dystocia | Full gestation | 7 | - |
| Premature birth | - | 3 | 1 |
| Total | | 19 | 2 |

volume of 10% NBF. Full term brains were sectioned once to allow access of the fixative to the ventricles. The brain was stored for three days, washed, and then cut.

**Processing of brains from different anatomical sites**

After proper fixation, thin coronal slices (3 mm) from posterior to anterior side of the brain were cut. Then parts of designated sites (cervical spinal cord enlargement behind medulla, medulla oblongata at the the obex, cerebellum at the caudal peduncle, pons, caudal colliculi, rostral colliculi, thalamus at the level of mammilary body, hippocampus with pyriform lobe and cerebrum with caudate nucleus) were taken to ensure examination of a wide range of neuroanatomical sites as per the method described by De Lahunta (1977) (Fig. 1). The morphological (gross and microscopic) lesions in brain induced by inherited such as dysraphic state, neuronal migration disorder (lissencephaly, pachygyria), encephaloclastic defects (porencephaly and hydranencepahly), malformation of the cerebellum (hypoplasia and atrophy) and hydrocephalus; diseases caused by bacteria, chlamydia, viruses ( BTV, BVD etc.), fungi, protozoa (toxoplasma, neospora); nutritional (Cu, Vit-$B_{1,}$ Vit-E, Vit-D deficiency; toxicosis (pesticides, chemicals,drugs); circulatory disturbances, hypomyelination and dysmyelination, if any, were taken into account during examination.

**Section preparation and staining**

The properly fixed sections were processed for making paraffin blocks and to get 5µ thick sections using standard procedures. The paraffin sections from each block were stained with hematoxylin and eosin (HE) and examined microscopically.

**Microscopic examination**

The brain section from designated sites in each case was examined at different neuroanatomical locations and the microscopic lesions were recorded (Table 3). The gross photographs of designated parts of the coronal sections were shot using digital camera. The microscopic photographs were taken with digital camera fitted with microscope.

**Etiological identification**

**Gram's staining**

The smear was made from fetal stomach content, air dried and fixed by heating. Gram's staining was done in the smear to demonstrate bacterial etiology.

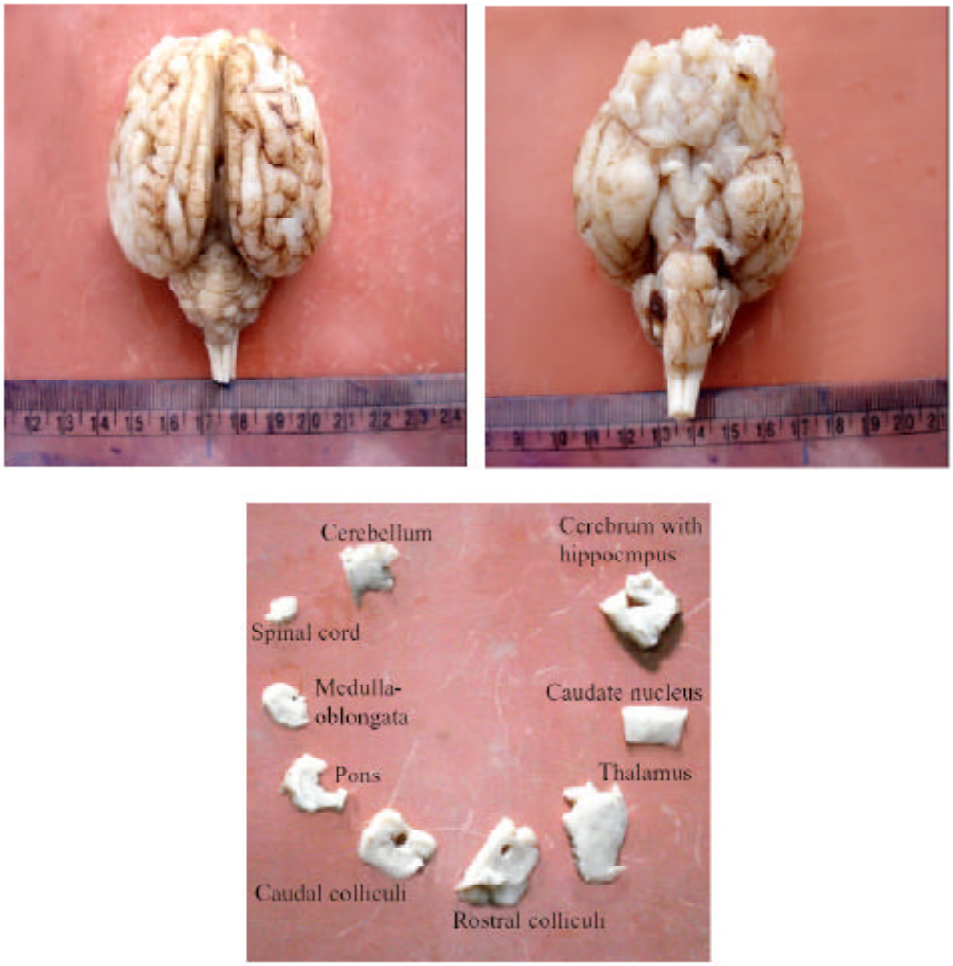



**Fig. 1 :** Dorsal and ventral view of cattle fetal brain (upper panel). The coronal or part of coronal sections of fetal brain at different anatomical sites used for histopathological studies (lower panel).

The fetal tissue/fluid such as lungs, liver, spleen, kidneys, brain and stomach contents were aseptically collected in sterile vials during post-mortem examination. Then the samples were got analysed in specific labs (Bacteriology Lab., CADRAD, IVRI; HSADL, Bhopal) for the presence of any specific etiological agents, affecting fetal brain development.

**Table 3: Different neuroanatomical sites of the bovine foetal brain for examination of any lesion.**

| **Anatomical Sites** | **Microscopic sites** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Spinal cord behind medulla (8) | Somatic afferent neurons (Dorsal gray column) | Visceral afferent neurons (Dorsal gray column) | Somatic efferent neurons (Ventral gray matter) | Visceral efferent neurons (Ventral gray matter) | Intermediate gray column | Dorsal funiculus | Lateral funiculus | Ventral funiculus |
| Medulla oblongat at the obex (8) | Hyposglossal nucleus | Vagus nucleus | Gracilis nucleus | Trigeminal nucleus | Inferiorior olivary nucleus | Raphe | Cuneatus nucleus | Formation reticularis |
| Cerebellum at the caudal peduncle (7) | Stratum moleculare | Purkinje cell layer | Stratum granulosum | Stratum medullare (tracts) | Basal cerebellar nuclei | Dentate nucleus | Nucleus globusus | – |
| Pons (6) | Abducent nucleus | Vestibularar nucleus | Trigeminal nucleus | Facial nerve nucleus | Superior olivary nucleus | Formation reticularis | – | – |
| Caudal colliculi (4) | Periaqudactal area | Red nucleus | Oculomotor nucleus | Substantia nigra | – | – | – | – |
| Rostral colliculi (4) | Periaqudactal | Red nucleus | Oculomotor nucleus | Substantia nigra | – | – | – | – |
| Thalamus at the level of mammillary body (4) | Habenular nucleus | Rostral thalamic nucleus | Mamillo thalamic tract | Mamillo tegmental tract (Formation reticularis) | – | – | – | – |
| Hippocampus at the pyriform lobe (5) | Fimbria fornicis | CA1 | CA2 | CA3 | Dentatus gyrus | – | – | – |
| Cerebrum with caudate nucleus (8) | Gray matter (Neuropil area) | White matter | Molecular layer | External molecular layer | External pyramidal layer | Internal granular layer | Internal pyramidal layer | Fusiform layer |

## B. Experimental study

### Reference virus

Challenge Virus Standard (CVS- 18) strain of rabies virus maintained in Biological Products Division of Indian Veterinary Research Institute, Izatnagar (U.P.) was used for making inoculms. The aliquot of titrated virus $LD_{50}$ $10^{3.7}$ per ml stored at -80$^0$C was diluted 1 in 100 using PBS to make 100 $LD_{50}$ 0.03 ml of inoculums for the experiment.

**Glass and plasticware**

All the glassware and plasticware were treated with 0.1% DEPC (Diethyl pyrocarbonate) treated water to remove the RNAse activity for RNA (Ribonucleic acid) work. The treated wares were also sterilized by autoclaving/hot air oven to maintain sterility.

**Chemicals and biologicals**

All the chemicals and the biologicals used in the study were of molecular and cell culture grade. Rabies antiserum of equine origin (Equirab 1500) from Bharat Serums and Vaccines limited, Ambernath, India was used in rabies infected mice. Details of other chemicals used in the study are given, wherever necessary.

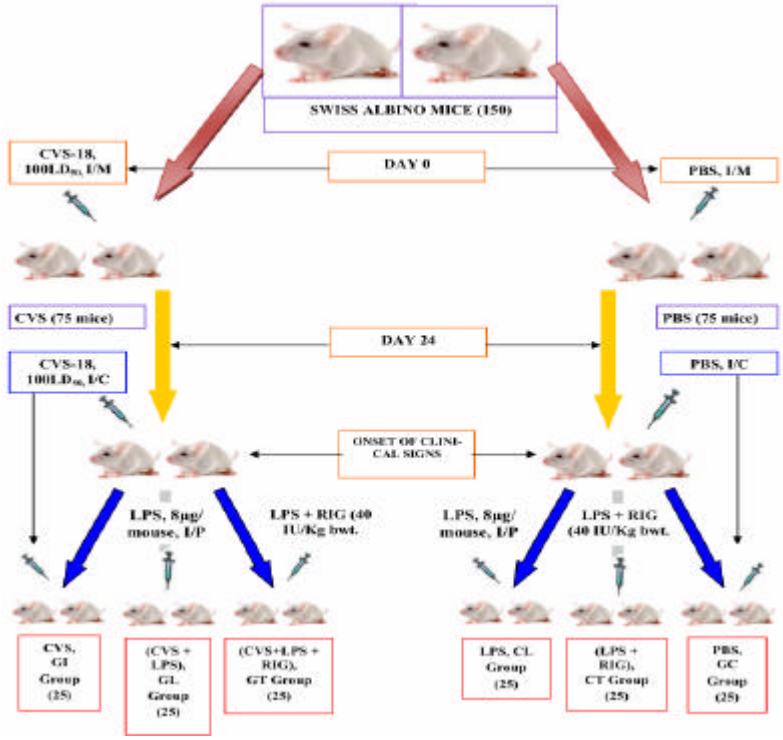
**Experimental animals**

A total of 150 Swiss albino mice of either sex, aged approx 4-5 weeks, were procured from Laboratory Animal Research Section (LARS), Indian Veterinary Research Institute, Izatnagar. The mice were kept in polypropylene cages and provided feed and water *ad libitum*. The mice were maintained as per the guidelines of Animal Ethics Committee of the institute. The experiment in mice was carried out as per the laid down procedures of CPCSEA and prior approval.

**Experimental plan**

Out of 150 mice, seventy five mice were inoculated with 100 $LD_{50}$ per 0.03 ml of CVS-18 strain on day 0 into masseter muscles with the intention to provoke certain degree of peripheral immune response as seen in natural cases of rabies and designated as CVS group. The CVS group was again inoculated with same dose intra-cerebrally on $24^{th}$ day for induction of clinical signs, since CVS-18 is highly adopted to brain and its peripheral inoculation does not produce the disease. On the day of onset of clinical signs ($6^{th}$ day), one-third of the mice (25/75) in CVS group were inoculated with LPS intra-peritoneally @ 8 µg/ mouse to modulate the blood-brain barrier and additional immunity, and designated as CVS+LPS group (GL). Then out of remaining 50 mice of CVS group, 25 mice were inoculated with LPS (8 µg/ mouse) + RIG (Rabies immunoglobulin) by intra-peritoneal and intra-venous routes, respectively and designated as CVS+LPS+RIG group (GT). The remaining 25 mice were kept as positive control and designated as CVS group (GI).

In 75/150 un-inoculated mice, PBS was injected in respective routes on respective days as done for CVS group and designated as PBS control (PBS). On day 6th, one-third (25/75) of the PBS group of mice were inoculated with LPS intra-peritoneally @ 8 µg/ mouse and designated as LPS control group (CL). Next one-third of mice (25/75) were inoculated with LPS (8 µg/ mouse) + RIG (Rabies immunoglobulin) by intra-peritoneal and intra-venous routes, respectively and designated as LPS+RIG control group (CT). The remaining one-third mice were kept as negative control and designated as PBS group (GC).




**Flow Chart of the expeirmental plan in Swiss albino mouse model**

I/M: intramuscular; I/C: intracerebral; I/P: intraperitoneal; IU: international unit, CVS: challange virus standard, LPS: lipopolysaccharide, RIG: Rabies Immunoglobulin, PBS: Phosphate Buffered Saline, LPS and antibody in control groups were injected at the same day as in infected group.

## Parameters studied

### Before LPS treatment

- After the first injection of CVS-18 in the masseter muscle, the mice were observed daily and clinical signs recorded in mouse history card. The blood samples were collected on days 0, 7th and 20th from retro-orbital plexus of both CVS and PBS groups, after ether anaesthesia. The NK cells and $CD4^+$/ $CD8^+$ cells kinetics were done in blood by Fluorescence Activated Cell Sorter (FACS) analysis and antibody response was estimated by Rapid fluorescent focal inhibition test (RFFIT) in serum.

- After second injection in brain (24th day after 1st injection) in mice, clinical signs were recorded daily. Later, on day 2nd of second inoculation 3 mice each were sacrificed from groups CVS and PBS. Brains were collected and preserved in 10% NBF and zinc fixative (BD Pharmingen, USA) for histopathology and immunohistochemistry, respectively. The brain tissue was collected in RNA Later (Ambion, USA ) for PCR.

**After LPS treatment**

- Three brains were collected from each group on next day of LPS inoculation (i.e. on 7th DPI after intra-cerebral inoculation) for detection of virus by conventional PCR and real time PCR. Besides, expression profile of TNF-a, CXCL10, IFN-ß, TLR3 and TLR4 mRNA using real-time PCR, histopathology and immunohistochemistry for detection of lesions, distribution of virus antigen by direct fluorescent antibody test (dFAT) and ß catenin expression in capillary endothelium were conducted in brain sections.
- Three mice in all the groups (GI, GL, GT, CL, CT and GC) were sacrificed by ether inhalation on 9th and 11th day (DPI to intra-cerebral inoculation). Thirteen mice in all the groups were retained till their death for survival study. The samples were collected similarly as above and subjected to above estimations.

**Flow Cytometry**

Flow cytometry was done for the evaluation of NK cells, $CD4^+$ and $CD8^+$ cells in blood in all the groups at 0, 7th, 20th day in intra-muscularly CVS challenged mice followed by at 2nd, 7th, 9th and 11th day in intra-cerebrally CVS challenged mice in same group in both blood and spleen (Fig. 11A and B). Following standard protocol was used to process the samples for FACS analysis:

- 50 µl of blood sample was taken in 1.5 ml micro centrifuge tube and diluted with 50µl of PBS (pH 7.4).
- 10 µl of rat anti mouse CD4: FITC/CD8: RPE cocktail antibodies (AbD SeoTec ) and 10 µl of rat anti mouse NKG2A antibody (AbD SeroTec ) were added to each sample and incubated at RT for 30 min in dark.

- The cells were washed twice with 1ml of PBS and spinned at 5000 rpm for 5 min.
- Supernatant was removed and 1 ml of erythrocyte lysis buffer was added to the pellet, vortexed and incubated for 7-10 min at RT.
- Tubes were spinned at 5000 rpm for 5 min.
- Supernatant was removed and the pellet was reconstituted with 0.5 ml of PBS and vortexed.
- Finally, the samples were subjected for FACS analysis (BD FACS Calibur Instrument, USA).

**Rapid fluorescent focus inhibition test (RFFIT)**

Blood was collected as per above schedule from mice and serum separated under aseptic condition in 500 µl of eppendorf tube, and were stored at -20ºC. Later, serum was inactivated by keeping in water bath at 56ºC for 30 min and used to assess antibody titer.

**Procedure**

1. **Serum dilution:** 30 µl of 10% GMEM was taken in a row of 10 wells in a 384 well cell culture plate. 30 µl of serum sample was added in the first well and mixed thoroughly and 30 µl was transferred to the next well. Likewise, a serial 2-fold dilution was made up to the last well and finally 30 µl was discarded from the $9^{th}$ well and $10^{th}$ well have only media (this makes 2-fold serum dilution from 1:4 to 1:4096). Similarly, 2-fold dilution of reference serum was prepared.
2. **Virus dilution:** PV strain of rabies virus, having titre of $1.29x10^7$ FFU/ml, was diluted 1:100 in chilled 10% GMEM and mixed thoroughly.
3. 30 µl of diluted virus was added to all wells containing serially 2-fold diluted serum and virus control well. Then, 384 well cell culture plate was incubated at 37ºC for 1 hr for virus neutralization in $CO_2$ incubator.
4. After the virus neutralization step, 30 µl of BHK-21 cells was added in all wells of virus-antibody mixture as well as in reference serum and virus control well, and incubated at 37ºC for 24 hrs in humidified chamber ($CO_2$ incubator).

5. After the incubation period of 24 hrs, the spent medium was discarded completely in wide mouth discard and the cell monolayer was washed with PBS (pH 7.2).

6. Cells were fixed with 80% chilled acetone (80/20 in PBS) and Terasaki plate was kept at -20ºC for 30 min.

7. Acetone was aspirated completely and the plate was air dried in room incubator at 37°C for 20 min.

8. After complete drying, rabies anti-nucleocapsid antibody conjugated with FITC (1/20) dilution in phosphate buffer (pH 7.2) was added at the rate of 10 µl per well to cover the monolayer entirely and plate was covered with aluminum foil.

9. Then, cell culture plate was incubated at 37ºC for 30 min. in humid chamber ($CO_2$ incubator).

10. Aspirated the conjugate and washed the plates with PBS. Then 20 µl of mounting fluid was added to all wells (50% Glycerine in PBS, v/v).

11. Plate was observed under fluorescent microscope and serum neutralization end point titer in terms of FFD50 was determined.

12. Antibody titer in test serum was calculated by comparison with the reference serum in IU/ml (300 IU/ml).

**Histopathology**

The brain samples were collected in 10% (NBF) and zinc fixative for histopathology and Immunohistochemistry, respectively. After proper fixation these were processed by paraffin embedded technique. The paraffin blocks were cut with microtome (Microme GmbH, Germany) to obtain 5 µm thick paraffin embedded coronal sections on glass slides. The coronal sections of cerebral hemispheres were selected at 3 levels, namely rostral, mid and caudal. The serial sections at these levels were stained by hematoxylin and eosin (H&E) technique (Luna, 1972) and selected as per a mouse stereotaxic atlas (Ref. http//:www.mbl.org). The lesions in selected sections of the brain were graded by histopathological score (HPS) system (Table 4). The sections at these levels were looked for various parameters: neuronal degeneration, gliosis, peri-vascular cuffing (PVC) and vascular changes. These were graded on the scale of 0 - 3 (0

= no change, 1 = mild change, 2 = moderate change, 3 = severe change). The maximum score was taken as the most severe inflammatory/immunological change.

**Table 4 : Histopathological score (HPS) system for evaluation of parameters in cerebral hemisphere as per mouse stereotaxic atlas at different intervals after intra-cerebral inoculation of CVS-18.**

| Anatomical Parameters | | 2 DPI | | 7 DPI | | 9 DPI | | 11 DPI | |
|---|---|---|---|---|---|---|---|---|---|
| sites | | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| **Rostral coronal section of cerebral hemisphere (section 14)** | | | | | | | | | |
| Gray matter | Neuronal degeneration | | | | | | | | |
| | Gliosis | | | | | | | | |
| | PVC | | | | | | | | |
| White matter | Nuclei degeneration | | | | | | | | |
| | Gliosis | | | | | | | | |
| | PVC | | | | | | | | |
| Meninges | Meningitis | | | | | | | | |
| Blood vessels | Congestion/Hemorrhage | | | | | | | | |
| | Total (24) | | | | | | | | |
| | Mean | | | | | | | | |
| **Mid coronal section of cerebral hemisphere (section 19)** | | | | | | | | | |
| Gray matter | Neuronal degeneration | | | | | | | | |
| | Gliosis | | | | | | | | |
| | PVC | | | | | | | | |
| White matter | Nuclei degeneration | | | | | | | | |
| | Gliosis | | | | | | | | |
| | PVC | | | | | | | | |
| Meninges | Meningitis | | | | | | | | |
| Blood vessels | Congestion/Hemorrhage | | | | | | | | |
| | Total (24) | | | | | | | | |
| | Mean | | | | | | | | |
| **Caudal coronal section of cerebral hemisphere (section 21)** | | | | | | | | | |
| Gray matter | Neuronal degeneration | | | | | | | | |
| | Gliosis | | | | | | | | |
| | PVC | | | | | | | | |
| White matter | Nuclei degeneration | | | | | | | | |
| | Gliosis | | | | | | | | |
| | PVC | | | | | | | | |
| Meninges | Meningitis | | | | | | | | |
| Blood vessels | Congestion/Hemorrhage | | | | | | | | |
| | Total (24) | | | | | | | | |
| | Mean | | | | | | | | |

Each parameter was assigned grade on scale 0-3, i.e. 0=no change, 1=mild change, 2=moderate change, 3=severe change. The score for each mouse at each time point was aggregated and divided by number of mice to obtain mean value. The higher score was taken as the criteria for severe pathological changes over the lower score. The parameters were graded for each anatomical site on total grade score of 24.

## Immunohistochemistry for ß-catenin

### Coating of glass slides:

Immunohistochemistry (IHC) involves prolonged exposure of sections to various solutions, antibodies and heat induced antigen retrieval, therefore, adequate adhesion was ensured using 3-aminopropyle- triethoxysilane (APES) (Sigma Aldrich, USA). The slides were washed in 2% detergent in pre-warmed distilled water (DW) for 30 min. The slides were thoroughly rinsed in DW followed by acetone and air dried. For coating, slides were immersed in 2% (v/v) APES in acetone for 30 min. Ultimately the slides were rinsed gently in acetone then in DW and air dried at $37^0$ C.

## b-catenin demonstration in brain capillaries

The paraffin embedded serial sections of cerebral hemisphere were taken on to amino propyle ethoxysilane (APES) coated slides. The sections were deparaffinized in xylene and rehydrated in descending grades of ethyl alcohol (100%, 90%, 80% and 70%) and brought to distilled water. Endogenous peroxidase activity was blocked by incubating for 10 min in 3% $H_2O_2$ in methanol and then washed thrice with PBS (pH 7.2), 5 min each. Later, the antigen retrieval was carried out by heat/boiling in citrate buffer for 15 min in microwave oven. The sections were cooled at room temperature and then incubated with normal goat serum for 20 min to avoid non specific binding. The slides were washed thrice in PBS and incubated with primary anti-β catenin polyclonal antibodies (AbD Serotec, UK) at 4°C overnight. After washing thrice with PBS for 5 min, slides were poured with biotinylated secondary anti-rabbit IgG antibody (Sigma, USA) and kept for 30 min at $37^0$C in an incubator. Sections were washed with PBS as above and incubated with avidin-biotin-peroxidase complex for 30 min (Rabbit ExtrAvidin peroxidase stainig kit, Sigma, USA). The sections were washed thoroughly with PBS and stained by using AEC staining kit (Sigma, USA) for 10 min. The sections were then rinsed briefly in distilled water and counter stained with Meyer's hematoxylin for 30 sec.

The sections were washed in water and mounted in aqueous mountant. All the steps were carried out in humidified chamber and as per the instruction of manufacturer. Proper negative and positive antibody controls were also run in parallel to test slides. The intensity of positive signals was comparatively graded by taking control mouse brain as positive control.

## Direct fluorescent antibody technique (dFAT)

The brain impressions on glass slides was taken from cerebrum, hippocampus and cerebellum. The impressions were air dried at room temperature and then fixed in coplin jars containing chilled acetone 2 hrs. After fixation, the impression area was and then the slides immersed in PBS (pH 7.2) for 5 min. The slides were then charged with anti-rabies FITC conjugate (FDI Fugirebio, USA) in encircled area and incubated in humidified dark chamber at 37 °C. The slides were washed thoroughly thrice with PBS in slide holding glass trough by creating current with magnetic stirrer. After washing, slides were examined under fluorescent microscope (Laborlux-S Leitz, USA) in 40x objective. The presence of dusty apple green fluorescence was taken as positive signal. During the process, positive and negative controls were run along with test slides.

## IFN –ß Analysis in serum:

The blood samples were collected at different time intervals as per experiment schedule, serum was separated and store at -20$^0$ C until analysis. ELISA kit (Mouse Interferon Beta ELISA Kit, PBL Biomedical Laboratories) was used in the analysis using the protocol provided by manufacturer.

**Procedure:**

1. **Preparation of Wash Buffer**: Diluted the 50 ml of 10x wash concentrate (Bottle A) with 450 ml of distilled water and stored at 4$^0$C.

2. Prepared 1:10 working stock of the Mouse IFN Standard by pipetting 10 µl of the Mouse IFN-ß Standard (Vial B) in 90 µl of sample diluent (Bottle C) and from this working stock, prepared the standard curve using the amounts in the table shown below:

| Tube No. | Working stock | $S_7$ | $S_6$ | $S_5$ | $S_4$ | $S_3$ | $S_2$ | $S_1$ | BL |
|---|---|---|---|---|---|---|---|---|---|
| Amount taken from tube to left (ml) | - | 0.020 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | - |
| Sample Diluent (ml) | - | 0.98 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 1.0 |
| Final Conc. (pg/ml) | 50000 | 1000 | 500 | 250 | 125 | 62.5 | 31.3 | 15.6 | 0 |

3. Samples of unknown interferon concentration to be tested were diluted in the sample diluents in 1:5 dilutions.

4. Placed 100 µl of the interferon samples and standard curve prepared in Step 2 in individual wells of the microtiter plate in duplicate.

5. Covered the microtiter plate with plastic plate sealer and incubated for 60 min in a closed chamber at 25°C. After incubation, washed three times with wash solution and blotted well after the final wash.

6. Added 100 µl of the Antibody Solution to each well and covered the microtiter plate with plastic plate sealer and incubated for 60 min in a closed chamber at 25°C. After incubation, washed three times with wash solution and blotted well after the final wash.

7. Added 100 µl of the HRP solution to each well and cover the microtiter plate with plastic plate sealer and incubated for 60 min in a closed chamber at 25°C. After incubation, washed three times with wash solution and blotted well after the final wash.

8. Added 100 µl of the TMB Substrate Solution (Bottle G) to each wel and incubated for 15 min in a closed chamber at 25°C.

9. After that added 100 µl of Stop Solution (Bottle H) to each well and mixed gently.

10. Then using a microplate reader, determined the absorbance at 450 nm within 5 minutes after the addition of the stop solution.

11. Unknown sample concentrations determined by extrapolation off the standard curve.

**Fluorojade B staining for degenerated neurons:**

Fluoro-Jade B ( Millipore) is a polyanionic fluorescein derivative which sensitively and specifically binds to degenerating neurons. In brief, the following protocol was used for paraffin embedded sections. The paraffin embedded sections were deparaffinized in xylene and rehydrated in descending grades of ethyl alcohol (100%, 90%, 80% and 70%) and brought to distilled water. The slides were then transferred to a solution of 0.06% potassium permanganate for 20 min. The slides were then rinsed in distilled water for 2 min. After that slides were kept in coplin jar containing working fluoro-jade B solution for 15 min. After 15 min in the staining solution, the slides were rinsed for one minute in each of three distilled water washes. Excess water was removed by briefly (about 15 s) draining the slides vertically on a paper towel. The slides were then made air dry. The dry slides were cleared by immersion in xylene and mounted by using DPX- a non-aqueous non-fluorescent plastic mounting media. The slides were then observed under fluorescence microscope.

**Isolation of total RNA from brain tissue**

Total RNA from brain tissue was isolated by RNeasy Lipid Tissue Mini Kit (QIAGEN, USA). Briefly, ~100 mg of brain tissue was taken in 1 ml of QIAzol lysis reagent and the contents were homogenized using Teflon glass homogenizer. The suspension was incubated for 5 min at RT. 200 µl of chloroform was added, mixed vigorously for 15 sec and incubated for 2-3 min at RT. Then, the contents were centrifuged at 13,000 rpm for 15 min at 4°C. After centrifugation, the colourless aqueous phase was transferred into a new (1.5 ml) microcentrifuge tube, to that 1 volume of 70% ethanol was added and vortexed. The sample was transferred to RNeasy mini spin column placed in a 2 ml collection tube, centrifuged for 15 sec at 10,000 rpm and flow-through was discarded. RNeasy column was washed with 700 µl of buffer RW1, and then the columns were treated with 500 ml of buffer RPE twice by centrifugation for 15 sec at 10,000 rpm each time. Finally, the RNA was eluted with 50 µl of nuclease free water by centrifuging at 10,000 rpm for 1 min and the RNA was stored at -20 °C until further use.

**cDNA preparation**

The RNA concentration in all the samples was quantified using NanoDrop ND-1000 spectrophotometer (Nanotechnologies, USA) and equal concentration of total RNA was taken

in all the groups for cDNA synthesis by using Promega Reverse Transcription Kit, following the protocol recommended by the manufacturer.

**Procedure:**

1. 1 µg of total RNA, were taken in 0.2 ml microcentrifuge tubes and incubated for 10 min at 70 °C. Then samples were spin briefly and placed over ice.

2. 20 µl reaction mixture was prepared by adding following reagents in order listed:

| Reagents | Amount |
|---|---|
| MgCl2, 25mM | 4 µl |
| Reverse transcription 10X Buffer | 2 µl |
| dNTP Mixture, 10 mM | 2 µl |
| Recombinant Rnasin(Ribonuclease inhibitor) | 0.5µl |
| AMV Reverse Transcriptase | 15 µl |
| Oligo$(dT)_{15}$ Primer | 0.5µg |
| mRNA | 1µg |
| Nuclease-free water to a final volume of | 20µl |

3. The reaction mixture was incubated at 42 °C for 15 min and then heated at 95 °C for 5 min to inactivate reverse transcriptase.

4. cDNA was then stored at -20 °C .

**Detection of rabies virus by RT-PCR**

PCR for N gene was carried out by mixing the following contents to make final volume of 25 µl reaction using 2.5x master mix (5 prime, USA).

| Component | Volume |
|---|---|
| 2.5x master mix | 10 µl |
| $MgCl_2$,10 mM | 1.0 µl |
| Primer Forward | 1 µl |
| Primer Reverse | 1 µl |
| Template cDNA | 2 µl |
| Nuclease free water | 10 µl |

The tube contents were mixed and centrifuged briefly to collect the contents. The N gene amplification was carried out using thermocycler (Mastercycler personal, Eppendorf, Germany) by following programme:

| Cycles | Time | Temperature (° C) |
|---|---|---|
| 1X | 5 min | 94 |
| 35X | 1 min | 94 |
| | 1 min | 53 |
| | 1 min | 72 |
| 1X | 10 min | 72 |

**Gel Electrophoresis**

1.5% agarose (Himedia, India) was prepared in 1X TBE buffer with ethidium bromide (0.5 µg/ml). The PCR product was run at 70V for 30 min. The bands were visualized in gel documentation system.

**Real Time PCR**

Cytokines (TNF-a, CXCL10, IFN-ß, TLR3 and TLR4) mRNAs expressions in brain samples were carried out by quantitative Real Time PCR (MX 3000 P System, Stratagene, USA) by using specific primers (Table 5). Real Time PCR reaction was performed in 96 well PCR plates using DyNAmo SYBR Green qPCR kit ( Finnzymes, Finland). The reaction mix was prepared by adding following reagents:

1. 10.0 µl of 2X master mix was taken in real time PCR tubes.
2. 1.0 µl of forward primer (10 pM of final concentration).
3. 1.0 µl of reverse primer (10 pM of final concentration).
4. 1.0 µl of cDNA (~ 50 ng final concentration).
5. Nuclease free water was added to adjust the final volume to 20µl.

The reagents were mixed gently without creating bubbles. The tubes were placed in the plate of the instrument and run on PCR programme as given below:

| Cycles | Time | Temperature (° C) |
|---|---|---|
| 1 | 10 min | 95 |
| | 30 sec | 94 |
| 40 | 30 sec | 56-62 |
| | 30 sec | 72 |

**Analysis of Real Time PCR results**

1. The arithematic mean of Ct values (m Ct) was calculated.
2. The Ct values were normalized using house keeping gene (ß- actin).

   ? Ct = mCt (target gene) - mCt (house keeping gene)
3. The Ct values were normalized using control group (GC).

   ? ? Ct = ? Ct (Experimental animal) - ? Ct (Control animal)
4. The relative quantity of target gene was calculated.

   Relative quantity = $2^{(-? ? Ct)}$
5. Transformed relative quantity of target gene was expressed in relative percentage of expression.

   Relative percentage of expression = relative quantity X 100

The data obtained were analysed by using the $2^{-(? ? Ct)}$ method (Livak and Schmittgen, 2001). Finally, the mean relative expression for each group were statistically analysed by one way ANOVA [least significance difference (LSD) and Dunccan's tests] for 't' distribution (P value) using GraphPad Prism 4 software.

**Table 5 : List of primers used for amplification of different genes by Real Time PCR.**

| Gene | Primer sequence | Primer length | Product (bp) | Annealing temp ($^{o}$C) | Product Tm | Reference |
|---|---|---|---|---|---|---|
| TNF-α | F 5' CCCTTTACTCTGACCCCTTT 3'<br>R 5' AACCTGACCACTCTCCCTTT 3' | 20<br>20 | 310 | 50 | 83.43±0.03 | Mckimmie *et al.*, 2005 |
| CXCL10 | F 5-' CAC CAT GAA CCC AAG TGC TGC CGT 3'<br>R 5'- AGG AGC CCT TTT AGA CCT TTT TTG 3' | 24<br>24 | 297 | 55 | 83.87±1.2 | Nagpal *et al.*, 2009 |
| IFN -ß | F5' CTGGAGCAGCTGAATGGAAAG 3'<br>R 5'CTTGAAGTCCGCCCTGTAGGT 3' | 21<br>21 | 50 | 60 | 76.90±0.05 | Auerbuch *et al.*, 2004 |
| TLR3 | F 5'GAAGCAGGCGTCCTTGGACTT 3'<br>R 5' TGTGCTGAATTCCGAGATCCA 3' | 21<br>21 | 330 | 62 | 79.60±0.03 | Mckimmie *et al.*, 2005 |
| TLR 4 | F5'TTCACCTCTGCCTTCACTACA 3'<br>R5'GGGACTTCTCAACCTTCTCAA 3' | 21<br>21 | 224 | 58 | 81.66±0.03 | Mckimmie *et al.*, 2005 |
| RabN | F5' AATCAGGTGGTCTCTTTGAAG 3'<br>R5'AGCCCAATTCCCTTCTACATC 3' | 21<br>21 | 329 | 50 | 82.72±0.42 | Self designed |
| RabN | F5' TATGAGTACAAGTACCCTGCC<br>R5' AGCCCAATTCCCTTCTACATC | 21<br>21 | 287 | 50 | - | Self designed |
| ß- actin | F 5' TCTAGGCACCAAGGTGTG 3'<br>R 5' TCATGAGGTAGTCCGTCAGG 3' | 18<br>20 | 460 | 56 | 90.26±0.18 | Mckimmie *et al.*, 2005 |

# 4 Results

## A. Spontaneous fetal brain affections

### Gross findings in brain

There were no specific gross lesions observed in brain in either of the cases. The brain samples preserved *in situ* appeared normal, except for different degree of autolysis. The foetuses were mostly of late gestation period.

### Microscopic findings in brain sections

In microscopic examination of different anatomical sites of the brain, the changes were mainly of vascular type. The microscopic lesions in majority included mild to moderate vascular engorgement (21/21 cases). However, in 9/12 cases haemorrhages were also evident. A few cases showed focal to diffuse gliosis (6/21 cases), mild perivascular cuffing (single cell) (1/ 21case), and ischemic neurons (2/21cases), nests of normal glial cells (Calleja cells) in sub-ependymal region of lateral ventricles (2/21 cases) and in dentate fascia of hippocampus (1/21 case), presence of intact external granular cells layer in cerebellum (1/21 case) and stray presence of Purkinje cells in internal granular layer (3/21 cases), as a part of developmental process. One case of compactly arranged neurons in cerebral cortex was also observed (Table 6 and 2 A to H.). The lesion were distributed in cerebellum (15/21 cases), brainstem (9/ 21cases), cerebral hemisphere/ caudate nucleus (8/21cases), thalamus (6/21cases), hippocampus (5/21cases) and in meninges (8/21cases) (Table 7).

**Table 6: Histopathological changes observed in central nervous system of aborted fetal brains**

| S. No. | Case No. | Congestion/ Engorgement | Hemorrhage | Perivascular cuffing | Necrosis | Gliosis | Neuronal degeneration | Other abnormality |
|---|---|---|---|---|---|---|---|---|
| 1 | 567A/10 | +++ | + | + | - | ++ | - | - |
| 2 | 590A/10 | ++ | ++ | - | - | - | - | - |
| 3 | 604A/10 | + | - | - | - | - | - | - |
| 4 | 660A/10 | +++ | - | - | - | + | - | - |
| 5 | 662A/10 | + | + | - | - | - | - | - |
| 6 | 4A/11 | + | - | - | - | + | - | - |
| 7 | 17A/11 | ++ | ++ | - | - | - | + | + |
| 8 | 27A/11 | + | - | - | - | - | - | - |
| 9 | 32A/11 | + | - | - | - | + | - | - |
| 10 | 36A/11 | + | - | - | - | - | - | - |
| 11 | 41A/11 | ++ | + | - | - | - | - | - |
| 12 | 45A/11 | ++ | - | - | - | - | - | - |
| 13 | 55A/11 | + | - | - | - | + | - | - |
| 14 | 78A/11 | + | + | - | - | ++ | + | + |
| 15 | 123A/11 | +++ | + | - | - | - | - | - |
| 16 | 139A/11 | + | ++ | - | - | - | - | + |
| 17 | 175A/11 | + | - | - | - | - | - | - |
| 18 | 190A/11 | ++ | + | - | - | - | - | - |
| 19 | 297A/11 | + | - | - | - | - | - | - |
| 20 | 311A/11 | + | - | - | - | - | - | - |
| 21 | 325A/11 | ++ | - | - | - | - | - | - |

**Table 7 : Distribution of histopathological changes in different anatomical region of the fetal brain**

| Necropsy No. | Gestation age (Month) | Spinal Cord | Cerebellum | Brain stem (Medulla, pons and Mid brain) | Thalamus | Hippocampus+ Pyriform lobe | Cerebral hemisphere+ Caudate nucleus | Meninges |
|---|---|---|---|---|---|---|---|---|
| 567A/10 | 8 | - | Stray presence of Purkinje cells in granular layer | - | - | Congestion and Gliosis | Subventricular collection of primordial cells | Blood vessels engorged, hemorrhages |
| 590A/10 | 8 | - | Congestion | Congestion, Hemorrhages and gliosis | - | - | Diffuse gliosis | Blood vessels engorged |
| 604A/10 | 9 | - | Congestion, focal hemorrhage | Congestion and focal hemorrhage | Congestion | | Congestion | - |
| 660A/10 | 8&1/2 | - | Roof nuclei neurons showing chromatolysis | - | congestion | - | - | Blood vessels engorged |
| 662A/10 | 8 | - | congestion | - | congestion | - | - | Blood vessels engorged |
| 4A/11 | 8 | - | Congestion | Focal gliosis | - | Congestion | - | - |
| 17A/11 | 9 | - | Congestion | Congestion | - | - | Congestion | - |
| 27A/11 | 5&1/2 | - | Purkinje cells in granular layer | Congestion, | | Congestion and gliosis | - | - |
| 32A/11 | 9 | - | - | Congestion, hemorrhage | Congestion, gliosis | Congestion and gliosis | Focal gliosis | Leptomenin geal blood vessels engorged with blood |
| 36A/11 | 9 | - | congestion | congestion | congestion | | congestion | Blood vessels engorged |
| 41A/11 | 9 | -Stray presence of Purkinje cells in granular layer- | | | | - | - | - - |

**Table 7 : Contd...**

| Necropsy No. | Gestation age (Month) | Spinal Cord | Cerebellum | Brain stem (Medulla, pons and Mid brain) | Thalamus | Hippocampus+ Pyriform lobe | Cerebral hemisphere+ Caudate nucleus | Meninges |
|---|---|---|---|---|---|---|---|---|
| 45A/11 | 9 | - | - | - | Multifocal gliosis | - | - | - |
| 55A/11 | 5 | - | Congestion | - | - | - | - | Congestion |
| 78A/11 | 9 | - | Congestion | - | - | Congestion | Neurons in cortex compactly arranged in columnar fashion | Severe blood vessels engorgement and hemorrhages |
| 123A/11 | 4 | - | - | - | - | - | - | - |
| 139A/11 | 7 | - | Below piamater primordial neurons present | - | - | - | Congestion | - |
| 175A/11 | 4 | - | - | - | - | - | - | - |
| 190A/11 | 7 | - | - | - | - | - | - | |
| 297A/11 | 6.5 | - | Congestion | Congestion | - | - | - | - |
| 311A/11 | 7 | - | - | - | - | | - | - |
| 325A/11 | 10 | - | Congestion | - | - | - | - | - |

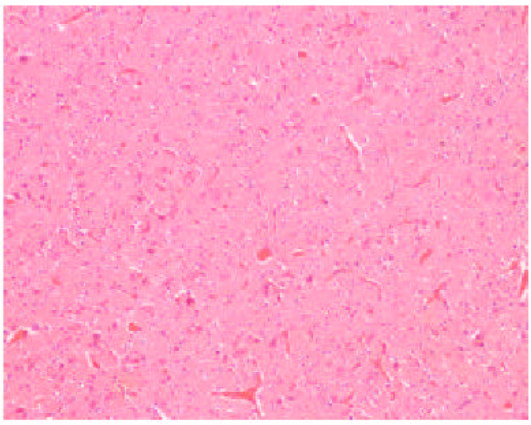


**Fig. 2A :** Micrograph of fetal brain showing vascular engorgement. H&E. X200

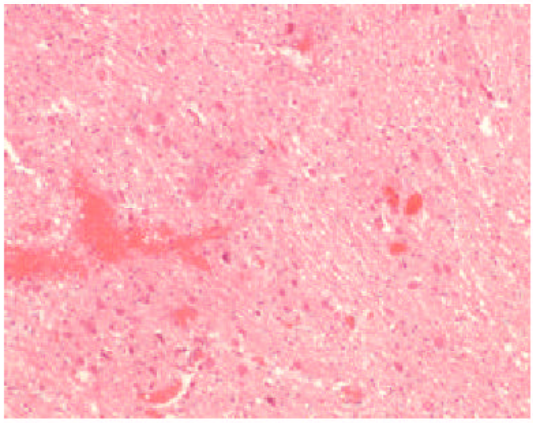


**Fig. 2B :** Micrograph of fetal brain showing haemorrhages. H&E. X200

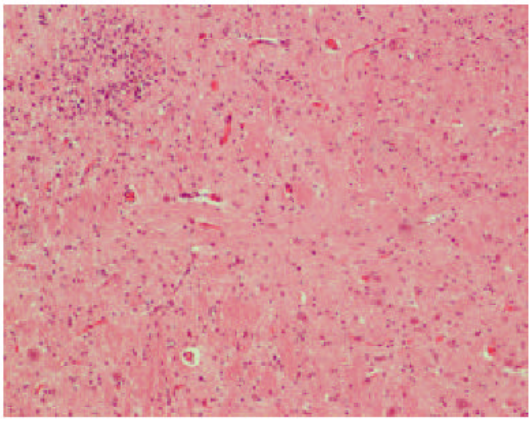


**Fig. 2C :** Micrograph of fetal brain showing focal gliosis in mid brain. H&E. X200

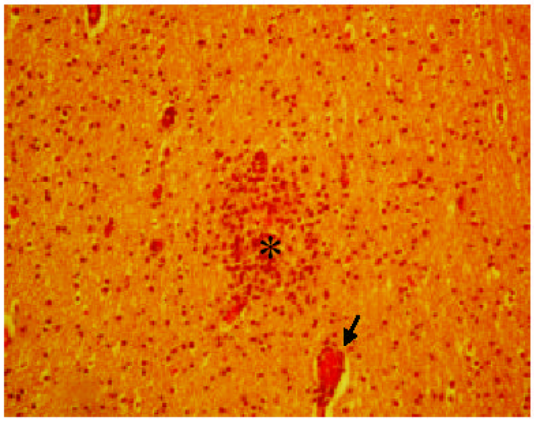


**Fig. 2D :** Micrograph of fetal brain showing focal gliosis (*) and perivascular cuffing (arrow). H&E. X200

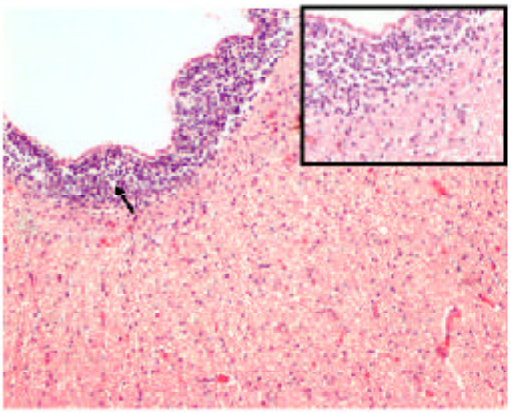


**Fig. 2D :** Micrograph of fetal brain showing nests of glia (Calleja cells in inset) in sub ventricular region (arrow). H&E. X200

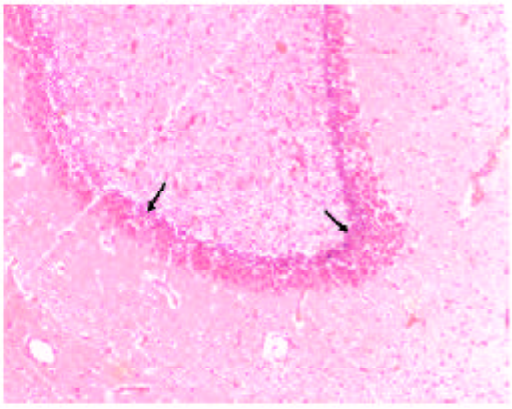


**Fig. 2E :** Micrograph of fetal brain showing nests of glia (Calleja cells) (arrow) in dentate region of hippocampus. H&E. X200

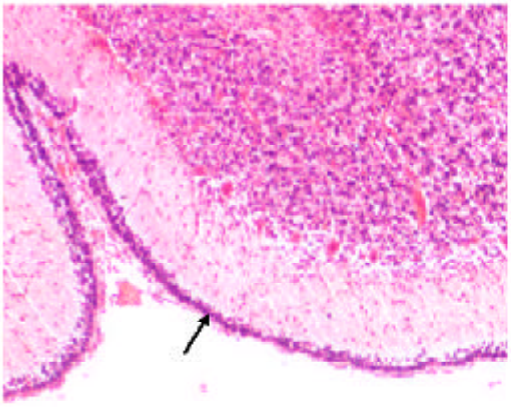


**Fig. 2F :** Micrograph of fetal brain showing presence of intact external granular layer (arrow) in cerebellum. H&E. X200

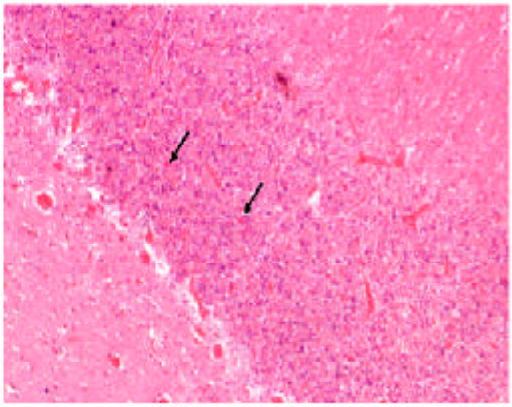


**Fig. 2G :** Micrograph of fetal brain showing stray presence of Purkinje cells (arrow) in granular layer in cerebellum. H&E. X200

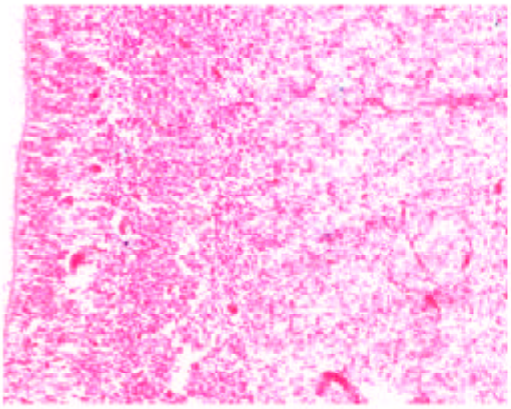


**Fig. 2H :** Micrograph of fetal brain showing compact neurons in cerebral cortex. H&E. X200

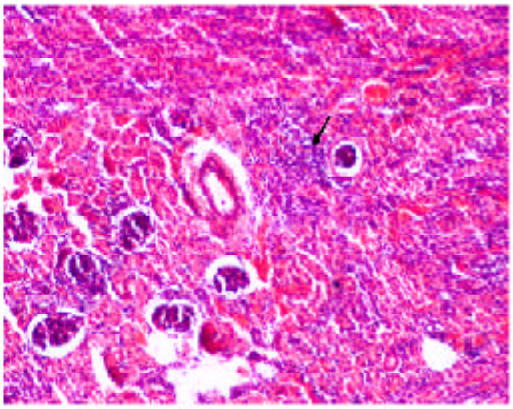


**Fig. 2I :** Micrograph of fetal kidney showing collection of lymphoid cells. H&E X200

**Microscopic findings in visceral organs**

In 8/21 cases, lungs, liver, spleen and kidneys did not show any specific changes. However, in remaining 13 cases there were vascular engorgement/haemorrhages in liver (7/13 cases), kidneys (6/13 cases) and lungs (6/13 cases), focal infiltration of mononuclear cells in four cases involved either, kidneys, liver, or lungs (Figs. 2I ).

**Etiological identification**

Seven out of 21 cases showed bacilli/coccobacilli on Gram's staining in stomach contents. However, on cultural examination, 4 out of 7 cases yielded *E. coli* and one case Candida spp. None of the samples were found positive for Brucella spp. The brain samples (14 cases) screened at HSADL, Bhopal for BVD virus, were found negative for the same by PCR.

## B. Experimental study

**Clinical signs and survival rate**

The mice (75/150) inoculated with 100 $LD_{50}$ CVS-18 strain of rabies virus into masseter muscle did not show any neurological signs up to 24 DPI. A few infected mice were sacrificed to detect the presence of virus in brain by dFAT and RT-PCR, which found negative for the same. Therefore, on 24 DPI, the same dose was injected intra-cerebrally. The clinical signs were noticeable by $6^{th}$ DPI in intra-cerebral infected group (Table 8, Fig. 3). Upon appearance of clinical signs (i.e. on $6^{th}$ day), one-third (25/75) of the mice were injected with LPS by intra-peritoneal route (GL group). The next one-third mice were injected with LPS+RIG by both intra-peritoneal and intra-venous routes, (GT group). The remaining one-third mice were kept as CVS positive control (GI group). Number of mice which showed clinical signs and mortality was less in the GT and GL compared to the GI (Table 9, Fig.4). The survivors were more in GT group (46%) followed by GL group (38%), as compared to GI group. In groups GT and GL, the clinical course was prolonged. Mice in the control groups CL (LPS), CT (LPS+RIG) and GC (PBS) did not show any clinical signs.

**Gross and histopathological lesions**

The mice sacrificed or died in all the six groups during the course of the experiment did not show any gross lesions. In GC group, the selected coronal sections of cerebral hemisphere

**Table 8 : Mouse clinical history card showing different clinical signs after LPS inoculation on the day of onset clinical signs.**

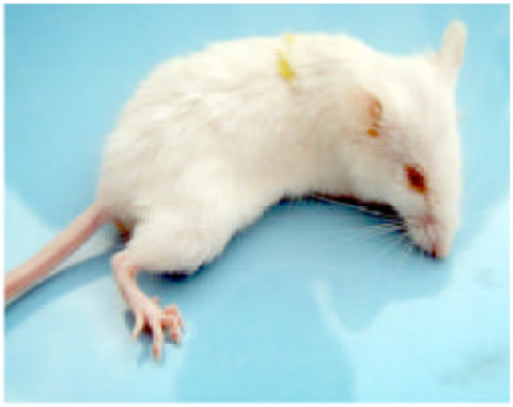
| Clinical parameters | Groups | No. of days post inoculation (DPI) LPS | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| **Ruffled fur** | CVS(GI) | 3 | 2 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | CVS+LPS(GL) | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | CVS+LPS+RIG(GT) | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | LPS(CL) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | LPS+RIG(CT) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | PBS(GC) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Tremor** | CVS(GI) | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | CVS+LPS(GL) | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | CVS+LPS+RIG(GT) | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | LPS(CL) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | LPS+RIG(CT) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | PBS(GC) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Incordination** | CVS(GI) | 0 | 1 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | CVS+LPS(GL) | 0 | 0 | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| | CVS+LPS+RIG(GT) | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | LPS(CL) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | LPS+RIG(CT) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | PBS(GC) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Paralysis** | CVS(GI) | 0 | 2 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | CVS+LPS(GL) | 0 | 1 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | CVS+LPS+RIG(GT) | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | LPS(CL) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | LPS+RIG(CT) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | PBS(GC) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Prostration** | CVS(GI) | 0 | 1 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | CVS+LPS(GL) | 0 | 0 | 1 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| | CVS+LPS+RIG(GT) | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | LPS(CL) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | LPS+RIG(CT) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | PBS(GC) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Death** | CVS(GI) | 2 | 1 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | CVS+LPS(GL)0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | CVS+LPS+RIG(GT) | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 |
| | LPS(CL) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | LPS+RIG(CT) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | PBS(GC) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 9 : Survival rate after LPS and LPS+RIG inoculation on the day of onset clinical signs.**

| Groups | Survival rate after LPS inoculation on the day of onset of clinical signs (At 6th DPI to CVS-18) | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
| CVS(GI) | 13 | 8 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| CVS+LPS(GL) | 13 | 9 | 7 | 6 | 6 | 6 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| CVS+LPS+RIG (GT) | 13 | 10 | 8 | 8 | 7 | 7 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| LPS(CL) | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 |
| LPS+RIG (CT) | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 |
| PBS(GC) | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 |

Ruffled fur

Posterior limb paralysis



Circling

Quadriplegia

Prostration

**Fig. 3 :** Clinical signs shown by rabies virus (CVS-18) infected Swiss albino mice during the course of disease.

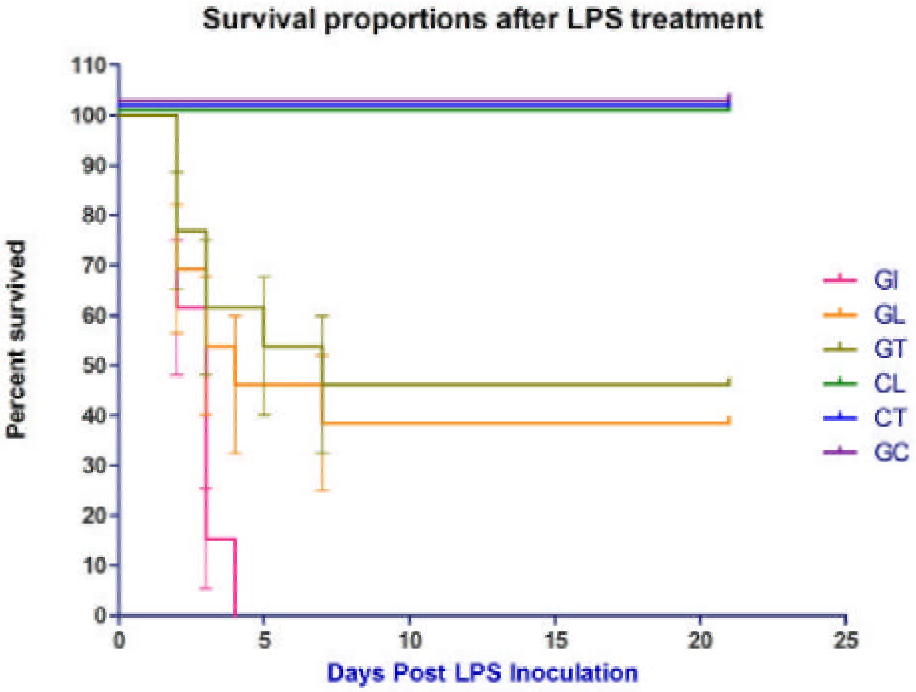



**Fig. 4 :** Survival rate of CVS-18 infected mice, treated with LPS @ (8µg/ mouse) (GL group) and LPS+RIG (40IU/Kg b.wt.) (GT group) on the day of onset of clinical signs.

did not show any microscopic lesions as evident by histo-pathological scores (HPS). In group GI on 2nd DPI, there was mild meningitis characterized by meningeal vessels engorgement and infiltration with mononuclear cells. The neurons in cerebral hemisphere were scattered and pyknotic with acidophilic cytoplasm. The hippocampal neurons (both dentate gyrus and CA1 and CA 2) appeared normal (Fig. 5). On 7th DPI, the cerebellar neurons appeared pyknotic and blood vessels in white matter were engorged .The extent of neuronal damage was more prominent in CA1 and CA2 layers with diffuse microgliosis and mild to moderate perivascular infiltration with mononuclear cells (Fig. 6,7 & 8). On 9th DPI, a severe neuronal degeneration in cerebral hemisphere and in the CA1 and CA2 regions, characterized by the distinct pyknosis was observed. Apoptotic bodies were also present in these areas. Majority of Purkinje neurons showed degenerative changes. The white matter between folia was loose with capillaries engorged with blood alongwith apoptotic bodies. The white matter in the thalamic/ hypothalamic area had similar reaction of perivascular cuffing, degenerated neurons and microgliosis (Fig. 9, 10 & 11). The HPS in cerebral hemisphere showed increasing trend (Gliosis, PVC, neuronal degeneration) from 2nd DPI onwards and reached maximum on 9th DPI. However, in LPS treated control groups (CL and CT), there were mild degree of vascular congestion with little mononuclear cell infiltration in lepto-meninges and the brain substance. On the contrary, the groups GT and GL showed mild pathological changes as compared with GI group (Table 10-12, Fig.12).

**Kinetics of $CD4^+$, $CD8^+$ and NK cells in blood after intra-muscular inoculation of CVS-18**

The kinetics of $CD4^+$, $CD8^+$ and NK cells was studied at different time intervals (Table 13-16, Fig. 13-17) in blood samples by flowcytometry.

The mean % of $CD4^+$, $CD8^+$ and NK cells and ratio of $CD4^+$ and $CD8^+$ cells in blood remained constant throughout the experiment in group PBS. In case of CVS group the value of $CD4^+$ decreased at day 7th and again revealed significant increase on day 20th.The same trend was also observed in $CD8^+$ and NK cells population. The ratio of $CD4^+$ and $CD8^+$ cells in CVS group decreased at day 7th and normalized on day 20th.

**Kinetics of $CD4^+$, $CD8^+$ and NK cells in blood after intra-cerebral inoculation of CVS-18**

After intra-cerebral inoculation of CVS-18 (On 24th day) to the same group the blood and spleen samples were used to study $CD4^+$, $CD8^+$ and NK cells kinetics at different time intervals.

**Table 10: Mean of HPS in adult Swiss albino mice brain sacrificed at 2nd, 7th, 9th and 11th DPI (intra-cerebral route).The combined distribution of pathological lesions in level 14.**

| Groups/ DPI | 2 | 7 | 9 | 11 |
|---|---|---|---|---|
| GI | 11 | 20 | 21 | - |
| GL | - | 15 | 15.5 | 14 |
| GT | - | 14.5 | 14 | 14.5 |
| CL | - | 1 | 1.5 | 1 |
| CT | - | 1 | 1 | 1 |
| GC | 0 | 0 | 0 | 0 |

**Table 11 : Mean of HPS in adult Swiss albino mice brain sacrificed at 2nd, 7th, 9th and 11th DPI (intra-cerebral route).The combined distribution of pathological lesions in level 19.**

| Groups/ DPI | 2 | 7 | 9 | 11 |
|---|---|---|---|---|
| GI | 10 | 19.5 | 20 | - |
| GL | - | 14 | 13.5 | 13.5 |
| GT | - | 14 | 13 | 14 |
| CL | - | 1 | 1.5 | 1 |
| CT | - | 1 | 1 | 1 |
| GC | 0 | 0 | 0 | 0 |

**Table 12 : Mean of HPS in adult Swiss albino mice brain sacrificed at 2nd, 7th, 9th and 11th DPI (intra-cerebral route).The combined distribution of pathological lesions in level 21.**

| Groups/ DPI | 2 | 7 | 9 | 11 |
|---|---|---|---|---|
| GI | 10 | 20 | 21 | - |
| GL | - | 14.5 | 13.5 | 13 |
| GT | - | 14 | 13 | 13.5 |
| CL | - | 1 | 1 | 1 |
| CT | - | 1 | 1 | 1 |
| GC | 0 | 0 | 0 | 0 |

**Table 13 : Mean ± SD percentage of $CD4^+$ cells in blood of adult Swiss albino mice after inta-muscular inoculation of CVS-18 @ 100LD50/ mouse at different time intervals.**

| Groups/ DPI | 0 | 7 | 20 |
|---|---|---|---|
| CVS | 27.65±2.01 | 14.39±0.93 | 25.22±0.81 |
| PBS | 17.09±1.33 | 16.62±0.96 | 17.59±1.05 |

**Table 14: Mean ± SD percentage of $CD8^+$ cells in blood of adult Swiss albino mice after inta-muscular inoculation of CVS-18 @ 100LD50/ mouse at different time intervals.**

| Groups/ DPI | 0 | 7 | 20 |
|---|---|---|---|
| CVS | 12.95±0.55 | 9.48±1.05 | 11.57±0.50 |
| PBS | 8.56±0.70 | 8.49±0.69 | 8.75±0.50 |

**Table 15 : Mean ± SD percentage of NK (CD16+ CD56+) cells in blood of adult Swiss albino mice after inta-muscular inoculation of CVS-18 @ 100LD50/ mouse at different time intervals.**

| Groups/ DPI | 0 | 7 | 20 |
|---|---|---|---|
| CVS | 5.72±0.78 | 10.39±0.77 | 8.16±0.51 |
| PBS | 5.15±0.53 | 5.71±0.68 | 4.91±1.21 |

**Table 16: Mean ± SD ratio of $CD4^+$: $CD8^+$ cells in blood of adult Swiss albino mice after intra-muscular inoculation of CVS-18 @ 100LD50/ mouse at different time intervals. Blood was analysed by FACS, using $CD4^+$FITC/$CD8^+$RPE cocktail of monoclonal antibodies.**

| Groups/ DPI | 0 | 7 | 20 |
|---|---|---|---|
| CVS | 2.12±0.01 | 1.54±0.02 | 2.10±0.08 |
| PBS | 1.95±0.05 | 1.93±0.03 | 1.77±0.24 |

**Table 17: Mean ± SD percentage of $CD4^+$ cells in blood of adult Swiss albino mice inoculated intra-muscularly followed by intra-cerebral route CVS-18 @ 100LD50/ mouse. Three mice in each group sacrificed and their blood was analysed by FACS, using $CD4^+$FITC/$CD8^+$RPE cocktail of monoclonal antibodies. Groups bearing common superscripts do not differ significantly (P<0.05P). GI (CVS-18, 30µl/mouse $100LD_{50}$), GL (CVS-18 + LPS 8µg/ mouse), GT (CVS-18+LPS+RIG 40IU/kg b. wt), CL (LPS), CT (LPS + RIG), GC (PBS).**

| Groups/ DPI | 7 | 9 | 11 |
|---|---|---|---|
| GI | $11.60\pm0.63^{bc}$ | $10.01\pm0.51^{d}$ | - |
| GL | $10.25\pm0.75^{c}$ | $11.77\pm0.38^{d}$ | $9.02\pm0.47^{b}$ |
| GT | $12.70\pm0.51^{b}$ | $18.77\pm0.75^{a}$ | $15.07\pm0.55^{a}$ |
| CL | $8.20\pm0.78^{d}$ | $17.00\pm0.45^{ab}$ | $15.78\pm0.51^{a}$ |
| CT | $9.92\pm0.64^{cd}$ | $15.38\pm0.96^{bc}$ | $10.31\pm0.76^{b}$ |
| GC | $15.51\pm0.50^{a}$ | $13.84\pm0.79^{c}$ | $14.93\pm0.65^{a}$ |

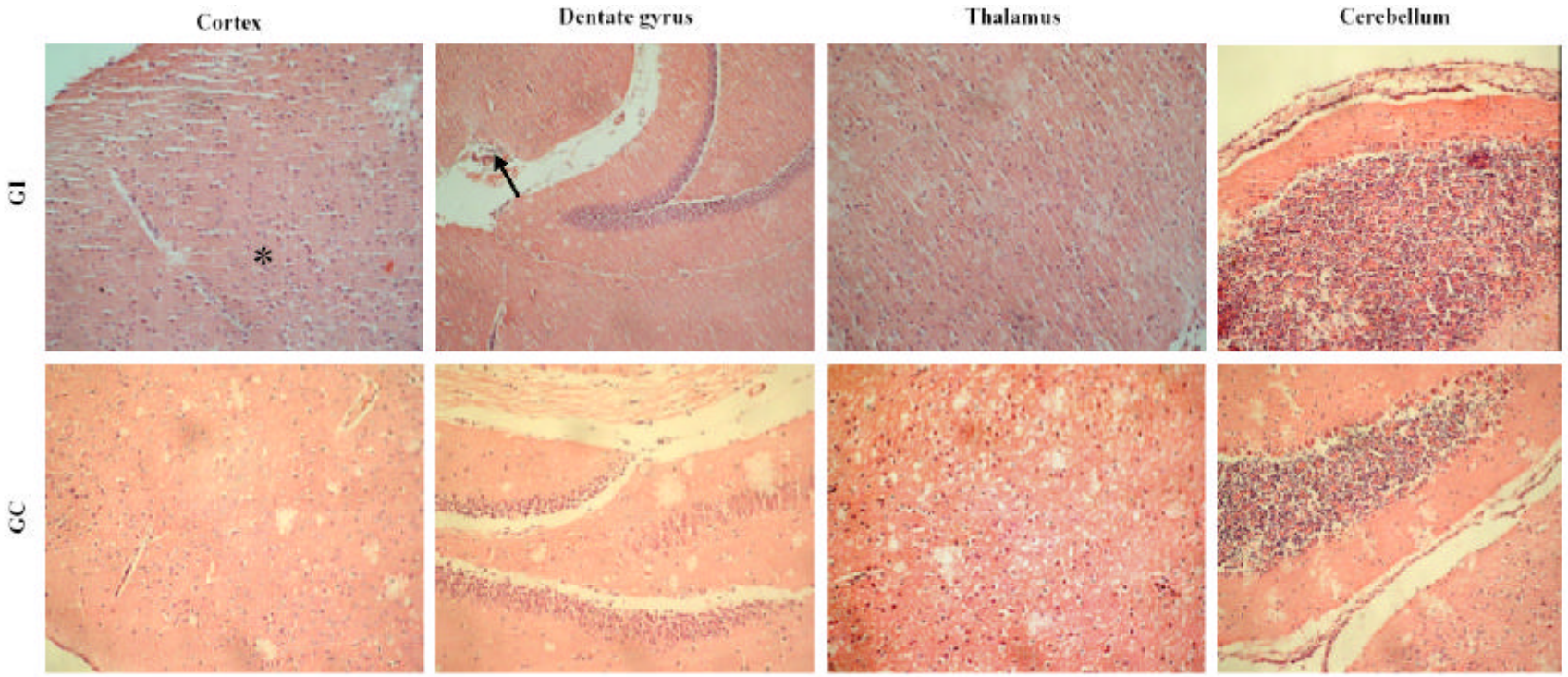
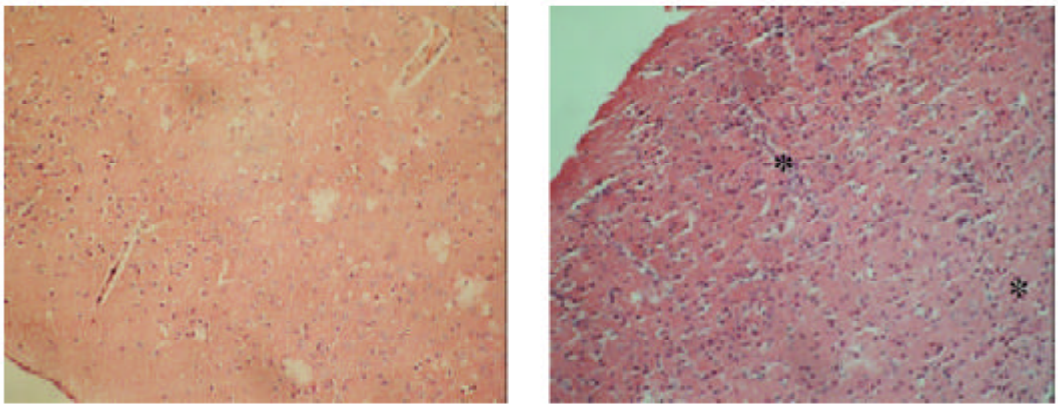
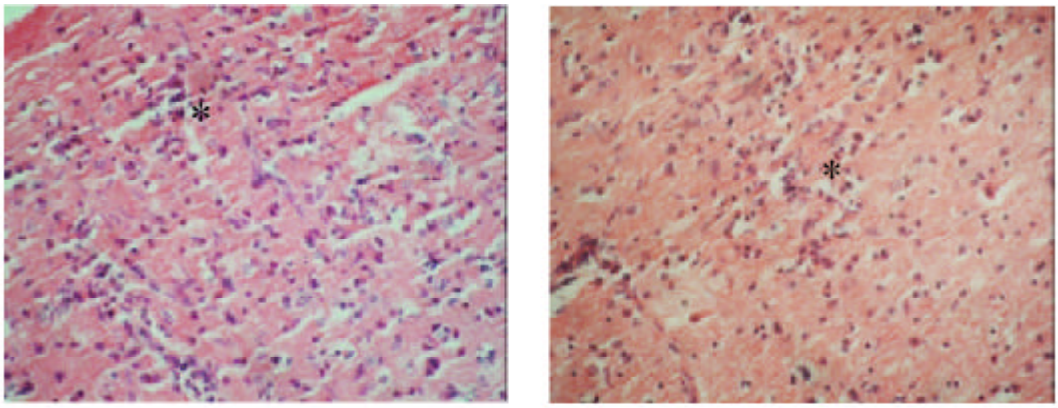



**Fig. 5 :** Brain sections of Swiss albino mice of GI (CVS-18, 30µl/mouse $100LD_{50}$) and GC (PBS) groups at 2nd day post infection by intra-cerebral route. The changes of perivascular infiltration (arrow) and gliosis (*) were mildly present in GI while absent in GC group. H&E x 200.



X 100, GC

X 100, GI



X 400, GI

X 400, GI

**Fig. 6 :** Sections of cerebral hemisphere showing diffuse gliosis (*) in GI group (CVS-18, 30µl/mouse $100LD_{50}$) at $7^{th}$ day post infection (intra-cerebral). GC (PBS). H&E.

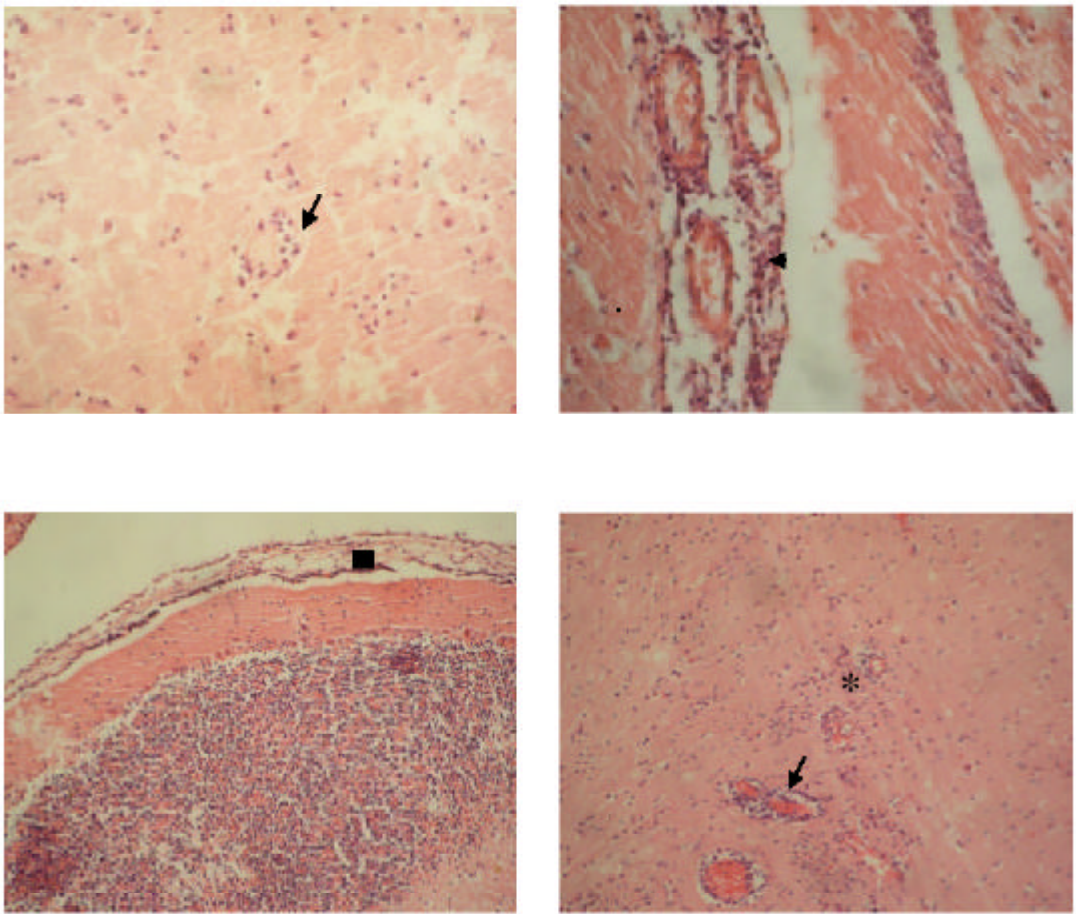


**Fig. 7 :** Sections of mice brain showing perivascular infiltration (arrow) and meningitis (arrow head) along with diffuse gliosis (*) in GI group (CVS-18, 30µl/mouse 100$LD_{50}$) at 7$^{th}$ day post infection (intra-cerebral). H&E. X 200.

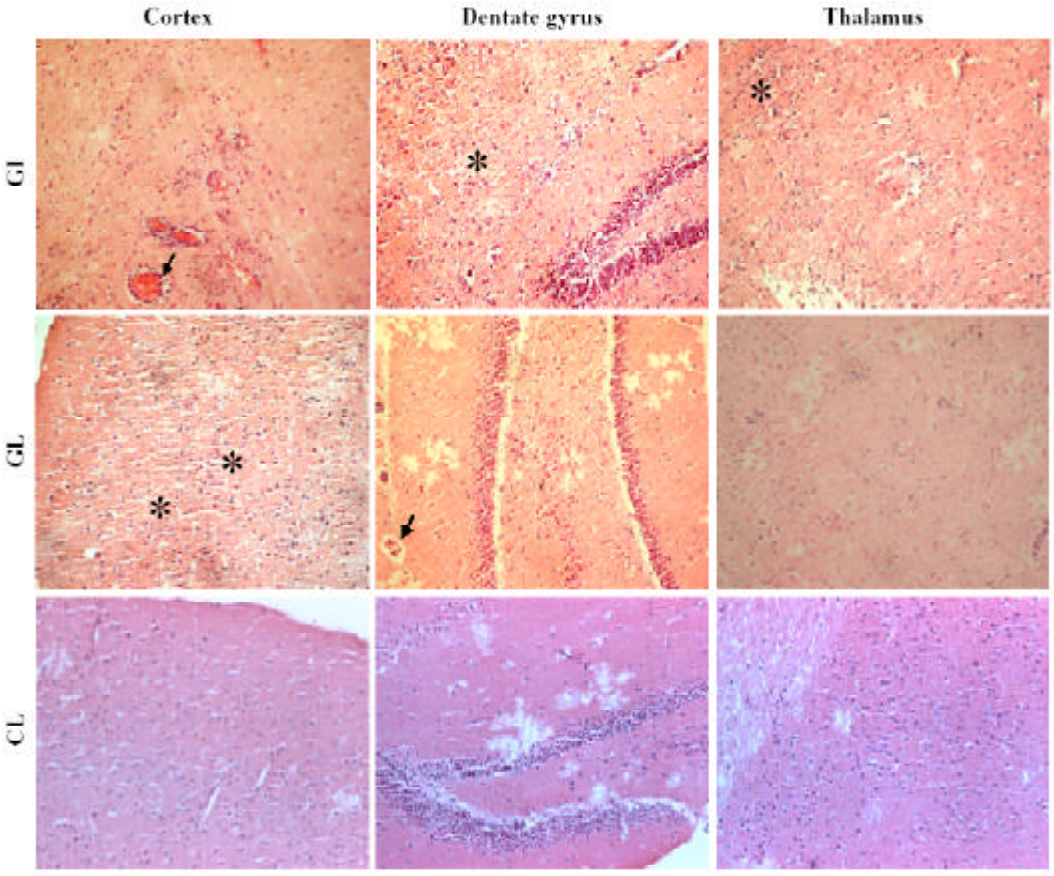



**Fig. 8 :** Cerebral hemisphere sections of mice brain in different treatment groups at 7th day post infection intra-cerebrally. GL group showed less gliosis (*) and PVC (arrows) compared to GI group. CL group showed only mild infiltration. H&E X 200. GI (CVS-18, 30µl/mouse $100LD_{50}$), GL (CVS-18 + LPS 8µg/mouse), CL (LPS).

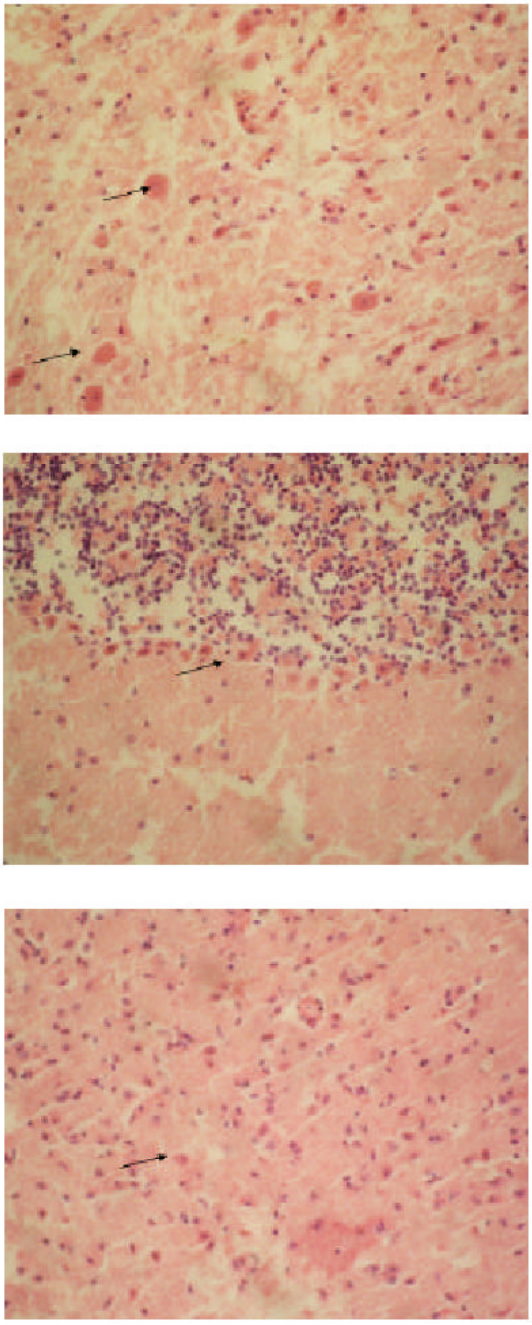


**Fig. 9 :** Sections of mice brain (cerebellum gray and white matter) showing pyknotic neurons with eosinophilic cytoplasm (arrows) in GI group (CVS-18, 30µl/ mouse100$LD_{50}$) at 9$^{th}$ day post infection (intra-cerebral). H&E. X 200.

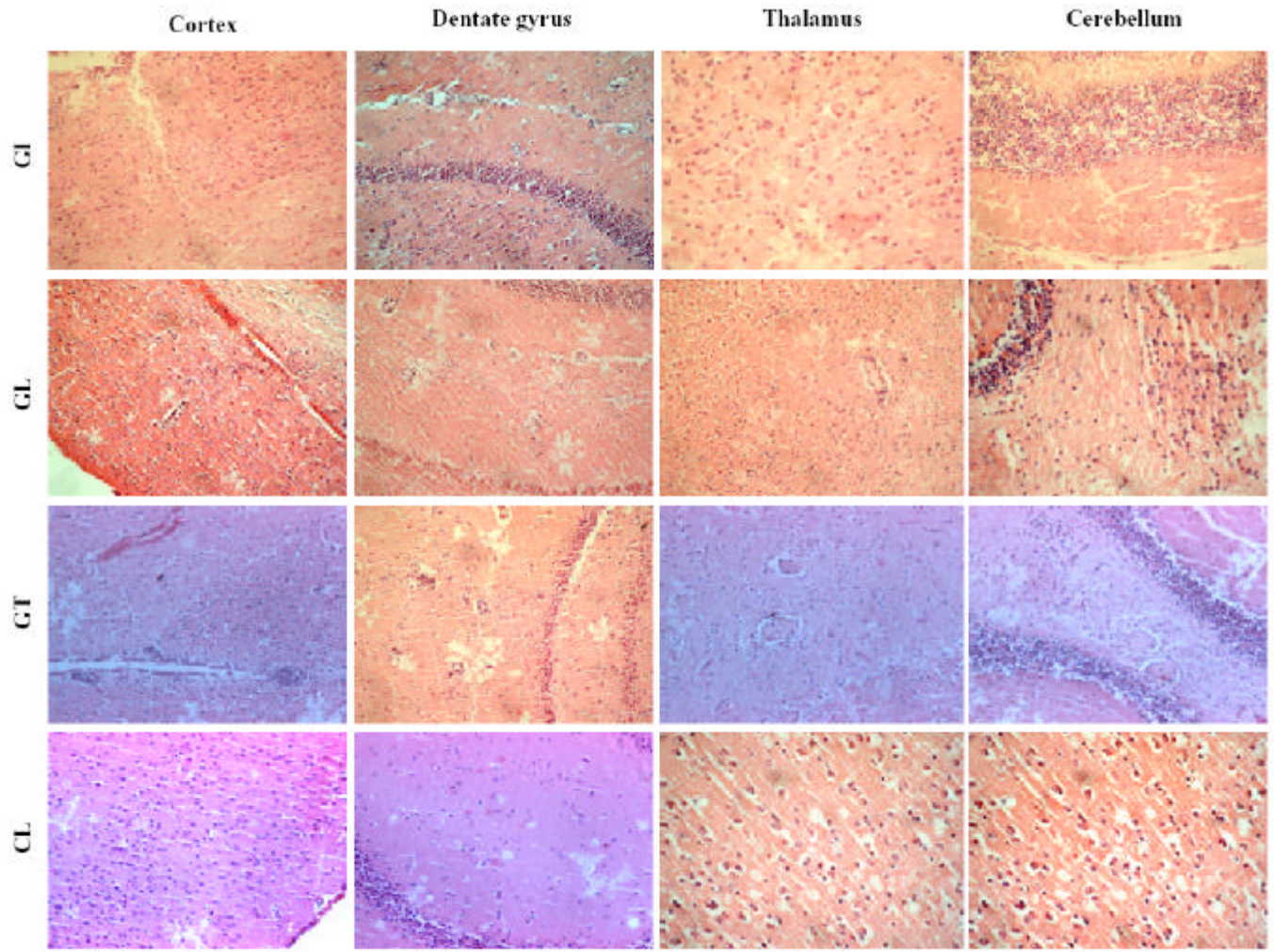



**Fig. 10 :** Sections of brains at different sites at 9th day post infection intra-cerebrally. The GT followed by GL group showed less pathological changes (gliosis, PVC and neuron degeneration) than GI group. H&E. X 200.

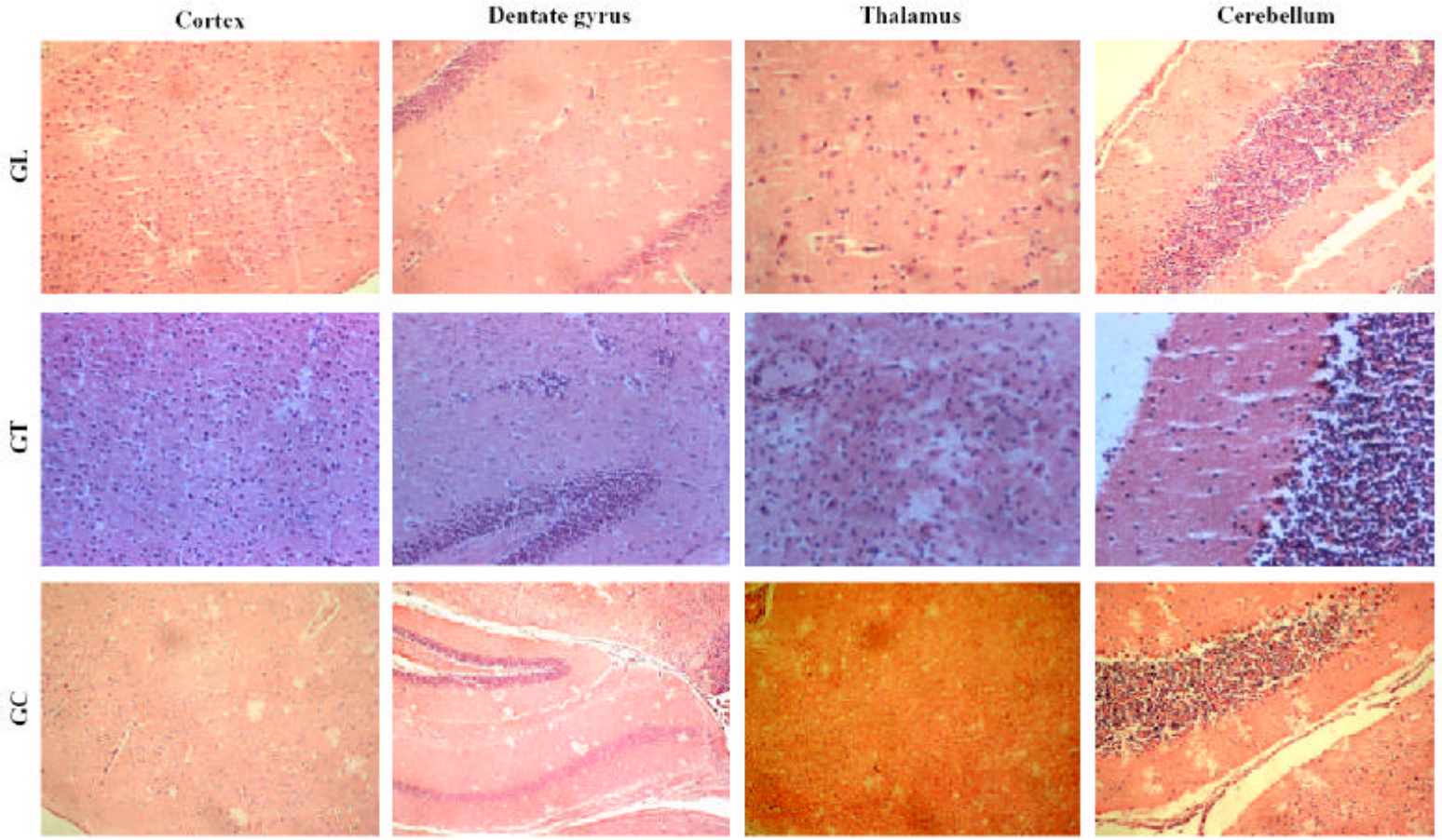
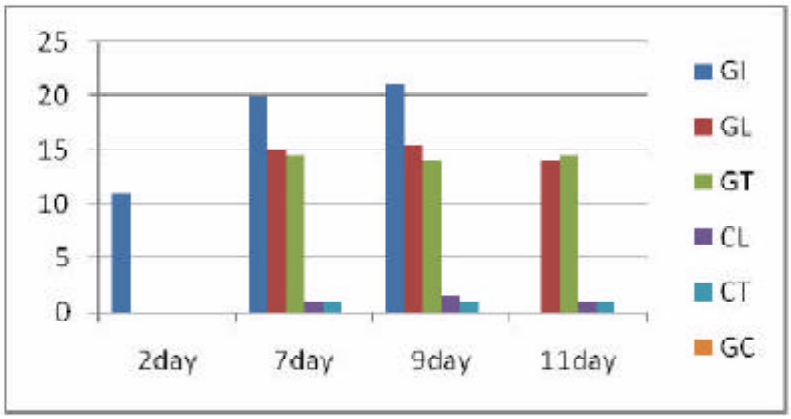
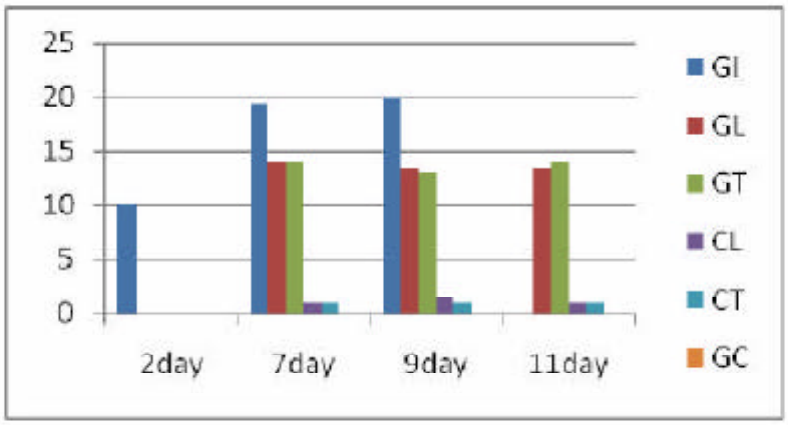


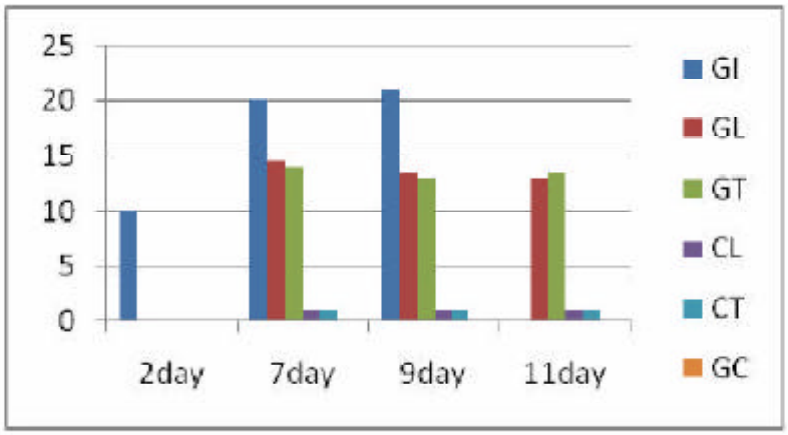

**Fig. 11 :** Sections of mice brains at different sites at 11th day post infection intra-cerebrally. The GT group showed slightly more cellularity (gliosis) in parenchyma than the GL group. H&E. X 200. GL (CVS-18 + LPS 8µg/mouse), GT (CVS-18+LPS+RIG 40IU/kg b.wt.), GC (PBS).




Rostral section




Middle section




Caudal section

**Fig. 12 :** Mean HPS of cerebral hemisphere (section rostral, middle and caudal) of Swiss albino mice inoculated inta-muscularly with CVS-18 @ 100LD50, 0.03ml/mouse followed by intra-cerebral route with same dose on 24th day. LPS and LPS + RIG inoculated on onset of clinical signs (on 6th DPI of intra-cerebral route). GL and GT groups indicated less pathology compared to GI group. GI (CVS-18, 30µl/mouse 100LD50), GL (CVS-18 + LPS 8µg/mouse), GT (CVS-18 + LPS + Rig 40IU/kg b.wt.), CL (LPS), CT (LPS + RIG), GC (PBS).

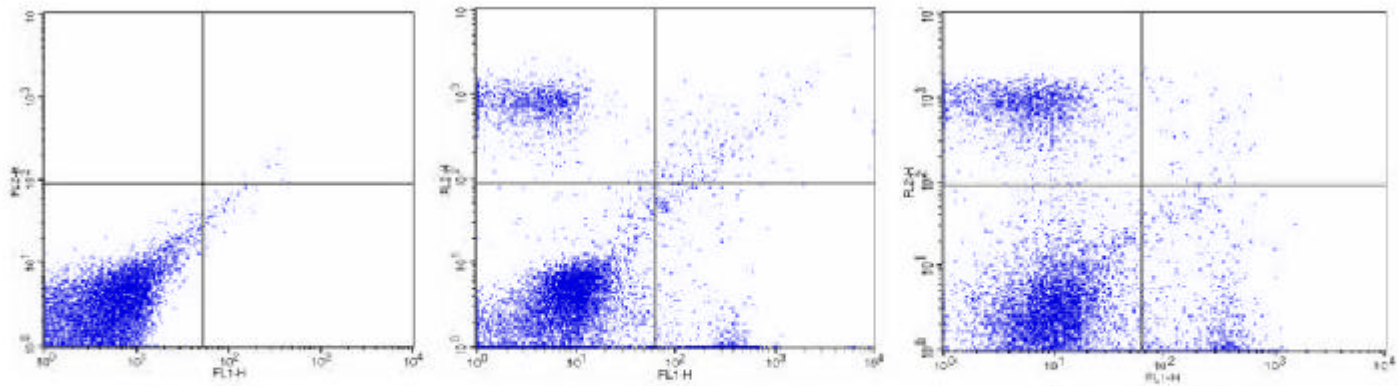



**Fig. 13A :** FACS analysis of blood and spleen samples on 2nd day post infection intra-cerebrally. FACS dot plots indicate $CD4^+$ population in lower right quadrants and $CD8^+$ population in upper left quadrants in test group (GT group- CVS + LPS + RIG).

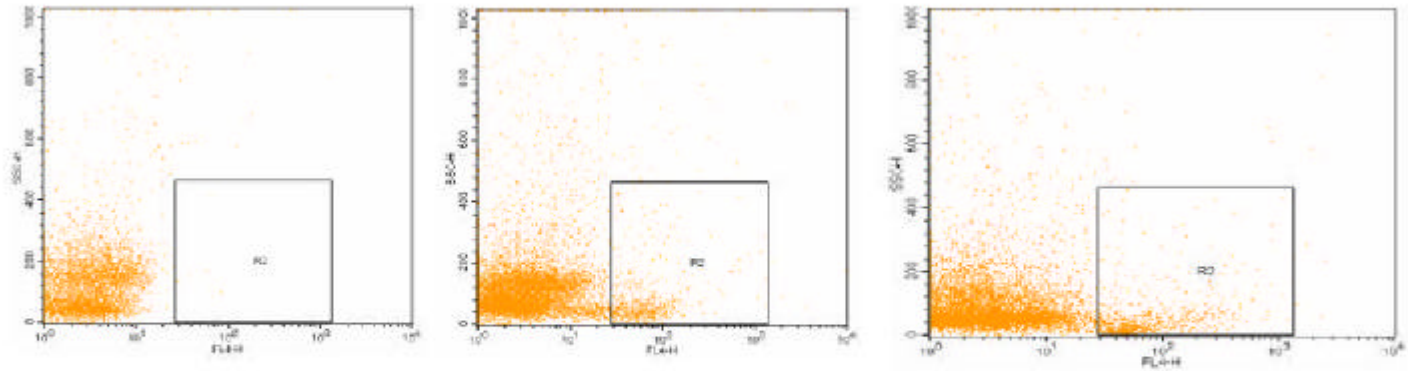



**Fig. 13B :** FACS analysis of blood and spleen samples on 2nd day post infection intra-cerebrally. FACS dot plots indicate NK cells population in test group (GT group- CVS + LPS + RIG).

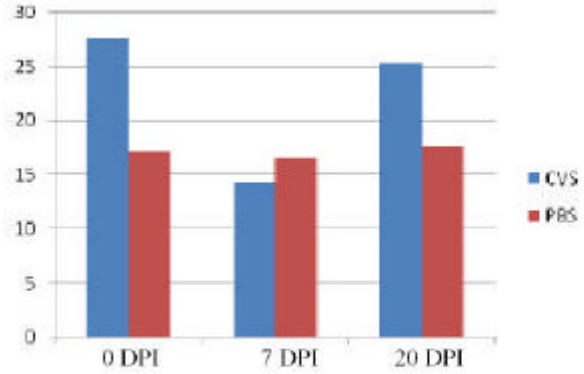



**Fig. 14 :** $CD4^+$ cells percentage in blood of adult Swiss albino mice at 0, 7th and 20th day after intra-muscular inoculation of CVS-18 @100LD50, 0.03ml/mouse.

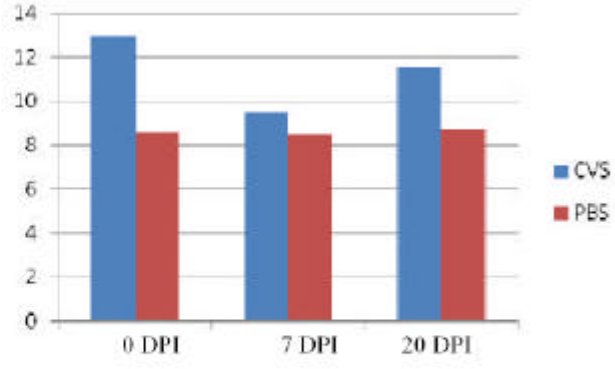



**Fig. 15 :** $CD8^+$ cells percentage in blood of adult Swiss albino mice at 0, 7th and 20th day after intra-muscular inoculation of CVS-18 @100LD50, 0.03ml/mouse.

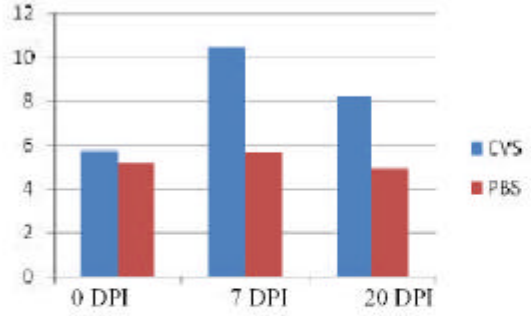



**Fig. 16 :** NK cells percentage in blood of adult Swiss albino mice at 0, 7th and 20th day after intra-muscular inoculation of CVS-18 @100LD50, 0.03ml/mouse.

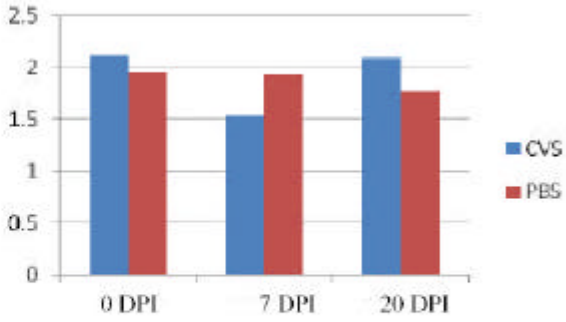



**Fig. 17 :** Mean ratio of $CD4^+$: $CD8^+$ cells in blood of adult Swiss albino mice after intra-muscular inoculation of CVS-18 @ 100LD50/ mouse at different time intervals. Blood was analysed by FACS, using $CD4^+$FITC/$CD8^+$RPE cocktail of monoclonal antibodies.

**$CD4^+$ cells in blood**

The mean % of $CD4^+$ cells on day $7^{th}$ was lower in groups GI, GL and GT compared to group GC and differed significantly. On day $9^{th}$, group GT showed higher value than all other groups and differed significantly. On day $11^{th}$, group GL showed lower value than all other group and differed significantly, except group CT (Table 17, Fig.18).

**$CD8^+$cells in blood**

The mean % of $CD8^+$ cells on day $7^{th}$ in group GT showed significant difference from all other groups. Groups GI and GL had almost the same value. Group GT had higher value than the initial and differed significantly within the groups on day $9^{th}$.On day $11^{th}$, group GL and GC showed almost similar value while, group GT differed significantly with higher value (Table 18, Fig. 19).

**NK cells in blood**

The mean % of NK cells on day $7^{th}$ in all groups was almost same, while it increased from day $7^{th}$ to day $11^{th}$ in group GC and decreased in group GI on day $9^{th}$. In groups GL, GT, CL, and CT, the values increased on day $9^{th}$ and differed significantly from group GC. On day $11^{th}$, groups GL and GT had lower value than group GC with significant difference (Table 19, Fig. 20).

**The ratio of $CD4^+$ and $CD8^+$ in blood**

The ratio of $CD4^+$ and $CD8^+$ on day $7^{th}$ was lower in group GI than all other groups with significance difference to groups GC and CT. On day $9^{th}$ and $11^{th}$, the ratio showed variable value, but there was no significant difference within the group at each point of interval (Table 20, Fig. 21).

**$CD4^+$ cells in spleen**

The mean % of $CD4^+$ cells on day $7^{th}$ in groups GI, GL and GT had lower value than group GC with significant difference. On day $9^{th}$, group GT has higher value than all other groups with significant difference. While groups GI, GL and GC showed no significant difference. On $11^{th}$ day, group GL showed lower value compared to all other groups with significance difference (Table 21, Fig. 22).

**Table 18: Mean ± SD percentage of $CD8^+$ cells in blood of adult Swiss albino mice inoculated followed by intra-cerebral route CVS-18 @ 100LD50/ mouse. Three mice in each group sacrificed and their blood was analysed by FACS, using CD4+FITC/CD8+RPE cocktail of monoclonal antibodies. Groups bearing common superscripts do not differ significantly (P<0.05P). GI (CVS-18, 30µl/mouse $100LD_{50}$), GL (CVS-18 + LPS 8µg/mouse), GT (CVS-18+LPS+RIG 40IU/kg b. wt), CL (LPS), CT (LPS + RIG), GC (PBS).**

| Groups/ DPI | 7 | 9 | 11 |
|---|---|---|---|
| GI | $9.72\pm0.59^{b}$ | $8.53\pm0.55^{c}$ | - |
| GL | $9.07\pm0.40^{b}$ | $9.90\pm0.36^{bc}$ | $8.51\pm0.63^{c}$ |
| GT | $11.84\pm0.79^{a}$ | $16.03\pm0.45^{a}$ | $14.40\pm0.52^{a}$ |
| CL | $8.70\pm0.75^{b}$ | $10.53\pm0.45^{b}$ | $10.37\pm0.58^{b}$ |
| CT | $5.23\pm0.71^{c}$ | $8.45\pm0.61^{c}$ | $6.12\pm0.65^{d}$ |
| GC | $7.87\pm0.85^{b}$ | $6.83\pm0.75^{d}$ | $8.87\pm0.33^{c}$ |

**Table 19: Mean ± SD percentage of NK ( $CD16^+$ $CD56^+$) cells in blood of adult Swiss albino mice inoculated intra-muscularly followed by intra-cerebral route with CVS-18 @ 100LD50/ mouse. Three mice in each group sacrificed and their blood was analysed by FACS, using CD16+ CD56+ monoclonal antibodies. Groups bearing common superscripts do not differ significantly (P<0.05P). GI (CVS-18, 30µl/mouse $100LD_{50}$), GL (CVS-18 + LPS 8µg/ mouse), GT (CVS-18+ LPS+ RIG 40IU/kg b. wt), CL (LPS), CT (LPS + RIG), GC (PBS).**

| Groups/ DPI | 7 | 9 | 11 |
|---|---|---|---|
| GI | $5.13\pm0.85^{a}$ | 2.93±0.60c | - |
| GL | $5.35\pm0.55^{a}$ | $9.58\pm0.67^{a}$ | $3.95\pm0.59^{b}$ |
| GT | $5.45\pm0.82^{a}$ | $9.80\pm0.70^{a}$ | $5.70\pm0.52^{ab}$ |
| CL | $5.71\pm0.38^{a}$ | $9.97\pm0.75^{a}$ | $5.25\pm1.13^{ab}$ |
| CT | $5.83\pm0.65^{a}$ | $6.22\pm0.60^{b}$ | $5.20\pm0.53^{ab}$ |
| GC | $5.60\pm0.60^{a}$ | $5.90\pm0.70^{b}$ | $6.17\pm0.80^{a}$ |

**Table 20 : Mean ± SD ratio of $CD4^+$ : $CD8^+$ cells in blood of adult Swiss albino mice inoculated intra-muscularly followed by intra-cerebral route with CVS-18 @ 100LD50/ mouse. Three mice in each group sacrificed and their blood was analysed by FACS, using $CD4^+$FITC/$CD8^+$RPE cocktail of monoclonal antibodies. Groups bearing common superscripts do not differ significantly (P<0.05P). GI (CVS-18, 30µl/mouse $100LD_{50}$), GL (CVS-18 + LPS 8µg/ mouse), GT (CVS-18+LPS+RIG 40IU/kg b. wt), CL (LPS), CT (LPS + RIG), GC (PBS).**

| Groups/ DPI | 7 | 9 | 11 |
|---|---|---|---|
| GI | $1.19\pm0.10^{c}$ | $1.36\pm0.19^{a}$ | - |
| GL | $1.33\pm0.20^{bc}$ | $1.42\pm0.23^{a}$ | $1.43\pm0.37^{a}$ |
| GT | $1.21\pm0.13^{bc}$ | $1.37\pm0.20^{a}$ | $1.40\pm0.35^{a}$ |
| CL | $1.20\pm0.26^{bc}$ | $1.81\pm0.20^{a}$ | $1.72\pm0.33^{a}$ |
| CT | $2.01\pm0.11^{a}$ | $1.64\pm0.19^{a}$ | $1.54\pm0.15^{a}$ |
| GC | $1.71\pm0.26^{ab}$ | $1.85\pm0.18^{a}$ | $1.63\pm0.05^{a}$ |

**Table 21: Mean ± SD percentage of CD4+ cells in spleen of adult Swiss albino mice inoculated intra-muscularly followed by intra-cerebral route with CVS-18 @ 100LD50/ mouse. Three mice in each group sacrificed and their blood was analysed by FACS, using $CD4^+$FITC/$CD8^+$RPE cocktail of monoclonal antibodies. Groups bearing common superscripts do not differ significantly (P<0.05P). GI (CVS-18, 30µl/mouse $100LD_{50}$), GL (CVS-18 + LPS 8µg/ mouse), GT (CVS-18+LPS+RIG 40IU/kg b. wt), CL (LPS), CT (LPS + RIG), GC (PBS).**

| Groups/ DPI | 7 | 9 | 11 |
|---|---|---|---|
| GI | $15.66\pm0.49^{b}$ | $13.02\pm0.64^{d}$ | - |
| GL | $11.57\pm0.46^{c}$ | $13.40\pm0.73^{cd}$ | $11.28\pm0.75^{c}$ |
| GT | $15.10\pm0.66^{b}$ | $22.73\pm3.81^{a}$ | $17.54\pm1.05^{a}$ |
| CL | $11.94\pm0.39^{c}$ | $18.67\pm0.44^{ab}$ | $17.54\pm0.56^{a}$ |
| CT | $12.16\pm0.70^{c}$ | $18.00\pm0.95^{bc}$ | $13.79\pm1.01^{b}$ |
| GC | $18.65\pm1.31^{a}$ | $15.44\pm0.96^{bcd}$ | $17.24\pm0.46^{a}$ |

Fig. 18-21: Percentage of $CD4^+$, $CD8^+$, NK cells and ratio $CD4^+$ : $CD8^+$ in blood of adult Swiss albino mice inoculated intra-muscularly followed by intra-cerebral route CVS-18 @ 100LD50/ mouse. The mice in each group sacrificed and their blood was analysed by FACS, using $CD4^+$FITC/$CD8^+$RPE cocktail of monoclonal antibodies.GI (CVS-18, 30µl/mouse $100LD_{50}$), GL (CVS-18 + LPS 8µg/mouse), GT (CVS-18+LPS+RIG 40IU/kg b. wt), CL (LPS), CT (LPS + RIG), GC (PBS).

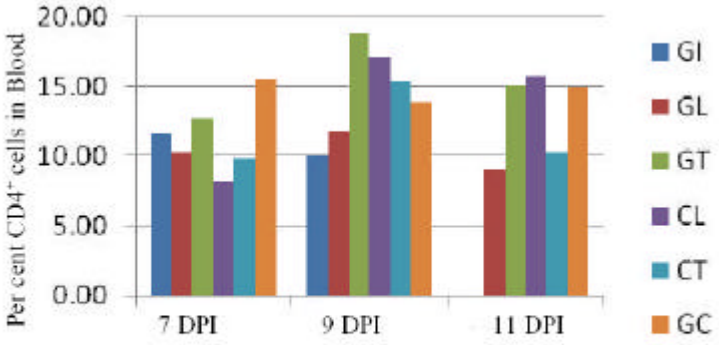



**Fig . 18**

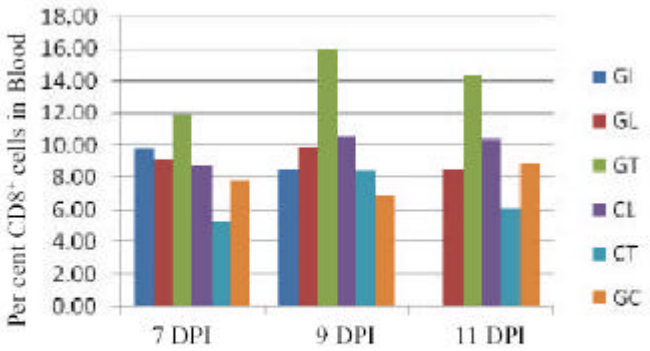



**Fig . 19**

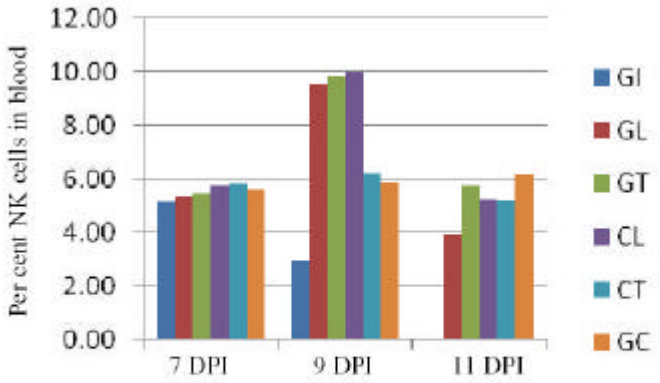



**Fig . 20**

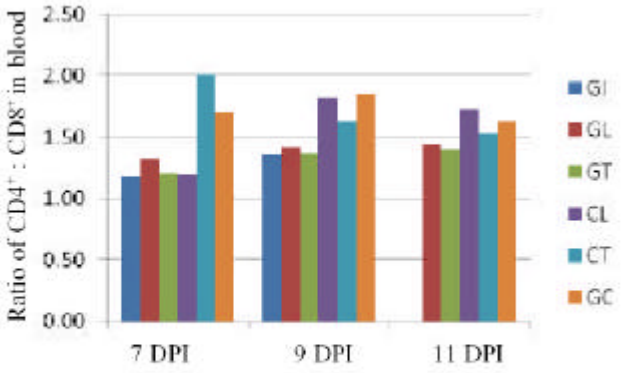



**Fig . 21**

**CD8$^+$cells in spleen**

On 7$^{th}$ day, the group GT revealed higher value as compared to all other groups with significant difference. The value increased in the groups GL, GT, CL, and CT, while it decreased in groups GI and GC on 9$^{th}$ day. On day 11$^{th}$, group GT significantly showed higher value than all other groups (Table 22, Fig. 23).

**NK cells in spleen**

The mean % of NK cells on 7$^{th}$ day significantly higher in group GT compared to all other groups and remained higher up to day 11$^{th}$.On day 9$^{th,}$ group GI revealed significantly lower value than all other groups. On day 11$^{th,}$ there was no significant difference within the group except in group GT (Table 23, Fig. 24).

**The ratio of CD4$^+$ and CD8$^+$ in spleen**

The ratio of CD4$^+$ and CD8$^+$ cells at each point of interval showed no significant difference within the groups (Table 24, Fig. 25).

**IFN-ß assay**

On 2$^{nd}$ DPI, GI showed higher concentration of IFN-ß (1.10E+02±0.71 pg/ml) than control group GC (60.50±0.71pg/ml). On 7$^{th}$ day after LPS inoculation, GL (1.07E+02± 0.57 pg/ml) and GT (1.08E+02±0.72 pg/ml) groups showed higher value than GI (62.75±01.06 pg/ml) group. The trend was same till the end of experiment with exception of GL group on 11$^{th}$ day (Table 25, Fig.26).

**Immunohistochemistry for ß-catenin**

The brain sections from GI, GL, GT, CL, CT and GC groups were demonstrated for modulation of blood brain-barrier (BBB) adhesion molecule ß-catenin. The positive signals were detected as red color in the endothelium lining of blood capillaries. The group GI showed positive signals, whereas group GL and GT did not show any signals. The negative control slides did not show any signals in the brain sections (Fig. 27).

**Rabies virus detection in brain by dFAT**

Rabies virus infected GL and GT groups showed less intensity and distribution of apple green fluorescence in comparison to GI group. The control groups (CL, CT and GC) did not show any positive signals (Fig. 28).

**Table 22: Mean ± SD percentage of $CD8^+$ cells in spleen of adult Swiss albino mice inoculated intra-muscularly followed by intra-cerebral route with CVS-18 @ 100LD50/ mouse. Three mice in each group sacrificed and their blood was analysed by FACS, using $CD4^+$ FITC/$CD8^+$ RPE cocktail of monoclonal antibodies. Groups bearing common superscripts do not differ significantly (P<0.05P). GI (CVS-18, 30µl/mouse $100LD_{50}$), GL (CVS-18 + LPS 8µg/ mouse), GT (CVS-18+LPS+RIG 40IU/kg b. wt), CL (LPS), CT (LPS + RIG), GC (PBS).**

| Groups/ DPI | 7 | 9 | 11 |
|---|---|---|---|
| GI | $10.92\pm0.34^{b}$ | $9.20\pm0.82^{c}$ | - |
| GL | $9.67\pm0.76^{bc}$ | $11.60\pm0.53^{b}$ | $9.21\pm0.86^{c}$ |
| GT | $14.10\pm0.66^{a}$ | $19.53\pm0.95^{a}$ | $15.78\pm0.40^{a}$ |
| CL | $9.64\pm0.41^{bc}$ | $13.30\pm0.75^{b}$ | $11.70\pm0.75^{b}$ |
| CT | $7.47\pm0.47^{d}$ | $11.86\pm0.81^{b}$ | $7.07\pm0.95^{d}$ |
| GC | $8.73\pm0.55^{cd}$ | $8.63\pm0.60^{c}$ | $9.43\pm0.60^{c}$ |

**Table 23: Mean ± SD percentage of NK ( $CD16^+$ $CD56^+$) cells in spleen of adult Swiss albino mice inoculated intra-muscularly followed by intra-cerebral route with CVS-18 @ 100LD50/ mouse. Three mice in each group sacrificed and their blood was analysed by FACS, using $CD16^+$ $CD56^+$ monoclonal antibodies. Groups bearing common superscripts do not differ significantly (P<0.05P). GI (CVS-18, 30µl/mouse $100LD_{50}$), GL (CVS-18 + LPS 8µg/ mouse), GT (CVS-18+ LPS+ RIG 40IU/kg b. wt), CL (LPS), CT (LPS + RIG), GC (PBS).**

| Groups/ DPI | 7 | 9 | 11 |
|---|---|---|---|
| GI | $6.39\pm1.44^{c}$ | $4.10\pm0.60^{d}$ | - |
| GL | $8.93\pm0.60^{ab}$ | $11.83\pm0.65^{b}$ | $5.82\pm0.65^{b}$ |
| GT | $10.38\pm0.71^{a}$ | $14.51\pm0.50^{a}$ | $9.58\pm0.95^{a}$ |
| CL | $7.62\pm0.50^{bc}$ | $11.98\pm0.74^{b}$ | $6.20\pm1.36^{b}$ |
| CT | $6.92\pm0.66^{bc}$ | $6.96\pm0.70^{c}$ | $6.93\pm0.95^{b}$ |
| GC | $8.09\pm0.85^{abc}$ | $7.96\pm0.95^{c}$ | $5.50\pm0.50^{b}$ |

**Table 24: Mean ± SD ratio of $CD4^+$: $CD8^+$ cells in spleen of adult Swiss albino mice inoculated intra-muscularly followed by intra-cerebral route with CVS-18 @ 100LD50/ mouse. Three mice in each group sacrificed and their blood was analysed by FACS, using $CD4^+$ FITC/$CD8^+$ RPE cocktail of monoclonal antibodies. Groups bearing common superscripts do not differ significantly (P<0.05P). GI (CVS-18, 30µl/mouse $100LD_{50}$), GL (CVS-18 + LPS 8µg/ mouse), GT (CVS-18+LPS+RIG 40IU/kg b. wt), CL (LPS), CT (LPS + RIG), GC (PBS).**

| Groups/ DPI | 7 | 9 | 11 |
|---|---|---|---|
| GI | 1.39±0.05[a] | 1.70±0.29[a] | - |
| GL | 1.44±0.24[a] | 1.39±0.24[a] | 1.62±0.39[a] |
| GT | 1.33±0.26[a] | 1.47±0.31[a] | 1.46±0.35[a] |
| CL | 1.35±0.11[a] | 1.69±0.29[a] | 1.75±0.26[a] |
| CT | 1.72±0.09[a] | 1.55±0.03[a] | 1.91±0.04[a] |
| GC | 1.85±0.29[a] | 1.89±0.10[a] | 1.58±0.24[a] |

**Table 25: Mean ± SD values of IFN-ß in serum of adult Swiss albino mice inoculated intra-muscularly followed by intra-cerebral route with CVS-18 @ 100LD50/ mouse followed by intra-cerebral route with same dose on 24th day.LPS and LPS+RIG inoculated on onset of clinical signs (on 6th DPI of intra-cerebral route). The Three mice in each group sacrificed and serum was collected for IFN-ß assay using ELISA kit. Groups bearing common superscripts do not differ significantly (P<0.05P). GI (CVS-18, 30µl/mouse $100LD_{50}$), GL (CVS-18 + LPS 8µg/mouse), GT (CVS-18+ LPS+ RIG 40IU/ kg b. wt), CL (LPS), CT (LPS + Rig), GC (PBS).**

| Groups/ DPI | GI | GL | GT | CL | CT | GC |
|---|---|---|---|---|---|---|
| 2 | 1.10E+02 ±0.71[a] | - | - | - | - | 60.50±0.71[b] |
| 7 | 62.75±1.06[d] | 1.07E+02 ±0.57[a] | 1.08E+02 ±0.72[a] | 71.49±0.69[b] | 68.78±0.31[c] | 57.67±0.47[e] |
| 9 | 66.89±0.16[d] | 84.11±1.27[a] | 80.43±0.81[b] | 70.49±2.10[c] | 60.94±0.09[e] | 63.95±0.08d[e] |
| 11 | - | 87.49±0.72[a] | 78.77±1.09[b] | 63.11±1.56[c] | 58.67±0.95[d] | 57.77±0.33[d] |

Fig. 22-25: Percentage of $CD4^+$, $CD8^+$, NK cells and ratio $CD4^+$ : $CD8^+$ in spleen of adult Swiss albino mice inoculated intra-muscularly followed by intra-cerebral route CVS-18 @ 100LD50/ mouse. The mice in each group sacrificed and their blood was analysed by FACS, using $CD4^+$FITC/$CD8^+$RPE cocktail of monoclonal antibodies.GI (CVS-18, 30µl/mouse $100LD_{50}$), GL (CVS-18 + LPS 8µg/mouse), GT (CVS-18+LPS+RIG 40IU/kg b. wt), CL (LPS), CT (LPS + RIG), GC (PBS).

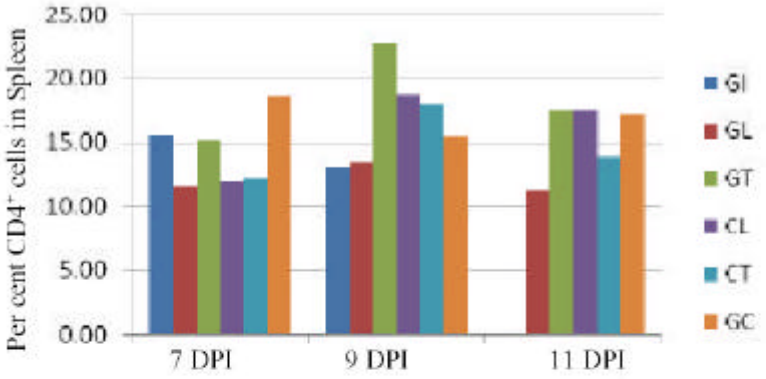



**Fig . 22**

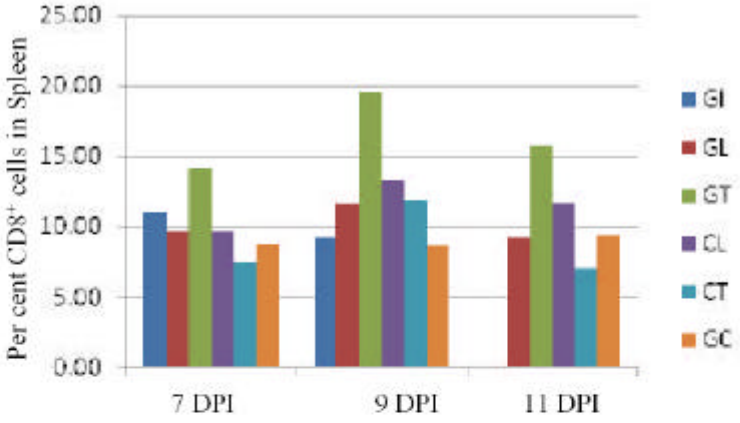



**Fig . 23**

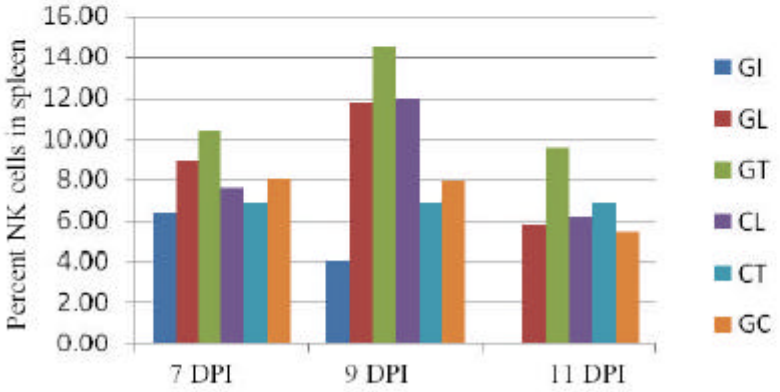



**Fig . 24**

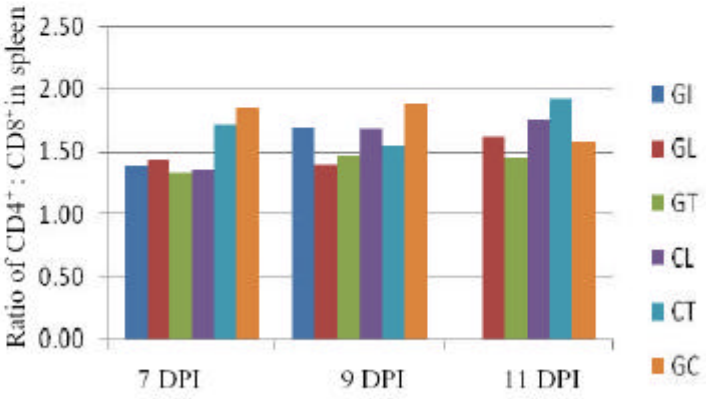



**Fig . 25**

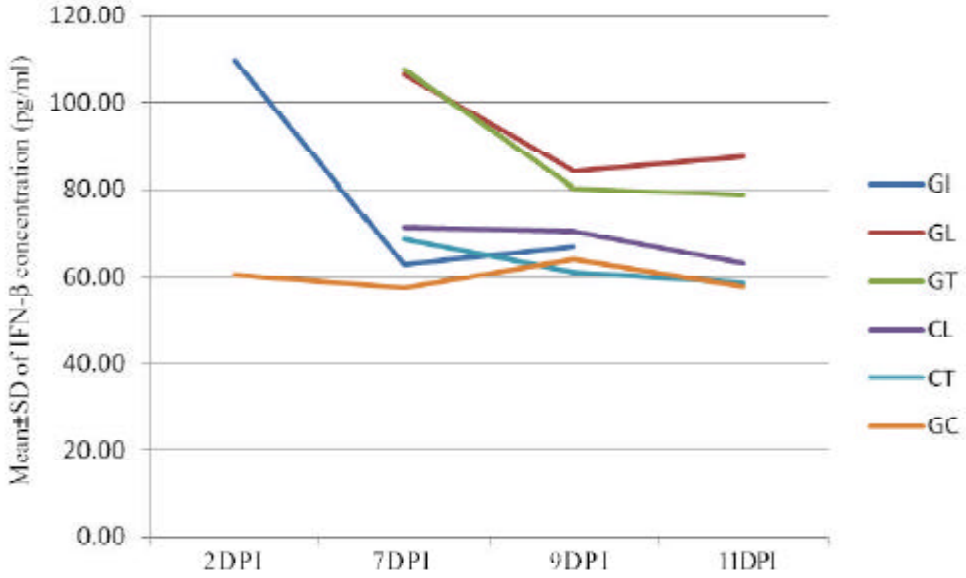



**Fig. 26 :** Swiss albino mice inoculated intramuscularly with CVS-18 rabies virus strain @ 100LD50 30µl/mouse followed by intracerebral challenge with same dose on 24th day. On appearance of clinical sings ( 6th day), the mice of GL, GT, CL and CT groups were inoculated with LPS @ 8µg/mouse intraperitoneally. The mice of GT and CT groups were injected with RIG in addition. The serum samples collected at different time intervals showed decrease concentration of IFN-ß by ELISA up to 9th day. Later, the concentration of IFN-ß remained static but higher in GL and GT groups than in GI group. GI (CVS-18, 30µl/ mouse 100LD50), GL (CVS-18 + LPS 8µg/mouse), GT (CVS-18 + LPS + RIG 40IU/kg bwt), CL (LPS), CT (LPS + RIG), GC (PBS).

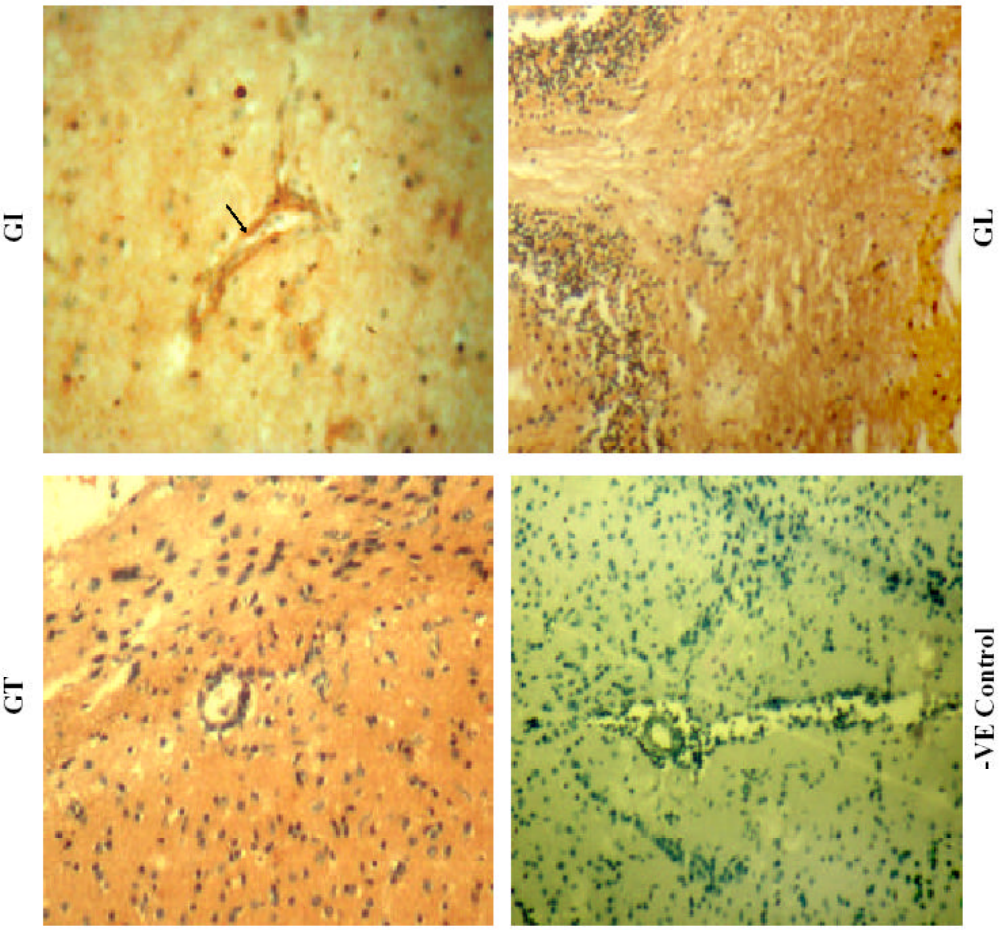



**Fig. 27 :** Sections of brain of Swiss albino mice showing no signals for β– catenin in the brain endothelial cells (BBB) of blood capillaries in groups GL and GT. GI group showed positive signals (arrow). GI (CVS-18, 30µl/mouse $100LD_{50}$), GL (CVS-18 + LPS 8µg/mouse), GT (CVS-18+LPS+RIG 40IU/kg bwt). IHC X 200.

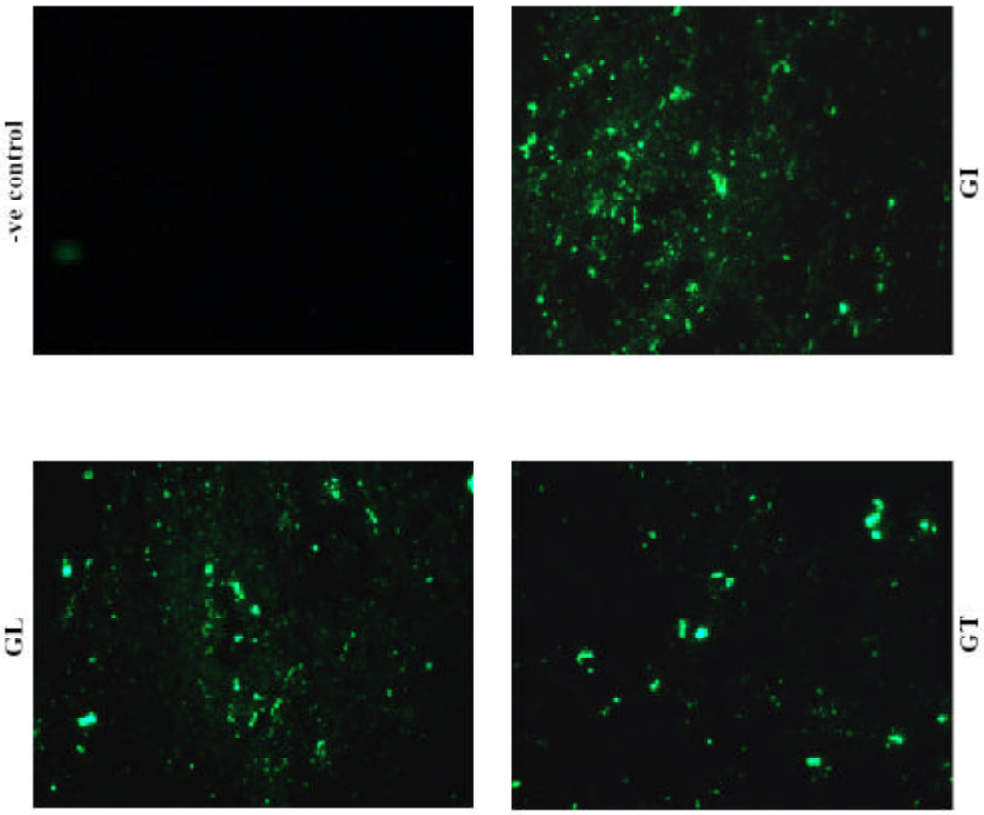



**Fig. 28 :** Swiss albino mice brain impression smears from caudal region of the cerebral hemisphere showing apple green fluorescent signals of rabies antigen. The GL and GT groups having less number of signals than the GI group on day 9. GI (CVS-18, 30µl/mouse 100LD50), GL (CVS-18 + LPS 8µg/mouse), GT (CVS-18+LPS+RIG 40IU/kg bwt), GC (PBS). dFAT. X200.

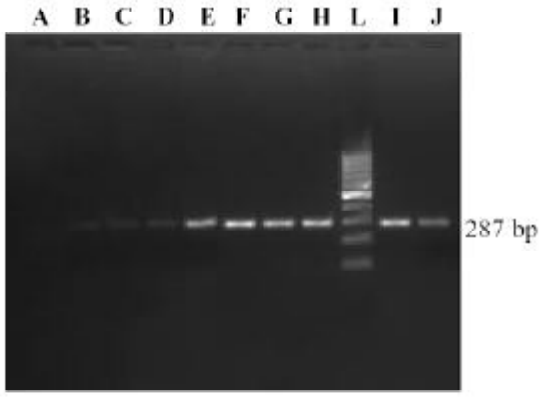



**Fig. 29 :** Demonstration of rabies virus in sacrificed Swiss albino mice by RI-PCR using N gene specific primers. Electrophoresis was done using 1.5% agarose gel to visualize 287 bp product. A (-ve), L (DNA marker 100 bp), B,C,D (2nd day), EFG (7th day) and H, I and J (9th day) brain samples.

**Detection of rabies viral N gene using conventional and Real Time PCR**

Rabies viral nucleoprotein (N gene) was detected in brain samples as early as on 2nd DPI in groups GI, GL and GT. The expected PCR amplicon of 287 bp was observed in 1.5% agarose gel as a confirmatory result (Fig. 29). This was further confirmed by Real Time PCR. The N gene amplicon showed Tm value 82.72±0.42 in dissociation curve (Fig. 30).

**Fluorojade B staining for degenerated neurons**

The mice brain sections of groups GI, GL and GT showed positive (bright green) signals for degenerated neurons. The signals were less in groups GL and GT as compared to group GI on 9th DPI (intra-cerebrally). Group GC showed no signals (Fig. 32).

**Detection of anti-rabies antibodies in peripheral blood**

The neutralizing antibodies against rabies in peripheral blood of CVS-18 inoculated mice by intramuscularly route could be detected in serum on 7th day on wards and upto 20th DPI (>0.1 IU).

**Expression of cytokines by Real Time PCR**

After each complete reaction in real-time PCR, the data were stored along with amplification plots and dissociation curves for each cytokines. The relative quantity of each cytokine was calculated after normalizing with β-actin house keeping gene (Tables 26-30, Figs. 33-37).

**TNF - a mRNA**

The relative expression of TNF-a was significantly higher up to 9th DPI (Intra-cerebrally) in group GI as compared with uninfected group (GC). From the day of LPS and LPS + RIG inoculation, there was decreased expression of TNF- a in treated groups (GL and GT) up to the end of experiment. Expression was more in GT group than the GL group and control groups.

**CXCL-10 mRNA**

The relative expression of CXCL-10 was significantly higher up to 9th DPI (Intracerebrally) in infected groups (GI, GL and GT) as compared with uninfected group

**Table 26-30: Mean ± SD of fold change for different cytokines in adult Swiss albino mice inoculated intra-muscularly with CVS-18 @ 100LD50/ mouse followed by intra-cerebral route with same dose on 24th day. LPS and LPS+RIG inoculated on onset of clinical signs (on 6th DPI of intra-cerebral route). The Two mice in each group sacrificed on different time points and the brains were processed to study mRNA expression.relative levels of cytokines were assayed by taking ß-actin as internal control by Real Time PCR. Groups bearing common superscripts do not differ significantly (P<0.05P). GI (CVS-18, 30µl/mouse 100$LD_{50}$), GL (CVS-18 + LPS 8µg/ mouse), GT (CVS-18+LPS+RIG 40IU/kg b. wt), CL (LPS), CT (LPS + RIG), GC (PBS).**

**Table 26 : TNF-**a

| **Groups/ DPI** | **2** | **7** | **9** | **11** |
|---|---|---|---|---|
| GI | 2.42±0.41$^{a}$ | 1.39±0.37$^{a}$ | 3.73±0.27$^{a}$ | - |
| GL | - | 1.46±.50$^{a}$ | 0.84±0.40$^{c}$ | 0.40±0.36$^{ab}$ |
| GT | - | 1.54±0.32$^{a}$ | 1.21±0.31$^{bc}$ | 0.80±0.30$^{ab}$ |
| CL | - | 1.41±0.37$^{a}$ | 1.95±0.21$^{b}$ | 0.17±0.05$^{ab}$ |
| CT | - | 1.90±.30$^{a}$ | 1.80±0.30$^{bc}$ | 0.45±0.30$^{ab}$ |
| GC | 1.00±0.00$^{b}$ | 1.00±0.00$^{a}$ | 1.00±0.00$^{bc}$ | 1.00±0.00$^{a}$ |

**Table 27 : CXCL-10**

| **Groups/ DPI** | **2** | **7** | **9** | **11** |
|---|---|---|---|---|
| GI | 1.80±.30$^{a}$ | 98.69±1.76$^{a}$ | 38.78±0.46$^{a}$ | - |
| GL | - | 53.63±0.52$^{c}$ | 8.62±0.68$^{c}$ | 2.04±0.37$^{ab}$ |
| GT | - | 72.68±0.60$^{b}$ | 20.65±0.66$^{b}$ | 1.29±0.38$^{bc}$ |
| CL | - | 0.33±0.32$^{d}$ | 0.37±0.14$^{d}$ | 0.36±0.35$^{cd}$ |
| CT | - | 1.41±0.42$^{d}$ | 0.07±0.08$^{d}$ | 2.96±0.20$^{a}$ |
| GC | 1.00±0.00$^{b}$ | 1.00±0.00$^{d}$ | 1.00±0.00$^{d}$ | 1.00±0.00$^{bcd}$ |

**Table 28 : IFN-**b

| Groups/ DPI | 2 | 7 | 9 | 11 |
|---|---|---|---|---|
| GI | 6.04±0.22[a] | 1.51±0.13[bc] | 0.51±0.07[b] | - |
| GL | - | 1.69±0.01[ab] | 0.61±0.01[b] | 0.26±0.01[c] |
| GT | - | 1.82±0.07[a] | 0.69±0.07[ab] | 0.09±0.01[d] |
| CL | - | 0.64±0.05[e] | 0.53±0.15[b] | 0.09±0.01[d] |
| CT | - | 1.37±0.09[c] | 0.39±0.06[b] | 0.53±0.05[b] |
| GC | 1±0.00[b] | 1±0.00[d] | 1±0.00[a] | 1±0.00[a] |

**Table 29 : TLR-3**

| Groups/ DPI | 2 | 7 | 9 | 11 |
|---|---|---|---|---|
| GI | 3.06±0.03[a] | 12.97±0.10[b] | 11.22±0.03[a] | - |
| GL | - | 14.24±0.03[a] | 2.17±0.04[c] | 0.18±0.03[c] |
| GT | - | 10.36±0.02[c] | 11.11±0.01[a] | 0.39±0.03[b] |
| CL | - | 3.06±0.07[e] | 4.14±0.02[b] | 0.17±0.03[c] |
| CT | - | 5.25±0.04[d] | 2.07±0.03[c] | 1.12±0.07[a] |
| GC | 1.00±0.00[b] | 1.00±0.00[f] | 1.00±0.00[d] | 1.00±0.00[a] |

**Table 30 : TLR-4**

| Groups/ DPI | 2 | 7 | 9 | 11 |
|---|---|---|---|---|
| GI | 3.76±0.27[a] | 1.83±0.21[a] | 1.14±0.47[bc] | - |
| GL | - | 1.14±0.06[abc] | 3.30±0.28[a] | 0.09±0.05[b] |
| GT | - | 1.39±0.33[ab] | 1.74±0.06[b] | 0.42±0.05[b] |
| CL | - | 0.44±0.06[c] | 0.62±0.07[c] | 0.10±0.06[b] |
| CT | - | 0.91±0.27[bc] | 0.96±0.15[bc] | 1.07±0.23[a] |
| GC | 1.00±0.00[a] | 1.00±0.00[bc] | 1.00±0.00[bc] | 1.00±0.00[a] |

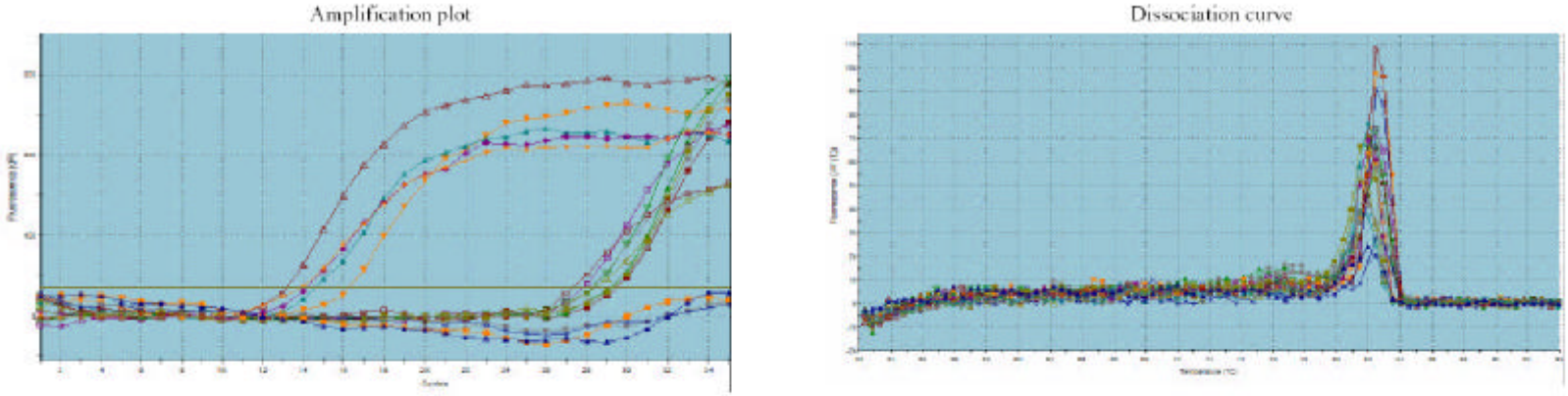



Fig. 30 : N gene amplification and dissociation plots of brain samples of Swiss albino mice. The mice brains in different groups at different time intervals were used for cDNA synthesis and from these, N gene was amplified using Real Time PCR. The Ct (threshold cycle) values on X-axis indicate amplification of the samples. Dissociation curve shows Tm value at 82.72±0.42. GI (CVS-18, 30µl/mouse 100LD50), GL (CVS-18 + LPS 8µg/mouse), GT (CVS-18+LPS+RIG 40IU/kg b.wt.), GC (PBS).

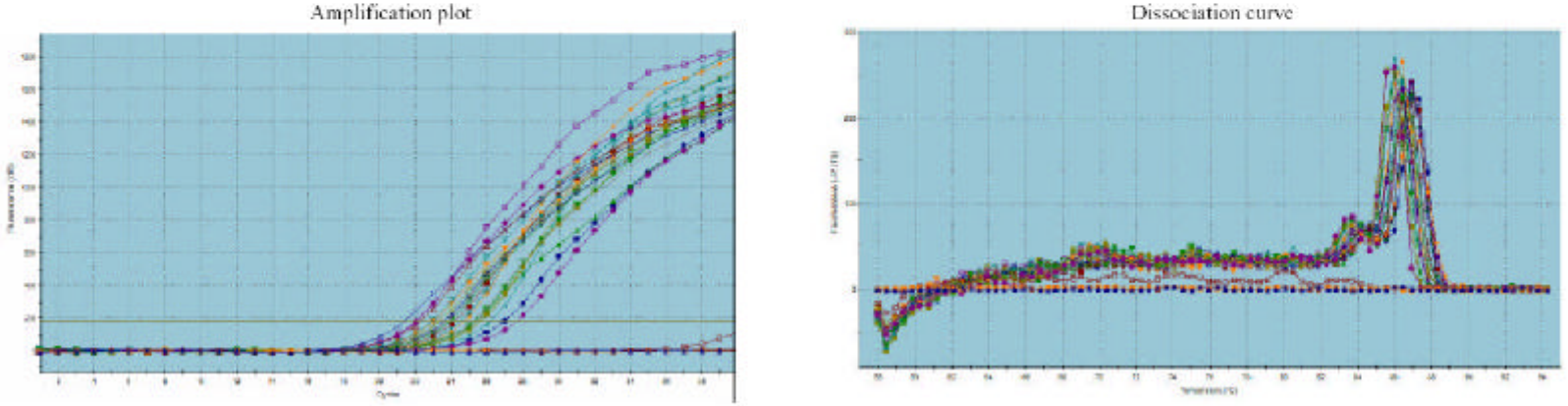



Fig. 31 : ß-actin amplification and dissociation plots of brain samples of adult Swiss albino mice inoculated CVS-18 intra-muscularly @100LD50/mouse followed by intra-cerebral route with same dose. The mice brains in different groups at different time points were used for cDNA synthesis and from these ß-actin gene was amplified using Real Time PCR. Melting temperature was 90.26±0.18.

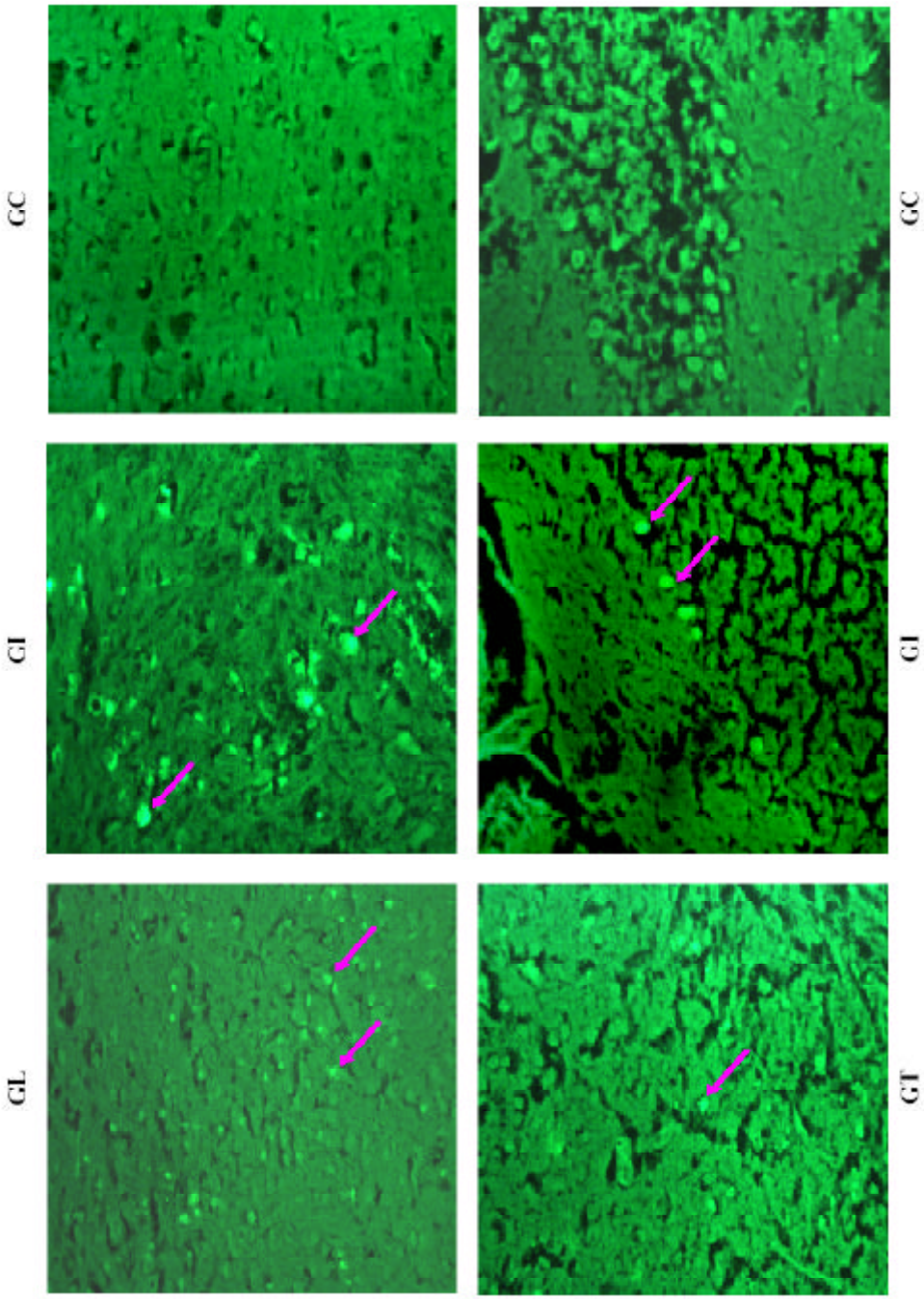



**Fig. 32 :** Sections of Swiss albino mice brain showing degenerated neurons (bright green color) in different groups at 9th day post infection intra-cerebrally. The signals were less in GL and GT groups than in GI group. Group GC showed no signals. Fluorojade B staining. X 200. GI (CVS-18, 30µl/ mouse 100LD50), GL (CVS-18 + LPS 8µg/ mouse), GT (CVS-18+LPS+RIG 40IU/kg b.wt.), GC (PBS).

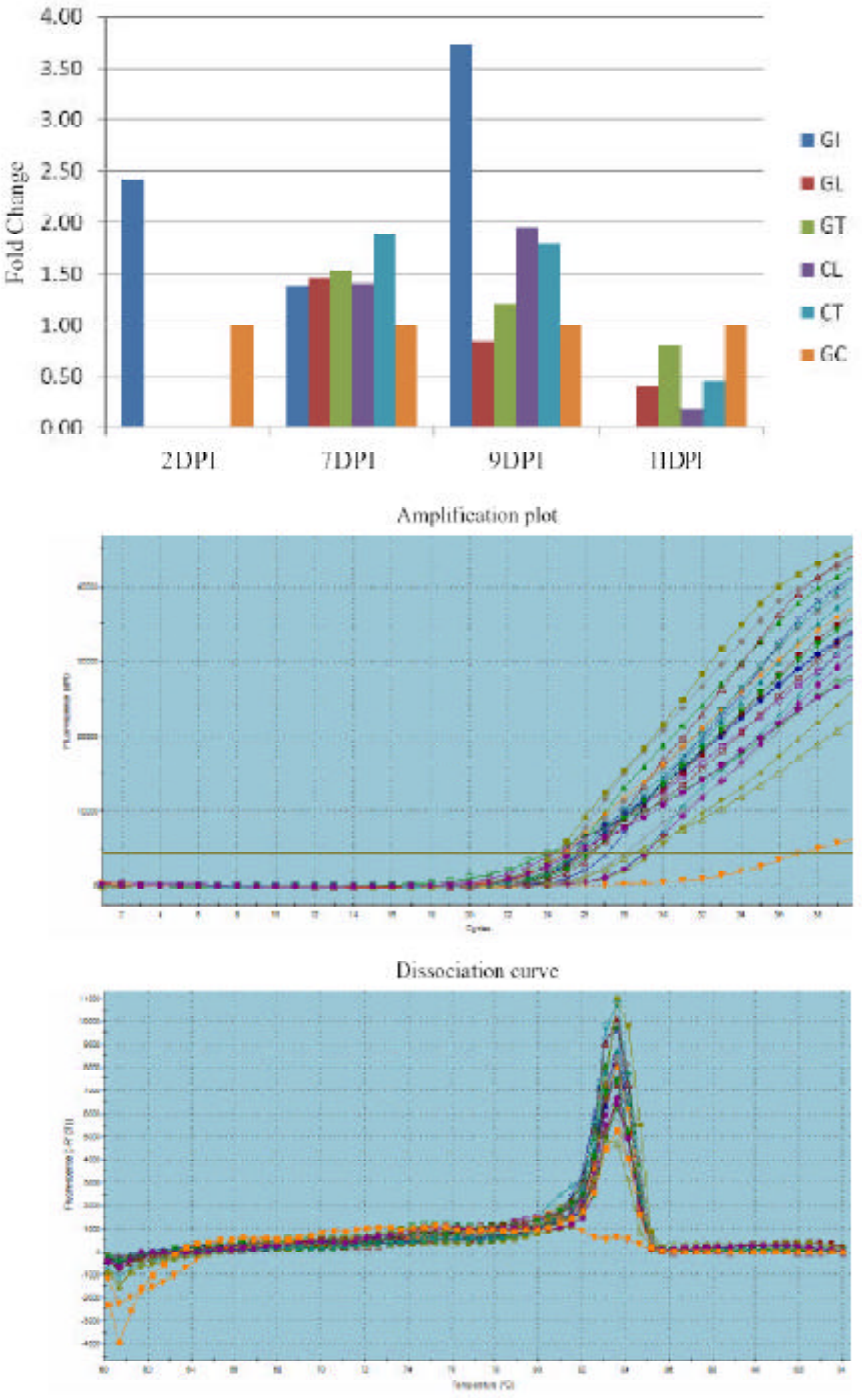



Fig. 33 : Mice brain collected on 2nd, 7th, 9th, & 11th DPI (Intra-cerebral) were assayed for relative levels of β-actin and TNF-α using Real Time PCR. The TNF-α mRNA expression was more in CVS inoculated group than control group before LPS inoculation. After LPS inoculation there was slight increase in TNF-á mRNA expression in GL and GT group compared to GI group and on 9th day GI group showed increased expression levels than GL & GT group. Dissociation plot showed melting temperature (Tm) at 83.43±0.03. GI (CVS-18, 30µl/mouse 100LD50), GL (CVS-18 + LPS 8µg/mouse), GT (CVS-18 + LPS + RIG 40IU/kg bwt), CL (LPS), CT (LPS + RIG), GC (PBS).

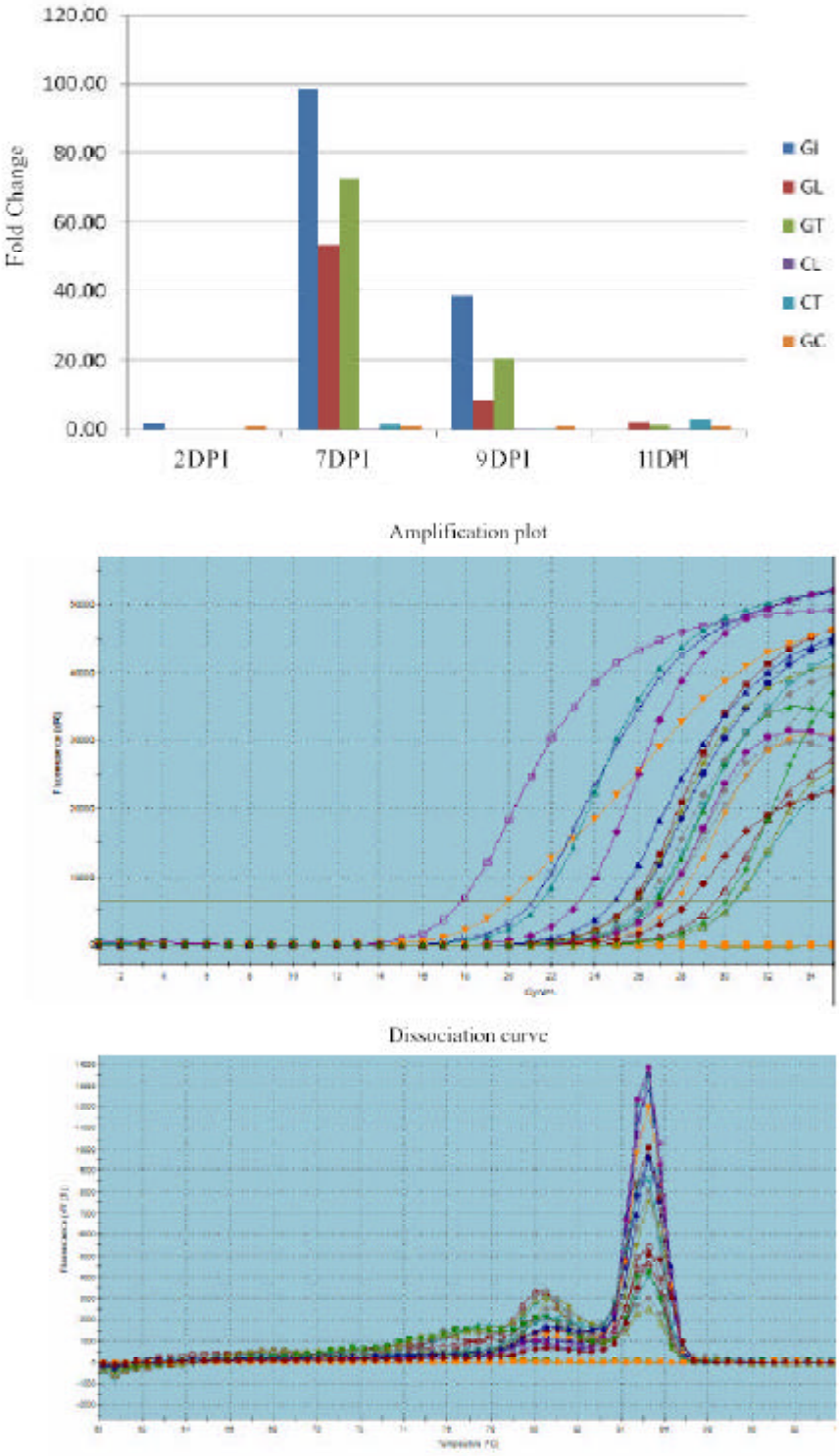



**Fig.34 :** Mice brain collected on 2nd, 7th, 9th, & 11th DPI (Intra-cerebral) were assayed for relative levels of ß-actin and CXCL10 using Real Time PCR.The CXCL10 mRNA expression was more in GI group than the GL and GT group. Dissociation plot showed melting temperature (Tm) at 83.87±1.2. GI (CVS-18, 30µl/mouse 100LD50), GL (CVS-18 + LPS 8µg/mouse), GT (CVS-18 + LPS + RIG 40IU/kg bwt), CL (LPS), CT (LPS + RIG), GC (PBS).

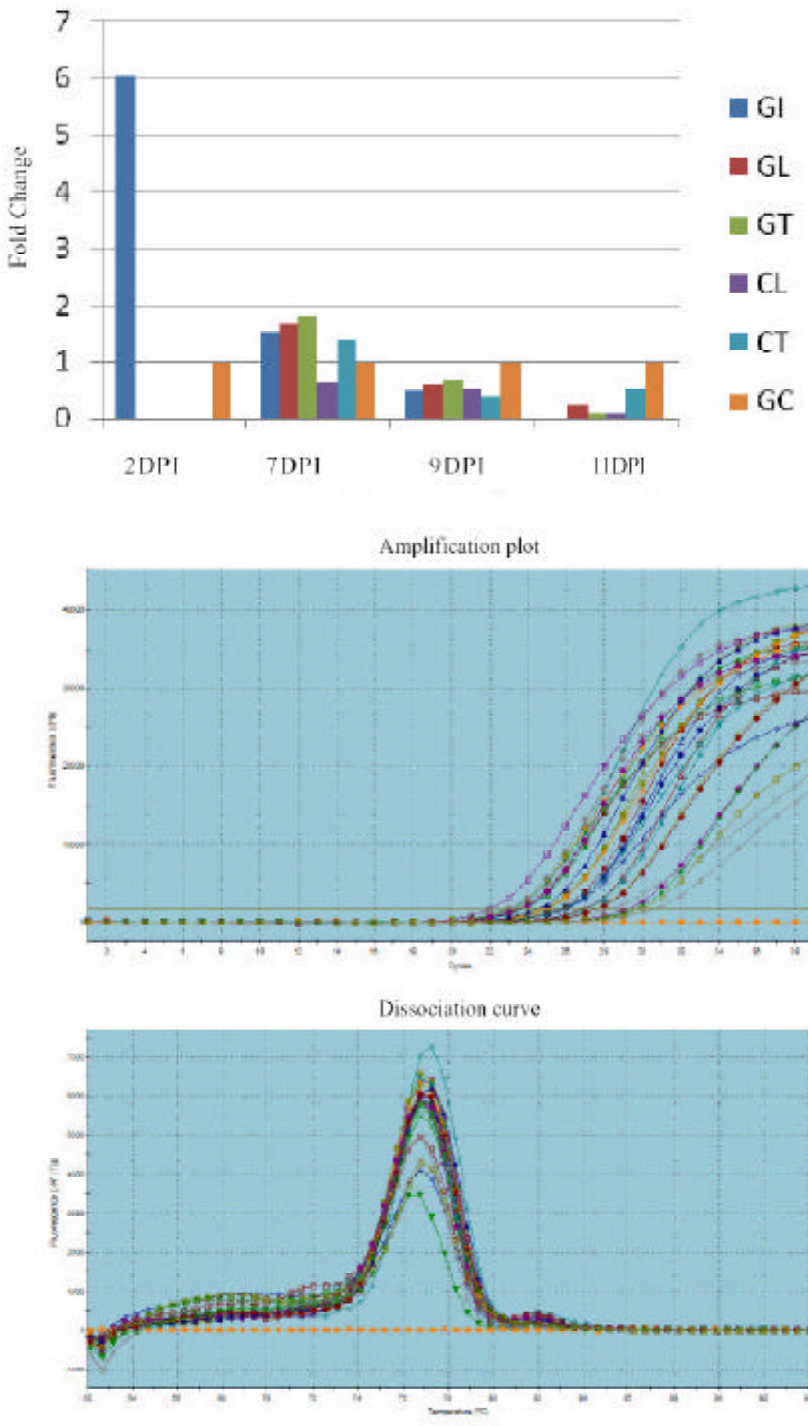



**Fig. 35 :** Mice brain collected on 2nd, 7th, 9th, & 11th DPI ( intra-cerebral) were assayed for relative levels of ß-actin and IFN-ß using Real Time PCR. The IFN-ß mRNA expression was more in CVS inoculated group than control group on 2nd day. On 7th day onwards, there was reduced in IFN-ß mRNA expression as disease progressed in all groups. Dissociation plot showed melting temperature (Tm) at 76.90±0.05. GI (CVS-18, 30µl/mouse 100LD50), GL (CVS-18 + LPS 8µg/ mouse), GT (CVS-18 + LPS + RIG 40IU/kg b.wt.), CL (LPS), CT (LPS + RIG), GC (PBS).

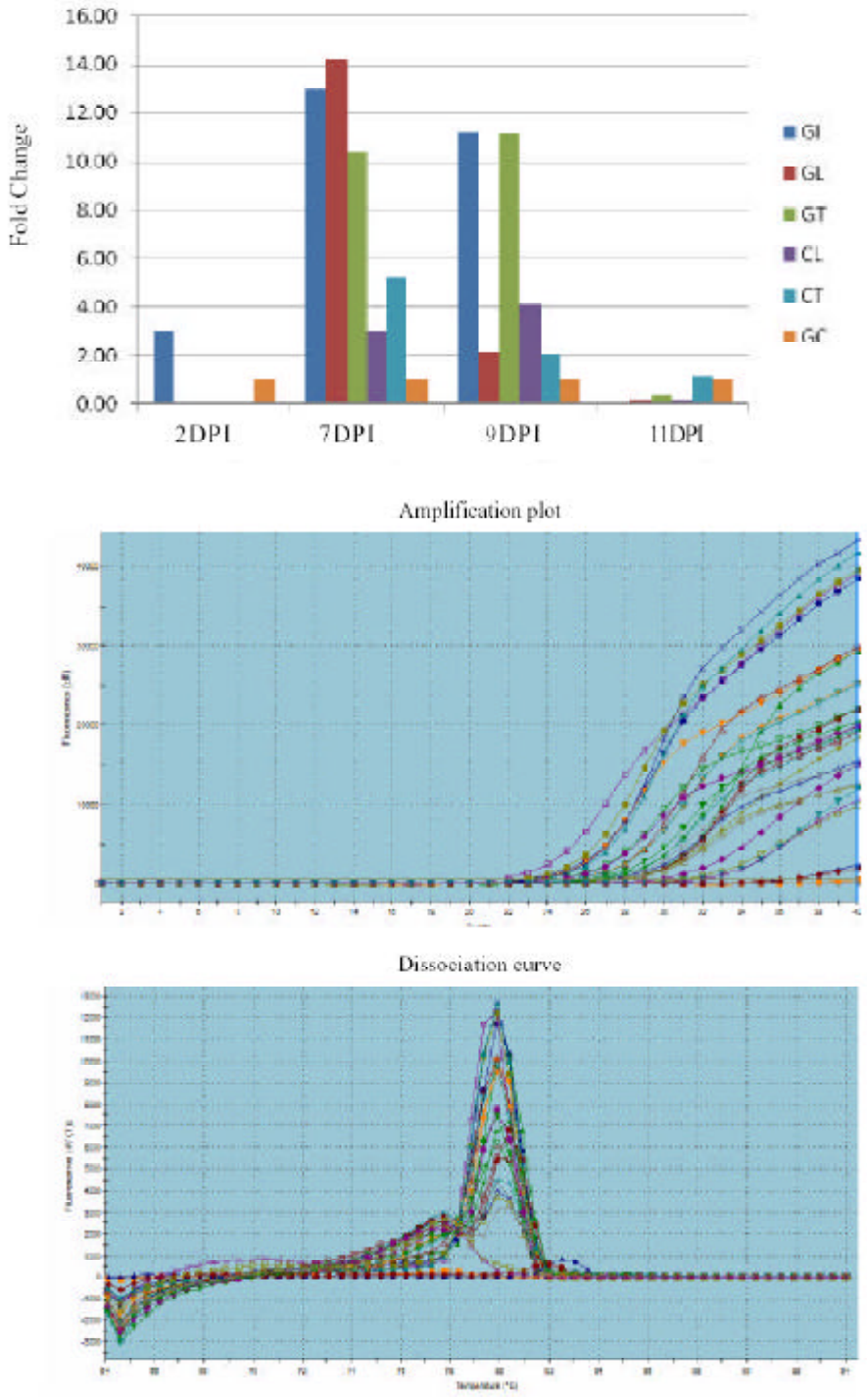



**Fig. 36 :** Mice brain collected on 2nd, 7th, 9th, & 11th DPI (intra-cerebral) were assayed for relative levels of ß-actin and TLR3 mRNA expression using Real Time PCR. The TLR-3 mRNA expression was more in CVS inoculated group than control group on 2nd day. On 7th day onwards there was increased in TLR-3 mRNA expression in GI and GT group. Dissociation plot showed melting temperature (Tm) at 79.60±0.03. GI (CVS-18, 30µl/mouse 100LD50), GL (CVS-18 + LPS 8µg/mouse), GT (CVS-18 + LPS + RIG 40IU/kg b.wt.), CL (LPS), CT (LPS + RIG), GC (PBS).

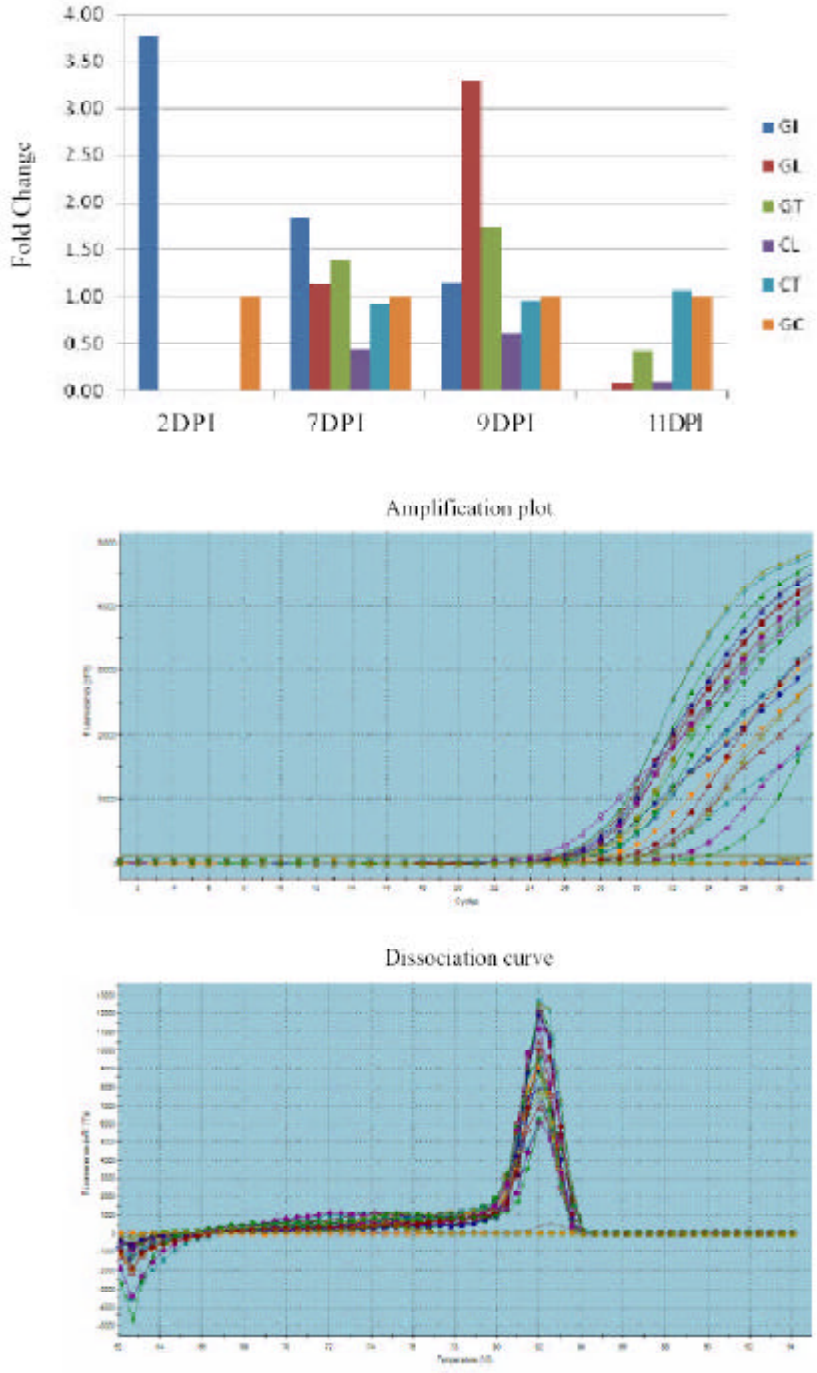



**Fig. 37 :** Mice brain collected on 2nd, 7th, 9th, & 11th DPI (intra-cerebral) were assayed for relative levels of ß-actin and TLR-4 expression using Real Time PCR. The TLR-4 mRNA expression was more in CVS inoculated group than control group on 2nd day. On 7th day onwards there was increased in TLR-4 mRNA expression in GL and GT group up to 9th day while decreased in GI group. Dissociation plot showed melting temperature (Tm) at 81.66±0.03. GI (CVS-18, 30µl/mouse 100LD50), GL (CVS-18 + LPS 8µg/mouse), GT (CVS-18 + LPS + RIG 40IU/kg b.wt.), CL (LPS), CT (LPS + RIG), GC (PBS).

(GC). From the day of LPS and LPS + RIG inoculation, there was decreased expression of CXCL-10 in treated groups (GL and GT) up to the end of experiment. The LPS+RIG treated group (GT) showed higher expression than the LPS treated group (GL) up to 9th DPI. On 11th day GL group showed higher expression than the group GT but did not differ significantly.

**IFN-ß mRNA**

The relative expression of IFN-ß was very high in infected group (GI) than the uninfected group (GC) on 2nd day. After LPS and LPS+RIG injection on day 6th of CVS injection (intra-cerebrally), a variation was observed between GI and treated groups (GL and GT, respectively). On day 7th, expression was more in groups GL and GT, than the GI and control groups. On day 9th, expression was less than the control group (GC) but still expression was more in treated group (GL and GT) than infected group (GI). Thereafter, on day 11th there was very less expression in all the groups except control group (GC).

**TLR – 3 mRNA**

The relative expression of the TLR - 3 was more in the infected group (GI) than the uninfected group (GC) on 2nd day. After LPS and LPS+RIG injection on day 6th of CVS injection (intra-cerebrally), a significant was variation observed between GI and treated groups (GL and GT, respectively) on 7th DPI. The expression was more in group GL than the group GI, GT and other respective controls. On day 9th, there was same expression in group GI and GT but significantly decreased expression in group GL. On day 11th, there was very low expression in all groups except group CT than the control group (GC).

**TLR – 4 mRNA**

The relative expression of the TLR - 4 was more in the infected group (GI) than the uninfected group (GC) on 2nd day. After LPS and LPS+RIG injection on day 6th of CVS injection (intra-cerebrally), no significant variation was observed between GI and treated groups (GL and GT, respectively) on 7th DPI. On day 9th, group GL showed significantly higher expression than the groups GI and GT. Expression was less in group CL than the other controls. There was low expression in all groups on 11th day except in groups CT and GC.

# 5 Discussion

The studies on spontaneous fetal brain affections in cattle and buffaloes are very limited in India and abroad. Therefore, the present study was carried out to generate some amount of histopathological information on common affections of brains in fetuses of cattle and buffaloes. The study involved systematic examination of brains in designated anatomical sites including spinal cord in a few cases. During the study, the brain specimens of 19/21 fetuses of cattle and 2/2 of buffaloes showed mostly congestion/hemorrhages, which could have been due to fragile nature of vasculature in fetus together with autolytic changes and hemodynamic disturbances during dystocia (Maxie and Youssef, 2007). The lesions of gliosis (focal/multi-focal) in fetal brains in 6/21 cases could be ascribed due to viral or toxic etiology as described by Zachary (2007). However, no etiological basis for gliosis could be established. The mild perivascular cuffing in one case could have been due to viral infection, but the same could not be verified. The neuronal degeneration in 2 cases is thought to be due to ischemic changes as a result of hypoxia/ hypoglycemia (Zachary, 2007).

The nests of primordial glial cells (Calleja cells) in sub-ependymal areas of lateral ventricles and in dentate fascia of hippocampus have been described as a part of normal developmental process. The relics of these cells act as replacement cells and are confused with inflammatory cells (Maxie and Youssef, 2007). The intact external granular cells in cerebellum observed in last trimester of fetus indicate normal developmental process. The literature says that these cells in external layer of cerebellum in fetus migrate to form the internal granular layer of the cerebellum and noticed till the birth of calves (Zachary, 2007). The bovine viral diarrhoea (BVD) virus has preference to the external granular layer, causes damage

of these cells resulting in cerebellar hypoplasia (Zachary, 2007). In our study, the external granular layer was found intact that finds further support from negative laboratory findings for BVD. The stray Purkinje cells in internal granular layer cells are supposed to have been due to retardation in migration as described by (Maxie and Youssef, 2007). The presence of compactly arranged neurons in cerebral cortex in a fetus of 9 months gestation as against distinct layers seen in adult brain might have been due to retardation in developmental process (Maxie and Youssef, 2007). The microscopic lesions occurred mostly in combinations, with maximum number in cerebellum followed by brainstem and cerebral hemisphere/caudate nucleus. While other neuron-anatomical sites showed approximately similar number of combinations. The frequency occurrence of engorgement/ hemorrhages and gliosis were more in comparison to developmental changes. In the present study, no etiological specific findings were recorded. The distribution of lesions was more in the cerebellum and brainstem regions indicating more susceptibility of these parts for different insults. However, this fact needs validation.

To assess the role of secondary infections on brain, fetal visceral organs, histopathology was done that showed vascular engorgement/hemorrhages in different organs, including focal infiltrations of mononuclear cells in kidneys, liver and in lungs. However, these changes were not in agreement with the isolation studies. This indicated that isolation made in the present study were simply contaminants. Moreover, Brucella sp organisms could not be detected in any of the cases. The reason for mononuclear cells infiltration in four cases remained unclear; however, possibility of other infections can not be ruled out.

**Experimental study**

Rabies is known to cause inevitable death in clinically infected hosts, if it is not protected properly with post-exposure prophylaxis (PEP). There is no validated therapy available once the clinical symptoms appear in the infected individual. *Hemachudha et al*. (2003) reported the use of human RIG therapy along with other supportive drugs to prolong the course of the disease. The studies of clinical cases revealed minimal or even no detectable amount of antibody at the end stage of the disease (Kesempimolporn *et al*., 1991). Once the virus enters the CNS, its spatial spread is very fast and unchecked by the immune effector cells and their cytokines, which are generated in the terminal stage of the disease. Moreover, peripherally

generated immune response may not reach the brain due to blood-brain barrier (BBB). This observation is consistent with the fact that rabies PEP (active and passive) fails to protect individuals from the disease, when administered after appearance of clinical signs of rabies. Roy *et al*. (2007) demonstrated that failure of opening of BBB causes no clearance of SHBRV from the CNS. The present study supports the hypothesis by demonstrating that modulating BBB permeability along with anti-rabies antibody facilitates prevention of clinical disease, delayed onset of clinical signs and prolongation of survival in CVS-18 infected Swiss albino mice after LPS treatment on the day of onset of clinical signs, probably due to influx of peripherally generated immune response and injected antibody from external source.

The present study revealed that mice injected intra-muscularly with 100 LD 50 dose with CVS-18 in masseter muscle did not develop clinical disease by 24$^{th}$ DPI. This could be due to requirement of more viral particles for intramuscular route than intra-cerebral route, or adaption of CVS-18 strain for neural tissue. CVS is a laboratory virus and found to result in non-fatal abortive disease. The restricted infection of CNS on peripheral injection of CVS is characterized by antibody production and action of inflammatory cytokines (Galelli *et al*., 2000). In the present study, the first injection was found to provoke some degree of peripheral immune response in mice as demonstrated by reduced $CD4^+/CD8^+$ ratio in FACS analysis and virus-neutralizing antibody production in RFFIT. The ratio of $CD4^+$ and $CD8^+$ cells was significantly decreased upto 7$^{th}$ DPI then showed increasing tendency up to 20$^{th}$ DPI. This indicates that peripheral cell mediated immunity was formed after intramuscular injection of CVS-18. The detection of antibodies by RFFIT in serum on 7$^{th}$ and 20$^{th}$ day after first I/m inoculation indicated formation of humoral immune response in periphery.

To develop the clinical disease, a second injection of CVS-18 was given by intra-cerebral route. After the first appearance of clinical signs in a few mice, the mice were injected with LPS and LPS+RIG to cause opening of the BBB and delivery of anti-rabies antibody generated peripherally as well as from external source. This resulted into more survivability in GT group followed by GL group and characterized by reduced number of clinical subjects and prolongation of clinical state, before death. In the first 2 days after LPS inoculation, the mortality pattern was almost similar in GI, GL and GT groups, since these animals were already in advanced stage of infection on the day of LPS inoculation. The difference in mortality rate

was evident from 3rd day onwards after LPS inoculation. On the 13th DPI (intra-cerebrally), the survival rate was 46% (6/13) in GT group, 38% (5/13) in GL group and no survivability in GI group. The similar study in same lab demonstrated delayed development of clinical signs and more survivors in Swiss albino mice when simultaneously inoculated with CVS I/C along with LPS I/P (Reddy, 2010). Shan, 2010 reported around 42% survivability in CVS-18 infected mice inoculated first intra-muscularly followed by intra-cerebral route after modulation of BBB permeability by LPS. The above observations could be due to enhanced BBB permeability as evident by no signals of ß-catenin in brain capillaries of GL and GT groups. This could have lead to influx of peripherally generated immune response along with injected antibody in brain. The above findings found support from increased expression of cytokines TNF-a, IFN-ß, TLR4 and decreased expression of CXCL10 in present study. Similar findings were recorded in cell culture studies (Singh and Jiang, 2004; Rosenberg *et al*., 2007) and in rat and mice using LPS (Qin *et al*., 2008 and Christopher *et al*., 2008).

The microscopic lesions in CVS infected brain sections included non suppurative meningitis, encephalitis, focal to diffuse gliosis and neuronal degeneration (Iwasaki and Tobita, 2002). In group GI, meningoencephalitic lesions of non-suppurative nature on 2nd DPI itself were observed, whereas these lesions appeared less in GL and GT groups as evident by respective histopathological scores (HPS). The lesions in GI group became more intense as the disease progressed compared to GL and GT groups. In GL and GT groups, the pathological alteration was less compared to GI group indicating some degree of viral clearance or delayed viral action as demonstrated by presence of less virus signals in dFAT. This was also reflected in prolonged clinical state in GL and GT groups. The lesser pathological changes in brain of GL and GT groups are corroborated with increased expression of neuroprotective cytokines including IFN-ß. Mononuclear cells, found especially in and around vessels, meninges and in neuropil in GI, GL and GT groups, indicated the clearance of virus by neutralizing antibodies with the help of T helper cells that played a major role in this process as reported by Jackson and Wunner (2007). The clearance of virus appeared more in GT group probably due to injected anti-rabies antibody. Dietschold *et al.* (1992) reported inhibition of both viral spread and RNA transcription *in-vitro* as well as *in-vivo* using *Mab* against rabies viral glycoprotein, resulting in virus clearance from CNS and protection in challenged laboratory rat.

Cell mediated immunity plays a vital role in limiting the intracellular pathogens, including viruses. There are many reports stating the role of both $CD4^+$ and $CD8^+$ T cells in antiviral immunity against rabies in brain (Hooper *et al.*, 1998; Marten, 2000). Rabies virus infection induces immune unresponsiveness (Camelo *et al.*, 2001, Torres-Anjel *et al.*, 1988), limits T cell infiltration into the brain (Roy and Hooper, 2008) and keeps the BBB tightly closed (Roy and Hooper, 2007). It also promotes the destruction of migratory $CD8^+$ T cells in the brain through the up regulation of immunoevasive proteins such as B7-H1 (Lafon *et al.*, 2008). The present study revealed more number of $CD8^+$ cells in GL and GT groups compared to GI group, indicating viral clearance. Bellur *et al.* (1981) reported recovery from rabies infection of the CNS by adoptive transfer of immune lymphoid cells.

Blood brain barrier is a selected physical barrier impermeable to most of the antibodies (Jackson and Wunner, 2007). It is demonstrated that functional immune response is developed in bat rabies and fixed virus strains and failure to open BBB and delivery of immune effectors to CNS leading to lethal outcome (Roy *et al*., 2007). Studies already proven that induction of increased blood brain permeability using immunization against myelin basic protein increased the survivability of rabies infected mice (Roy *et al*., 2007). LPS has been found to increase BBB permeability in different animal models, including newborn pigs, rats and mice (Temesvari *et al*., 1993; Veszelka *et al*., 2003 and Christopher *et al*., 2008). LPS activated microglias induce dysfunction of BBB in microvascular endothelial cells co-cultured with microglia (Sumi *et al*., 2009). LPS also induces pro-inflammatory cytokines TNF-a and type-1 interferons. Salkeni *et al.* (2008) demonstrated that LPS impairs BBB P- glycoprotein function in mice through TNF-a activation. Brain endothelial cells express LPS receptors TLR-4, TLR-2 and CD14 (Quan *et al*., 2002) and mediate the effects of peripheral LPS in the CNS (Singh and Jiang, 2004). LPS can have many effects on BBB function, including its disruption (Watkins *et al*., 1995), inducing cytokine release (Reyes *et al*., 1999; Verma *et al*., 2006), altering its permeability to virus and viral proteins (Banks *et al*., 1999; Dohgu and Banks 2008), increasing immune cell adherence and passage (De Vries *et al*.,1994;Persidsky *et al.*, 1997), and altering its transport systems ( Nonaka *et al*., 2005; Banks *et al*., 2008). The LPS and TNF-a modulate P-gp activity, first suppressing it through the endothelin-1B receptor and later stimulating P-gp expression through an NF-kB pathway (Bauer *et al*., 2007; Hartz *et*

*al*., 2006). The increased survivability of GL and GT groups in the present study is indicative of LPS effect on BBB and cytokine production. There was higher expression of TNF-a, TLR4 and IFN-ß in GL and GT groups than control up to7th DPI. This indicates that these cytokines definitely have played role as antiviral mechanisms in GL and GT groups. Similar observations have also been reported by earlier workers in rabies infected brain using semi-quantitative RT-PCR (Ware *et al*., 1996; Bsibsi *et al*., 2002) and quantitative real time RT-PCR (Shivasharanappa, 2008).

Cytokines are the key regulatory mediators involved in the host immune response to immunological agents, also in brain injury and in the communication between the immune system and CNS. One of the most important cytokines of adaptive and innate immunity is tumor necrosis factor alpha (TNF-a), a proinflammatory cytokine produced by T cells and cells of the monocyte/macrophage lineage, including microglial cells (Bette *et al.,* 2003). There is evidence that CNS inflammatory processes contribute to the clearance of virus from the CNS (Morimoto *et al*., 2001). TNF-a has direct cytotoxic and immuno-modulatory effects (Wang *et al.,* 2002). In the present study, the mRNA expression of TNF-a in brain tissue, was more in group GI before LPS inoculation. The expression of TNF- a was higher in GL and GT groups than GI group after 24 h of LPS inoculation. But on 9th day, expression again shot up in GI group which might be indicating cytotoxic effect of TNF. LPS induced the release of TNF- a within 4 h of inoculation (Reimer *et al.,* 2008). TNF is a major mediator of LPS pathophysiological sequelae which is found in high levels in the serum at 1-2 h after LPS challenge and disappears at 5 h (Zuckerman *et al.,* 1989).

In the present study, there was significant increase in CXCL10 mRNA expression on 7th DPI (Intra-cerebrally) in CVS-18 infected mice. CXCL10 and its receptor CXCR3 have been shown to function in host resistance to virus infection by regulating the trafficking of activated inflammatory T cells (Dufour *et al.,* 2002).The important role of CXCL10 in the innate immune response has also been found to inhibit viral replication at early stage of infection through modulating natural killer (NK) cells trafficking and the delivery of NK cell-derived IFN-? (Mahalingam *et al.,* 1999). The increased CXCL10 expression was most likely to occur in microglia, astrocytes and neurons as these cells are the principal responders to CNS damage and infection (Nakamichi *et al.,* 2004). The expression was significantly down regulated

in GL and GT groups from day 9$^{th}$ to end of experiment, indicating role of LPS and anti-rabies antibody in these treated groups. This might be due to decreased viral load in brain parenchyma as a result of neutralizing antibody generated internally as well as injected from external source.

The interferons are group of secreted cytokines that elicit distinct antiviral effects. The expression of the IFN-β mRNA was very high in GI group on 2$^{nd}$ day, indicating generation of innate immune response and decreases with progression of disease. After LPS inoculation, expression was high in GL and GT groups. LPS induces a strong IFN-mRNA response within a short time-frame. The IFN-stimulated genes (ISGs), (ISG56) and IFN-inducible protein 10, are strongly induced by LPS (Reimer *et. al.*, 2008). These responses are associated with NF-κB and IRF3 activation. NF-κB contributes to IFN-ß expression (Schafer, *et. al.,* 1998). IFN-ß assay in serum was in conjunction with the findings of mRNA expression in different groups at different time intervals. In GI group, initially concentration was very high and later decreased with the progression of disease. After LPS inoculation, concentration of IFN-β was more in GL and GT groups than GI. It might be due to activation of NF-κB pathway by LPS leading to induce a type-I IFN response as well as the release of TNF (Reimer *et al.*, 2008).

TLRs play an important role in the innate host defense against invading microorganisms by recognizing pathogen- associated molecular patterns (PAMPs). TLR-3 recognizes the dsRNA generated as an intermediate in the viral replication (Alexopoulov *et al.,* 2001). TLR-3 expression study showed marked increase in TLR-3 mRNA expression in GI, GL and GT groups as compared to GC group. Glial cells have been identified as the major producers of TLR- 3 and initiators of inflammation in the CNS (Farina *et al.,* 2005). Glial cells (microglia) proliferation was a major change in pathology.LPS and dsRNA interacting with TLR-4, TLR-3, or Mda-5 activate different signaling pathways using various adaptor proteins leading to subsequent activation of TNFR-associated factor family member-associated NF-κB activator-binding kinase 1 (TBK-1) and IFN regulatory factor 3 (IRF3) for the induction of type-I IFN and IFN-stimulated genes (ISGs), such as RANTES, ISG56, or IFN-inducible protein 10 (IP-10) (Grandvaux *et al.,* 2002) in turn help in clearance of virus from GL and GT groups.

Cytokines induced by LPS might have protected the mice through different mechanisms, such as (i) direct inhibition of virus replication in neurons; (ii) accelerated clearance of RV

infection via the induction of brain inflammatory processes, which open the BBB to allow access of immune effectors such as RV-neutralizing antibodies and T cells to the infected neurons; and (iii) enhanced microglial activation, which likely contributes to the immune defense (Lokensgard *et al*., 2001; Phares *et al*., 2006).

The CVS-18 infected mice brain sections (cerebrum, hippocampus and cerebellum) revealed positive signals from 7$^{th}$ day onwards for degenerated neurons in Fluorojade-B staining. Control groups did not show any signals. The decreased signals in GL and GT groups compared to GI group attribute to virus clearance mediated by LPS and injected anti-rabies antibody. However, between GL and GT groups there was not much difference in positive signals. Although, the staining time and dye concentration were reduced, but the method was as rapid and reliable as the recommended staining protocol. The anti-rabies antibody injected from external source might have protected the mice through different mechanisms, such as (i) direct neutralization of virus (ii) inhibition of spread of virus from cell to cell (iii) inhibition of rabies virus RNA transcription (iv) endocytosis of anti-rabies antibody by virus infected cells( Ramakrishna *et al.*, 2003; Dietzschold *et al.*, 1992).

The histopathological changes seen in the terminal stage of the disease indicated severe degeneration of neurons owing due to the viral replication in neurons. If the viral replication is checked in neurons, the present treatment might enhance the survival rate.

# 6 Summary and Conclusions

The successful parturition in animals is hampered due to various biotic and abiotic factors that directly or indirectly affect the dam, the placenta or the fetus to cause abortion. Amongst various teratogens, viral pathogens at particular stage of gestation are known to affect specific cell populations of brain to cause congenital malformations. Of late, many protozoan parasites have also been incriminated to cause fetal brain lesions, resulting into abortions. Since, the information on fetal brain pathology is very meager in available literature, therefore, the present study was undertaken to garner some information on the subject. A total of 21 fetal brains of cattle (19) and buffaloes (2) of different gestation length (majority > 6 months) were collected from organized dairy herd. Grossly, no congenital or developmental anomalies were observed in any of the cases. However, in microscopic examination vascular engorgement (21/21 cases) and hemorrhages (9/21 cases) were predominantly found, which could be due to fragile nature of fetal brain, autolysis and hemo- dynamic disturbances during dystocia. In a few cases, focal to diffuse gliosis (6/21 cases), mild perivascular cuffing (1/21 case), ischemic neurons (2/21 case) were also observed, indicating some viral insult to the brain. The other observations included nests of glia cells (islands of Calleja) beneath the ependyma of the lateral ventricles (2/21 cases) and in the dentate fascia of the hippocampus (1/21 case), primordial external granular cells layer in cerebellum (1/21) and stray presence of Purkinje cells in internal granular layer (3/21), as a part of developmental process. No case of BVD was detected in any of the 14 brain samples tested. The brain lesions occurred mostly in combination, with maximum number in cerebellum followed by brainstem, cerebral hemisphere/ caudate nucleus, while other neuro-anatomical sites showed approximately similar number of combinations. The frequency occurrence of engorgement/ hemorrhages and gliosis were more

in comparison to primordial cells of developmental process (7/21 cases). In the present study, no congenital malformations of brain were detected. In visceral organs of the fetuses, the lesions were mostly of non specific type (engorgement and hemorrhages), except in few cases there were infiltrates of mononuclear cells either in lungs, liver, or kidneys. However, in these cases no etiology could be established.

Rabies is a major viral zoonoses and causes almost 100% fatality in clinically affected subjects. Till date there is no validated therapy available to prolong the life of rabies affected subjects, due to the low degree of immune responses, and if develops, it is too late. Moreover, the virus evades host immune system to cause entry into neurons, replication and disturbance in neuronal function. It is further known that both innate (type-1 interferons, NK cells) and adaptive (both cellular and humoral) immune responses generated peripherally remain inadequate, that also do not find entry into brain in the early phase of the clinical signs due to intact blood-brain barrier (BBB). The BBB opens late in clinical course, by that time irreversible damage has already done. Therefore, it was hypothesized that if BBB permeability is modulated transiently during the early stage of clinical disease using LPS followed by administration of antirabies antibody of equine origin, it might help in prolonging the survival of the infected host by some degree of viral clearance through influx of peripherally generated immune response and injected anti-rabies antibody. The use of LPS has been found to modulate BBB permeability, mediated through toll like receptor-4 (TLR-4). Besides, LPS also induces neuro-protective cytokines (type-1 interferons) through IFN-stimulated genes. To prove the hypothesis, a total of 150 young Swiss albino mice were used. Half (75/150) of the mice were injected with 0.03 ml of 100 $LD_{50}$ per mouse of CVS-18 suspension intramuscularly in masseter muscle. The infected mice observed for 24 days and did not show any clinical signs till 24$^{th}$ day. Hence, a second dose was inoculated intra-cerebrally with same dose. On the 6$^{th}$ day, a few mice started showing clinical signs. The same day, the infected group was randomly divided into three groups of 25 mice each. One group was inoculated with 8 µg of LPS intra-peritoneally per mouse and named as GL (CVS+LPS). Another group inoculated with LPS (8µg/mouse) and anti-rabies antibody; and was designated as GT (CVS+LPS+RIG). The remaining mice were kept as CVS control and named as GI group. Same time, the uninfected mice were inoculated with LPS (25/75), LPS+RIG (25/75) and PBS (25/75), and were designated as GL, GT and

GC controls. Thirteen mice in each group were left out for survival study at the end of the experiment. The mice in all groups were observed for clinical signs and survivability. The clinical and histopathological scores (sections 14, 19 and 21 as per stereotaxic mouse brain atlas), kinetics of $CD4^+$, $CD8^+$ and NK cells in blood as well as in spleen by FACS and antibody levels in serum by RFFIT, demonstration of ß-catenin by IHC, detection of virus in brain tissue by dFAT and by conventional and Real Time PCR and expression of TNF-a, CXCL10, IFN-ß, TLR3 and TLR4 cytokines by Real Time PCR were studied at $2^{nd}$, $7^{th}$, $9^{th}$ and $11^{th}$ day after intra-cerebral inoculation.

The animals which were in advanced stage of clinical signs on the day of LPS inoculation died in groups GI, GL and GT on the next day itself, without showing any response to LPS and anti-rabies antibody. Thereafter, significant changes were observed in development of clinical signs and mortality pattern. The mortality in GL and GT groups of mice was less compared to GI group and clinical stage was prolonged in these groups, as supported by histopathological scores using parameters like neuronal degeneration, gliosis and vascular changes. The survival in GT and GL groups was 46% (6/13) and 38% (5/13), respectively, as compared to GI group. The survivability and prolonged clinical course before death in GL and GT groups could be due to influx of peripherally produced immune molecules and cells into brain along with injected anti-rabies antibody. This might have resulted into some degree of virus clearance and decreasing its load and spread in the brain as evident by dFAT signals. The LPS might have contributed to virus clearance through induced expression of IFN-ß and TNF-a in GL and GT groups over GI group.

The ratio of $CD4^+$ to $CD8^+$ cells almost remained constant throughout the experiment in GC group, while in CVS-18 inoculated group, the ratio was significantly decreased on $7^{th}$ DPI and normalized by $24^{th}$ DPI. The NK cells revealed significant higher value on $7^{th}$ day followed by decreased in population on $20^{th}$ DPI. This indicated that peripheral cell mediated immunity had generated after intramuscular injection of CVS-18. Detection of anti-rabies antibodies in serum indicated development of humoral immunity in periphery, and might have played role in clearance of virus in brain after BBB was made open as evident by ß-catenin immuno-staining. The neuronal degeneration was less in GL and GT groups as compared to GI group that might be due to decrease viral load resulted due to loosening of BBB and the effect of ant-rabies antibody.

In the present study, there has been higher expression of TNF-a, IFN-ß, TLR3 and TLR4 in CVS group than PBS group, before LPS inoculation. After LPS inoculation on 6th DPI (intra-cerebrally), GL and GT groups exhibited increased expression of TNF-a, IFN-ß and decreased expression of CXCL10 compared with GI group. The variability in expression was observed both in TLR-3 and TLR-4 cytokines. The IFN-ß assay by ELISA supported the findings of Real Time PCR. Anti-rabies antibody along with LPS might have protected the infected mice through different mechanisms as reported, such as (i) direct inhibition of virus replication in neurons; (ii) accelerated clearance of rabies virus infection via the induction of brain inflammatory processes, which opens the BBB to allow access of immune effectors such as rabies virus-neutralizing antibodies and T cells to the infected neurons; and (iii) enhanced microglial activation, which likely contributes to the immune clearance (iv) inhibition of cellular spread of virus (v) uptake of anti-rabies antibody by infected cells. The survivability and milder pathology in mice in GL and GT groups as compared to GI group could be due to modulation of BBB permeability by LPS and virus clearance by both peripherally generated and external source of anti-rabies antibody and LPS associated cytokines.

However, to completely overcome the viral replication in neurons and other cells, there is necessity to use siRNA approach along with above treatments for possible prolongation of survival of the host by compromising some degree of neuronological deficit.

## Conclusions

a) There is a need to study fetal brain lesions in more number of aborted cases together the correlation of lesions with etiology using immunohistochemical staining and other molecular based techniques

b) In rabies experimental study, it was found that antirabies antibody from external source along with modulation of BBB by LPS and its associated cytokines had some role in prolongation of clinically affected mouse model.

c) The survivability was more in GL (38%) and GT (46%) groups compared with GI group. Even in GT group, survivability was more than the GL group.

d) The pathological changes on different time points were lesser in GL and GT groups as compared to GI group.

e) The decreasing trend in $CD4^+/CD8^+$ ratio, increase in NK cells population and presence of anti-rabies antibodies in peripherally inoculated CVS-18 mice prior to LPS inoculation, indicated generation of cell mediated and humoral immunity in periphery.

f) LPS inoculation on the day of onset of clinical signs resulted in opening of blood-brain barrier in GL and GT groups as evident by the absence of signals of ß-catenin compared to GI group.

g) There was increased expression of TNF- a and IFN -ß and decreased expression of CXCL10 in GL and GT groups compared to GI group after LPS inoculation.

h) The IFN –ß concentration was higher in GL and GT groups as compared to GI group.

i) Neural degeneration was less in GL and GT groups as compared to GI group, as evident on Fluorojade-B staining.

# 7 Mini Abstract

To record the spontaneous fetal brain lesions, a total of 21 fetuses (19 cattle, 2 buffaloes) of different gestation length were collected from an organized dairy farm. Grossly, no congenital malformations in brains were observed. However, on microscopic examination vascular engorgement (21/21 cases) and hemorrhages (9/21 cases) were predominantly found. In a few cases, focal to diffuse gliosis (6/21 cases), mild perivascular cuffing (1/21 case), ischemic neurons (2/21 case) were observed. The other microscopic observations included: nests of glia cells beneath the ependyma of the lateral ventricles (2/21 cases) and in the dentate fascia of the hippocampus (1/21 case), primordial external granular cells layer in cerebellum (1/21), stray presence of Purkinje cells in internal granular layer (3/21), compact neurons in cerebral cortex (1/21 case), as a part of developmental process. No case of BVD was detected in any of the 14 brain samples tested. The brain lesions occurred mostly in combination, with maximum number in cerebellum followed by brainstem, cerebral hemisphere/ caudate nucleus, while other neuro-anatomical sites showed approximately similar number of combinations. The frequency occurrence of engorgement/ hemorrhages and gliosis were more in comparison to primordial cells (7/21 cases) of brain development. In addition, the lesions involving visceral organs other than brain were mostly of non specific type, except in few cases there were infiltrates of mononuclear cells either in lungs, liver, or kidneys. To study the combined effect of LPS and rabies immunoglobulin, a total of 150 young Swiss albino mice were used. Half (75/150) of the mice were injected with 0.03 ml of 100 $LD_{50}$ per mouse of CVS-18 suspension intramuscularly in masseter muscle. The infected mice observed for 24 days and did not show any clinical signs till 24$^{th}$ day. Hence, a second dose was inoculated intra-cerebrally with same dose. On the 6$^{th}$ day, a few mice started showing clinical signs. The same day, the infected group was randomly divided into three groups of 25 mice each. One group was inoculated with 8 µg of LPS intra-peritoneally per mouse and named as GL (CVS+LPS). Another group inoculated with LPS (8µg/mouse) and anti-rabies antibody, and was designated as GT (CVS+LPS+RIG). The remaining mice were kept as CVS control and named as GI group. Same time, the uninfected mice were inoculated with LPS (25/75), LPS+RIG (25/75) and PBS (25/75), and were designated as GL, GT and GC controls. Thirteen mice in each group were kept for survival study. The mice in all groups were observed for clinical signs and survivability. The clinical and histopathological scores, kinetics of $CD4^+$, $CD8^+$ and NK cells in blood as well as in spleen by FACS and antibody levels in serum by RFFIT, demonstration of ß-catenin by IHC, detection of virus in brain tissue by dFAT and by conventional and Real Time PCR and expression of TNF-a, CXCL10, IFN-ß, TLR3 and TLR4 cytokines by Real Time PCR were studied at 2$^{nd}$ ,7$^{th}$ ,9$^{th}$ and 1$^{th}$ day after intra-cerebral inoculation. The animals which were in advanced stage of clinical signs on the day of LPS inoculation died in groups GI, GL and GT on the next day itself, without showing any response to LPS and anti-rabies antibody. Thereafter, significant changes were observed in development of clinical signs and mortality pattern. The mortality in GL and GT groups of mice was less compared to GI group and clinical stage was prolonged in these groups. The survival in GT and GL groups was 46% (6/13) and 38% (5/13), respectively, as compared to GI group. The survivability and prolonged clinical course before death in GL and GT groups could be due to influx of peripherally produced immune molecules and cells into brain along with injected anti-rabies antibody. The LPS might have contributed to virus clearance through induced expression of IFN-ß and TNF-a as seen presently in GL and GT groups over GI group. The ratio of $CD4^+$ to $CD8^+$ cells almost remained constant throughout the experiment in GC group, while in CVS-18 inoculated group; the ratio was significantly decreased on 7$^{th}$ DPI and normalized by 24$^{th}$ DPI. The NK cells revealed significant higher value on 7$^{th}$ day followed by decreased in population on 20$^{th}$ DPI. This indicated that peripheral cell mediated immunity had generated after intramuscular injection of CVS-18. Detection of anti-rabies antibodies in serum indicated development of humoral immunity in periphery, and might have played role in clearance of virus after in fluxing thorough modulated blood-brain barrier (BBB). The neuronal degeneration was less in GL and GT groups as compared to GI group that might be due to decrease viral load. In the present study, there has been higher expression of TNF-a, IFN-ß, TLR3 and TLR4 in CVS group than PBS group, before LPS inoculation. After LPS inoculation on 6$^{th}$ DPI (intra-cerebrally), GL and GT groups exhibited increased expression of TNF-a, IFN-ß and decreased expression of CXCL10 compared with GI group. The variability in expression was observed both in TLR-3 and TLR-4 cytokines. The survivability and milder pathology in mice in GL and GT groups as compared to GI group could be due to modulation of BBB permeability by LPS and virus clearance by both peripherally generated and external source of anti-rabies antibody and LPS associated cytokines.

From the above studies, it was concluded that there is a need to study fetal brain lesions involving more number of aborted cases together with correlation of lesions with etiology using immunohistochemical staining and other molecular based techniques. Further, in rabies pathogenesis, the LPS and RIG have found to show some role in viral clearance to the extent that caused prolongation of life of infected mouse model. However, to completely overcome the viral replication in neurons and other cells, there is necessity to use siRNA approach along with above treatments for possible prolongation of life of the host by compromising some degree of neurological deficit.

# 8 लघु सारांश

गाय, एवं भैसों के भ्रूणों में प्राकृतिक कारणों से मस्तिष्क में होने वाली विकृतियों को अध्ययन करने के लिए 21 भ्रूणों (19 गायों, 2 भैसों) को संघठित डेयरी फार्म से एकत्र किया गया। अध्ययन में कोई स्थूल विकृति नहीं पाई गई। हालांकि सूक्ष्मदर्शी परीक्षण में संवहनी रक्त भराव (21/21), एवं रक्तस्त्राव (9/21) पाये गये। कुछ मामलों में केन्द्रित ग्लायोसिस, मामूली पेरीवैस्कुलर कफिंग एवं क्षतिग्रस्त न्यूट्रॉन पाये गये। कुछ में विकासीय परिवर्तन जैसे पार्श्व वेन्टिकल्स के नीचे और हिप्पोकैम्पस में ग्लाया कोशिकाओं की उपस्थिति, बाहरी सेरीबेलम परत में दानेदार कोशिकाओं की उपस्थिति, सेरीबेलम की आंतरिक परत में परिकिंजे कोशिकाओं की उपस्थिति और सघन न्यूरॉन पाये गये। 14 मस्तिष्क नमूने परीक्षण में बीवीडी का कोई मामला नहीं पाया गया। सहज विकृतियां जैसे (संवहनी रक्त भराव एवं रक्त स्राव) मस्तिष्क के विकास की मौलिक कोशिकाओं (7/21) की तुलना में अधिक थे। इसके अलावा अन्य अंगों में गैर विशिष्ट प्रकार के कुछ मामले (जैसे रक्त भराव, रक्तस्राव) के अतिरिक्त कुछ मामलों में लसिका कोशिकाओं की उपस्थिति पाई गई। रेबीज रोग जनन में लाइपोपॉलीसैकेराइड और रेबीज इम्यूनोग्लोब्यूलिन के प्रभाव को अध्ययन करने के लिए 150 चूहों पर प्रयोग किया गया। चूहों के आधे समूह (75/150) को सीवीएस–18.0.03 मिली ली. मांसपेशी में दिया गया। संक्रमित चूहों को चौबीस दिनों तक निगरानी में रखा गया और इस सममान्तराल में रेबीज रोग का कोई लक्षणन नहीं दिखा। इसलिए सीवीएस–18 की दूसरी खुराक मस्तिष्क में दी गई। छठें दिन कुछ चूहों ने रेबीज बिमारी के लक्षण दिखाने शुरू किये। इसीदिन चूहों को तीन समूहों में विभाजित किया गया। एक समूह को एलपीएस (8 मिली.ग्राम/चूहा), दूसरे समूह को एलपीएस और एंटीरेबीज एंटीबॉडी दी गई और क्रमशः जीएल और जीटी समूह का नाम दिया गया। बचे हुए चूहों (25/75) को नियन्त्रण समूह (जीआई) के रूप में रखा गया। इसी समय असंक्रमित चूहों को तीन नियन्त्रण समूह सीएल, सीटी और जीसी के रूप में रखा गया। सभी समूहों में तेरह चूहें सरवाइवेवीलटी के लिए रखे गये। मस्तिष्क में सक्रमण देने के बाद सीडी$4^+$, सीडी$8^+$ और एन के कोशिकाओं का अध्ययन फैक्स विधि से, एंटी रेबीज, एंटीबाडी का स्तर आरएफएफआईटी से, बीटा–कैटेनिन का प्रदर्शन इम्यूनो हिस्टोकेमेस्ट्री से, विषाणु का परीक्षण डीफैट, पीसीआर और रीयल टाइम पीसीआर से, साइटोकॉइन्स टीएनएफ–अल्फा, सीएक्ससीएल–10, आईएफएन–बीटा, टीएलआर–3 और टीएलआर–4 की अभिव्यक्ति रीयल टाइम पीसीआर से अध्ययन की गई। एलपीएस और एंटीरेबीज एंटीबॉडी (बाह्य स्त्रोत) का प्रभाव बीमारी के अन्तिम चरण के चूहों में नहीं पाया गया। इसके पश्चात, जीएल और जीटी समूहों में जीआई की तुलना में मृत्युदर और बीमारी की अवधि कम पाई गई। जीटी और जीएल समूहों में सरवाइवेबीलिटी क्रमशः 46(6/13) और 38 प्रतिशत (5/13) पाई गई। जीएल और जीटी समूहों में जीआई की अपेक्षा विषाणुओं का निष्कासन अधिक देखा गया जोकि एटी–रेबीज एंटीबॉडी (बाहयस्रोत) और एलपीएस उत्प्रेरित साइटोकॉइन्स एवं रक्त मस्तिष्क बाधा के खुलने के कारण संभव हुआ। सीडी$4^+$ और सीडी$8^+$ कोशिकाओं का अनुपात पूरे प्रयोग के दौरान लगभग समान रहा। मांसपेशी के द्वारा संक्रमित समूह में यह अनुपात सातवें दिन कम पाया गया और पुनः 24वें दिन लगभग सामान्य हो गया। इससे परिधीय रोग क्षमता के उत्पन्न का संकेत मिला। सीरम में रेबीज प्रतिरोधी एंटीबॉडी की उपस्थिति हयूमोरल प्रतिरोध क्षमता को दर्शाती है। वर्तमान अध्ययन में टीएनएफ–अल्फा, आईएफएन–बीटा, टीएलआर–3 और टीएलआर–4 की उच्च अभिव्यक्ति संक्रमित समूह में नियन्त्रण समूह की तुलना में लाइपोपॉलिसैकेराइड के इंजेक्शन से पहले ज्यादा पाई गई। लाइपोपॉलीसैकेराइड के इंजेक्शन के बाद जीएल और जीआई समूहों में जीआई समूह की तुलना में टीएनएफ–अल्फा और आईएफएन–बीटा की अभिव्यक्ति बढ़ी पाई गई जबकि सीएक्ससीएल–10 की अभिव्यक्ति कम थी। टीएलआर–3 और टीएलआर–4 की अभिव्यक्ति में परिवर्तनशीलता पाई गई। जीएल और जीटी समूहों के चूहों में कम विकृति संभवता रक्त मस्तिष्क बाधा के खुलने, परिधिय विकसित प्रतिरोध क्षमता और एंटीरेबीज एंटीबाडी (बाहयस्रोत्र) के मस्तिष्क में पहुचने की वजह से हुई। उपरोक्त अध्ययन से यह निष्कर्ष निकला कि गाय एवं भैंस के भ्रूणों से संबंधित अध्ययन में अधिक संख्या में नमूनों की जरूरत है और इनसे जुड़ी विकृति के कारणों को पता लगाने के लिए इम्यनोहिस्टोकेमस्ट्री और अन्य आणविक आधारित तकनीकों को प्रयोग करने की आवश्यकता है। इसके अतिरिक्त संक्रमित चूहों की सरवाइवेबीलिटी में एलपीएस और एंटीरेबीज एंटीबॉडी की साकारत्मक भूमिका पाई गई। हालांकि पूरी तरह से विषाणु के रोग कारक क्षमता को रोकने के लिए उपरोक्त उपचार के साथ एसआई–आरएनए आधारित तनकीक के प्रयोग करने की आवश्यकता है।

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# *Appendix*

**Ethidium bromide stock solution (10 mg/ml) (10 ml)**

| | |
|---|---|
| Ethidium bromide | 100 mg |
| DD $H_2O$ | |
| To make | 10 ml |

Store in dark, cool place
pH adjust to 7.2 with 4 M NaOH
Autoclave at 15 PSI x 15 minutes
Store at 4°C

**Phosphate Bufferred Saline (PBS) (pH 7.2) (1 litre)**

| | |
|---|---|
| Sodium chloride (NaCl) | 8.0gm |
| Disodium hydrogen phosphate (Na¬2HPO4) | 1.16gm |
| Potassium chloride (KCl) | 0.2gm |
| Potassium dihydrogen phosphate (KH2PO4) | 0.2gm |
| Add double distilled water to make | 1000ml. |

Check pH and adjust to 7.2Sterilized by autoclaving for 15 minutes at 15 psi
Store at room temperature.

**0.1% DEPC treated water**

| | |
|---|---|
| DEPC | 1 ml |
| DD H2O | |
| To make 1 litre | |

Incubate overnight at room temperature
Autoclave at 15 psi x 15 minutes
Store at room temperature

**Ethidium bromide staining solution for gels (1 litre)**

| | |
|---|---|
| Ethidium bromide | 500 mg |
| DD H2O | 100 ml |

**6 X Gel loading buffer**

| | |
|---|---|
| Bromophenol blue | 0.25% |
| Xylene cyanol FF | 0.25% |
| Sucrose in H2O | 40% (w/v) |

**5X TBE (pH 8.0) ( 1 litre)**

| | |
|---|---|
| Tris base | 54 g |
| Boric acid | 27.5 g |
| 0.5 M EDTA (pH 8.0) | 20 ml |
| DD $H_2O$ to make 1 litre | |

# *Vitae*

| | | |
|---|---|---|
| Name | : | **Dr. Awadh Narayan Kushwaha** |
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| B.V.Sc. & A.H. | S.V.V.U. Tirupati | College of Veterinary science, Rajendranagar, Hyderabad. | 7.85 | 2009 | 1st |

Fellowship awarded : IVRI Fellowship

Member of professional societies:

1. Registered in Veterinary Council of India
2. Registered in Uttar Pradesh Veterinary Council
3. Life member of Indian Association of Veterinary Pathologist