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# COMPARISON OF APOPTOSIS IN STREET RABIES VIRUS ISOLATES AND CHALLENGE VIRUS STANDARD BY FLOWCYTOMETRY USING TUNEL STAINING METHOD

**H. Prabhavathy**
Assistant Professor, Madras Veterinary College, TANUVAS, Chennai-600 007

**Abstract:** Rabies is an avertable viral disease caused by the rabid animal to the warm blooded animals especially human. The animals which are mainly reported as causes of rabies are; dogs, raccoons, skunks, bats, and foxes. A study was undertaken to compare the ability of Indian street rabies virus isolate and challenge virus Standard to induce apoptosis in murine neuroblastoma cell line. Thirty Indian rabies virus isolates from infected street dogs were tested by FAT used to infect Murine Neuroblastoma cell line (MNA) along with Challenge Virus Standard (CVS). Expression of Caspase-1, Bad and Glycoprotein genes in MNA cells in comparison with CVS. TUNEL staining method used to study the apoptotic index of Street rabies virus and CVS was measured by using Flow cytometer.
**Keywords:** Rabies, Glycoprotein, Apoptosis, Apoptotic gene, Tunel staining method, Flow cytometry

## INTRODUCTION

Rabies is most feared neurotropic diseases in human that affects the central nervous system and is almost invariably fatal encephalomyelitis in human and animals (Dietzschold et al., 1996). Rabies is enzootic and is a serious economic problem in India. Rabvies virus is an enveloped bullet shaped virus that belongs to the genus Lyssavirus in the family Rhabdoviridae (Pringle 1991). The genome of the rabies virus is a non segmented negative sense RNA that codes for five proteins namely the nucleoprotein, Phosphoprotein, Matrix protein, Glycoprotein and the virion – associate transcriptase protein (Tordo etal., 1988). Neuroinvasiveness is the major defining characteristics of a classical rabies virus infection. Street rabies virus strain are highly neuroinvasive (Schnell et al., 2005). Viral Glycoprotein is an important contributor to rabies virus pathogenicity (Dietzschold et al., 1985).

Apoptosis is active physiological process of cellular self destruction which plays an important role in producing cell death in rabies virus infection of cultured cells. Ability of a rabies virus to induce apoptosis in primary neuronal cultures correlated inversely with its pathogenicity (Morimoto et al., 1999).

Present study was carried out to compare the Apoptosis induced by Indian street virus isolates from infected street dogs and Challenge virus standard.

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## MATERIALS AND METHODS

Thirty Indian street rabies suspected samples and impression smears from hippocampus and different regions of brain were collected in sterile containers at Department of Veterinary pathology, Madras Veterinary college, Chennai confirmed positive by FAT, were stored at -80C for infecting MNA cells. The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. 10% suspension of rabies positive brain samples was prepared in DMEM with 2% FBS for infecting MNA cells in 24 well plates for 24 and 48 hrs post-infection.RNA isolation from 24 and 48 hrs post-infection using Trizol method. Complementary DNA was synthesized with the total RNA isolated from infected MNA cells using Superscript III first strand synthesis system for RT-PCR. RT-PCR was performed with the Glycoprotein gene and single step RT-PCR was performed with primer CASPASe-1 and BAD gene. The cDNA synthesized was used to amplify the glycoprotein gene. Single step RT-PCR for the amplification of Caspase-1 and Bad gene.

Rabies virus infected cells (1-2x106) were resuspended in 0.5Ml of 10mM sodium phosphate, 150mM sodium chloride. The cell suspension was added to 5mL of 1% paraformaldehyde in PBS and placed in ice. The cells were centrifuged and washed the cells in 5mL of PBS repeatedly.

Positive, negative control cells and rabies virus infected cells samples were resuspended and 1 ml aliquots of control suspension were removed and placed in 12 x 75 mm flow cytometry centrifuge tubes. The cells were resuspended in wash buffer, centrifuged and cell pellet was resuspended in 50uL of the DNA labellling solution. The cells were incubated in the DNA labeling solution for 60 minutes and add rinse buffer. Supernatant was removed and the cells resuspended in 1ml of rinse buffer. The cell pellet was resuspended in 0.1 ml of antibody solution. The cells were incubated with Fluroscein – PRB-1 antibody solution.

10% suspension of positive brain samples was prepared in Dulbecco's medium with 2% FBS. The positive samples were used to infect MNA cells and used for RNA extraction. cDNA was synthesized with total RNA isolated using Superscript III first strand synthesis system for RT-PCR. cDNA was used to amplify the Glycoprotein gene, amplification of BAD gene and Caspase-1 gene. Cell fixation by Apo-Br-DU procedure. The cells in propidium iodide/RNase solution were analyzed by flow cytometry within 3 hours of staining.

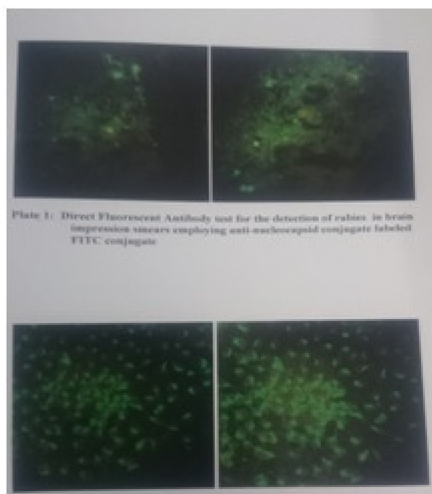
## RESULTS AND DISCUSSION

Twenty samples were found positive for rabies by FAT using rabies anti-nucleocapsid antibody conjugate. Positive samples revealed areas of brilliant green fluorescence. Positive

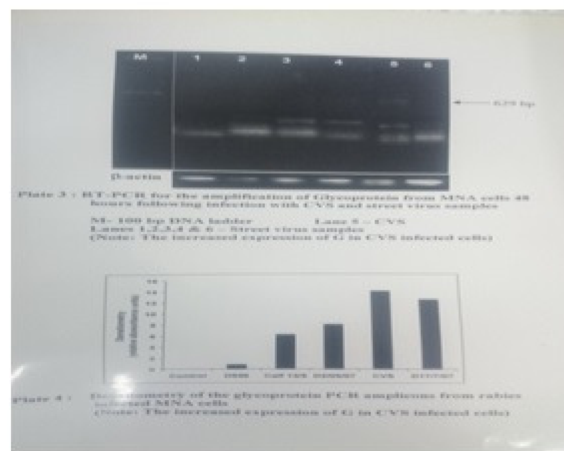
samples were used for infecting murine neuroblastoma cell line. Three passages were performed in the MNA cells and direct FAT was performed to detect the infectivity in the cells. Four street rabies virus were and CVS used for the study to determine their ability to induce apoptosis. Fluorescent microscopic examination of infected cover slip cultures of the samples revealed multiple fluorescent foci, 48 hours post infection.

RNA isolation and RT-PCR for amplification of the Glycoprotein gene and Single step RT-PCR for CASPASE-1 and BAD gene. The densitometry values for glycoprotein gene lie between 600bp to 700bp of molecular weight marker. The expression levels of Glycoprotein were higher in CVS infected cells than street virus. Single step RT-PCR is performed for amplification of CASPASE-1 and BAD gene. The densitometry values for Caspase-1 gene lie between 400 to 500 bp and For BAD gene lie between 400 bp to 500bp. The expression level of Caspase and BAD gene is highest in Street rabies virus and lowest in CVS.

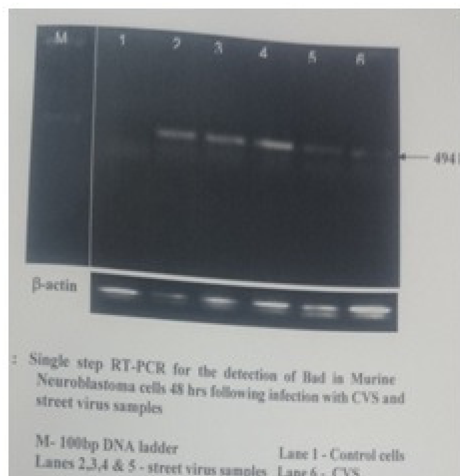
The street virus and CVS induced apoptosis in MNA cells were assessed using APO-BRDU kit. The labeled cells were analysed by flowcytometry. CVS infected MNA cells exhibited lower levels of apoptotic cells when compared to street rabies virus infected MNA cells.



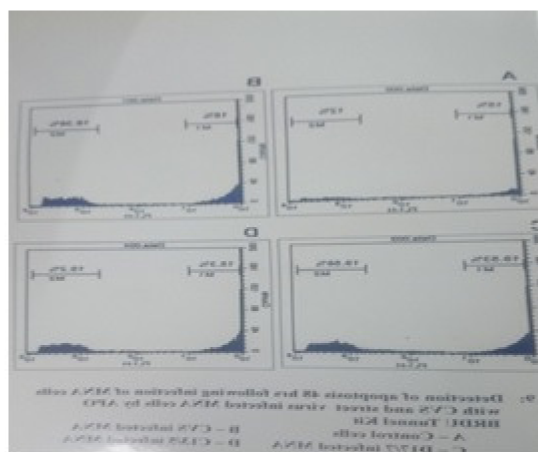
**Direct Fluorecent Antibody Test**



**RT-PCR amplification of Apoptotic genes**




**Single step-RT-PCR**



**APOBRDU Tunnel Analysis**

Apoptosis or programmed cell death is a process were by individual cells undergo systemic cell destruction in response to wide variety of stimuli (Choi and Benveniste, 2004). Rabies is a negative sense RNA virus has been shown to induce apoptosis (Reid and Jackson 2001). In natural cases of rabies virus infection, neuropathological changes are not prominent (Iwasaki and Tobita, 2002). Apoptosis is induced by rabies virus in cell culture. Expresssion levels of glycoprotein and proapoptotic gene were assessed at 48 hrs post infection (Ubol et al., 2005). In CVS strain lowest level of proapoptotic gene expression exhibited highest viral glycoprotein gene expression. In Street rabies virus highest level of proapoptotic gene expression and lowest level of glycoprotein gene. The pathogenicity of different rabies isolates dependent on the glycoprotein. The expression level of glycoprotein have been negatively correlated with apoptosis. Viral glycoprotein gene expression inhibits the proapoptotic genes (Alcami and Koszinowski, 2000) so virus infected cells are not killed. This enables the virus to persisat in thed neurons of the host. All street rabies virus isolates had lower levels of glycoprotein gene expression with concomitant increased proapoptotic gene expression. This would reflected in the varying degrees of apoptosis inhibition in the host.

DNA cleavage in apoptotic cells was detected by TUNEL assay (Theerasurakarn and Ubol, 1998). CVS and street rabies virus infection in MNA cells induced similar proportion of apoptotic cells. It was not possible to quantify the cells expressing the viral glycoprotein using flow cytometry due to non availability of glycoprotein specific monoclonal antibody. Hence apparently it appears that the increased or decreased expression level of proapoptotic

gene was not reflected in the actual apoptotic cell population. Viral gene expression and apoptotic gene expression are related.

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