

## Original Article

# Localization of West Nile Virus in monkey brain: double staining antigens immunohistochemically of neurons, neuroglia cells and West Nile Virus

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**Abstract:** West Nile virus (WNV) can cause encephalitis or meningitis that affects brain tissue, which can also lead to permanent neurological damage that can be fatal. To our knowledge, no consistent double immunohistochemical staining of neurons, neuroglia cells, and WNV has yet been reported. To establish a method for performing double-label immunohistochemical detection of neurons, neuroglia cells and WNV, examining the pathological characteristics of WNV-infected neurons, neuroglia cells, and investigating distribution of WNV in monkey brain, paraffin-embedded monkey brain tissue were retrospectively studied by immunohistochemical staining of neurons, neuroglia cells and WNV. Antibodies against neuron-specific enolase (NSE), glial fibrillary acidic protein (GFAP) and WNV were used to develop the method of double-label immunohistochemical staining, which allowed independent assessment of neuron status and WNV distribution. A range of immunohistochemical WNV infection in monkey brain was observed in both neurons and neuroglia cells in terms of the thickness of lesion staining, and the WNV staining was slightly higher in neuroglia cells than in neurons. All these findings suggest that WNV invasion in the brain plays a crucial role in neurological damage by inducing central nervous system (CNS) cell dysfunction or cell death directly.

**Key words:** West Nile virus (WNV), encephalitis, meningitis, double immunohistochemical staining, neurons, neuroglia

## Introduction

West Nile virus (WNV) is a single-stranded RNA arbovirus of the Flavivirus family with the potential to cause meningoencephalitis [1]. Humans and other mammals are incidental hosts with transmission through bites of infected mosquitoes. WNV is a neurotropic virus that causes encephalitis in humans and a variety of animals [2]. It also can cause a spectrum of illness, which includes WN fever, chorioretinitis, acute flaccid paralysis syndrome and fatal meningoencephalitis. The clinical manifestation of WNV infection is well defined, but the mechanism of pathogenesis of WNV infection has not been elucidated completely. Previous studies have proved that WNV could infect and induce cytopathic effect (CPE) in

various cell cultures of human, primate, rodent and insect origin. In humans, as well as in experimental animal studies, a lethal infection of WNV, can trigger both necrosis and apoptosis in WNV-infected cells and brain tissue [3]. All these data suggest WNV can invade neurons and directly cause central nervous system (CNS) damage.

Recent investigations have revealed much information about the development and structure of CNS, and some of the CNS elements and markers can be useful in diagnostic procedures [4]. The cytoplasm of neurons and neuroglia cells contains many enzymes and organelles which are useful in the identification of these cells in routinely fixed and embedded biopsy material. Neuron-specific

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enolase (NSE) and glial fibrillary acidic protein (GFAP) were adopted for identification of neurons and neuroglia cells, respectively in this study.

Immunohistochemical staining augments the sensitivity and specificity of morphological studies. However, in this study, we established a double immunohistochemical staining method and more definitively analyzed CNS damage with WNV infection.

### Materials and methods

#### *Tissue sections*

Formalin-fixed, paraffin-embedded monkey brain and liver tissues with WNV infection were preserved in our lab. Normal, healthy liver sections were used as negative controls.

#### *Antibodies and developing solutions*

Primary antibodies including mouse monoclonal antibody against NSE (BBS/NC/VI-H14, Signet), GFAP (6F2, Signet) were used, and WNV-infected mouse immune ascetic fluid were provided by Dr. Robert Tesh of University of Texas Medical Branch, Galveston, TX. Alkaline phosphatase-labeled goat antibody to mouse IgG (H+L) (Kpl) was used as a secondary antibody. Immunohistochemistry kit AEC HC-3119-05 (InnoGenex, CA) was prepared for WNV staining. AEC substrate system (DAKO K0696) and HistoMark@BLUE substrate system (Kpl) were used for visualization of alkaline phosphatase-labeled reagents or HRP-labeled reagents. Target retrieval solution (DAKO S1699) was also used for retrieving antigen in immunohistochemistry.

#### *Histological and immunohistochemical staining*

Double immunohistochemical staining was performed as follows: Formalin-fixed, paraffin-embedded tissue sections were cut at 4  $\mu$ M, heated at 58°C for 1 hour, deparaffinized in 2 stations of xylene for 5 minutes each, and rehydrated in 2 stations of absolute alcohol, 95% alcohol, 70% alcohol for 5 minutes each. To decrease the endogenous peroxidase inherently present in tissue, the slides were put into a hydrogen peroxide station (3% H<sub>2</sub>O<sub>2</sub>) for 30 minutes at room temperature (RT) and then into deionized water. Antigen retrieval was carried out for 30 minutes with 10% pre-

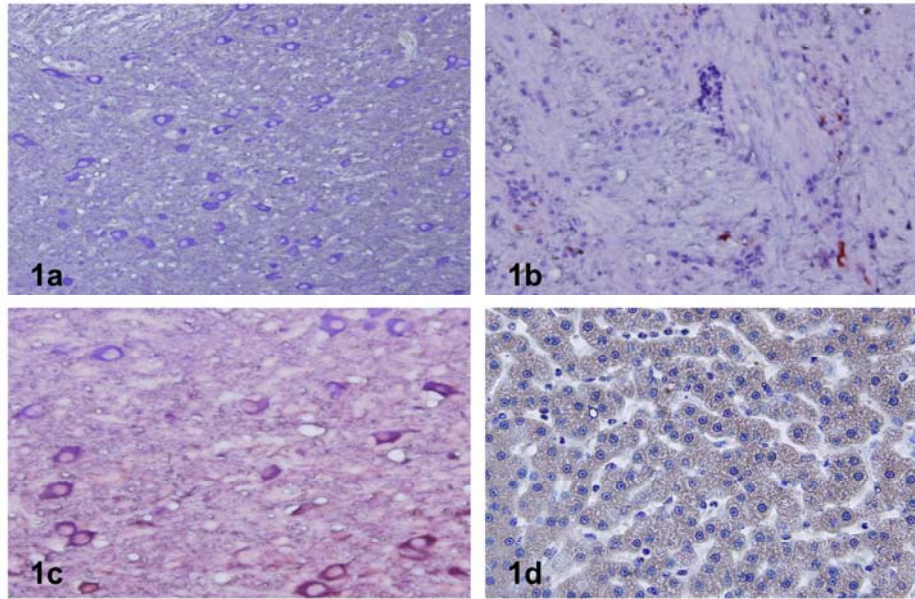
warmed target retrieval solution in 90°C water bath. After cooling down for another 20 minutes at RT, slides were blocked with 10% FBS (Gibco) at 4°C overnight. Following procedures were performed for optimal staining conditions: first staining, visualization with AP developing solution; blocking with 10% FBS at 4°C overnight; second staining, visualization with HRP developing solution. The detailed protocol for this staining is shown below.

#### *Double immunohistochemical staining procedure*

Primary staining protocol: (NSE and GFAP): After blocking, slide is rinsed 5 minutes in reagent quality water; Soak in 0.1 M Tris-HCl for 5 minutes (NOTE: Inorganic phosphate inhibits alkaline phosphatase activity. Avoid use of PBS or any solution containing phosphates.); Treat sample with primary antibody diluted in Tris-HCl for 1 hour; Wash sample with Tris-HCl (0.1% Tween) for 5 minutes two times; Incubate sample with AP-labeled secondary antibody diluted in Tris-HCl for 40 minutes at RT; Wash with Tris-HCl, and prepare substrate solution during this step; Discard excess buffer and cover sample with substrate solution; Incubate 10 minutes at RT in the dark; Rinse slide 5 minutes in reagent quality water; Air dry, observe by microscopy and take photo of image; Block with 10% FBS at 4°C overnight.

Secondary staining protocol (WNV): Prepare Labeled Antibody (150  $\mu$ l per slide), add primary antibody (1:100) to Dako S0809 Ab Diluent; Label by adding Biotin Labeling Reagent (1/10 volume) to tube; Incubate at RT for 30 minutes; Add Mouse Block to primary Ab-Biotin labeling tube ( Ab Diluent, Biotin Labeling Reagent and Mouse Blocking Reagent should be in the proportion of 8:1:1); Incubate 3 minutes at RT. (must be used within 15 minutes.); Add Ab-Biotin-Mouse block mix to slide; Incubate 2 hours at RT; Wash sample with Tris-HCl (0.1% Tween) for 5 minutes twice; Add Horseradish Peroxidase-Streptavidin Conjugate to cover sample; Incubate 30 minutes at RT; Wash with Tris-HCl for 5 minutes twice; Cover sample with AEC substrate; Incubate 10 minutes at RT in the dark; Rinse slide 10 minutes in reagent quality water; Mounting: use Supermount on sample and cover slide with glass; Read slide by microscopy and take photo of image; Store slide in folder in dark.

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**Figure 1.** Immunohistochemical detection of NSE and WNV antigen in brain of WNV-inoculated monkey. The photomicrographs show strong cytoplasmic staining of neurons and WNV antigen, 1a: NSE staining (blue color), 1b: WNV antigen staining (red color, hematoxylin counter stain), 1c: Double staining of NSE and WNV antigen, 1d: Double staining of NSE and WNV antigen in monkey liver (hematoxylin counter stain), negative control. Magnification: 20 X.

### *Immunohistochemical analysis*

Staining was evaluated on the basis of sensitivity and specificity of the target proteins by comparing with the negative control. The optimal staining conditions were selected to establish a double staining method in mammal brain tissues. Microscopic evaluation was adopted with visualization of the entire lesion. The location staining among monkey brain cells were compared among several slides.

### **Results**

#### *Double immunohistochemical staining*

Both NSE and GFAP stained intensively ganglion cell cytoplasm with very good technical quality, while the nucleus remained negative. Double immunohistochemical staining contributed to the identification of neurons and neuroglial cells in monkey brain slides. In combination with WNV staining, we can disclose the distribution of WNV in neurons and neuroglial

cells. The detailed results are shown as follows.

#### *NSE and WNV*

The locations of positive staining for NSE, WNV were summarized in **Figure 1**, showing different staining patterns including NSE (**Figure 1a**), WNV (**Figure 1b**), NSE and WNV (**Figure 1c**). Monkey liver tissue staining was treated as negative control to evaluate tissue specificity of the primary antibody against NSE and WNV, respectively (**Figure 1d**).

#### *GFAP and WNV*

The locations of positive staining for GFAP, WNV were also summarized in **Figure 2** showing different staining patterns including GFAP (**Figure 2a**), WNV (**Figure 2b**), GFAP and WNV (**Figure 2c**). Monkey liver tissue staining was treated as negative control to evaluate tissue specificity of the primary antibody against GFAP and WNV, respectively (**Figure 2d**).

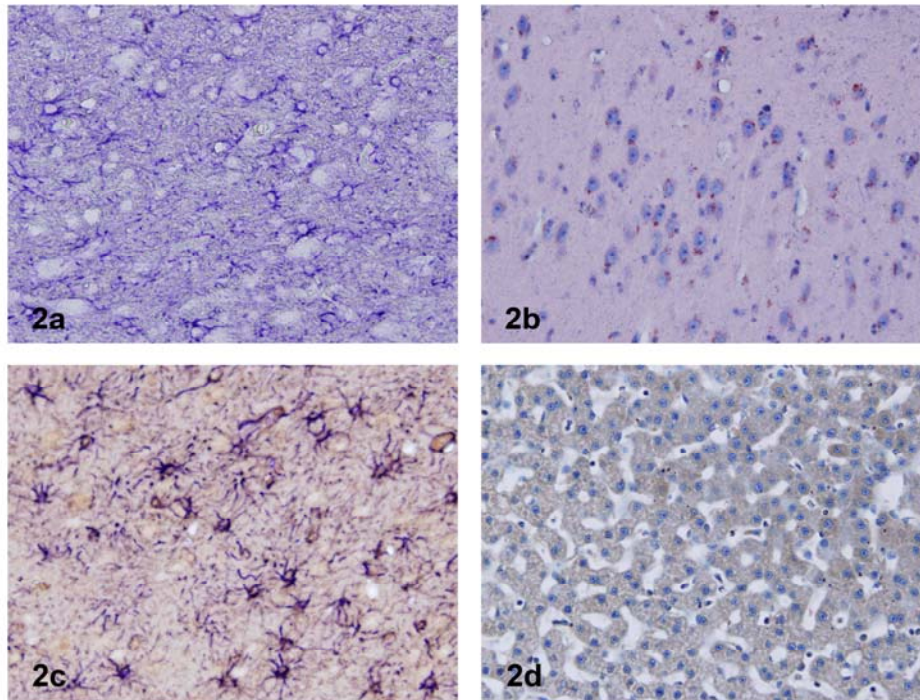
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### Discussion

In order to adequately evaluate WNV-infected CNS, a variety of histological techniques are needed. The double staining method for monkey brain with WNV infection has been established in this study.

NSE is a glycolytic enzyme found in neurons and neuroendocrine cells and is currently used to identify neurons [5-7], and GFAP is a member of the intermediate filament family that provides support and strength to cells. Several

of astrocytes, and in the interactions of astrocytes with other cells, which are required for the formation and maintenance of the insulating layer (myelin) that covers nerve cells. Additionally, GFAP may assist in maintaining the protective barrier that allows only certain substances to transport between blood vessels and the brain (blood-brain barrier). Since WNV can cross the blood-brain barrier to reach the brain, and interfere with normal central nervous system functioning, the detection of GFAP can contribute to disclose the distribution of WNV in glial cells. In this study, we



**Figure 2.** Double staining of GFAP and WNV antigen in brains of WNV-inoculated monkey. The photomicrographs demonstrate strong cytoplasmic staining of neuroglial cells and WNV antigen, 2a: GFAP staining (blue color); 2b: WNV antigen staining (red color, hematoxylin counter stain); 2c: Double staining of GFAP and WNV antigen; 2d: Double staining of GFAP and WNV antigen in monkey liver (hematoxylin counter stain), negative control. Magnification: 20 X.

molecules of GFAP protein bind together to form the main intermediate filament found in specialized brain cells called astrocytes. Astrocytes are star-shaped cells that support the functions of nerve cells in the brain and spinal cord (CNS). If the CNS is injured through trauma or disease, astrocytes react by rapidly producing more GFAP [8, 9]. Although its function is not fully understood, GFAP may be involved in controlling the shape and movement

adopted NSE and GFAP as indications of WNV-infected neurons and neuroglial cells in monkey brains.

The WNV is a mosquito-borne flavivirus of the Japanese encephalitis (JE) serocomplex group that causes lethal encephalitis in humans and horses. Several mechanisms have been proposed as its pathogenesis: (1) immune-mediated mechanisms include antibody-mediated



injury to CNS cells infected with virus [10-12, 22]. (2) mosquito-borne viruses cause encephalitis by inducing neuronal apoptosis [13, 14]. (3) WNV invasion into brain plays a crucial role in neurological damage and induces CNS cells dysfunction or death directly [3, 15, 22]. Previous studies showed that hamsters infected with WNV developed a brief viremia, lasting 5 to 6 days, and many animals had clinical signs of acute CNS injury (somnolence, muscle weakness, paralysis, tremors, and loss of balance) with a substantial number of deaths occurring on days 7 to 14 after inoculation [3, 16-18, 22]. Based on these experiments, we proposed that CNS cells dysfunction and death are caused directly by WNV infection, which is the main mechanism of neuronal damage. But the exact mechanism by which WNV initiates the cell death pathway is not yet clear. In this study, we observed that WNV localized in both neurons and glial cells. In addition, the quantities of WNV distributed in glial cells were more than in neurons, which is not consistent with previous studies in human patients with WNV infection [19-21]. In WNV-infected human studies, no WNV was identified in glial cells, which derived from an immunocompromised patient, and a kidney-transplanted patient with WNV infection. However, our studies were performed with monkey brain, which is experimentally inoculated with WNV.

All together, these observations suggest that WNV migrated through the blood-brain barrier and infected neurons and glial cells, and the inflammatory infiltration (perivascular inflammation and microgliosis) was a secondary response to neuronal damage, which was consistent with previous findings [3, 22, 23]. Therefore, further studies of double staining will be necessary to elucidate the precise pathogenic mechanism of WNV infection.

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