International Journal of Sciences: Basic and Applied Research (IJSBAR)

ISSN 2307-4531
(Print & Online)

http://gssrr.org/index.php?journal=JournalOfBasicAndApplied

Dengue Virus Type 3 (DENV-3) Distribution in Tissues of Pig-tailed Macaque (*Macaca nemestrina*) Post Infection Using Immunohistochemistry Technique

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Abstract

Nonhuman primates play as an indispensable animal model in biomedical research for studying a variety of human health issues, diseases and disorders, therapies, and preventive strategies. In this study, we used pig-tailed macaques (Macaca nemestrina) as an experimental animal to study dengue type-3 virus (DENV-3) infection. We evaluated DENV-3 distribution and replication sites after a primary infection in all collected tissue by immunohistochemistry to localize viral protein. Significant gross lesions were not seen in any of the examined pigtailed macaques. However, microscopic lesions were present in variable degrees of severity in multiple tissues: liver, stomach, spleen and lymph nodes as evaluated by histopathological analysis. Viral protein was demonstrated in reactive lymphoid cells in spleen, thymus, axillary lymph node, inguinal lymph node, mesenteric lymph node and submandibular lymph node. In general, evidence for the presence of viral protein in various tissues after DENV-3 infection reveals that M. nemestrina is susceptible to the infection and could serve as a good alternate model to evaluate the replication of dengue virus in tissues.

Keywords: DENV-3; *Macaca nemestrina*; immunohistochemistry.

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1. Introduction

Dengue Hemorrhagic Fever (DHF) is a disease caused by arbovirus infection (*arthropode-borne viral disease*) which becomes a problem of public health, especially in tropical and sub-tropical countries [1]. In humans, DHF is caused by the infection of Dengue virus (DENV) which taxonomically belongs to the family Flaviviridae and genus *Flavivirus* with four serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) which have been successfully isolated. The four serotypes are reported to cause similar symptoms in people [2]. The replication of the virus depends on *Aedes aegypti* as the main transmission vector [3,4]. In general, *in vivo* research related to DENV infection and its pathogenesis is still limited due to the lack of experimental animal models that can give an overall description of the disease resembling humans [5]. Most of the reported histophatological descriptions of fatal DENV infections in humans indicate that liver, spleen, and lymph node are the organs target of DENV infections [6-8]. The observed lesions were in the forms of hemorrhage and damages to the liver, spleen, lungs and kidneys, mononuclear inflammatory cell infiltration in the lungs and kidneys [7,9]. Reports of other cases mention that DENV infections can cause lesions in the tissue of kidneys, heart, and central nervous system, which are hemorrhage, edema and infiltration of inflammatory cells [11-14], hyaline membrane formation in the lungs, acute tubular necrosis of the kidneys and destruction of heart muscle fibers [15]. The development of appropriate animal models for studying DENV infection and related diseases is a major challenge because the virus does not naturally infect species other than humans. Nonhuman primate is a very important animal model in biomedical research, particularly for studying a variety of human health problems, especially related to the treatment and prevention strategies. Given the physiological and immune responses of nonhuman primate against viral infections similar to those found in humans, it is possible that research on nonhuman primate DENV infection may help to understand the infections of the virus in humans. Some species of nonhuman primates that are used as animal models in research related to DENV infection include rhesus monkey (*Macaca mulatta*) [4,16], baboon (*Papio anubis*), long-tailed macaque (*Macaca fascicularis*), vervet monkey (*Chlorocebus aethiops*) [17], chimp (*Pan troglodytes*), marmosets (*Callithrix* sp.) [18] and pig-tailed macaque (*Macaca nemestrina*) [19,20]. Although there were no clinical signs detected in DENV infected-nonhuman primates, researchers could show that these animals were susceptible to DENV infection with viremia level reported to be lower than 100 pfu mL^{-1} [21]. To understand the pathogenesis of DENV infection, the use of animal models is needed to reveal the distribution of viruses on tissues, such as the histopathological findings in human tissues infected with DENV. The genetic similarity of *M. nemestrina* to human had been reported to have consequences in their role as hosts to the same pathogens; this could mean that there is critical molecules shared by *M. nemestrina* and human being used by pathogens in their life cycle. Thus *M. nemestrina* often are the only species that can be infected by human pathogen [20]. The aim of this study is to obtain a distribution profile of DENV-3 at a cellular level by immunohistochemistry technique to *M. nemestrina* post-infection with dengue virus type 3 (DENV-3).

2. Materials and Methods

2.1. Animal

Specimens used in this research are parts of Pamungkas and his colleagues 2011 study [20] and the protocol has

been approved by Animal Care and Use Committee (ACUC) of Primate Research Center, Institut Pertanian Bogor with ACUC number P.09-08-IR. A total eight animals (5 males and 3 females *M. nemestrina* aged from four to six years) were inoculated with DENV-3 through the low-dosage subcutaneous (10^4 pfu mL^{-1}), high-dosage subcutaneous (10^7-10^8 pfu mL^{-1}), intravenous (10^7-10^8 pfu mL^{-1}), and intradermal (10^7-10^8 pfu mL^{-1}). Euthanasia and necropsy were performed on the second day after virus inoculation to all animals humanely.

2.2. Tissues collection

Tissue samples were collected from each animal, including liver, kidney, spleen, thymus, lung, pancreas, heart, adrenal gland, salivary gland, thyroid gland, mammary gland, testis, prostate gland, seminal vesicle, uterus, ovary, urinary bladder, abdominal skin, axillary lymph node, inguinal lymph node, submandibular lymph node, mesenteric lymph node, intestine, bone marrow and brain. Tissue samples were preserved in 4% paraformaldehyde upon use.

2.3. Histopathological analysis and immunohistochemistry

Sections of 5 µm thickness of tissue samples were cut and mounted for hematoxylin and eosin staining. For immunohistochemistry, sections were treated with 3% H_2O_2 diluted in methanol for 30 minutes. For antigen retrieval, tissue sections were treated with trypsin diluted in $CaCl_2$ for 1 hour at 37^{o}C. For detecting the DENV-3 protein (NS1 protein), an anti-Dengue Virus (D1-11): sc-65659 mouse monoclonal antibody (Santa Cruz Biotechnology, Inc) was used in a dilution of 1:50 at 4^{o}C overnight. After incubation, the tissue sections were washed with phosphate-buffered saline (PBS) and treated with biotinylated detection antibody (Starr Trek Universal HRP Detection System, Biocare Medical®). The sections were then treated with 3.3'-diaminobenzidine tetrachloride (Biocare Medical®) as a chromogen for 3 minutes and stained with Harris's hematoxylin. Negative controls were obtained by the omission of primary antibodies, which were substituted by PBS.

2.4. Data analysis

A descriptive analysis was performed on the results of hematoxylin eosin staining to determine the tissue morphology in general and lesions caused by DENV-3 infection. The analysis of the results of immunohistochemical staining was done semi-quantitatively to determine the location and distribution of the protein in the organs of *M. nemestrina* which infected by DENV-3. The degree of the viral protein was determined on 400 magnification, based on the number of cells containing protein per field for each organ with scores: + or low (1-5 cells with protein/field), ++ or moderate (6-10 cells with protein/field), +++ or high (more than 10 cells with protein/field).

3. Results and Discussions

The presence of DENV-3 in the tissues of *M. nemestrina* in this study was detected by immunohistochemical staining using monoclonal antibodies against DENV serotypes 1,2,3 and 4. The tissues which showed a positive reaction (+) to the immunohistochemical staining were the spleen, thymus, axillary lymph node, inguinal lymph

node, submandibular lymph node, and mesenteric lymph node, as presented in Table 1.

Table 1: The distribution of DENV-3 protein in various tissues of *M. nemestrina*

Tissues	Low-dosage subcutaneous				High-dosage subcutaneous				Intravena				Intradermal			
	1.5797(♀)		1.6395(♂)		1.5799(♂)		F9004(♀)		M9008(♂)		1.5808(♂)		1.6087(♂)		1.6136(♀)	
	PCR	IHC	PCR	IHC	PCR	IHC	PCR	IHC	PCR	IHC	PCR	IHC	PCR	IHC	PCR	IHC
Abdominal skin	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-
Mammary gland	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bone marrow	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Spleen	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-
Thymus	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
Lung	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Axillary ln	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
Inguinal ln	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Submandibular ln	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
Mesenteric ln	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
Intestine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Brain	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pancreas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Adrenal gland	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-
Kidney	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Heart	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Salivary gland	-	-	+	-	-	-	-	-	+	-	-	-	+	-	+	-
Thyroid gland	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-
Liver	-	-	-	-	+	-	-	-	+	-	+	-	-	-	-	-
Prostate gland	NA	NA	-	-	-	-	NA	NA	+	-	-	-	-	-	NA	NA
Testis	NA	NA	-	-	-	-	NA	NA	-	-	-	-	-	-	NA	NA
Seminal vesicle	NA	NA	-	-	+	-	NA	NA	-	-	-	-	-	-	NA	NA
Uterus	-	-	NA	NA	NA	NA	-	-	NA	NA	NA	NA	NA	NA	-	-
Ovary	-	-	NA	NA	NA	NA	-	-	NA	NA	NA	NA	NA	NA	-	-
Urinary bladder	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- : negative reaction
+: positive reaction
ln: lymph node
NA: not applicable
IHC: immunohistochemistry data
PCR data refer to Pamungkas and his colleagues (2011)

The DENV-3 protein observed in lymphoid cells of the tissue appeared to be randomly distributed on parenchyme (Figure 1). The Reverse Transcriptase PCR (RT-PCR) analysis toward *M. nemestrina* tissue infected by DENV-3 in previous research [20] showed that some tissues expressed DENV-3, including abdominal skin, bone marrow, spleen, thymus, pancreas, adrenal gland, kidney, heart, salivary gland, thyroid gland, liver, prostate gland, axillary lymph node, inguinal lymph node, submandibular lymph node and mesenteric lymph node.

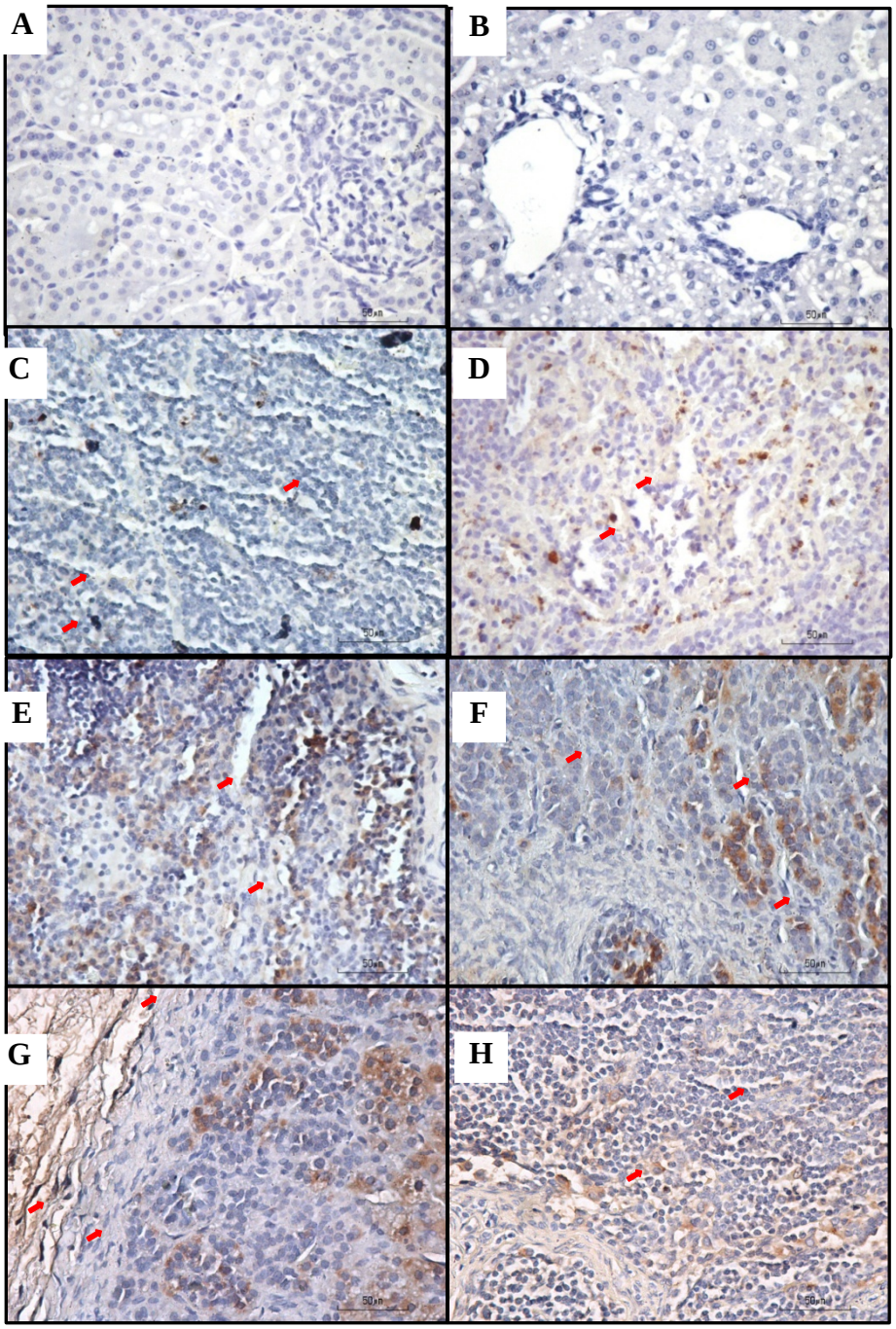

Figure 1: DENV-3 protein distribution was not detectable in liver (A) and kidneys (B). DENV-3 proteins (arrow) were detected in splenic lymphoid cells (C), thymus (D), axillary lymph node (E), inguinal lymph node (F), mesenteric lymph node (G) and submandibular lymph node (H) through random distribution in the parenchyma (immunohistochemical staining).

The tissues which showed a positive reaction with the immunohistochemical technique were lymphoid organs suggesting that the organs of the immune system could be the target of DENV-3. DENV-3 infection cycle begins with the attachment of the virus to the target cells through the interaction between viral surface proteins and receptor molecules on the surface of the cells found on dendritic cells, which are the main target of the replication of DENV-3 *in vivo* [21]. After the capture of antigen in the tissues through phagocytosis or

endocytosis, dendritic cells migrate through the blood vessels or the lymphatic vessels and circulates into the lymphoid organs, which were taken and then recognized by T lymphocytes. The tissues' ability to identify the virus depends on the distribution and the number of receptors on the cell surface [22]. This is important information that is used in the development of antiviral compounds . Virus presence in the lymphoid organs was also indicated by the presence of tissue damage as observed with hematoxylin eosin staining. Histopathological analysis (Figure 2) on all collected tissues showed the infiltration of a large number of lymphocytes and plasma cells in the multifocal area of stomach and liver; also sinusoidal histiocytosis in lymph nodes with mild to moderate severity.

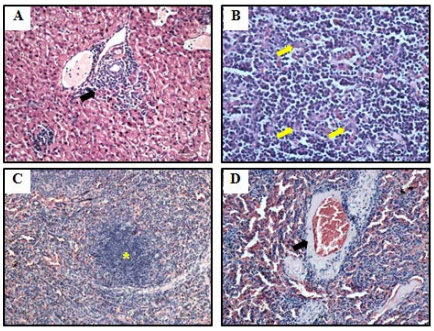

Figure 2: The infiltration of lymphocyte and plasma cells perivascular (arrows) was observed in the liver (A) and stomach. Histiocytosis (arrows) in the lymph node sinusoids (B). Depletion of lymphoid follicles (C, asterisks) and dilation of blood vessels (arrow) or congestion (D) were observed in the spleen parenchyme (hematoxylin eosin staining).

Table 2: The degree of DENV-3 protein on the tissue of *M. nemestrina*

Tissues	**Low-dosage subcutaneous**	**High-dosage subcutaneous**	**Intravena**		**Intradermal**	
	1.5797 (♀)	**1.5799** (♂)	**M9008** (♂)	**1.5808** (♂)	**1.6087** (♂)	**1.6136** (♀)
Spleen	+++	+++	+++	-	+++	-
Thymus	-	-	-	-	+	-
Axillary lymph node	-	-	-	-	+++	-
Inguinal lymph node	+++	-	-	-	-	+++
Submandibular lymph node	-	-	-	-	+++	-
Mesenteric lymph node	-	-	-	+++	-	-

+ (low) : 1-5 cells with antigen/field
++ (moderate) : 6-10 cells with antigen/field
+++ (high) : >10 cells with antigen/field
- : negative reaction

The depletion of lymphoid follicles and blood vessel dilation (congestion) with mild to moderate severity is seen in the spleen parenchyme of some animals. The number of cells infected by DENV-3 was counted semi-quantitatively according to the number of cells that expressed DENV-3 protein for each tissue by immunohistochemistry assay (Table 2). Quantification showed that DENV-3 protein was high in spleen and all lymph nodes tissues with score of (+++), while the number was low in thymus with score of (+). Although the lymphoid tissues are preferred in DENV-3 infection, the DENV-3 protein was detected at various levels in all tissue analyzed. This might be because each tissue has different morphology. In spleen tissue, DENV-3 protein was found at high level in red pulp which a major structure of spleen parenchyma. The red pulp contains macrophages which are antigen presenting cells that present DENV-3 antigen on their surface resulting to the high level of protein detected on spleen. In lymph nodes tissues, the DENV-3 protein was evenly distributed on lymphoid cells which are the major cells of lymph nodes parenchyma tissue. In thymus tissue, DENV-3 protein was localized on cells surrounding the cortex area (Figure 7). This finding is consistent with previous study of Gubler and his colleagues (1997) which mentioned that DENV protein was localized in cortex and was not observed in medulla of thymus. Thymus is a primary lymphoid organ, but the parenchyma is dominated by stromal cells which are not antigen presenting cells thus the number of DENV infected cells were low [23].

4. Conclusion

DENV-3 protein can be detected in the tissues of the spleen, thymus, axillary lymph node, inguinal lymph node, submandibular lymph node, and mesenteric lymph node by immunohistochemistry technique. In addition, cell damage caused by DENV-3 infection is most commonly found in the spleen. The distribution of DENV-3 can be found at the cellular level on *M. nemestrina* post-infection. This suggests that *M. nemestrina* is a susceptible host of DENV-3 and could serve as a good alternate model to evaluate the replication of dengue virus in tissues. Some tissues which are positive RT-PCR did not show protein expression with immunohistochemistry. This result might be because the replication stage of DENV-3 at the time of analysis was mainly at transcriptional level and the protein expressed was still very low. Further research on prolonging euthanasia time after viral inoculation in animal model will be useful to describe the viral distribution in tissue and the pathological effect.

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