








Rapid Communication
Infectious Diseases



Serological evidence of West Nile viral infection in archived swine serum samples from Peninsular Malaysia

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 OPEN ACCESS

Received: Jul 18, 2020

Revised: Jan 21, 2021

Accepted: Jan 26, 2021

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ABSTRACT

West Nile virus (WNV), a neurotropic arbovirus, has been detected in mosquitos, birds, wildlife, horses, and humans in Malaysia, but limited information is available on WNV infection in Malaysian pigs. We tested 80 archived swine serum samples for the presence of WNV antibody and West Nile (WN) viral RNA using ID Screen West Nile Competition Multi-species enzyme-linked immunosorbent assay kits and WNV-specific primers in reverse transcription polymerase chain reaction assays, respectively. A WNV seroprevalence of 62.5% (50/80) at 95% confidence interval (51.6%–72.3%) was recorded, with a significantly higher seroprevalence among young pigs (weaner and grower) and pigs from south Malaysia. One sample was positive for Japanese encephalitis virus antibodies; WN viral RNA was not detected in any of the serum samples.

Keywords: West Nile virus; pigs; Malaysia; ELISA; RT-PCR

INTRODUCTION

West Nile virus (WNV), a zoonotic single-stranded RNA virus in the genus *Flavivirus*, is an important emerging neurotropic arbovirus. It is a major cause of viral encephalitis in birds, humans, and horses and has also been reported in dog, wolf, sheep, and alpaca [1-3]. In nature, the virus is maintained in a cycle between the avian reservoir hosts and ornithophilic *Culex* mosquitos, and wild migratory birds are important in the spread of the virus [4]. Mosquitos are infected following a blood-meal on birds harboring the virus, and they, in turn, amplify and pass on the virus to other birds and also to incidental (dead-end) hosts – including non-avian species [5]. Following infection, most infected humans remain asymptomatic, but a few may develop a fatal neurological disease.

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Funding

This research was funded by Universiti Putra Malaysia (UPM) research grants UPM 700-2/1/GP-IPM/2016/9510500 and UPM 700-2/1/GP-IPS/2017/9547800.

Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Yasmin AR; Data curation: Mohammed MN; Formal analysis: Mohammed MN; Funding acquisition: Yasmin AR; Investigation: Mohammed MN; Methodology: Yasmin AR; Supervision: Yasmin AR, Noraniza MA, Ramanoon SZ, Arshad SS, Mohammed HO; Visualization: Yasmin AR; Writing - original draft: Mohammed MN; Writing - review & editing: Yasmin AR, Noraniza MA, Ramanoon SZ, Arshad SS, Faruku B, Mohammed HO.

First discovered in Africa in 1937, the virus is now distributed globally and is endemic in parts of Africa, the Middle East, Europe, Asia, and America [6]. Among members of the *Flavivirus* genus, which include important pathogens like dengue virus, Japanese encephalitis virus, St. Louis encephalitis virus, and yellow fever virus, WNV has the broadest host range, which includes more than 200 bird species and almost 30 species of animals, including horses, cattle, cats, dogs, sheep, and wildlife [7]. Except for birds, other vertebrate hosts, including humans, are dead-end hosts and are not important in the transmission of WNV due to their low virus titer during the viremic phase [5]. Pigs do not develop clinical symptoms following infection with WNV and are not involved in the virus' maintenance and transmission [8].

The detection of WNV among animals and livestock is an early sign of the virus' presence and transmission. During the historic WNV outbreak in 1999–2000 that marked its first appearance in the Western Hemisphere, deaths in animals, particularly in birds and horses, preceded human cases [3]. There is evidence of WNV infection in Malaysia, including birds, mosquitos, humans, and birds, and preliminary results from our ongoing research on WNV in animals and livestock reveal WNV infection among macaques, bats, and horses [9]. Therefore, we conducted this study to determine the presence of WNV-specific antibodies and West Nile (WN) viral RNA in swine serum samples from Peninsular Malaysia.

MATERIALS AND METHODS

Serum samples

A batch of 80 swine sera submitted to the Faculty of Veterinary Medicine, Universiti Putra Malaysia in 2016 and archived at -80°C was used in this study. The batch was made up of 40 samples from northern Peninsular Malaysia and 40 samples from southern Peninsular Malaysia. The samples were obtained from pigs of different groups, including weaners (n = 10), growers (n = 30), gilts (n = 10), and sows (n = 30). **Table 1** shows the distribution of the samples used in this study. The serum samples were stored at -80°C in a freezer (Sanyo Ultra Low, Japan), and all tests were conducted in a Class II biosafety cabinet (Esco, Singapore).

Antibody detection by WNV C-enzyme-linked immunosorbent assay (ELISA) and Japanese encephalitis virus (JEV) double antibody sandwich-ELISA

For the detection of WNV antibody, an ID Screen West Nile Competition Multi-species (IDvet, France) ELISA kit was used. The kit detects antibodies (IgG class) specific to WNV envelope protein (pr-E) by performing competitive ELISA. Because the WNV E protein cross-reacts with the antigen of the endemic JEV [10], a DAS ELISA kit – Porcine JE-IgG ELISA kit (SunRed Biotechnology, China) – was used to screen samples against the endemic cross-reacting JEV. The tests were carried out as instructed by the kit manufacturers, and the microplates were read in duplicate at 450 nm (TECAN Infinite M200, Switzerland). Both tests were validated and interpreted according to the instructions of each kit's manufacturer.

Table 1. Distribution of serum samples used to determine the prevalence of West Nile virus infection in pigs in Peninsular Malaysia

Category	Number of serum samples		Total sample size
Location	Northern Peninsular Malaysia	40	80
	Southern Peninsular Malaysia	40	
Age Group	Young	Weaner	80
		Grower	
	Adults	Gilt	
		Sow	

Molecular WNV viral RNA detection using reverse transcription polymerase chain reaction assays (RT-PCR)

Total RNA was extracted from the serum samples using TRIure (Bioline, UK). For detection of WNV RNA, the conserved region between the capsid protein (C) and pre-membrane (prM) of the WNV genome was amplified by RT-PCR (MyTaq One-step RT-PCR Kit, Bioline, USA) in a Mastercycler gradient thermal cycler (Eppendorf, Germany) with an expected amplicon size of 470 bp. A synthetic gene of the amplified region of the WNV genome was used as the positive control in this study. The primer pairs used in the reaction were CprM F 5'-ATG TCT TCA GGG TCA TTT CC-3' and CprM R 5'-CCA ATA CGT TTC GTC TTG G-3', and the protocol was carried out as previously described [9]. The PCR products were stained with GelRed Nucleic Acid Gel Stain (Biotium, USA), subjected to gel electrophoresis in 1.5% agarose with a Tris acetate-EDTA (TAE) buffer, and viewed using a UV transilluminator (Bio-Rad, USA).

Data analysis

Prevalence and the associated 95% confidence interval (CI) were determined, and Fisher's exact test was performed using GraphPad Prism version 9.0.0 (GraphPad Software, USA).

RESULTS

Seroprevalence of WNV

Out of the 80 serum samples analyzed, 50 tested positive for anti-WNV IgG antibodies, as shown in **Table 2**. Furthermore, the anti-WNV IgG-positive samples were analyzed for the presence of the cross-reactive JEV antibodies; however, only one of the serum samples tested positive. The WNV seroprevalence in this study was 62.5% (50/80) with a 95% CI of 51.6–72.3%. The distributions of seropositive pigs by location and age group are shown in **Fig. 1**. Of the 40 samples from the northern region of Peninsular Malaysia, 18 tested positives for WNV, including all five weaners and 13 growers. The only sample that tested positive for JEV antibody was a weaner from the northern region. Thirty-two samples from the southern region were positive for WNV antibody, and all eight WNV-negative samples were from sows. None of the samples from the southern region were positive for the JEV antibody. Overall, the WNV seroprevalence among pigs originating from the southern region was significantly higher ($p = 0.0024$) than that for pigs from the northern region.

The highest seroprevalence was detected among weaners (100%), followed by growers (93.3%), gilts (50%), and sows (23.3%). Furthermore, WNV seroprevalence was significantly greater ($p = 0.0001$) in young pigs (weaners and growers), with a prevalence of 95%, than in adults (gilts and sows), which had a prevalence of 30%.

Table 2. Prevalence of WNV and JEV antibodies among pigs from different locations and age groups

Location			Total tested with WNV C-ELISA	WNV C-ELISA positive	Total tested with JEV DAS ELISA	JEV DAS ELISA positive
Northern Peninsular Malaysia	Age group	Weaner	5	5	5	1
		Grower	15	13	13	0
		Gilt	5	0	0	0
		Sow	15	0	0	0
Southern Peninsular Malaysia	Age group	Weaner	5	5	5	0
		Grower	15	15	15	0
		Gilt	5	5	5	0
		Sow	15	7	7	0
Total			80	50	50	1

The table shows the total number of samples screened for WNV and JEV antibodies. Samples that tested positive for WNV were screened for JEV. WNV, West Nile virus; JEV, Japanese encephalitis virus; ELISA, enzyme-linked immunosorbent assay; DAS, double antibody sandwich.



Fig. 1. Map of Peninsular Malaysia showing the geographic distribution of WNV seropositive pigs categorized by age group. WNV, West Nile virus.

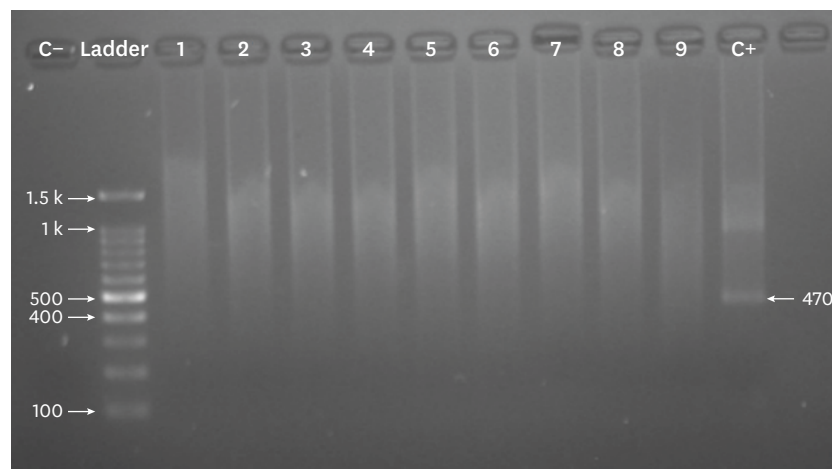


Fig. 2. Results of RT-PCR for the detection of West Nile viral RNA. C- (negative control): PCR mix with water; C+ (positive control): synthetic plasmid gene of the conserved region of the West Nile virus between the capsid protein (C) and pre-membrane (prM); lanes labeled 1 through 9 are samples from swine.

Molecular prevalence of WN viral RNA

Based on the RT-PCR results, all of the samples in this study were negative for WN viral RNA (Fig. 2).

DISCUSSION

In Malaysia, limited data are available on the prevalence of WNV among livestock, and although WNV had previously been reported in farmed swine elsewhere [8], no study of this nature

had been carried out on pigs in Malaysia. Recent reports show evidence of WNV presence in Malaysia, including in birds and wildlife, suggesting that the virus might be in circulation in the country [9,11]. The presence of WNV in livestock could give an insight into the epidemiology of the virus, which may prove useful in preventing future outbreaks of the disease.

Our study is of significance as it is the first to report evidence of WNV infection in farmed pigs in Malaysia. A previous study in Spain reported a prevalence of 2.7% (6/177) among Iberian pigs [12], while another study among feral pigs in the USA reported a prevalence of 22.5% (50/222) [13]. In a different study, a prevalence of 15.4% (43/279) was reported among intensively managed farm pigs in Serbia [8]. The sampling method in this study could have contributed to the relatively high prevalence compared to those in the aforementioned studies. More information, including mosquito and bird abundance around pig farms and exposure of the pigs to mosquitoes, is required to elucidate the reasons for the relatively high WNV seroprevalence recorded in our study.

The samples used in this study originated from states in the northern (Perlis, Kedah, Penang, and Perak) and southern (Negeri Sembilan, Melaka and Johor) regions of Peninsular Malaysia. The results indicate that WNV presence is quite widespread, with a higher prevalence in the southern states. This suggestion is in line with other studies that have reported differences in WNV prevalence due to variations in local conditions and agricultural practices; differences that could be due to variations in local climate and landscape, vector distribution, bird populations and diversity, and farm management practices [12,14,15].

We also observed that WNV seroprevalence among younger pigs was significantly higher than in adults, with young pigs having three times the seroprevalence of adult pigs. This agrees with the report by Teehee et al. [16] showing that after experimental infection of pigs with the virulent N99 WNV strain, young pigs were more likely to become infected than older pigs. The high seroprevalence observed in weaners and growers could result from passive transfer of maternal antibodies through colostrum from previously exposed sows to suckling piglets; however, to the best of our knowledge, maternal transfer of WNV antibodies in pigs has not been studied.

In this study, WNV RNA was not detected, similar to the results reported by Gutiérrez-Guzmán et al. [12]. This could be because, following WNV infection, pigs develop transient viremia that lasts between 48 and 60 h [16]; thus, allowing a narrow window of opportunity for the detection of the virus in serum. In future studies, nasopharyngeal swabs or tissue samples from the central nervous system might be more suitable for detecting WNV RNA.

In summary, based on the detection of WNV-specific antibodies, we show that pigs in Malaysia have been exposed to WNV. However, we did not detect WN viral RNA in our samples. Despite posing no significant health risks to pigs, our result highlights the need for increased vigilance in the surveillance of WNV in Malaysia.

ACKNOWLEDGMENTS

The authors express their gratitude to the Department of Veterinary Services (DVS) Malaysia for facilitating the process of WNV notification and reporting to the World Organization for Animal Health (OIE).

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